

Neuroprotective Effect of Zhen Tian Wan on Pial strip-Induced Cognitive Impairments and anti-oxidant status in Rats

Ahmadu P.U.^{1,2*}, Limin-Hu¹, Guozhen L¹, Adah D¹, Peter, Bai J¹, Salawu O.A²

¹Key laboratory for Pharmacology of Chinese medicine, Tianjin university of traditional Chinese medicine, TUTCM, 88 Yuquan Road, Nankai District, Tianjin, China P.R

²Department of Pharmacology and Toxicology, National Institute for Pharmaceutical Research and Development (NIPRD), P.M.B 21, Garki, Abuja, Nigeria

Email: peterjahmadu@gmail.com

Phone: +2348056585359

ABSTRACT

Zhen Tian Wan (ZTW), decoction consisting of seven herbs including Rhizoma ligustici, Radix Angelicae sinensis, Ledebouriella sesloides, Radix Angelica pubescentis, Flos carthami, Ramulus uncaria cumuncis and Radix Angelica dahuricae, has been widely used in traditional Chinese medicine (TCM), as treatment for headaches, migraine, premenstrual syndrome (PMS), and in soothing the nervous systems. The objective of the study was to investigate the neuroprotective effects of the formula using the Pial-strip model and Morris water maze analysis in rats. Doses of 1600, 3200 and 6400 mg/kg body wt orally were used. Dihydroergocristine 0.4 mg/kg p.o was used as the reference standard. The contents of malondialdehyde (MDA) and nitric oxide (NO), and the activity of superoxide dismutase (SOD) in the hippocampus and cortex were measured using thiobarbituric acid, nitrate reductase and xanthine-xanthine oxidase spectrophotometric methods, respectively. ZTW 1600- 6400mg/kg daily doses to pial strip-lesioned rats for 36 d, from day 6-42 after pial strip significantly reduced the prolonged latency and increased the swimming time spent within the target quadrant. The increased contents of MDA and NO and the decreased activities of SOD induced by the pial strip were significantly improved. ZTW significantly reduced the level of free radicals in pial stripped rats. ZTW can improve learning and memory function and it possess

anti-oxidant activity. ZTW may be beneficial in the treatment of vascular dementia.

Keywords: Zhen Tian Wan; pial strip; lesion; Memory; Morris water maze; free radicals

INTRODUCTION

A variety of deficits in learning and memory function have been demonstrated in the brain of animals after injury by craniotomy especially at the hippocampus region [1, 2, and 3]. Pial strip is known to produce severe histopathological damage and related behavioural deficits including cognitive and motor disorders, some of which continues to progress beyond the time of initial insult [2]. Certain pathophysiological changes like neuronal damage, hippocampal sclerosis, glial cell activation and proliferation [1, 4, 5] and abnormality in the level of metabolites have been associated with vascular dementia [6]. Lesion created affects the cerebral innervations especially the arteries supplying the parietal cortex, hippocampus and the striatum [7]. The hippocampal neurons which play important role in learning and memory processes [8, 9, and 10] are susceptible to lesion produced by removal of the pial matter. In addition, lesions of the cortex, hippocampus and striatum could result in cerebral ischemia which is a well-established cause of severe deficits of learning and memory in diverse behavioural tasks [7, 11].

Pial strip have also been associated with certain degree of loss of cholinergic neurons

including the levels of acetylcholine (ACh), and choline acetyltransferase (ChAT). Cholinergic neurons originating from the medial septum project to areas such as the cortex and the hippocampus [10] play a role in cognitive function. Many studies have demonstrated relationship between learning and memory functions and the cholinergic system in experimental animals [11, 12].

Oxidative stress have been associated with many neuronal function impairments [7, 11], including memory and learning functions of the hippocampus [1].

In TCM, many herbal drugs and prescriptions have been used clinically for the treatment of stroke, Alzheimer's disease and vascular dementia [13]. Several studies have shown neuro-protective effects of ginseng, *Uncaria ramulus et unculus*, *chuan xiong* and *xin nao shutong* [14,15,16]. Some of the active components of ZTW, such as *Uncaria Ramulus et Unculus*, *Rhizoma ligustici wallichii* have been reported to possess protective effects on ischemia-induced neuronal injury. Another component, *Hong hua* (safflower) was reported to be protective on reactive oxygen species (ROS) and oxidative stress in many parts parts of the body including the brain [17, 18, and 19].

Zhen Tian Wan is a decoction consisting of seven herbs: *chuan xiong* Rhizome 800mg, *Angelica* root 800mg, *Ledebouriella* root 800mg, *Pubescent angelica* root 800mg, *Safflower saffron* 800mg, *Uncaria* stem 800mg, *Dahurian angelica* root 800mg.

According to the theory of TCM, ZTW is classified under drugs used for promoting blood circulation and relieving of blood stasis. In addition to its analgesic properties, it is used to promote healthy nervous systems. Also, many researchers have studied not only singular herbal compound or active components, but also decoctions from a combinations of herbs for treatment of vascular dementia [20, 21, 22, and 23].

Although research work on some of the components of ZTW demonstrated neuroprotective effects on ischemia-induced stroke [24, 16], its neuro-protective actions on learning and memory impairment has not been studied. The aim of the present study was to determine the effect of ZTW on learning and memory in *Pial Strip* (lesion) –induced amnesia in rat using the Morris water maze. The neuroprotective effects and its anti-oxidant effects on the brain were studied by analyzing superoxide dismutase (SOD) activity, contents of malondialdehyde (MDA), and nitric oxide (NO) on the brain.

Materials and Methods

Animals

Adult male white wistar rats weighing 240-280g were obtained from Experimental Animal Center of Tianjin university of Traditional Chinese medicine, Tianjin, china. All animals were housed in groups of six with continuous access to food and water and were maintained on a 12h light /dark cycle regulated at 23°C room temperature. The experiments were carried out in accordance with the prevention of cruelty to Animals Acts 1986 C 14 and NIH guidance for the care and use of laboratory animals for experimental procedures.

Reagents

The reagent kits for measurement of malonedialdehyde (MDA) and nitric oxide (NO), and contents of Superoxide dismutase (SOD) were purchased from the Nanjing Institute of Jiancheng Biological Engineering. Other reagents were of analytical grade.

Apparatus

Morris Water Maze tank was purchased from Chinese Academy of Medical Sciences, Beijing, China with an attached camera (PANASONIC MADE IN JAPAN) that had Tamoron lens; 3.0- 8mm 1:1.0.

An electrophotometric machine (model Infinite M200) purchased from Switzerland, were also used.

Temperature controller CMA/Microdialysis (model CMA/150) used to regulate the

temperature of the rats during surgery was purchased from Sweden.

Preparation of ZTW solution

Granules of ZTW were obtained from San Jiu Medical and Pharmaceutical, Company Limited, Shenzhen, Guangdong Province, China. The dose of ZTW administered were 1600, 3200, and 6400mg/kg groups respectively. Fresh solution of ZTW was made using sufficient volumes of normal saline each day. Based on the weight, dose for each animal was calculated and administered orally.

Pial Strip Surgery

Rats were anaesthetised with chloral hydrate 0.3mg/kg. After head shaving, each rat was placed in a stereotaxic instrument with the head secured by non-puncture ear bars. In all surgeries head position was adjusted to place bregma and Lambda in the same horizontal plane. Under aseptic conditions, a midline incision was made on the skull and the surface was cleared. Using stereotaxic coordinate derived from Paxinos and Watson (2007), 6-8 small holes were drilled round just within the portion to be removed, and with the aid of forceps craniectomy was done. A sterile cotton bud was used to scrub the exposed portion of the hippocampus to ensure removal of the Pial and Dura matter after which tiny vessels and the meninges were removed [2, 9]. Throughout the surgical procedure, body temperature was monitored by inserting rectal thermometric probe and maintained at 37 ± 0.5 °C. Animals were then housed in a cage with heating lamp to maintain the temperature at 29 ± 1 °C for another 1 hr to counteract any possible hypothermic effect.

Experimental Design

Rats were divided into five groups (8 rats/group). The experimental group was treated with graded doses of ZTW (1600, 3200, and 6400) mg/kg (ZTW + Lesion) respectively. The standard reference used was Dihydroergocristine 0.4mg/kg (HDG + Lesion). The control group received normal saline 5mg/kg (N/S + Lesion) for six weeks after craniotomy. ZTW, HDG and normal

saline were administered orally everyday in the morning. The model group was not treated with any drug for six weeks after the craniotomy. The water maze tests were performed in the sixth week after the craniotomy. The brain of the animals were harvested thereafter for determination of anti-oxidants effects analysis.

Water Maze Task

The water maze consisted of a circular tank (2.0m in diameter, 0.35m high) constructed of aluminum panel. The water was maintained at a temperature of 22 ± 2 °C, and was made opaque by addition of 1kg of powdered skimmed milk. During testing in the water maze, a platform 15cm in diameter was located 1.5cm below the water surface in one of four locations in the pool, approximately 50cm from the side wall. The maze was surrounded by many cues external to the water. A video camera was mounted in the ceiling above the pool and was connected to video recorder and tracking device (PANASONIC, MADE IN JAPAN), which permitted online and off-line automated tracking of the path taken by the rat. The animals were subjected to four trials per session. The rats in all the groups were trained to locate the hidden escape platform, which remained in a fixed location throughout the study. The trials lasted for a maximum of 60s, and the latency and swim distance to find the submerged platform were recorded. The animals were tested in this way for 4 days, and were given a probe trial on the 5th day. For the probe trials, the platform was removed from the pool and the rats were released from the quadrant opposite where the platform had been located. The length of the trial was 60s, and thereafter, the rats were taken out of the pool. The proportion of time and swim distance the rat spent searching for the platform in the training quadrant, were recorded and used as a measure of retention. The swimming time and distance within only a 30cm circular zone around the previous platform, that is, not in the whole quadrant, were recorded.

Measurements of contents of MDA and NO and activities of SOD

The assay was done according to instructions manual and other published work by Pari, L., *et al*, 2004. Under chloralhydrate (0.3mg/kg) anaesthesia the rats were decapitated following 5 weeks of drug administration and behavioural probe on day 42 after surgery. The brain were homogenized in cold saline to obtain 10% homogenates to assay MDA and NO contents, and 1% homogenates for SOD activity measurement using thiobarbuturic acid ,nitrate reductase and Xanthine-xanthine oxidase spectrophotometric methods respectively. The SOD activity was expressed as the difference in nitrite content between the control in the test kit and sample. The nitrite content was calculated according to the standard curve, $Y = 0.06683 - X = 0.009373$, where Y is the absorbance, and X is the concentration of nitrite (mmol/ L). The protein content was determined using the Coomassie blue protein-binding method, using bovine serum albumin as a standard. A small amount of the protein sample was combined with the assay agent, thoroughly mixed and briefly incubated and thereafter, the absorbance was measured at 595nm. The protein concentration is estimated by reference to absorbances obtained for a series of standard (bovine serum albumin) protein dilutions.

Statistical Analysis

The data were expressed as mean \pm SEM. Group difference in latency in the Morris

water maze were analysed using one-way analysis of variance (ANOVA) with repeated measures. Group differences in the probe trials and biochemical assays were similarly evaluated using one-way ANOVA followed by Dunnet's T3 test.

Results

Water Maze Task

The mean latency to find the hidden platform from all the groups showed that N/S + Lesion group had worst performance (60.00s, 54.77s, and 44.14s) respectively compared to the control based on the significantly increased latencies to finding the hidden platform (Fig.1 .A). The average latency on day 4 for model was significantly ($p < 0.05$) higher than that of the control group (39.21 ± 19.21 Vs 3.69 ± 1.90 s) respectively. The standard reference drug's group (HDG + Lesion), ($n=8$) had latency of 5.04 ± 3.5 s. The latencies for the ZTW + Lesion (1600, 3200 and 6400) mg/kg body wt doses were 6.00 ± 4.63 s, 5.93 ± 5.21 s, 4.02 ± 2.83 s respectively and were significantly ($p < 0.05$) decreased compared to the model group.

Individual comparison of the HDG, the test groups with the model group showed statistically significant difference at ($p < 0.05$). However, individual comparison of the latencies of the test groups ZTW +Lesion (1600, 3200, 6400mg/kg) doses with the standard reference (HDG) did not show statistically significant difference.

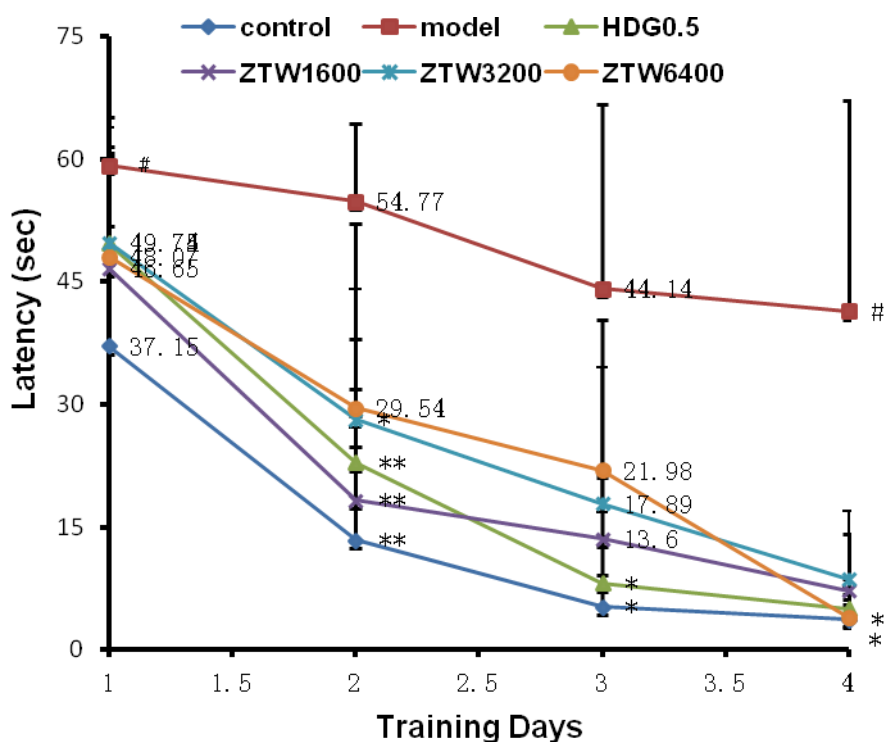


Fig. 1.A: Mean latency to find the hidden platform; ZTW-treated rats and HDG-treated rats highly significantly ($p < 0.05$ – $P < 0.01$) reduced latency to finding the hidden platform compared to the model group from day 1-3 of the training respectively

The swim distance covered by the model group was significantly ($p < 0.01$) reduced compared to the control [241.33 ± 56.26] cm Vs [383.41 ± 84.26] cm respectively. The swim distance covered by the rats in reference standard group was 296.79 ± 53.85 cm. Rats administered with ZTW (1600, 3200 and 6400) mg /kg body weight covered $312.53 \pm$

68.26 cm, 314.20 ± 55.12 cm and 332.49 ± 80.09 cm respectively and were significantly ($p < 0.05$) higher than distance covered by rats in the model group(Fig.1B), however, not with the HDG group. Comparison of model Vs control groups showed significantly reduced ($p < 0.01$) swim distance than the control.

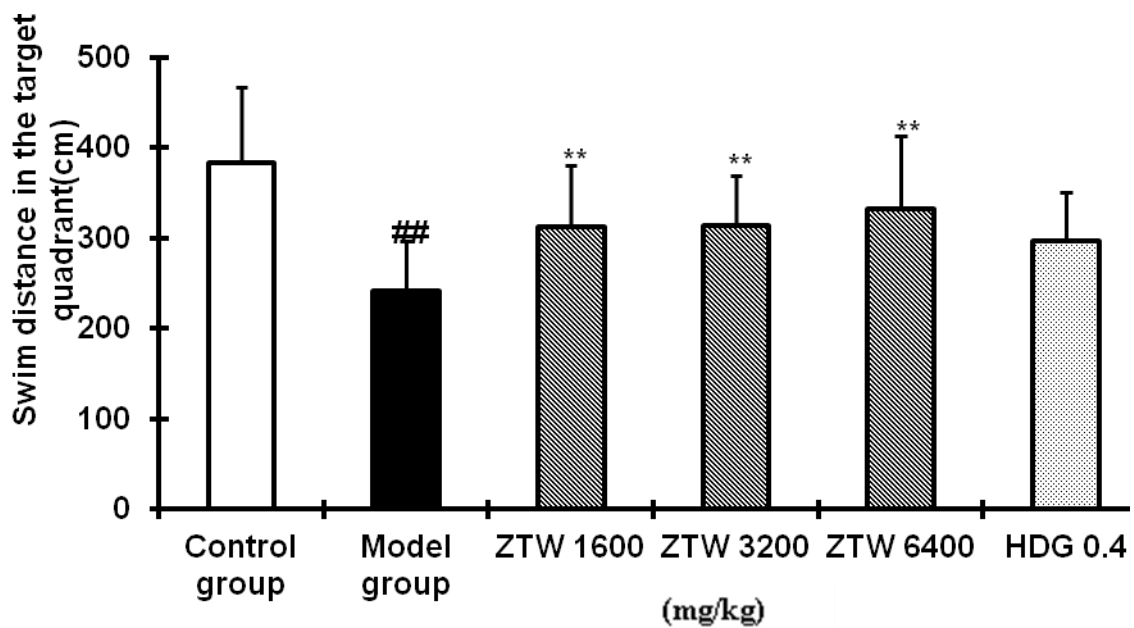


Fig. 1.B: Swimming Distance in the target quadrant in 60 seconds probe trial (no platform). Rats in all ZTW groups highly significantly ($p < 0.01$) respectively longer distance within the target quadrant compared to the model.

On the 5th day, the platform was removed and learning and memory test was carried out as previously done. The average time spent by rats in the model group was 14.37 ± 0.6 sec. while the control group spent 25.80 ± 1.78 sec. The HDG group spent 20.02 ± 1.79 sec on the average. The time spent by rats that received graded doses of ZTW (1600, 3200, 6400) mg/kg, were 19.61 ± 2.53 s, 24.08 ± 2.48 s and 23.91 ± 2.51 s respectively (Fig. 1C). The lesion

caused by removal of the pial matter caused severe impairment of spatial cognitive function for the water maze task. Also, the test revealed that time spent by the model group around the platform compared with those of HDG (0.4mg/kg), and ZTW groups (1600,3200,and 6400mg/kg), were significantly reduced ($P < 0.01$), indicating that lesion as a result of the pial strip impaired learning and memory functions in the rats.

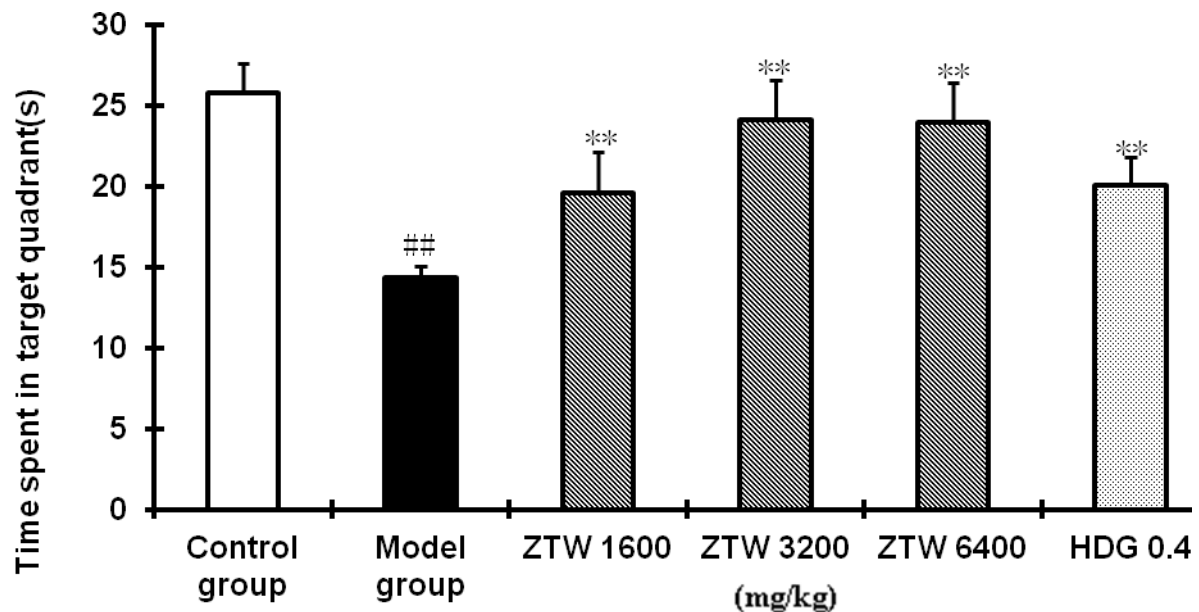
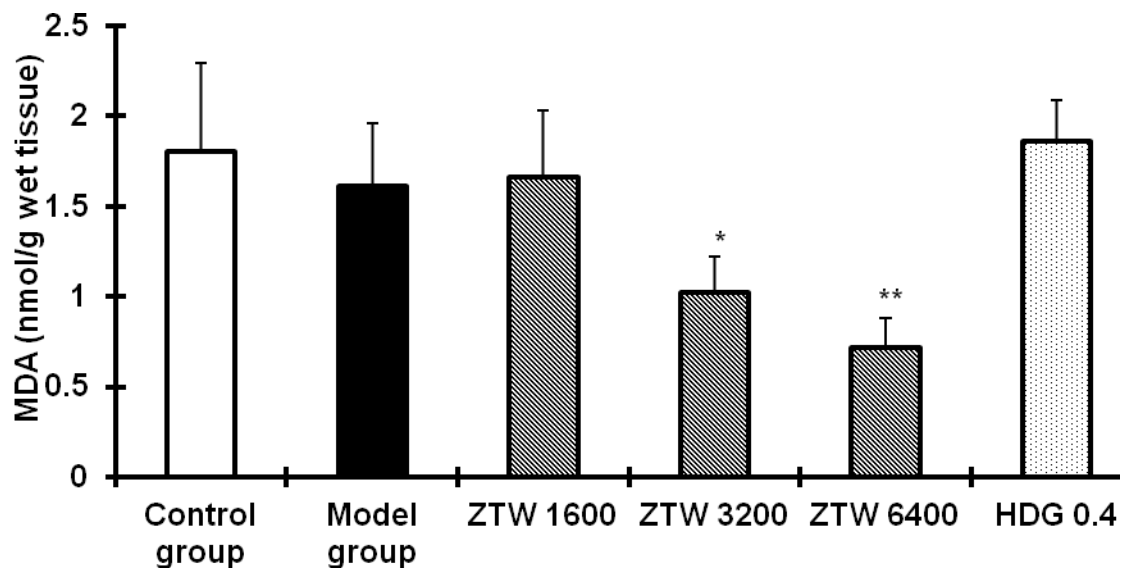


Fig.1.C: Time spent in the target quadrant without the platform; the time spent by rats in ZTW groups were significantly ($p < 0.01$) respectively compared to the model group. Rats in the reference standard drug group, (HDG 0.4mg), significantly ($p < 0.01$) spent more time in the target quadrant compared to the model.

3.2 Measurements of Contents of MDA and NO, and Activities of SOD

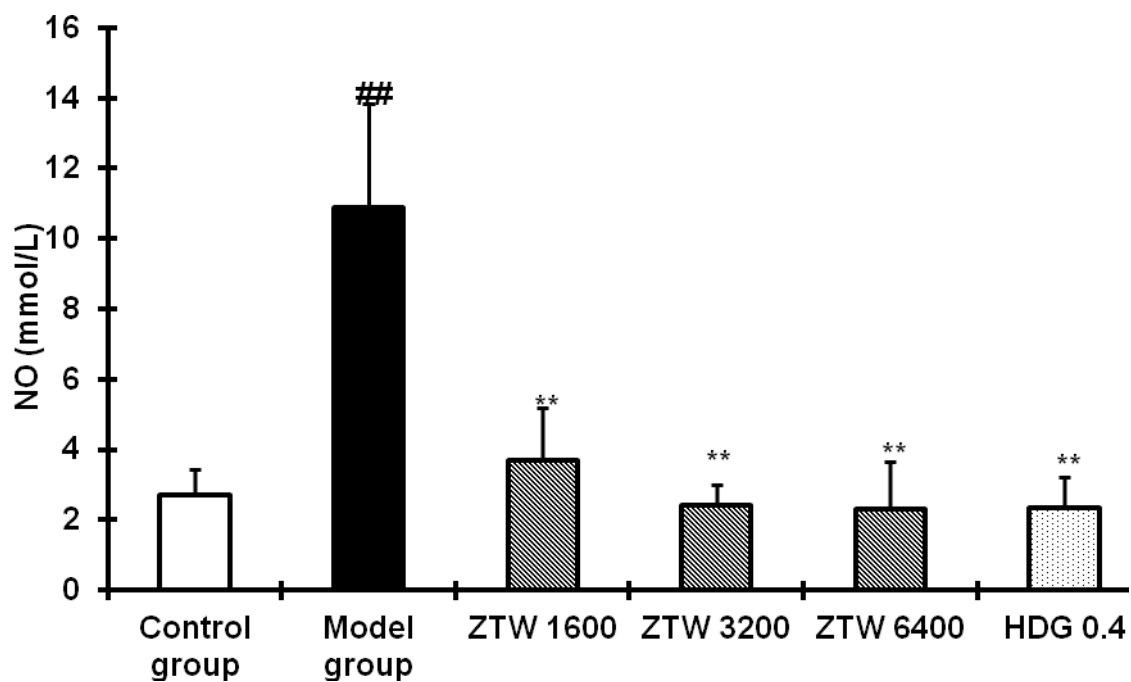
Compared with the Control group, the contents of MDA and NO significantly increased in the brain of the model group. Daily administration of ZTW 36 days (3200, 6400 mg/kg) significantly ($P < 0.05$ - $P < 0.01$) reversed the increase in MDA and NO contents respectively (Figs. 2A and 2B). The standard reference however, showed no significant

effects on the MDA content. Also, SOD activity was decreased in the model group ($p < 0.01$) compared to the control. Furthermore, the SOD activity on the brain of rats in ZTW groups (3200, 6400mg) significantly ($p < 0.01$) increased compared to the model group (Fig. 2C). Also, administration of ZTW at dose of 1600g/kg did not show significant effects.



Mg /kg

Fig. 2A: Pial Strip-induced lesion significantly increased the levels of MDA in the model group compared to ZTW 3200mg/kg group ($p < 0.05$); and ZTW 6400mg/kg ($p < 0.01$) respectively reduced the content of MDA after 36 days of ZTW administration post-surgery.



mg/kg

Fig. 2B: Pial Strip-induced lesion significantly increased the NO contents in the model group. ZTW 1600, 3200, and 6400mg/kg significantly reduced NO contents after 36 days of treatment post-surgery compared to the model group ($p < 0.01$) respectively.

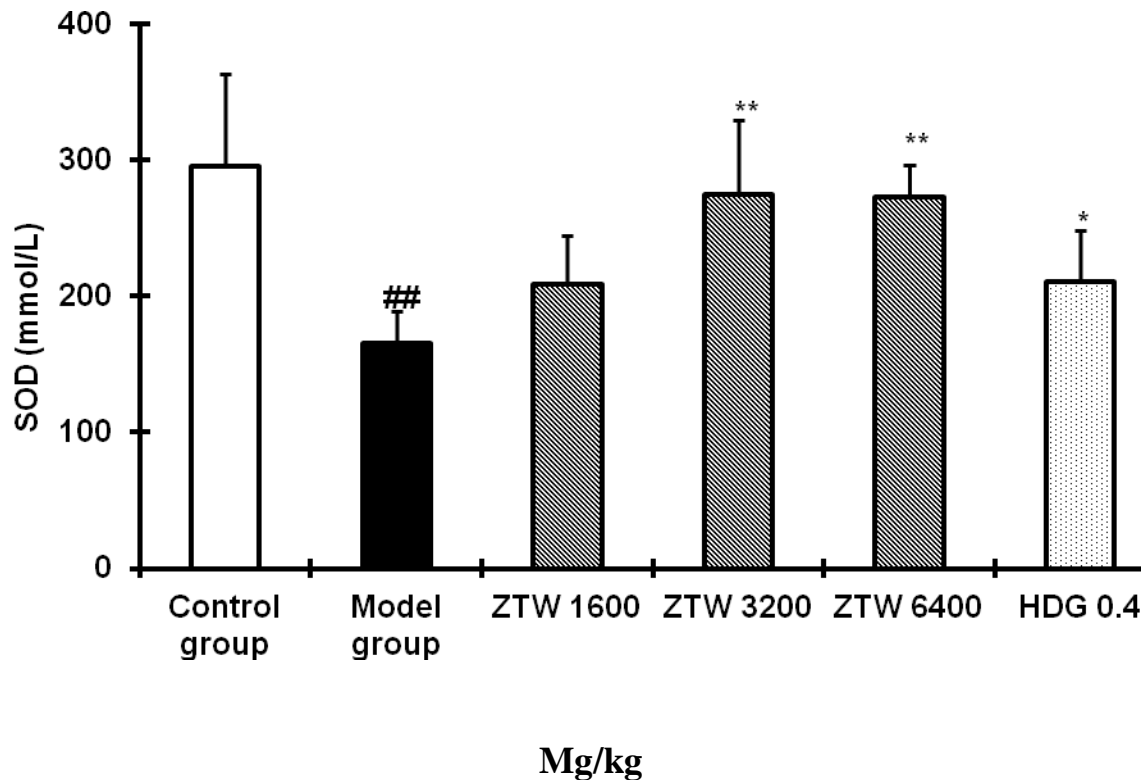


Fig.2C: ZTW 3200 and 6400mg/kg significantly increased SOD activity compared to the model group ($p < 0.01$) respectively of treatment post-surgery. Model group received no drug.

Discussion and Conclusion

The results showed that the Pial strip which involved removal of the pial matter, dura matter and the meninges produced lesion specifically at the hippocampus region of the rats brain, thereby causing deficits in cognitive and behavioural functions in the rats [2, 7, 11, 13]. Post-treatment with ZTW improved lesion-induced learning and memory deficits in the water maze (Figs.1A, 1B, and 1C), an effective method used in studying cognitive and learning disorder in the rodents [5, 1]. Since spatial map is necessary for cognitive task, Morris water maze is similar to nonverbal tests of cognitive function which are sensitive

in detecting senescence and memory disorders in clinical setting [20, 21]. In Morris water maze test, the platform trial detects rat learning acquisition. In doing this, the rats use many approaches like circling the tank at a certain distance from the edge, to locate the platform and form memory by way of repeated training. The trial probe that tests the rat memory status and it is a more reliable measurement of the accuracy of memory.

Pial strip produces direct lesion and insult on the hippocampal region of the rat brain, leading to erosion of meninges and micro arteries and veins and this could cause neuronal and striatum damage [2, 6, 7, 11]

similar to global ischemia-induced neuronal deaths. This produces in addition, release of free radicals that exert oxidative stress contributing to neuronal cell death. These changes may contribute to learning and memory impairment. In this study therefore, the lesion produced by stripping of the pial matter, and the removal of the meninges and small arteries had severely impaired learning performance in the Morris water maze task over a period of six weeks. The results conform with others who report increase in swimming time required to locate the hidden platform which was associated with neuronal impairment especially at the hippocampal region and the cortex with reduced swimming time within the target quadrant[32,]. ZTW has been used in TCM as remedy for treatment of vascular headaches, and maintenance of healthy nervous system that depend on the neuronal functions. This may serve as validation of such claims that it maintains and regulate Qi and resolve blood stasis or enhance vascular blood flow. Furthermore, report from several studies on chuan xiong, a component of ZTW showed that it has neuroprotective effects against ischemia-induced stroke in rats. In this study, rats with the pial strip lesion treated with ZTW performed better than rats in model group in acquisition and memory retention as shown by reduced latency and increased swim time within the quadrant where the platform was located. The reference drug HDG similarly attenuated cognitive impairment and neuronal damage caused by pial strip.

There is increasing evidence that parietal craniotomy could affect mast cell integrity in the sub-adjacent dura matter, contralateral histamine in the dura matter and cortex, vascular permeability of cerebral cortical blood vessels. This results into breakdown of the, leading to trauma in the brain [24, 25, and 26]. In this study, Pial strip is a procedure that involves artificial injury on the skull (craniotomy) in the absence of penetrating brain injury which can activate dural mast cells and possibly elevate cortical histamine resulting in inflammation and neurological trauma and eventually ,oxidative stress [8,9].

There is also a direct rupturing of the mid cerebral arteries innervations, especially on the hippocampus with a reduced supply of blood and glucose to this region which leads to slow death of neurons[24].These injuries may have developed over a period of over 5 weeks. These results showed that ZTW could reduce oxidative stress as shown by the increase in SOD activity and the reduction in the contents of MDA, and NO respectively. Reports have shown that release of these free radicals plays important roles in neuronal degeneration and death, and are involved in pathophysiology of neuronal death in neuro-degenerative diseases [13, 26, 31]. There is greater tendencies for the brain to go into peroxidation due to its high oxygen consumption rate, high level of lipid and a relative inadequate level of antioxidant enzymes when compared with other tissues of the body [26, 27]. Under conditions of inadequate oxygen supply to the brain, lipid peroxidation takes place yielding malonaldehyde (MDA), a highly reactive substance known to be involved in cytotoxicity and death of neurons [27]. Also NO plays important roles in oxidative stress by mediating in the reaction between superoxide and nitric oxide yielding the very reactive peroxynitrite as the end product. The Pial Strip therefore, caused the increase in the MDA and NO contents due to direct brain tissue damage, and initiation of oxidative stress with resultant release of free radicals. SOD has been reported to play essential role in maintaining the physiological levels of oxygen and hydrogen peroxide by enhancing dismutation of oxygen radicals [5]. SOD acts in concert with other enzymes like catalase, glutathione peroxidase, and reduced glutathione (GSH) to combat reactive oxygen species (ROS)-mediated damage. The decrease in the level of SOD therefore is as a result of increased level of hydrogen peroxide and oxygen by auto oxidation and glycation [28]. Furthermore, increase in SOD level protects other antioxidant enzymes such as catalase and glutathione peroxidase against inactivation by oxygen anions [29, 30]. Treatment with ZTW increased the activities of SOD and decreased the contents of MDA and NO, and this may

help to control the free radicals. ZTW is a registered Chinese herbal formula that has been in use in the treatment of headaches and migraine. In an unpublished study, ZTW proved to reverse both ischemia-induced and pial-strip-induced elevation of acetylcholinesterase enzymes (AChE) in the rats' brain as shown in the water maze learning and memory task, and this may be in line with its previous use in the maintenance of healthy nervous system. High level of AChE in the CNS has been associated with memory impairments due to excessive hydrolysis of acetylcholine (ACh), making it unavailable for synaptic transmission necessary for cognitive function. From the results of this study, ZTW proved to ameliorate memory deficits caused by pial strip lesion as can be seen in the significantly reduced latency to finding the hidden platform by the rats during the water maze performance test. The significantly increased swim distance in the target quadrant as well as total distance which were significantly higher than swim distance seen in rats of model group showed that ZTW enhances and or ameliorates damaged memory function. Spatial memory and learning have been associated with CA1 region of the hippocampus and the prefrontal cortex [13, 17]. Therefore, ZTW possess components that may have positive effects on both prefrontal cortex and the Hippocampus. ZTW may also improve cholinergic transmission via maintenance of optimal level of acetylcholinesterase enzymes in the brain.

Treatment with ZTW (1600, 3200 and 6400mg/kg per d for 36 days) could decrease MDA and NO contents and increase SOD activity in the rats' brain. This effects correlates with reduction of cognitive deficits and neuronal damage and these effects of ZTW in improving cognitive impairments may be due to reduction of neuronal damage and improving the scavenging activities of antioxidant enzymes.

This study revealed that ZTW at doses 1600mg/kg and 6400mg/kg produced significant improvements in learning and memory and thereby improved cognitive function. It may also reduce neuronal damage

and improves the anti-oxidant activity in cerebral ischemic rat. In addition to other studies, it suggests that the neuroprotective action in pial strip –induced deficits may slow or prevent the physiological pathway of cerebro-vascular dementia.

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