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Neuroprotective effects of vitamin E in cold induced cerebral injury in guinea pigs

Prerna Badhe, Jayant Thorat, Batuk D Diyora, Ravikrishna Mamidanna, Parag Sayal, Suvarna Badhe & Alok K Sharma
Department of Neurosurgery, Lokmanya Tilak Municipal Medical College & General Hospital, Sion, Mumbai 400 022, India

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Significant reduction in hemorrhage (10 v/s 13), necrosis (2 v/s 4), cavitations (7 v/s 13), neuronal degeneration, perivascular and parenchymal inflammatory infiltrate (7 v/s 11) were observed in Vitamin E treated cold induced head injury in guinea pigs, evaluated post injury using the modified Benderson's scale. The results suggest that Vitamin E is highly effective in promoting clinical and histopathological recovery in cold induced head injury in guinea pigs.

Keywords: Antioxidant, Head injury, Neuroprotection, Vitamin E

Head injury accounts for almost 50% of trauma-related deaths and affects the most productive age groups of life. The use of antioxidants in the treatment of trauma needs special attention as it can prevent secondary neuronal degeneration, which is responsible for the rise in mortality and morbidity of severe head injury patients and perhaps prevent the long term sequelae of brain trauma.

During recent years free oxygen radical generation and lipid peroxidation have been considered to be responsible for secondary auto destructive injury. The membrane lipids in the brain and nervous system are especially enriched in cholesterol and polyunsaturated fatty acid, which can be attacked by oxygen free radicals. Vitamin E is a well-known free radical scavenger and the most effective antioxidant in the lipid phase. The use of antioxidants in treatment of trauma needs to be explored as it can prevent secondary neuronal degeneration, which is responsible for the morbidity and mortality in severe head injury and limit the long-term sequelae of brain trauma.

Traumatic neural injuries of the brain cause tissue damage through primary and secondary mechanisms; of which, delayed secondary injury initiated by oxygen free radicals has a major contributory role[1-4]. This concept of secondary or auto destructive injury factors has provided the theoretical basis for evaluation of an antioxidant as a pharmacological intervention[5,6]. Vitamin E, a lipid soluble agent, crosses the blood brain barrier with ease and is a highly efficient antioxidant[7,8].

Cold induced head injury can be produced by dipping a copper plate in a cooling mixture of acetone and dry ice to achieve a freezing surface temperature of -50° ± 2° C and then applying it to the exposed dura over the frontal motor cortex of the animal for one minute. The aim of the present study is to evaluate the neuroprotective effects of pretreatment of Vitamin E in promoting neurological and histopathological recovery in cold induced cerebral trauma in guinea pigs.

Materials and Methods

All the experiments were conducted in the pharmacology experimental laboratory in accordance with rules and regulations of the animal research committee on animal research and experimentation. The animals were maintained as per standard guidelines (INSA). Immunized and conditioned 30 adult guinea pigs (English Short hair) of either sex, with average age of 3-4 months and weighing 450-550 g were obtained from the Haffkine Institute, Mumbai. They were randomly divided into two groups: control group (Group A) and study group (Group B), each consisting of 15 animals with no differences in the gender, age, weight or distribution. The study group (B) was fed a daily dose of 20 mg of Vitamin E per day via a metallic feeding tube, for 5 days pre-operatively and 5 days post-operatively, just prior to their regular feeds. The control group (A) was fed 0.5 cc of normal saline in a similar way.

Phone: 91 2224024713
Fax: 91 22 24025521
E-mail: prernabadhe@yahoo.co.in

The animals in both groups were anesthetized by 20 mg/kg thiopentone sodium (ip). Pulse, temperature and respiration were monitored carefully. After 15 min, an ip injection of 50 mg/kg Cefotaxime was given. The scalp was cleaned and shaved. Under aseptic precautions, a vertical skin incision over scalp was taken between the outer canthus of eye and ear on left side. The bone was removed near zygomatico-frontal suture line to create a craniectomy that exposed a 5 mm area over frontal lobe just posterior to coronal suture. A copper plate with an area measuring 4 mm square was dipped into a cooling mixture of acetone and dry ice to achieve a freezing surface temperature of -50° ± 2° C was then applied to the exposed dura over the frontal motor cortex for 1 min. The dura was covered with surgicel and wound was closed in single layer. The wounds were cleaned and dressed with antibiotic coverage postoperatively for 5 days.

All the guinea pigs were neurologically evaluated post injury using the modified Benderson's scale[9] and were graded on a scale of 0 – 6, at regular intervals of 6, 24, 48, 72, 96, and 120 h (Table 1). Subsequently, animals were given ip injection of 2 ml of 2% Evans blue. After 5 days, animals were sacrificed humanely by an overdose (ip) of thiopentone sodium. Immediately after death, transcardiac infusion of 100 cc normal saline followed by 10% formalin solution was injected. A wide craniectomy was done bilaterally to expose full surface of the brain. The brain was removed in toto and examined grossly. Brain sections were taken through the most representative part of the lesion and were stained with haematoxylin and eosin. A neuropathologist, who was blinded to the source of animal groups, graded the histological features as 1+, 2+ or 3+ in each category of hemorrhage, necrosis and cavitation. The other features looked for were the presence of neuronal degeneration and extent of perivascular and parenchymal inflammatory infiltrate by neutrophils, reactive astrocytes, macrophages and gitter cells. Mean recovery index and microscopic findings were compared between the control and study groups and appropriate conclusions drawn. Statistical evaluation was done. Mean values were compared as arithmetic mean ±SD and compared with Chi square test. Level of significance was set at $P<0.05$.

Results

There was no mortality in either of the groups. In the immediate post-operative period, 6 of the Group A animals developed tonic-clonic seizures associated with urinary and fecal incontinence. Only 4 animals in the Group B had similar seizures. The convulsions stopped spontaneously and no specific drug therapy was given. There was no mucosal cyanosis and the oxygen saturation (measured by Ohmeda pulse oximeter) in the right ear was normal. All animals recovered from anesthesia and convulsions at 6 h post operatively. On calculation of the mean recovery index , significant differences ($P< 0.05$) were noted in neurological recovery at 6, 24, 48, 72, 96 and 120 h between Group B (vitamin E treated group) and Group A (control group), (Table 2). The neurological grade of deficit at 6 h, in the post operative period, in the group A was 6 in 5 and 5 in 10 animals whereas in

Table 2 —Comparison of mean neurological grades between vitamin E study group (B) and control (A) group*

Groups	Post-op (hrs)						
(n=15)	6	12	24	48	72	96	120
Group A (Control)	5.33	5.33	4.47	4.00	3.33	3.00	2.67
Group B (Vit E)	5.27	4.80	4.00	3.13†	2.40†	1.87†	1.47†

Chi square test
† $P< 0.05$ (significant)
The grades were arrived at by obtaining the mean of the grades of all the animals in that particular sub-group.

Table 1 — Modified Benderson's scale for neurological grades in guinea pigs

Grade 0	Normal power, gait and righting reflex (full recovery)
Grade 1	Gait normal. Forelimb flexed on affected side. On holding upside down, there are equal clawing movements of both forelimbs.
Grade 2	Runs in a straight path without deviation. On pulling by rump, it makes equal clawing movements on both sides of floor to escape. On holding upside down, there is decrease in clawing on affected side. Righting reflex normal. Equal resistance to lateral push.
Grade 3	Runs straight, but slow. On pulling by ramp, there is decreased clawing on affected side. On holding upside down, there is turning of head to paretic side with flexion of affected forelimb.
Grade 4	Righting reflex grossly impaired (more than 4 attempts). On lateral punch, the animal falls easily without offering resistance.
Grade 5	Makes circling motions as it attempts to walk. Head turned to paretic side even while resting. Righting reflex absent.
Grade 6	Complete hemiplegia lying on the paralyzed side. Drowsiness and diminished blinking (no recovery)

group B it was 6 in 2, 4 in 5, and 5 in 8 animals. Thus the mean neurological grade was 5.33 and 5.27 in control and vitamin E treated groups respectively (P=0.35). At 12 h the difference was insignificant. (P=0.010). At 24 h group A showed neurological deficit of 5 in 8, 4 in 6 and 3 in only 1 animal. In contrast to this, group B had grades of 4 in 7, 5 in 4, and 3 in 4 animals. The mean neurological grades was not significant (P=0.039). At 48 h, group A had grades 4 in 9, 5 in 3 and 3 in 3 animals whereas group B, had grades 4 in 6, 2 in 4, and 5 in 5 animals. The mean neurological deficit score was statistically significant. (P=0.0019). At 72 h group A had grades 4 in 7, 3 in 6, and 2 in 2 animals while group B had lowest grade of 1 in 2, 4 in 1, 3 in 6 and 2 in 6 animals. There was significant improvement over the previous observation at 48 h, it was statistically highly significant (P=0.00136). At 96 hr a mean neurological grade of 3.00 *vs* 1.87 in group A *vs* group B was noted which was statistically significant (P=0.00018). As compared to the previous observation period, the neurological grade had improved by 0.33 *vs* 0.53 for the group A and B respectively. At 120 h in the control group, grades 3 in 9, 4 in 1, 2 in 4 and 1 in 1 animals were noted. Whereas group B had grade 2 in 8, 1 in 6 and 0 in 1 (i.e. normal) score. The mean neuurological score was significant (P<0.00003). The group B had shown an improvement with the difference of 0.40 over the previous score as compared to the group A of animals who had shown a difference of 0.33, thus although the group B had improved outcome because of their lower deficit and early recovery, there was only slight difference in their rate of improvement, suggesting an early neuroprotective effect following trauma and the secondary neurological insults. The group B animals recovered faster neurologically as evidenced by the mean neurological grade as compared to the controls. This trend was more evident at 48 h postoperatively and continued up to 120 h. On gross examination with Evan's Blue, the spread seemed to be lighter in the Group B indicating a less severe form of lesion in this group as against the control. The Group A of animals displayed hemorrhages (defined as any hemorrhages larger than 2 mm and visible to the naked eye) in 7 out of 15 animals as compared to 4 in the Group B. Necrosis was seen in 9 out of 15 control animals as compared to 5 of the Group B animals. The histopathological evaluation also showed significant differences between the two groups. The group B showed a significant reduction in haemorrhages, cavitation, oedema, reactive astrocytes and infiltration by macrophages, neutrophils and gitter cells; as compared to the group A (Table 3). Hemorrhages were found in 10 *vs* 13, animals. (P< 0.05). Necrosis was graded as 1+, 2+ and 3+. Necrosis was seen to be greater with the depth of the lesion, 2+ in 4 gray white matter lesions and 3+ in white matter lesions in the control group. The group B on the other hand had only 2 animals with white matter lesions, who had 2+ necrosis and 1+ necrosis in 6 animals. 2+ perivascular inflammatory infiltration was seen in 11 control animals v/s 1+ in 3 vitamin E treated animals (Fig. 1, 2).

Discussion

Vitamin E is a free radical scavenger and efficiently breaks the chain of oxygen free radical induced lipid peroxidation in the cell membrane of the cell as well as subcellular structures such as mitochondria and lysosomes[10-12]. The cold induced cerebral injury model was chosen as the duration and intensity of injury can be well controlled and a repeatable end point can be obtained. The vitamin E group animals recovered faster neurologically as evidenced by the lowered mean neurological grades as compared to the controls. This trend was evident at 48 h postoperatively (3.13/4.00) and continued up to 120 h (1.47/2.67) respectively (P<0.05). The Vitamin E group also showed significant reduction in the frequency and degree of hemorrhage (10 *vs* 13), necrosis (2 *vs* 4), cavitation (7 *vs* 13), neuronal degeneration, perivascular and

Table 3 — Comparison of microscopic examination findings between vitamin E study group (B) and control (A) groups

Microscopic examination	GroupA (n=15) Group B (n=15)		Mean Grade	1+	2+	3+
Hemorrhages	Group A	13	2.3	-	05	08
	Group B	10	0.9	07	03	00
Cavitations	Group A	13	1.3	07	06	
	Group B	07	0.5	07	00	
Oedema	Group A	11	1.0	07	04	
	Group B	06	0.4	06	00	
Necrosis	Group A	14	1.4	07	07	
	Group B	06	0.4	06	00	
Macrophages	Group A	11	1.1	06	05	
	Group B	07	0.7	04	03	
Reactive	Group A	11	1.1	06	05	
Astrocytes	Group B	03	0.3	02	01	
Neutrophil	Group A	10	0.9	06	04	
Infiltration	Group B	02	0.2	01	01	
Gitter Cell	Group A	13	1.3	06	07	
Infiltration	Group B	08	0.5	08	00	

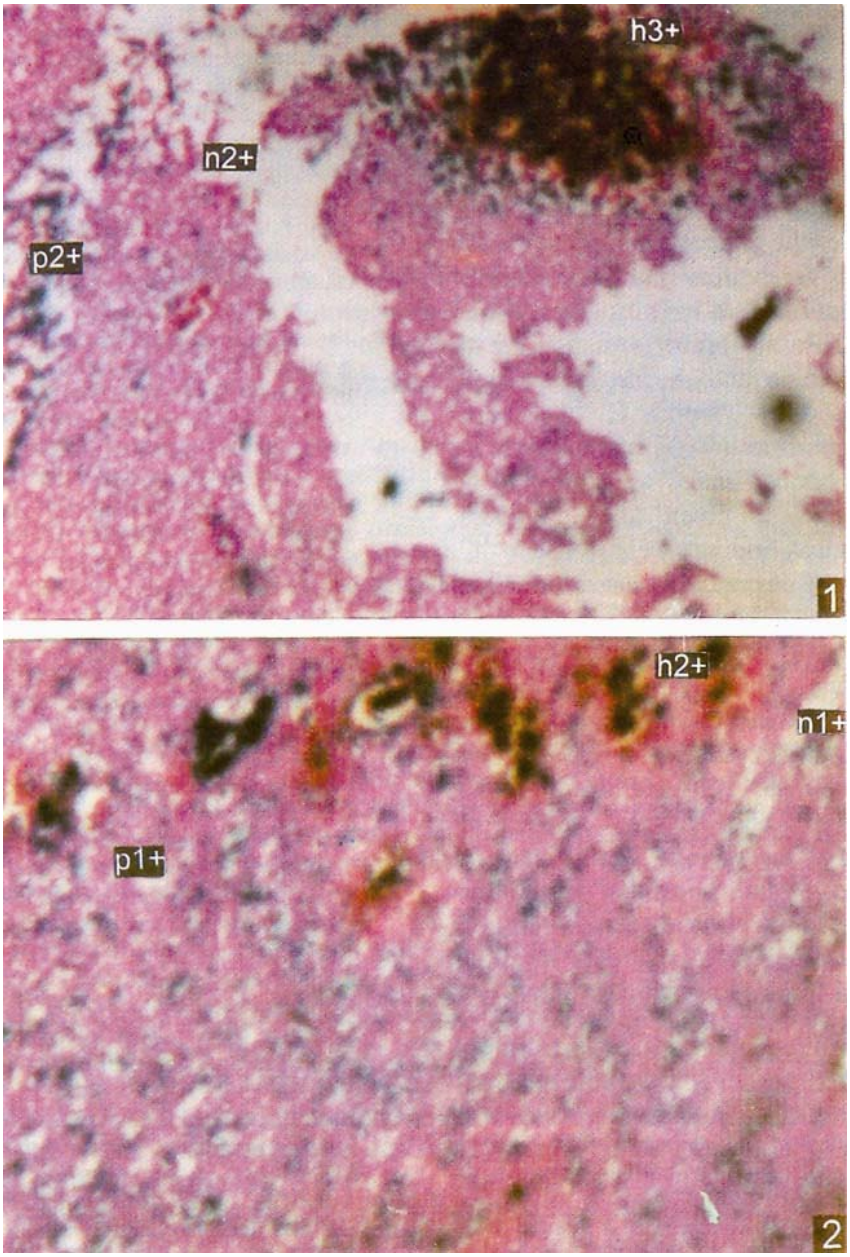

Fig. 1 — Section from cerebral cortex at the site of cold induced injury in Group A showing areas of hemorrhage (h) 3+, necrosis (n) 2+, perivascular inflammatory infiltrate (p) 2+ [100X, H&E]

Fig. 2 — Section from the cerebral cortex at the site of cold induced injury in Group B showing areas of hemorrhage (h) 2+, necrosis (n) 1+, perivascular inflammatory infiltrate (p) 1+ [100X, H&E]

parenchymal infiltrates, infiltration by macrophages, reactive astrocytes, neutrophils and gitter cells (7 *vs* 11) as compared to the control group ($P<0.05$), suggesting their better neurological outcome. The vitamin E treated group recovered earlier as they were pretreated with vitamin E, affording neuronal protection from lipid peroxidation, which immediately follows trauma[13,14]. The vascular lining endothelium afforded protection as evidenced by the diminished hemorrhagic response both at the macroscopic and microscopic levels. This amounts to diminished tissue destruction and reduced inflammatory response to trauma[15]. Neutrophil accumulation in the acute phase of inflammatory

response causes an increase in cerebral blood flow and therefore increased edema raising the intracranial tension[16]. Activated neutrophils synthesize oxygen radical (O^-) and have been reported to contribute to cerebrovascular disturbances after trauma, including cerebral vascular edema and subsequent decreased blood flow[17]. According to Kainova *et. al*[18], the addition of Fe^{2+} induces the greatest lipid peroxidation stimulation within the cerebellum and brain stem of Vitamin E deficient animals. It has also been found that alpha tocopherol in the brain is responsive to vitamin E supplementation and the degree of lipid peroxidation is reduced by vitamin E[19-22]. Studies have shown that administration of an anti-oxidant agent within 3 hr of brain damage was found to be neuroprotective whereas if given after 3 hours, differences in histological findings were not seen[23,24]. These data provide proof of the lipid peroxidation and free radical induced secondary irreversible damage and provide the basis of pretreating with the anti-oxidants before trauma. Thus, Vitamin E therapy protected against oxygen free radical induced lipid peroxidation, afforded membrane protection at cellular and subcellular levels, resulting in lesser inflammatory response and tissue destruction. Although high levels of the vitamin were given, no side effects were observed indicating that it is safe to all organs of the body. In the clinical setup, vitamin E can be easily administered in all patients awaiting elective neurosurgical procedures and to high-risk groups of head trauma.

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