IMPAIRMENT OF BLOOD-BRAIN BARRIER (BBB) IN RAT BY IMMOBILIZATION STRESS : ROLE OF SEROTONIN (5-HT)

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Summary : Influence of immobilization stress on blood-brain barrier (BBB) was studied in rats using Evans blue as barrier tracer. 7-9 hr of immobilization had increased the permeability of BBB mainly in younger rats. This increased permeability was significantly reduced with p-CPA, indomethacin or vinblastine pretreatment. Theophylline treatment caused early extravasation of Evans blue dye. The increased level of serotonin in immobilization and infusion of 5-HT in model experiments suggest a causative role of serotonin in the increased permeability of BBB induced by immobitization stress.

Key words : immobilization stress, serotonin (5-HT), blood-brain barrier (BBB), indomethacin, vinblastine, parachlorophenylalanine (p-CPA), Evans blue, theophylline, horseradish peroxidase (HRP)

INTRODUCTION

Blood-brain barrier (BBB) maintains a suitable fluid environment for brain which is very essential for the normal functioning of neural tissues. BBB is characterised mainly by the presence of inter-endothelial tight junctions and absence of any vesicular transport (10). Recent studies have shown that BBB is not a passive static barrier, rather it functions as a dynamic regulatory barrier (17). Various physiological and pathological conditions have been shown to increase the permeability of BBB to various tracers (17, 28, 29). Recently, stressors such as foot-electroshock, starvation, training in water maze have also been reported to enhance the cerebrovascular permeability to various tracers (1,28). We have reported earlier that immobilization stress also induced extravasation of Evans blue in cerebral tissues of rats (18).

However, the mechanism(s) underlying increased barrier permeability in stress is not well understood. Immobilization is known to elevate 5-HT in plasma (23) as well as in brain (6, 25). Rise in brain 5-HT particularly in cerebral cortex has been reported following foot-electroshock and pentylenetetrazol induced convulsions (9). Increased 5-HT has been demonstrated in cerebral vessels and brain in response to mechanical trauma and in ischaemic conditions in the affected area of the cerebral cortex (16, 24), and the association of these conditions with increased BBB permeability have already been reported by other workers (3, 5, 20, 28). Infusion of 5-HT into systemic circulation or into cerebral tissues or cerebral ventricles have been shown to increase the permeability of BBB to various protein tracers (4, 21, 26, 27, 29).

Therefore, the present study was undertaken to examine the relationship between the level of 5-HT in plasma and brain tissue and increased permeability of BBB at different periods of immobilization stress. Further, the model experiments have been carried out to examine the permeability of BBB with the dose of 5-HT, which was almost equal to the serotonin level observed in plasma associated with increased permeability of BBB following immobilization stress.

MATERIALS AND METHODS

Experiments were carried out on inbred Charles Foster albino rats of either sex at an ambient temperature range of 18° to 29°C. Animals were housed at controlled temperature (22° to 24°C) with 12.00 hr light and 12.00 hr dark schedule. They had free access to food and water *ad libitum*.

Immobilization : Immobilization of rats was carried out on wooden board in prone position and limbs were fixed with adhesive tape. 8 rats (45-80 *g*) were immobilized for 2-4 hr; 24 rats (40-80 *g*) and 12 rats (200-260 *g*) were immobilized for 7-9 hr; and 8 rats (50-80 *g*) were immobilized for 14-16 hr. 12 non-restrained rats (40-80 *g*) served as control.

Evaluation of increased BBB permeability : After completion of stress period Evans blue dye (2%, 3 *ml/kg*) was injected into right jugular vein under urethane anaesthesia (0.8 *g/kg, i.p.*) in all rats except in 6 rats (subjected to 8-9 hr of immobilization), in which Evans blue dye was injected without anaesthesia, 5 min before termination of stress period, through an aseptically implanted polythene cannula into right jugular vein two days before the experiment. This was done to examine whether urethane anaesthesia has any influence itself on BBB permeability.

5 min after dye injection 0.9% saline was perfused through the heart and brain was removed and the penetration of Evans blue into cerebral tissues was examined by naked eye. Evans blue in brain was measured according to Harada *et al.* (11).

5-HT estimation : The estimation of 5-HT in plasma and brain tissue was done in separate groups of rats after different periods of immobilization. In this group simultaneous measurement of BBB and 5-HT was not possible to be carried out because of the obvious reason that injection of Evans blue and brain perfusion would have interfered with the estimation of 5-HT.

Thus, immobilization was carried out for 4 hr in 5 rats (60-75 *g*) another group of 4 rats (71-78 *g*) and 5 rats (260-280 *g*) were immobilized for 8 hr; same was carried out in another 4 rats (70-78 *g*) for 14 hr. After completion of stress period the animals were anaesthetized with urethane. The blood samples were collected in heparinized syringe through cardiac puncture and centrifuged immediately for separation of plasma. One *ml* of plasma was diluted in 8 *ml* of ice-cold 0.4 N perchloric acid (PCA) and the brain tissues (cerebral and cerebellar cortices) were homogenized in 8 *ml* of ice-cold 0.4 N PCA. The plasma and the homogenized brain tissues were centrifuged at 900 x g for 10 min and 4 *ml* of supernatant from each of them was collected and 5-HT was measured fluorometrically in Aminco Bowman Spectrophotofluorometer (MD, USA) according to Snyder *et al.* (19).

To avoid the changes in 5-HT concentration due to circadian rhythm, the corresponding control animals were sacrificed at the same time period with immobilized rats.

Drug treatment :

Since increase in BBB permeability following immobilization was observed mainly in younger rats (50-80 *g*), all drug treatments were carried out in this age group. Following drugs were used in this study :

(a) *Parachlorophenylalanine* (*p-CPA*) : p-CPA (Sigma Chemical Co., USA) was injected 100 *mg/kg, i.p.* daily for three consecutive days in 10 rats (49-80 *g*). 24 hr after the last injection of p-CPA animals were immobilized for 8-9 hr. BBB was studied in 6 rats and 5-HT was measured in 4 rats.

(b) *Indomethacin* : 10 *mg/kg* indomethacin (Sigma Chemical Co., USA) was injected intraperitoneally in 20 rats (75-80 *g*) 30 min before subjecting the animals for immobilization for a period of 8-9 hr. A second dose of indomethacin (10 *mg/kg, i.p.*) was injected

4.5 hr after the commencement of stress to sustain its action. After the completion of stress period, the permeability of BBB was studied in 16 rats and 5-HT was determined in 4 rats.

(c) *Vinblastine* : Vinblastine (Eli Lily Co., Indianopolis, USA) 0.7 *mg/kg* was injected into right jugular vein under ether anaesthesia in 8 rats (60-80 *g*). 48 hr after the administration of drug the rats were immobilized for 8-9 hr. Half of the animals were tested for BBB permeability and in other half; 5-HT was determined.

(d) *Theophylline* : 12 rats (60-68 *g*) were immobilized for 4.5 hr. Aminophylline (Burroughs Wellcome & Co. Pvt. Ltd., Bombay, India), 10 *mg/kg* was injected intraperitoneally, 15 min after the animals were subjected to immobilization. BBB was tested in 8 rats and 5-HT estimation was done in 4 rats.

5-*HT infusion* : In 10 rats (60-75 *g*) and in 6 rats (220-280 *g*) under urethane anaesthesia 5-HT (Sigma Chemical Co., USA), 10 *μg/kg/min* was infused for a total period of 8 min into right jugular vein through a constant infusion pump (INCO, India). Evans blue dye (2%, 3 *ml/kg*) in 14 rats and HRP (Type I, Centron Research, India) 2%, 5 *ml/kg* in 2 rats (68-71 *g*) was injected 5 min before 5-HT infusion. In 6 rats (65-80 *g*) Evans blue dye was injected 2 hr after 5-HT infusion to study the reversibility of increased BBB permeability induced by 5-HT. 0.9% saline was perfused in animals which received Evans blue and demonstration of HRP in cerebral tissues was done according to the modified method of Malmgren and Olsson (15) as described by Dey *et al.* (8).

RESULTS

Results are shown in Table I and II.

Table I shows that 2-4 hr of immobilization were not very effective in increasing the permeability of BBB to Evans blue. Thus, only one rat out of 8 showed extravasation of the dye. On the other hand 7-9 hr of immobilization have significantly increased the permeability of BBB in 19 out of 24 younger rats (40-80 *g*) but when stress is continued for a longer period (upto 14-16 hr), the increased permeability of BBB appeared to diminish. Older rats (200-260 *g*) were more resistent to show the increased permeability of BBB in comparison to younger rats (40-80 *g*) following 8-9 hr of immobilization (Fig. 1).

Increased permeability to dye was observed mainly in cingulate, occipital and parietal cerebral cortex as well as in vermis portion of cerebellar cortex. No difference in penetration or distribution pattern in dye was observed in rats in which Evans blue dye

TABLE I : Effect of immobilization and infusion of 5-HT on the permeability of BBB in rat.

Treatment	*No. of observations*	*Body weight range (g)*	*increased BBB permeability*	
			Yes	*No*
Control	12	40-80	—	12
Immobilization (2-4 hr)	8	45-80	1	7
Immobilization (7-9 hr)	24	40-80	19	5
Immobilization (14-16 hr)	8	50-68	3	5
Immobilization (8-9 hr)	12	200-260	4	8
Immobilization + Indomethacin (8-9 hr)	16	75-80	3	13
Immobilization + p-CPA (8-9 hr)	6	49-80	—	6
Immobilization + vinblastine (8-9 hr)	4	60-80	—	4
Immobilization + theophylline (4.5 hr)	8	60-68	7	1
5-HT 10 *μg/kg/min* 8 min	10	60-75	10	—
5-HT 10 *μg/kg/min* 8 min	6	65-80	—	6
5-HT 10 *μg/kg/min* 8 min	6	220-280	1	5

*Evans blue dye was injected 2 hr after 5-HT infusion.

TABLE II : Effect of immobilization on plasma and brain 5-HT in rat.

Treatment	*No. of observation*	*Body wt. range (g)*	*Serotonin (mean±S.D.)*	
			Plasma (μg/ml)	*Brain (μg/g)*
Control	8	50-78	0.29±0.08	0.68±0.06
Immobilization (4 hr)	5	60-75	0.37±0.15	0.81±0.15
Immobilization (8 hr)	4	71-78	2.34±0.53	1.62±0.37
Immobilization (14 hr)	4	70-75	0.91±0.02	0.71±0.12
Immobilization (8 hr)	5	260-280	0.41±0.38	0.77±0.20
Immobilization + p-CPA (8-9 hr)	4	50-75	0.41±0.03	0.44±0.01
Immobilization + indomethacin (8 hr)	4	78-80	0.47±0.07	0.92±0.19
Immobilization + vinblastine (8 hr)	4	65-78	1.89±0.08	0.88±0.09
Immobilization + theophylline (4.5 hr)	4	60-65	0.77±0.17	0.72±0.04

was injected before urethane anaesthesia in comparison to other rats in which Evans blue was injected after urethane anaesthesia, indicating that urethane anaesthesia itself had no influence on BBB permeability.

Measurement of 5-HT showed a close parallelism between increase in BBB permeability and increase in plasma and brain 5-HT in younger rats following different

periods of immobilization. Thus, no significant increase in plasma or brain 5-HT was observed after 4 hr of immobilization but profound increase in 5-HT (6-8 fold) was observed in younger rats (60-80 *g*) after 8 hr of immobilization which was not evident following 14 hr of immobilization. Older rats (260-280 *g*) in contrast to younger rats showed least response to stress-induced increase in 5-HT either in plasma or in brain, only one rat out of 5 showed marked increase in 5-HT (Fig. 1).

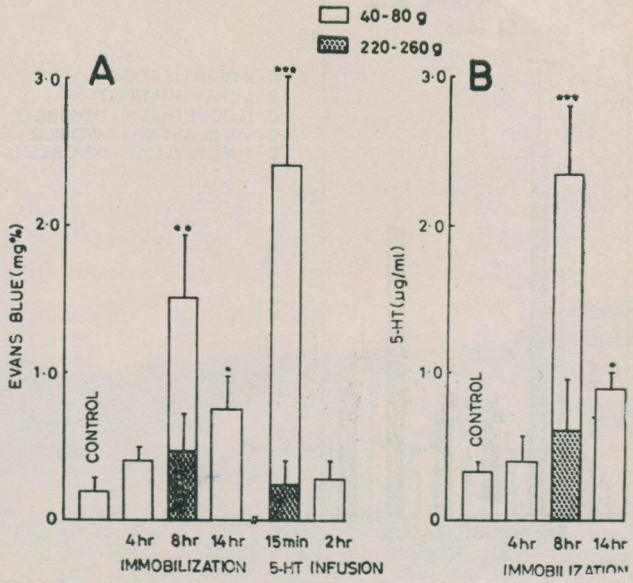

Fig. 1 : (A) Shows penetration of Evans blue in brain following different periods of immobilization in younger rats and in older rats (following 8 hr of immobilization); and infusion of 5-HT (10 *µg/kg/min* for 8 min) into right jugular vein in younger and in older rats. Figures below the bars indicate time interval between sacrifice of animals after completion of infusion.

(B) Shows increase in 5-HT in plasma following different periods of immobilization in younger rats and in older rats (following 8 hr of immobilization) (* $P<0.05$; ** $P<0.02$; *** $P<0.001$).

p-CPA pretreatment has completely blocked the rise in 5-HT following stress and prevented the extravasation of Evans blue dye in brain. Indomethacin pretreatment has significantly reduced the increased permeability of BBB. Only 3 rats out of 16 rats (75-80 *g*) showed staining of cerebral tissues. Rise in 5-HT was also significantly reduced in plasma as well as in brain. Vinblastine pretreatment has completely blocked the extravasation

of dye in all rats but rise in 5-HT in plasma and brain was not significantly altered. Theophylline treatment caused early extravasation of Evans blue dye in cerebral tissues. Thus 7 out of 8 rats showed increase in BBB permeability to dye after 4.5 hr of immobilization. A slight elevation of plasma 5-HT was noted in theophylline treated rats. (Table II, Fig. 2).

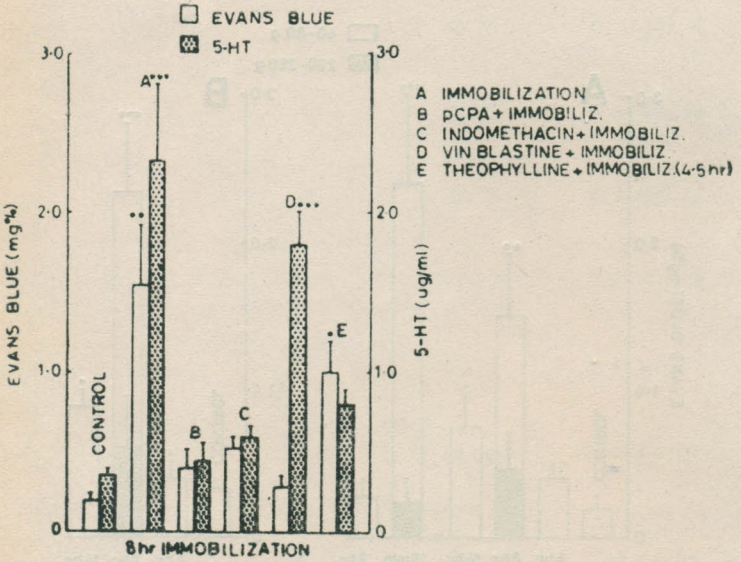

Fig. 2 : Shows drug influence on penetration of Evans blue in brain and level of 5-HT in plasma following 8 hr of immobilization in younger rats except in E in which immobilization was done for 4.5 hr only. (* $P<0.05$; ** $P<0.02$; *** $P<0.001$).

Infusion of 5-HT (10 *μg/kg/min* for 8 min) which approximated the increase in 5-HT in plasma after 8-9 hr of immobilization showed increase in permeability of BBB in all younger rats (60-75 *g)* after 15 min of 5-HT infusion, as evidenced by extravasation of Evans blue and HRP (Fig. 1, 3), while in older rats (220-280 *g)* only one rat out of 6 showed extravasation of Evans blue. No increase in BBB permeability was observed when Evans blue dye was injected 2 hr after the end of 5-HT infusion. This suggests that increased permeability of BBB induced by 5-HT is reversible in nature (Table I).

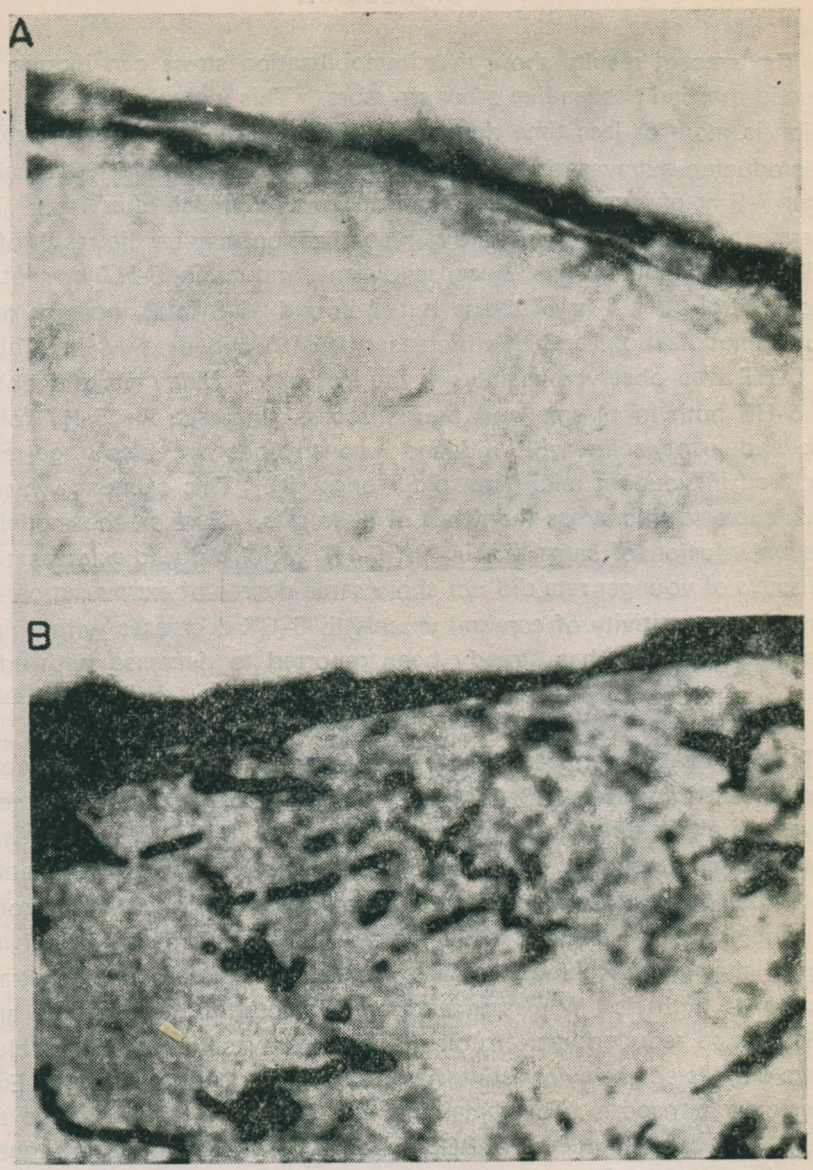

Fig. 3 : (A) Control experiment shows absence of exudation of HRP in rat parietal cerebral cortex following 15 min after saline infusion into right jugular vein. HRP (Type I, 2%, 5 *ml/kg* j.v.) was injected 5 min before saline infusion (X 650).

(B) Shows exudation of HRP across cerebral vessels into rat parietal cerebral cortex following 5-HT (10 *µg/kg/min* for 8 min) infusion into right jugular vein. HRP was injected into jugular vein 5 min before 5-HT infusion. Animal was sacrificed 15 min after the end of 5-HT infusion (X 650).

DISCUSSION

The present results show that immobilization stress can induce extravasation of Evans blue in cerebral tissues like other stressors. This increased permeability following immobilization is more or less time dependent. Thus, 2-4 hr of immobilization was not effective in producing extravasation of dye but significant increase in permeability of BBB occurred after 7-9 hr of immobilization which tends to diminish after 14 hr (Table I). This increased permeability of BBB induced by immobilization may be the result of the increase in 5-HT following immobilization, because plasma and brain 5-HT do not differ much in respect to control after 4 hr of stress but profound (6-8 fold) increase of it occurred after 8 hr of immobilization, and this increased level declined at 14 hr (Table II). This increase in 5-HT was observed mainly in younger rats. Older rats did not show such increase in 5-HT both in plasma and brain tissues. Increase in 5-HT following foot-electroshock and pentyleneterazol induced convulsions is also age-related. More increase occurred in younger mice than older ones (9). This explains why younger rats are more vulnerable to this stress than that of older rats. Further, the model experiments show that even infusion of same amount of 5-HT which induced extravasation of tracers in cerebral tissues of younger rats did not show same degree of extravasation in older rats. This suggests that senstitivity of cerebral vessels to 5-HT decreases with aging. Density of cerebrovascular receptors has already been reported to decrease with advancing age (22).

5-HT increases the permeability of BBB to protein tracers by enhancing vesicular transport across cerebral vessels (26, 27, 28, 29). 5-HT is known to stimulate pinocytosis in cerebral vessels either directly or through cAMP (17, 26, 27, 29). Increased vesicular transport rather than junctional widening can be accounted for extravasation of Evans blue dye in cerebral tissues following immobilization stress which is evident from vinblastine treatment. Vinblastine- an amitotic drug which inhibits polymerization of microtubules thus interfering their normal functions, has blocked completely the penetration of dye in brain inspite of high rise in plasma and brain serotonin. Vincristine, another vinca alkaloid, is known to reduce hypertension induced extravasation of Evans blue dye (14). These workers suggested that vincristine has reduced the extravasation of Evans blue by inhibiting vesicular transport. The remaining dye entered in brain is due to junctional widening (17). Thus total blockade of BBB permeability with vinblastine suggests vesicular transport a major sole route for dye transfer in immobilization. Indomethacin pretreatment has been reported to reduce hypertension induced extravasation of Evans blue dye (13). Indomethacin pretreatment in our study has reduced the rise in plasma and brain 5-HT also. This suggest again a direct relationship between increased 5-HT and increased BBB

permeability. Indomethacin is a prostaglandin synthetase inhibitor drug and reduction in 5-HT level by indomethacin suggest that prostaglandins stimulate 5-HT synthesis which has already been reported by other workers (8, 12). Complete blockade of increased BBB permeability and rise in 5-HT by p-CPA- a 5-HT synthesis inhibitor drug- further confirms 5-HT mediated increase in BBB permeability induced by immobilization.

Theophylline which prevents the destruction of cAMP by inhibiting phosphodiesterase enzyme (2) thus leads to accumulation of cAMP, has caused early extravasation of Evans blue inspite of slight rise in plasma serotonin. This suggests that cAMP which has accumulated, increased the vesicular transport leading to the extravasation of dye. Serotonin is known to stimulate cAMP synthesis and infusion of cAMP has already been shown to cause enhanced transfer of HRP throguh vesicles (26, 27).

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