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The Effects of Ethanol Oral Administration and Toluene Inhalation Exposure on Plasma Free Large Neutral Amino Acids in Wistar Rats

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Abstract

To investigate the effects of ethanol or tuluene on the large neutral amino acids (LNAA) and plasma ratios tryptophan/LNAA and tyrosine/LNAA, known under physiological conditions to predict the brain concentrations of tryptophan and tyrosine respectively, a study was carried out involving Male Wistar rats divided into alcohol (ethanol) 20 rats, and toluene inhalation 20 rats, for a period of two months. Using t test ethanol oral administration caused consistent and significant increase in plasma branched chain amino acids (BCAA). Ethanol also caused a significant decrease in plasma ratio of tryptophan/LNAA. Toluene showed no effect on the BCAA as well as the plasma ratio of tryptophan/LNAA, but caused a significant increase in plasma ratio of tyrosine/LNAA. Tryptophan is the precusor of cerebral indolylamine 5-hydroxytryptamine (5-HT), which is believed to be involved in neurotransmission and regulation of mood and possibly also certain other behaviours. Therefore the plasma amino acid characteristics observed in this study may contribute to sustaining or accentuating mood and behavioural changes seen in alcoholics. More research is needed in this area to help in varifying these findings especially in human subjects who are alcoholics or those exposed to toluene.

Introduction

Alcohol (ethanol) is consumed by a large proportion of adult population world wide, Sullivan et al.(1). In addition to the physiological changes that occur with alcohol (2-5), there are a number of important emotional consequences. With modest intake, at a peak or decreasing blood alcohol levels, most people, both alcoholic and normal, experience sadness, anxiety, irritability and a whole host of resulting interpersonal problems (6-9). At persistent high doses alcohol can cause almost any psychiatry symptom including intense sadness, auditory hallucinations and even intense anxiety (10). On the other hand, studies of operant behaviour following toluene inhalation less than 1,500 ppm have indicated increased rates of responding on Sidman avoidance (11) and differential reinforcement at low rate schedules (12). In contrast a study has shown the absence of neurostructural changes in the rat brain following inhalation exposure at a concentration less than 1,500 ppm (13). Hence

the absence of neurostructural changes (13) and behavioural effects reported in studies involving humans (14-16), may suggest central nervous system toxicity limited to behaviourally significant neurochemistry. Toluene is one of the commonly used industrial solvent especiallly in glues, paints, paint thinners and other petroleum products (17-20).

In trying to explain how alcohol or toluene causes the above symptoms, we studied their long term effects on large neutral amino acids (LNAA), notably valine, isoleucine, leucine, phenylalanine, tryptophan (try) and tyrosine (tyr) as well as their effects on the tryptophan/LNAA and tyrosine/LNAA ratios. In normal populations depressive symptoms which may associate with a disturbed brain serotoninergic function (21) are positively correlated with the proportion of protein to carbohydrate in the diety (22,23). Ingestion of protein produces changes of the plasma amino acid concentrtions that roughly reflect the LNAA composition of the protein and because tryptophan is scarce in the protein relative to other LNAA the plasma ratio of try/LNAA Will decrease (24). Tryptophan is the precusor of cerebral indolylamine 5-hydroxytryptamine (5-HT), which is believed to be involved in neurotransmission and regulation of mood and possibly also certain other behaviours (25). Ingestion of pure carbohydrate though devoid of amino acids is known to induce a slight increase of plasma ratio of tyr/LNAA and moderate increase of try/LNAAT ratio (26,27).

Tryptophan and tyrosine are transported from blood to brain by a carrier mechanism specific for those two and the other large neutral amino acids (28,29). Due to the competitive nature of the amino acid transport, it is the molar ratio in plasma of tryptophan to the sum of the other LNAA that under physiological conditions best predicts the brain tryptophan concentration and plasma ratio of tyrosine/LNAA which best predicts the brain tyrosine concentration, Fernstrom and Faller (30). Serotonin is synthesized from and at a rate that parallels the brain concentration of tryptophan, the other is noradrenaline the formation of which in part depends on the brain concentration of its precursor amino acid, tyrosine (31).

Material and methods

The study was divided into alcohol (ethanol) experimental group and purified toluene vapour (99%) inhalation experimental group. All animals were monitored by taking daily body weight during the study period of two months.

a) Alcohol group involved 20 male Wistar rats 6 weeks old (120-156 g) purchased from Ryukyu Biotech company, Okinawa, Japan. Before use the animals were housed individually in each wire mesh metabolic cage for at least one week in the animal laboratry room on 12/12 hr light/dark cycle, constant temperature of 25 degree centigrade and constant humidity (60-65%). They were allowed free access to food and water both before and during the experiment which lasted for two months. During the experiment, the control, PF, the paired fed rats (8 in total), on isocaloric glucose ingestion, through oesophageal tube, received food equal to the amount taken previous day by the correspon-ding paired rat in the alcohol ingestion group L1 (8 rats, two rats died in the course course of the experiment), received ethanol 2.5 g/kg body weight through the oesophageal tube. L2 (4 rats), received ethanol 1.25 g/kg body weight through the oesophageal tube. Alcohol and glucose were administered

daily starting at the same time (1 p.m.) daily throughout the experiment.

b) Toluene vapour inhalation exposure experiment involved 20 male Wister rats 10 weeks old (250-300 g) Purchased from Ryukyu Biotech company, Okinawa, Japan. Before use the animals were housed randomly in groups of two in the metabolic wire mesh cages measuring 26x38x20 cm for at least 10 days in the same animal laboratory described above. The control (Ce), 10 rats received clean air inhalation exposure and toluene exposed (Te), 10 rats received 900 ppm purified toluene vapour in the exposure chamber. Rats were exposed in groups of ten in 60 litres stainless steel exposure chambers. Animals moved freely in the chambers on the flat surface of a wire mesh. The desired exposure atmosphere of toluene vapour (900 ppm) was generated by dilution the solvent saturated airstream with clean air. The diluted toluene vapour was introduced into the chamber at the top at a flow rate of 1000 mls per minute and exhausted at the bottom, as described earlier (32,33). The chamber was ventilated 16 times per hour with a fresh air. The control chamber was ventilated with a stream of clean air only. The temperature inside the chambers (26-27 degree centigrade) slightly exceeded that of the laboratory, maintained at 25 degree centigrade, while the humidity (55-60%) corresponded to the ambient one. Concentration of toluene vapour inside the chamber was monitored by gas chromatograpgy, at 30 minutes interval. Exposure started at the same time everyday (11 a.m.), 6 hr per day, 5 days per week for 60 days (two months). In the exposure chambers animals had free access to water only while in the wire mesh metabolic cages, in groups of two per cage, they had access to free food and water.

On the 30th day, before alcohol and glucose administration and toluene inhalation exposure, and on the the 60th day (end of alcohol and toluene experiments) tail 50 microlitre blood samples were collected from all rats using a micropipet, for large neutral amino acids analysis, using high performance liquid chromatograpy (HPLC). The blood was immediately centrifuged at 1,500 rpm for 15 minutes and stored at -70 degree centigrade till analysis 3 weeks later by using HPLC method.

Procedure for high perfomance liquid chromatographic separation of amino acids in blood were as explained elsewhere (34). 50 microlitres of whole blood sample collected from the rat tail using a micropipets was mixed with 500 microlitres of 70% ethanol containing Gama-amino-n-butyric acid (500ng/ml) as an internal standard solution in a test tube. The mixture was vigorously shaken and its supernatant was evaporated to dryness after centrifugation at 1,500 rpm for 15 minutes at a room temperature. The residue was stored at -70 degree centigrade till analysis two weeks later when it was dissolved in 300 microlitres of sodium lauryl sulphate solution (SLS). 50 microlitre of the solution mixture was injected into the high Performance liquid chromatography (HPLC) by an autosampler. Detailed HPLC analytical methods as previously described elsewhere (34), with a flow rate of 1.2 ml/min and 250 by 4 mm HPLC column internal diameter. Later in the same day (on the 60th day) all animals were killed by decapitation. All animals in both alcohol and toluene experimental groups were put on the same powder diety with the following composition; casein 25%, cellulose 4%, corn oil 5%, corn starch 60%, vitamin mixture 2% and salt mixture 4%, details as described elsewhere (35). All

Table 1 Effect of ethanol oral administration on plasma individual large neutral amino acids in male Wistar rats in (micromole/litre).

Amino acids	Animal groups PF (n=8)	L1 (n=6)	L2 (n=4)
Tyrosine			
1 month	109.83± 7.17	136.32±19.87**	128.04± 8.83
2 months	72.85±17.11	89.96±20.42	104.86±17.11
Valine			
1 month	188.65±34.14	251.81±42.68**	233.03±23.05
2 months	205.72±25.61	263.76±20.49***	233.03±22.19
Isoleucine			
1 month	97.59± 2.29	127.32±9.91***	118.17±11.44
2 months	87.68±13.72	120.46±16.77**	110.55±11.44
Phenylalanine			
1 month	75.68±10.90	76.89± 9.69	72.65± 7.26
2 months	49.64± 7.87	75.68±21.79**	60.54± 7.87
Leucine			
1 month	67.09± 6.86	174.59±20.58***	113.60±21.35
2 months	135.71±19.82	179.93±22.87**	167.73±13.72
Tryptophan			
1 month	71.99± 2.94	83.74± 2.94***	84.72± 6.37
2 months	62.68± 3.92	70.03± 6.86*	63.66± 1.47

Mean values in (micromole/litre) and ±SD are given. n=sample number. *p <0.05, **p <0.01, ***p <0.001 indicate significance of difference in plasma amino acids between L1 and PF. No significant difference between L2 and L1.

compositions were obtained from Oriental Yeast Co., Tokyo, Japan.

All the reagents and solvents used were of high analytical grade and obtained from Wako Pure Chemicals Industries Ltd, Japan.

Statistical analysis were performed using Student's t test, when P values were equal to or less than 0.05, the results were considered to be significant.

The experiment in animals, used in this

Table 2 Effect of toluene inhalation expsure on plasma individual large neutral amino acids in male Wistar rats in (micromole/litre).

Amino acids	Animal groups	
	Ce (n=10)	Te (n=10)
Tyrosine		
1 month	76.16±24.84	99.34±14.35*
2 months	71.75±31.46	101.55±24.28*
Valine		
1 month	219.38±33.29	250.96±47.80
2 months	175.84±28.17	156.21±46.95
Isoleucine		
1 month	116.65±14.49	128.08±15.25
2 months	111.31±34.31	109.02±16.77
Phenylalanine		
1 month	89.60±10.29	95.05± 9.08
2 months	68.41±24.82	67.20±23.61
Leucine		
1 month	201.27±27.45	218.05±24.40
2 months	216.52±53.37	185.26±60.99
Tryptophan		
1 month	111.16±13.71	115.57± 12.24
2 months	289.94±67.58	289.90±74.92

Mean values in (micromole/litre) and ±SD are given. n=sample number. *p <0.05, indicate significance of difference in plasma tyrosine between Ce and Te. Other amino acids showed no significant difference between Ce and Te.

study, followed the Institutional (University of the Ryukyus) and National (Japan) Research Council criteria for the care and use of laboratory animals in research.

Results

Ethanol caused a significant increase in the plasma levels of branched chain amino acids (BCAA) notably valine ($P<0.01, 0.001$),

Table 3 Effect of ethanol on plasma ratios of try/LNAA and tyr/LNAA in (%).

Animal group	Plasma ratio try/LNAA		tyr/LNAA	
PF (n=8)				
1 month	(538.84)	13.4±0.55	(501.0)	21.9±1.43
2 months	(551.60)	11.4±0.71	(541.43)	13.5±3.17
L1 (n=6)				
1 month	(766.93)	10.9±0.38***	(714.35)	19.1±2.78*
2 months	(729.79)	9.6±0.94**	(709.86)	12.7±2.88
L2 (n=4)				
1 month	(665.49)	12.7±0.95a**	(622.17)	20.6±1.42
2 months	(676.71)	9.4±0.22	(635.51)	16.5±2.69

Mean values in (%) and ±SD are given. n=sample number.
try: tryptophan, tyr: tyrosine, LNAA: large neutral amino acids.
( ) indicate the sum of LNAA.
*p <0.05, **p <0.01, ***p <0.001 indicate significance of difference in plasma ratio try/LNAA and tyr/LNAA between L1 and PF, a**p <0.01 indicates significant difference between L2 and L1.

isoleucine (P<0.001,0.01) and leucine (P<0.001, 0.01) at one and two month respectively, Table 1. Tyrosine showed a significant increase in the first month results (P<0.01) and no significant change observed in the second month results. Phenylalanine showed no significant change in the first month results but was significantly increased in the second month results (P<0.01). The results for tryptophan showed significant increase (P<0.001) in the first month and a marginal increase (P<0.05). No ethanol dose related significant difference observed (no significant difference between L1 and L2, a high and low dose respectively), Table 1.

No significant main effect for the dose of toluene inhalation exposure were observed on the plasma branched chain amino acids, Table 2. Tryptophan and phenylalanine (both aromatic amino acids) also showed no

Table 4 Effect of toluene on plasma ratios of try/LNAA and tyr/LNAA in (%).

Animal group	Plasma ratio try/LNAA	Plasma ratio tyr/LNAA
Ce (n=10)		
1 month (703.06)	15.8±1.95	(738.06) 10.3±3.36
2 months (643.83)	45.0±10.49	(862.02) 8.3±3.64
Te (n=10)		
1 month (791.48)	14.6±1.55	(807.71) 12.3±1.78
2 months (619.24)	46.8±12.09	(807.59) 12.6±3.01**

Mean values in (%) and ±SD are given. n=sample number.
try: tryptophan, tyr: tyrosine, LNAA: large neutral amino acids.
( ) indicate the sum of LNAA.
**p <0.01 indicates significant difference in plasma ratio tyr/LNAA between Ce and Te.

significant change. Tyrosine (aromatic amino acid) however showed small but significant increase in plasma concentration levels ($P<0.05$, both for first and second month results), Table 2.

Mean plasma ratio tryptophan/LNAA was significantly decreased by ethanol administration, Table 3. A marked decrease was observed in the first month ($P<0.001$). The decrease observed in the second month was also very significant ($P<0.01$). Ethanol administration showed a significant dose related difference on tryptophan/LNAA in the first month ($P<0.001$), however insignificant difference was observed in the second month results. A small but a significant effect of ethanol oral administration on plasma ratio tyrosine/LNAA at one month was observed (the ratio was decreased by ethanol), Table 3.

No significant main effect of the toluene inhalation exposure on the plasma ratio tryptophan/LNAA, Table 4. For the plasma ratio tyrosine/LNAA there was a significant main effect for the dose of toluene inhalation at two months ($P<0.01$).

Discussion

This study has shown a significant increase of the plasma branched chain amino acids in alcohol administered rats. This finding is in clear consistent with previous studies (36-38). They observed that the pattern of the branched chain amino acids in alcoholics were different from non alcoholics (the branched chain amino acids were increased by alcohol ingestion). The finding of the aromatic amino acids in this experiment was very variable and inconsistent. Another previous study observed no change in the plasma levels of the aromatic amino acids following alcohol administration (39). Therefore in this study while the total change of the aromatic amino acids was small and variable, the branched chain amino acids showed marked and consistent increase following alcohol administration within the period of our study.

In this experiment toluene inhalation exposure showed no effect at all on the plasma branched chain amino acids. No effect also on the plasma concentration of the aromatic amino acids(phenylalanine and tryptophan) except for tyrosine which showed small but significant increase. Ludersdorf et al. (40) in his short term exposure study for only 3 hours in human subjects noticed a decrease in tyrosine and isoleucine plasma levels. This discrepancy can be explained by the fact that our experiment was carried for long term and that the previous study involved mixture of organic solvents.

Ethanol oral administration in this study decreased significantly the plasma ratio of tryptophan/LNAA. Neuromodulator, serotonin, is synthesized from and with a rate that parallels the brain concentration of the precursor amino acid, tryptophan (31). The molar ratio in plasma of tryptophan to the sum of the other LNAA, under physiological conditions best predicts the brain concentration of tryptophan (30). This therefore may imply that ethanol interfered with the synthesis of serotonin in the brain.

This effect may be attributed to the decreased peripheral tissues (mainly skeletal muscles) utilization of the branched chain amino acids. The plasma branched chain amino acids as explained earlier were very much increased by alcohol administration in this study. The role of an increased proteolysis in producing increased levels of the branched chain amino acids can be inferred from some studies which indicate that skeletal muscles undergoes structural and biochemical changes following ethanol consumption even if the administered diet is adequate (41-43). In the contrast, the increased uptake of the branched chain amino acids into the peripheral tissues caused an increased plasma ratio of tryptophan/LNAA (44, 45). In normal populations depressive symptoms which may associate with a disturbed brain serotoninergic function (21) were positively correlated with the proportion of protein to carbohydrate in the diet (high protein diety decreased the plasma ratio of tryptophan/LNAA) which was positively associated with the depressive symptoms) (22, 23). A dose related significant difference was observed in the first month results in which high ethanol administration showed more decreased plasma ratio of tryptophan/LNAA. Two months results however showed no difference between high and low ethanol administration, suggesting that the long term effect of ethanol on the neuromodulator (serotonin) is independent of the dose (concentration).

In the present study ethanol administration hardly affected the plasma ratio of tyrosine/

LNAA to any significant degree. Small significant decrease in plasma ratio of tyrosine/LNAA was observed only in the first month results.

Toluene inhalation exposure showed no significant effect on the plasma ratio of tryptophan/LNAA in this study. Acute exposure of rats to 1,000 ppm toluene caused an increase in brain 5HT (serotonin) (46). The present study indicated that long term exposure of less than 1,000 ppm toluene cause no significant effect on the plasma ratio of tryptophan/LNAA.

The present study has indicated that long term toluene inhalation caused a significant increase in the plasma ratio tyrosine/LNAA. It is known that noradrenaline (neuromodulator) is synthesized from and with a rate that parallels the brain concentration of the precursor amino acid, tyrosine and that the molar concentration of tyrosine to the sum of the other LNAA under physiological conditions best predict the brain tyrosine concentration (30). Our finding is supported by another previous study in which dopamine and norepinephrine were increased in the brain after inhalation of 500 ppm or 1,000 ppm toluene by male rats (47). Toluene inhalation has been shown to produce its own pattern of discrete changes in noradrenaline and dopamine turnover within the hypothalamus and that the results supported the view that the changes in catecholamine levels and turnover were not due to toxic effect and stress inducing actions of toluene (48). The results in the present study may help to explain how toluene causes the disturbance in the turnover of the catecholamines.

In conclusion the results in the present study indicated that ethanol oral administration causes a significant increase in the plasma concentration of the branched chain amino acids and a decrease in plasma ratio of tryptophan/LNAA (which predicts the brain concentration of tryptophan). Toluene demonstrated no effect on the LNAA except for tyrosine which showed small but a significant increase in the plasma levels. Long term toluene inhalation in this study demonstr ated a significant increase effect on plasma ratio of tyrosine/LNAA (which predicts the brain concentration of tyrosine). Therefore the plasma amino acid characteristics observed in this study may contribute to sustaining or accentuating mood and behavioural changes seen in alcoholics. More research is needed in this area to help in varifying these findings especially in human subjects who are alcoholics or those exposed to toluene.

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