International Journal of Pharmacological Research www.ssjournals.com
ISSN: 2277-3312 Journal DOI:10.7439/ijpr

Ornithine aspartate attenuates thioacetamide induced hepatic encephalopathy through GABA-benzodiazepine receptors

A. K. Najmi[*1], K. K. Pillai[1], S. N. Pal[2], Aftab Ahmad[3], A. S. Nazmi[4] and Mohd. Akhtar[1]

[1]*Department of Pharmacology, Faculty of Pharmacy, Hamdard University, New Delhi 110 062, India*
[2]*World Health Organization, 20, Avenue Appia, CH-1211 Geneva 27, Switzerland*
[3]*Health Information Technology Department, Jeddah Community College, King Abdul Aziz University, Jeddah-21589, Kingdom of Saudi Arabia*
[4]*Department of Pharmacy, Oman Medical College, Muscat, Oman*

Corresponding author*:
Dr. A.K. Najmi
Department of Pharmacology
Faculty of Pharmacy,
Hamdard University, New Delhi 110 062, India
E-mail: aknajmi@hotmail.com

Abstract
The aim for this study was to investigate the effect of L-ornithine-L-aspartate (OA) in combination with benzodiazepine agonist (diazepam) and antagonist (flumazenil) in thioacetamide (TAA) induced hepatic encephalopathy (HE) in mice. HE was induced in mice by administration of TAA (25mg/kg, i.p.) thrice at 24 hours interval. OA (2gm/kg, p.o.), diazepam (4mg/kg, i.p.) and flumazenil (5mg/kg, i.p.) were administered in different combinations along with TAA and the mice were observed for motor activity, grip strength, traction test and mortality. TAA administration produced stage II – III of HE in mice with highly significant reduction in motor activity. OA protected the animals significantly in TAA and TAA plus diazepam treated groups as was done by flumazenil. Taken together, the findings of the present study suggest that OA actions in TAA induced HE in mice may be mediated through GABA-benzodiazepine receptors.
Keywords: Ornithine aspartate, GABA-benzodiazepine receptors, hepatic encephalopathy, thioacetamide, mice

1. Introduction
L-ornithine-L-aspartate (OA) is a stable salt of natural amino acids ornithine and aspartic acid. It has been studied extensively and has been proven to be effective in both experimental as well as clinical set up of HE. OA produces this beneficial effect in HE by reducing elevated levels of ammonia in blood and brain[1,2].

Hepatic encephalopathy (HE), a neuropsychiatric syndrome arising from acute or chronic liver dysfunction, is related to an increase in blood and brain ammonia. HE is characterized by neuropsychiatric manifestations ranging from a slightly altered mental status to coma. It may include psychomotor dysfunction, impaired memory, increased reaction time, sensory abnormalities, poor concentration and in severe form coma. This complication is a result of the failure of the liver to detoxify toxins originating in the intestine. The pathogenesis is multifactorial and ammonia, manganese, aromatic amino acids, free fatty acids, marcaptans, serotonin, GABA have been implicated[3-8].

It has been surmised that impaired motor functions and lowered levels of consciousness in HE are due to increased GABAergic tone[9,10]. Functions of postsynaptic $GABA_A$ receptors are altered specifically in HE[5,11-13]. Endogenous benzodiazepine ligands (endozepines) are also elevated in HE[12,13] and modulate the activity of these receptors[13,14,15].

Cumulated evidence suggests that OA act by reducing elevated ammonia concentration in HE, but whether it also acts through GABA-benzodiazepine receptors has not been reported. Hence, the present investigation was designed to find out whether the effects of OA in TAA induced HE in mice are also mediated through GABA-benzodiazepine receptor complex.

2. Materials
2.1. Drugs
Diazepam (Ranbaxy, India), Flumazenil (Roche, USA), Ornithine Aspartate (Systopic, India) and Thioacetamide (GS Chemicals, India) were used in the study.

2.2. Animals
Albino mice of Wistar strain (either sex, 25 –30 gram), procured from Central Animal House Facility of Hamdard University were used. The animals were housed in plastic cages at an ambient temperature of 25±2 ^{0}C on a 12:12 h light dark cycle. Food and water were provided *ad libitum* and mice were acclimatized for 1 week before the study. Mice were randomly divided into different groups of 6 animals each. All experimental procedures carried out on animals were

approved by the Institutional Animal Ethics Committee (JHAEC). All the experiments were performed from 7.00 am to 11.00 am.

3. Methods

3.1. Experimental protocol

The animals were randomly divided into different groups of 6 animals each. Test drugs; ornithine aspartate (2 gm/kg, p.o.), diazepam (4 mg/kg, i.p.), flumazenil (5 mg/kg, i.p.), were administered along with TAA (25 mg/kg, i.p.), in different combinations thrice (for three days) at 24 hours interval as per the scheme (treatment plan) given below.

Treatment Plan

Group I NS (1 ml/kg, p.o.) + DW (1 ml/kg, i.p.) thrice at 24 hours interval
Group II TAA (25 mg/kg, i.p.) + DW (1ml/kg, p.o.) thrice at 24 hours interval
Group III BZD (4 mg/kg, i.p.) + FLU (5 mg/kg, i.p.) thrice at 24 hours interval
Group IV TAA (25 mg/kg, i.p.) + FLU (5 mg/kg, i.p.) thrice at 24 hours interval
Group V TAA (25 mg/kg, i.p.) + OA (2 gm/kg, p.o.) thrice at 24 hours interval
Group VI TAA (25 mg/kg, i.p.) + BZD (4 mg/kg, i.p.) thrice at 24 hours interval
Group VII TAA (25 mg/kg, i.p.) + OA (2 gm/kg, p.o.) + FLU (5 mg/kg, i.p.) thrice at 24 hours interval
Group VIII TAA (25 mg/kg, i.p.) + BZD (4 mg/kg, i.p.) + OA (2 gm/kg, p.o.) thrice at 24 hours interval
Group IX TAA (25 mg/kg, i.p.) + BZD (4 mg/kg, i.p.) + FLU (5 mg/kg, i.p.) thrice at 24 hours interval
Group X BZD (4 mg/kg, i.p.) + FLU (5 mg/kg, i.p.) + OA (2 gm/kg, p.o.) thrice at 24 hours interval

After 24 hours of the last treatment of the respective groups, animals were observed for motor activity, grip strength, traction test, tail clip test and mortality to assess the progression of hepatic encephalopathy.

3.2. Motor activity

The spontaneous motor activity of rats was recorded by placing the animal in a photoelectric actimeter (actophotometer). This apparatus consisted of a square chamber and the activity of the animal was measured by light beams connected to a photoelectric cell. The total number of beam breaks was measured for 6 minutes[16].

3.3. Grip strength

Grip strength of animals in all the 3 groups was measured as an indicator of neuromuscular function after 1hour of their respective treatments. The grip strength meter was positioned horizontally. Mice were held by the tail and lowered towards the apparatus. Mice were allowed to grasp smooth metal wire mesh (fore limbs only) and were then pulled backward in the horizontal plane. The force applied to the bar at the moment the grasp was released was recorded as the peak tension (kg). The test was repeated 5 times with in the same session and the highest value from the 5 trails was recorded as grip strength for that animal[17].

3.4. Traction test

This test was carried out as per the procedure reported by Anca *et al.* (1993). Animals were suspended by their hind legs from a taut metal wire and the time taken to bring their front paws up to the wire was recorded. Animals were considered to have passed or failed the test according to whether this did or did not occur with in 5 seconds. Failure was considered to be synonymous with muscle relaxation[18].

3.5. Tail clip test

This test was carried out as per the procedure described by Esan *et al.*[19] with slight modification. Briefly, a bulldog clamp was placed approximately 1 inch from the base of the tail. Animals were considered to have passed the test or not based on whether the animals responded to the clip placement by turning or biting at the clip within 15 seconds.

3.6. Mortality

Number of animals died from the start of the treatment, up to 24 hours after the last dose were recorded and reported as number of animals died/ total number of animals per group.

3.7. Statistical analysis

Results are expressed as mean±SEM. Variation present in a set of the data was estimated by ANOVA followed by Dunnet's post hoc test. $P<0.05$ was considered significant.

4. Results and Discussion

Normal control animals (group I) showed 390.16±32.6 number of beam breaks and 0.0% mortality. TAA 25 mg/kg for 3 days (group II) reduced the motor activity of mice to 75.28±16.66 number of beam breaks with 16.0% mortality. When TAA and diazepam were given together (group VI) the motor activity was 85.5±38.5 number of beam breaks but the mortality was increased to 66.6%. It indicates the susceptibility of TAA induced encephalopathic mice to exogenously administered benzodiazepine agonist, highlighting the involvement of GABA-benzodiazepine receptors. When OA was administered (groups V) along with TAA for 3 days highly significant ($P<0.01$) improvement in motor activity was observed with 0.0% mortality. Flumazenil (group IV), restored the motor activity significantly. When OA was administered along with TAA and flumazenil (groups VII) significant increase in motor activity was observed with 16.6%

of mortality. OA administration with diazepam and TAA (group VIII) produced significant (P<0.05) recovery of motor activity but 16.6% mortality. Flumazenil, when administered with diazepam and TAA (group IX) produced significant improvement in motor activity and protected all the animals from mortality. No significant change was observed in diazepam and flumazenil treated group (group III) and in diazepam, flumazenil and OA treated group (group X) when compared with group I. Effect of the above treatments on muscle grip, traction test and tail clip test were largely non-significant and variable.

GABA, the major inhibitory neurotransmitter in the CNS was proposed as a cause of HE by number of investigators in 1980's. It has been postulated that increase in GABAergic tone could be the reason for the impairment of motor function and lowered level of consciousness in HE[9,10,20]. Under normal circumstances, excitatory glutamatergic tone is balanced by GABA induced inhibitory tone and modulated through GABA receptors. These receptors represent ligand gated chloride channels that permit neuroinhibition through hyperpolarization of postsynaptic membranes. Functions of postsynaptic $GABA_A$ receptors are altered in these conditions specifically[5,11,13]. In addition to this, elevated levels of endogenous benzodiazepines (endozepines) also modulate the activity of GABA-benzodiazepine receptor complex[14,15]. It was also suggested that elevated levels of ammonia enhance GABAergic neurotransmission and synergistically augment the action of benzodiazepine receptor agonists[10].

In our study, the increase in mortality of animals in group VII (TAA+BDZ) points to the involvement of GABA-benzodiazepine receptors in TAA induced HE. Our study corroborates the earlier reports in this line of work[12,21]. Protection afforded to animals of group IX (TAA+BDZ+OA) from mortality and significant restoration of motor activity highlights the possible involvement of GABA-benzodiazepine receptor complex.

Table 1-Effect of ornithine aspartate with diazepam and flumazenil in TAA induced HE in mice on photoactometer (no. of beam breaks), Grip strength (Kg), traction test, tail clip test (number of animals failed the test/total no. of animals), mortality (number of animals died/total no. of animals).

Group	Treatment	Beam breaks	Grip strength	Traction test	Tail clip test	Mortality
I	**NS (1ml/kg)**	390.16±32.60	0.145±0.0056	0/6	0/6	0/6
II	**TAA alone**	75.28±16.66**	0.136±0.0091 ^{ns}	3/6	3/6	1/6
III	**BZD+FLU**	314.0±39.37^{ns}	0.123±0.0072 ^{ns}	0/6	0/6	0/6
IV	**TAA+FLU**	308.66±63.04*	0.134±0.011 ^{ns}	1/3	1/3	1/6
V	**TAA+OA**	313.0±93.52**	0.130±0.0068 ^{ns}	1/6	1/6	0/6
VI	**TAA+BZD**	85.5±38.5^{ns}	0.130±0.0040 ^{ns}	1/2	2/2	4/6
VII	**TAA+OA+FLU**	250.5±27.49*	0.129±0.0098 ^{ns}	1/4	1/4	1/6
VIII	**TAA+BZD+OA**	283.4±42.19*	0.150±0.0079 ^{ns}	1/5	1/5	1/6
IX	**TAA+BZD+FLU**	224.0±21.21*	0.140±0.021 ^{ns}	1/6	2/6	0/6
X	**BZD+FLU+OA**	304.4±49.07 ^{ns}	0.129±0.012	0/6	0/6	0/6
F ratio		3.868	0.598			

Data expressed as mean ± SEM, n=6
Significance of difference for groups I, IV, V, VI, VII, VIII and IX was evaluated with respect to group II by one-way ANOVA followed by Dunnet's post hoc test.
Groups III and X were compared with group I.
(*P<0.05, **P<0.01, ns=non significant
Abbreviations in the table;
TAA –Thioacetamide, NS-Normal Saline, BZD-diazepam, FLU-Flumazenil, OA-ornithinr aspartate

5. Conclusion and future studies

On the basis of above results it may be proposed that OA acts via GABA-benzodiazepine receptors in TAA induced HE in mice. Further studies are needed to confirm the above hypothesis.

Acknowledgments

Gift sample of flumazenil from Roche, USA is gratefully acknowledged.

References

1. Gebhardt R, Beckers G, Gannitz F, Hampt W, Jonitza D, Klein S, Scheja L. Treatment of cirrhotic rats with L-ornithine-L-aspartate enhances urea synthesis and lowers serum ammonia levels. *J Pharmacol Exp Ther* 1997; 283:1-6.
2. Kircheis G, Wettstein M, Dahl S, Haussinger D. Clinical efficacy of L-ornithine-L-aspartate in the management of hepatic encephalopathy. *Metab Brain Dis* 2002; 17: 453-462.
3. Butterworth RF, Gignere JF, Michand J, Lavoei J, Pomeir-Layrargues G. Ammonia: key factor in the pathogenesis of hepatic encephalopathy. *Neurochem Pathol* 1987; 6: 1-12.

4. Butterworth RF. The neurobiology of hepatic encephalopathy. *Semin Liver Dis* 1996; 16: 235-244.
5. Hazell AS, Butterworth RF. Hepatic encephalopathy: an update of pathophysiologic mechanism. *Proc Soc Exp Biol Med* 1999; 222:99-112.
6. Jones EA, Basile AS. The involvement of ammonia with the mechanisms that enhances GABAergic neurotransmission in hepatic failure. *Adv Exp Med Biol* 1997;420:75-83.
7. Vaguero J, Chung C, Cahill M, Blei AT. Pathogenesis of hepatic encephalopathy in acute liver failure. *Semin Liver Dis* 2003; 23:259-269.
8. Blei T. Albumin dialysis for the treatment of hepatic encephalopathy. *J Gasteroenerol Hepatol* 2004;19(S7):224-229.
9. Anderson B. A proposed theory for the encephalopthies in Reye's syndrome and hepatic encephalopathy. *Med Hypoth* 1984;15:415-420.
10. Basile AS, Jones EA. Ammonia and GABAergic neurotransmission on interrelated factors in the pathogenesis of hepatic encephalopathy. *Hepatology* 1997;25:1303-1305.
11. Albrecht J, Jones EA. Hepatic encephalopathy: molecular mechanisms underlying the clinical syndrome. *J Neurol Sci* 1999;170:138-146.
12. Yurdaydin C, Gu ZQ, Nowa G, Fromm C, Holt AG, Basile AS. Benzodiazepine receptor ligands are elevated in an experimental model of hepatic encephalopathy. *J Pharmcol Exp Ther* 1993;265:565-571.
13. Stewart CA, Reivich M, Lucey MR, Gores GJ. Neuroimaging in hepatic encephalopthy. *Clin Gasteroenterol Hepatol* 2005;3:197-207.
14. Mullen KD, Szanter KM, Kaminsky-Russ K. Endogenous benzodiazepine activity in body fluids of patient with hepatic encephalopathy. *Lancet* 1990;336:81-83.
15. Gitlin N. Hepatic encephalopathy. In: Hepatology: a textbook of liver diseases. Ed.: Zakim, D., Boyer, T.D. 3rd ed., Vol I. WB Saunders, Philadelphia, USA, 1996, pp. 605-617.
16. Renault O, Huard GC, Meil H, Steibing S, Le Bourn S, Boulouard M. Synthesis and CNS activity of 3-amino-3-arylpropionic acid derivatives. *Pharm Pharmacol Commun* 1999;5:217-223
17. Meyer OA, Tilson HA, Byrd WC, Riley MT. A method for routine assessment of forelimb and hindlimb grip strength of rats and mice. *Neurobehav Toxicol* 1979;1:233-236.
18. Anca JM, Lamela M, Gato MA, Cadavid I, Calleja JM. Activity on the Central Nervous System of *Himanthalia elongata*; Part II. *Planta Medica* 1992;59:101-105.
19. Esan Z, Dejani Larson KR, Taylor J, Dejani NE, Shawan T, Neeleman SD, Taylor MS, Dayton MT, Mir NG. 1', 1'-Dimethylheptyl-D-8-tetracannabinol-11-oic Acids. A novel, orally effective cannabinoid with analgesic and anti-inflammatory properties. *J Pharmcol Exp Ther* 1999;29:31-38.
20. Schafer DF, Pappas SL, Brody LE, Jacobs R, Jones EA. Sequential changes and visual evoked potentials in a rabbit model of hepatic encephalopathy. I. Sequential change and comparisons with drug-induced comas. *Gasteroenterology* 1984;86:540-545.
21. Zimmerman C, Ferenci P, Pifl C. Hepatic encephalopathy in thioacetamide induced acute liver failure in rats: characterization of an improved model and study of amino acidergic neurotransmission. *Hepatology* 1989;9:594-601.