

## ORIGINAL ARTICLE

## INCREASE IN HEPATIC QUINOLINIC ACID CONCENTRATIONS IN ALCOHOL WITHDRAWN RATS

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**Background:** Behavioral associated disturbance involves excitotoxic quinolinate in alcohol withdrawal syndrome in man due to increase availability of tryptophan. In present study we investigated alcoholism related clinical features in relation to tryptophan and 5-HT levels in rat's model. **Methods:** Locally bred male Wistar rats, weighing 200–250 g were housed separately into 6 animals/ group with 12 h light: dark cycle at room temp  $22\pm 3$  °C. They were given diet *ad libitum*, for three days then alcohol 8% (v/v) was added into the liquid diet. Matched control rats of each group were given maltose-dextrin as a substitute of alcohol. Alcohol withdrawal syndrome was assessed after 7 hours by replacing the alcohol-containing liquid diet with tap water. **Results:** Alcohol withdrawal group showed significant increase ( $p<0.001$ ) in holo, apo, and total tryptophan 2, 3 dioxygenase enzyme activities, no significant change in brain tryptophan and 5HIAA however significant decrease ( $p<0.001$ ) in brain 5HT was observed when compared with chow controls. Both alcohols administered and withdrawal groups showed significant rise in serum corticosterone by  $p<0.05$  and  $p<0.001$  respectively. Liver quinolinic acid concentrations were increased significantly ( $p<0.01$ ) with robust increase in alcohol withdrawn rats. **Conclusion:** We conclude that the excitotoxin tryptophan metabolite quinolinic acid of peripheral origin plays significant role in the behavioral manifestation of the alcohol withdrawal syndrome. Tryptophan metabolites should be targeted to develop new strategies in the progress of pharmacological interventions related to alcoholism.

**Keywords:** Ethanol; Quinolinic acid; Tryptophan metabolism; Alcohol withdrawal; tryptophan 2, 3 dioxygenase; 5–hydroxytryptamine; Rats

**Citation:** Bano S, Ara I, Naseem W. Increases in hepatic quinolinic acid concentrations in alcohol withdrawn rats. J Ayub Med Coll Abbottabad 2019;31(3):346–50.

## INTRODUCTION

Ethanol exerts both excitatory and inhibitory actions on behavior and physiological functions. Depressant effects on the central nervous system (CNS) may involve disturbance at the cellular and molecular levels, which are the major causes of acute alcohol intoxication. Depression of the CNS occurs as a result of accumulation of ethanol in the blood at a rate far exceeding that of removal of the drug from the circulation.<sup>1</sup> Blood ethanol concentration exceeds 80 mg/dl accounts for acute alcohol intoxication which require approximately 5h for achieving total elimination of ethanol from the circulation.<sup>2</sup> Numerous brain regions with diverse function play role in controlling emotions and monoaminergic systems in the brain respond region-specifically to ethanol treatment.<sup>3</sup> Serotonin influences mood, thinking and even behavior such as alcohol drinking.<sup>4</sup> Restriction from chronic alcohol consumption results in up regulation of brain and causes severe anxiety and seizure.<sup>5,6</sup>

In brain alcohol interacts with serotonergic neurotransmission. Acute alcohol exposure alters 5–HT function and increase serotonergic neurotransmission.<sup>7,1</sup> Serotonin is the neurotransmitter involved in various behavioral functions and is also implicated in disorders

like depression, anxiety, obsessive-compulsive disorders and probably in drug addiction.<sup>8</sup> In CSF of alcoholics levels of 5–hydroxyindoleacetic acid, were lower as compared to non-alcoholics, predicting they may also have low brain serotonin levels.<sup>9</sup>

Tryptophan (Trp) is metabolized in man by 4 known pathways, all of which produce metabolites of significant biological importance both in the brain and elsewhere in the body. Approximately 90% of the TRP in the liver is catabolized through hepatic kynurenine-nicotinic acid pathway. Tryptophan 2,3 dioxygenase (TDO) is the main enzyme in the liver in this pathway. It is considered that the overall rate of TRP degradation and the metabolic flux of the intermediates that are synthesized through this pathway are controlled by the TDO.<sup>10</sup> Neuro-active substances serotonin; quinolinic acid, kynurenine and kynurenic acid are the metabolites of tryptophan, which is an essential amino acid. Quinolinic acid, a neurotoxin is an agonist at NMDA receptors in the CNS and possibly involved in alcohol withdrawal syndrome. It has been shown that NMDA receptors may be involve in alcohol withdrawal seizures in animals that can be reduced by NMDA receptor antagonists.<sup>11</sup> Tryptophan metabolites, Quinolinic acid is a NMDA receptor agonist while kynurenic acid is a NMDA antagonist.<sup>12</sup> Quinolinic acid occurs naturally in

the mammalian brain and has remarkable *in-vivo* properties of an excitotoxin.<sup>13</sup> Less is known about the potential effects alcohol on tryptophan metabolite in central nervous system and periphery. Present study aims to investigate tryptophan metabolism and in particular involvement of excitotoxin quinolinic acid on behavioral manifestation of alcohol withdrawal syndrome.

## MATERIAL AND METHODS

All animal treatments were carried out in agreement with the national research council for the care and use of laboratory animals (1996). Ethical approval was taken from research ethics committee, University of Karachi. Adult male Wistar rats, weighing 200–250 g were housed 6 per cage in a quiet and temperature controlled (22±3°C) environment with 12 h dark: 12 h light cycle. Rats were given an alcohol-free liquid diet *ad libitum*, for three days. For three days rats were kept in liquid diet without alcohol freely. The ethanol concentration was increased to 8% for up to 21 days. Rats of matched control group were given maltose dextrin as a substitute of alcohol in liquid diet. As well as, all rats of control group were fed with solid lab chow *ad-libitum*.<sup>14</sup>

Assessment of alcohol withdrawal was done as described in detail earlier.<sup>15</sup> Separate group of rats were used for biochemical determination and for the evaluation of seizures due to ethanol withdrawal. Rats were killed by decapitation and perfused livers were rapidly removed and frozen in liquid nitrogen until analysis. Blood alcohol concentration was carried out by an enzyme-based method of Badawy & Aliyu<sup>16</sup> The blood ethanol concentration was determined in rats (non-withdrawn, 0 h) in mg /dl 138±35, 233±40 and 306±43, was calculated as means ± SEM of six rats.

Tryptophan 2,3 dioxygenase was determined by method as described earlier.<sup>17</sup> Serum corticosterone levels were quantified by the method of Glick *et al.*<sup>18</sup> using spectrofluorimetry.

Brain indoles (tryptophan, 5-HT, 5HIAA were analyzed by Anderson *et al.*<sup>19</sup> Whole brains were removed and washed with ice cold normal saline and kept at -70 °C until analysis. Homogenization of rat brains (1–2 g) was carried in 4.0 ml of 0.1 M HClO<sub>4</sub> (400 µl of 1 M NaHSO<sub>4</sub>/L). The brain homogenate was sonicated at 0–4 °C for 15 sec. After adding 0.5 ml of 4.0 M HClO<sub>4</sub> and vortex, the samples were centrifuged at 10,000g for 10 minutes and the clear supernatant stored in polyethylene tubes for HPLC analysis. Stock solutions (10 mg/100 ml) were made in distilled water with 0.1% ascorbate added. Serotonin (100 ng/ml), 5HIAA (100ng/ml) and tryptophan (1000 ng/ml) diluted standards were injected to assess the retention time of the metabolites. Quinolinic acid was analyzed in liver and brain by rapid isocratic HPLC procedure.<sup>20</sup> Briefly, the mobile phase was a methanol: sodium dihydrogen

phosphate mixture (27:73, v/v) at pH of 2.0 with flow rate of 1.2 ml/min. The system was run isocratically using 4 µ reverse-phases C<sub>18</sub> column (250×4.6 mm). A standard quinolinic acid (1 µg/ml) was used to calibrate at the start of each run. The HPLC system consisted of a UV detector (SPD-20A, Shimadzu), pump (LC 20 AT) and an injector (20 µl loop) connected to the LC computerized program.

## RESULTS

Table-1 shows effects of alcohol withdrawal on TDO enzyme activity. Significant increase ( $p<0.001$ ) in holoenzyme, apoenzyme and total enzyme activity was observed when chow control was compared with alcohol withdrawal group of rats. However, 8% ethanol administration/matched controls showed decreased total ( $p<0.05$ ) and apo- enzyme activity ( $p<0.001$ ), though holo-enzyme showed significant rise ( $p<0.01$ ) in activity. Effect of liquid diet showed significant rise ( $p<0.001$ ) in holoenzyme and total enzyme, however apoenzyme activity showed insignificant change. Ethanol administration showed remarkable increase ( $p<0.001$ ) in holoenzyme activity, while great reduction ( $p<0.05$ ) in total and ( $p<0.001$ ) apoenzyme activity.

Table-2 shows the effects of brain tryptophan, 5HT and 5HIAA levels on alcohol consumption and withdrawal in rats. When alcohol withdrawal group of rats was compared with chow control, no significant change in brain tryptophan and 5HIAA was observed, however significant decrease ( $p<0.001$ ) in brain 5HT was observed. Liquid diet (containing milk only) group when compared with chow control group, insignificant change in brain 5HT and 5HIAA was observed however remarkable rise ( $p<0.001$ ) in brain tryptophan was observed. 8% ethanol administration showed great reduction ( $p<0.05$ ) in brain tryptophan levels, however great rise ( $p<0.001$ ) in brain 5HT was observed when compared with matched controls (Maltose dextran). Maltose dextran 8% ethanol showed remarkable decrease ( $p<0.001$ ) in brain 5HIAA levels. Alcohol withdrawal group of rats showed remarkable increase ( $p<0.001$ ) in corticosterone concentration when compared with chow control (Table-1). 8% ethanol administration showed significant rise ( $p<0.05$ ) in corticosterone concentration when compared with matched controls. Also, liquid diet (containing milk) consumption increased ( $p<0.01$ ) corticosterone levels.

Table-3 shows the effect of alcohol administration and subsequent withdrawal on cytotoxic tryptophan metabolite brain quinolinic acid. Data analyzed by students *t*-test shows that 8% alcohol administration and its subsequent withdrawal had no effect on brain quinolinic acid however liver quinolinic acid concentrations were increased significantly ( $p<0.01$ ) in alcohol administered and withdrawal group with robust increase in the latter group.

**Table-1: Effects of alcohol consumption and withdrawal on TDO Activity**

Groups	TDO enzyme activities ( $\mu$ moles of Kynurenine formed /hr/gm wet wt. of liver)		
	Holoenzyme	Total enzyme	Apo enzyme
Chow control	1.7 $\pm$ 0.11	4.5 $\pm$ 0.33	2.8 $\pm$ 0.25
Liquid diet	4.0 $\pm$ 0.08***	7.0 $\pm$ 0.19***	3.0 $\pm$ 0.19
Maltose dextrin	1.58 $\pm$ 0.05	3.67 $\pm$ 0.08	2.09 $\pm$ 0.11
8%Ethanol diet	2.8 $\pm$ 0.31**	3.3 $\pm$ 0.38*	0.5 $\pm$ 0.14***
Alcohol withdrawal	4.26 $\pm$ 0.19***	9.86 $\pm$ 0.29***	5.59 $\pm$ 0.30***

All values are mean  $\pm$  SEM for each group (n=6) animals. Statistical analysis was performed using student's t test. The significance of the difference is indicated by \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.01

**Table-2: Effects of alcohol consumption and withdrawal on brain indoleamines**

Groups	Brain tryptophan (ug/g)	Brain 5HT (ug/g)	Brain 5HIAA (ug/g)	Corticosterone (ug/g)
Chow control	2.5 $\pm$ 0.08	0.72 $\pm$ 0.01	0.40 $\pm$ 0.008	41.2 $\pm$ 1.0
Liquid diet	3.30 $\pm$ 0.07***	0.72 $\pm$ 0.02	0.41 $\pm$ 0.01	49.0 $\pm$ 2.1**
Maltose dextrin	3.5 $\pm$ 0.14	0.71 $\pm$ 0.02	0.50 $\pm$ 0.01	41.9 $\pm$ 1.22
8% Ethanol diet	3.05 $\pm$ 0.09*	0.80 $\pm$ 0.01***	0.37 $\pm$ 0.01***	50.0 $\pm$ 3.0*
Alcohol withdrawal	2.3 $\pm$ 0.06	0.60 $\pm$ 0.02***	0.30 $\pm$ 0.007	72.09 $\pm$ 1.2***

All values are mean $\pm$  SEM for each group (n=6) animals. Statistical analysis was performed using student's t test. The results obtained in liquid diet controls were compared with those in solid diet controls (chow control), whereas those obtained with 8% ethanol or at 7 h after its withdrawal were compared with the corresponding matched liquid diet (The significance of the difference is indicated by \* $p$ <0.05, \*\* $p$ <0.001 \*\*\* $p$ <0.01

**Table-3: Effects alcohol withdrawal on brain and liver quinolinic acid**

Quinolinic Acid (pmoles/gwet wt.)	Matched Control	8% Alcohol	Alcohol Withdrawal
Brain	86 $\pm$ 4	90 $\pm$ 3	82 $\pm$ 5
Liver	220 $\pm$ 23	345 $\pm$ 31*	537 $\pm$ 43**

Experimental details are as described in Materials and Method section. Values are means $\pm$ SEM of 6 rats per group. 8%Alcohol administered and alcohol withdrawal groups were compared with matched controls. The significance of the differences is indicated by \* $p$ <0.01; \*\* $p$ <0.001.

## DISCUSSION

Ethanol withdrawal group of rats showed dramatic increase in hepatic tryptophan 2,3 dioxygenase activity. This induction is hormonal type-activation mechanism, which is specifically related to increased corticosterone levels by ethanol withdrawal. Earlier, investigating the mechanism of substrate or hormonal type induction phenomenon of TDO enzyme, it was suggested that circulating corticosterone increases apo-TDO enzyme activity at mRNA expression level while increase in holo-TDO enzyme activity is specifically related to substrate (tryptophan) or co-factor (heme) type mechanism of induction.<sup>21,22</sup> Therefore it appears that consistent rise in holo-enzyme in the present findings may determine TDO induction by substrate or co-factor. Tryptophan 2,3 dioxygenase is a main determinant of peripheral tryptophan metabolism. In the brain tryptophan metabolism is controlled by tryptophan hydroxylase enzyme, which is a rate-limiting enzyme in 5HT biosynthesis pathway. The enzyme tryptophan hydroxylase remains unsaturated with its substrate tryptophan. It is suggested that tryptophan uptake to

the brain largely depends on its binding with albumin and as well as its competition with five large neutral amino acids. Degradation of tryptophan through kynurenine nicotinic acid pathway following TDO induction also determines cerebral uptake of the amino acid as reported previously.<sup>23,24</sup> Therefore the present results clearly represent the induction of TDO in withdrawal caused no change in brain tryptophan levels that is linked with the decreased 5HT and 5HIAA levels when compared with chow control suggesting increased neuronal firing activity due to anxiety during withdrawal.

Ethanol diet consumption decreased TDO total and apo-enzyme activity. This decrease is specific to apo-enzyme activity but not to holo-enzyme activity. An earlier investigation on chronic ethanol administration in drinking water is in accordance with our present results. These authors reported that this decrease in total enzyme activity is associated with consequent enhancement of brain serotonin synthesis, whereas subsequent withdrawal may induce the opposite effects.<sup>25</sup> It is possible that hepatic tryptophan increases saturation of apo tryptophan 2,3 dioxygenase by tryptophan (substrate) that results into increased holoenzyme activity and decreased apo and total enzyme activity. As indicated in the results increased corticosterone by ethanol administration did not induce enzyme activity, though induction of TDO by corticosterone has been suggested to synthesize apo-enzyme at its mRNA expression level.<sup>14</sup> Therefore it can be concluded that

chronic ethanol administration failed to induce TDO enzyme at mRNA expression level that is indicated by decreased total enzyme. Similarly, a decreased brain tryptophan level with lower 5HT turnover by ethanol administration was observed when compared with matched controls (Table-2). It can be suggested that alcohol administration by liquid diet procedure altered TDO enzyme activity that could result in decreased tryptophan levels and as well as 5HT turnover.

As far as the effects of liquid diet itself are concerned, our results showed increased TDO enzyme activity which could be the result of increased corticosterone levels or liquid diet itself. The present results clearly indicate that liquid diet also caused remarkable changes on tryptophan metabolism as enhancement of hepatic TDO activity and other aspects of tryptophan disposition and administration of ethanol into liquid diet were shown to overcome these effects.

Stimulation of liver TDO activity during withdrawal may be a significant factor of the AWS. Oretti and its coworker<sup>26</sup> found that the time course of induction of TDO activity and gene expression reflected very closely to the severity of AWS. Cessation of alcohol consumption after prolonged period may cause activation of excitatory NMDA glutamate receptor which manifest symptoms of alcohol withdrawal for example hyper excitability and other behavioral changes<sup>27</sup> and apart from glutamate itself tryptophan metabolite quinolinic acid may be the strong indirect acting endogenous activator of NMDA receptors<sup>12</sup>. The possible involvement of quinolinic acid in the AWS has already been hypothesized<sup>28</sup> and induction of TDO activity during AW may provide the link as stated earlier at least 50% of brain quinolinic acid is of peripheral origin<sup>29</sup> and elevation of brain levels of this excitotoxin could therefore occur following TDO activity. The robust increases in liver quinolinic acid in the present study are in agreement with those reported above.

## CONCLUSION

Prolong alcohol consumption in rats may cause inhibition of liver TDO resulting in increased synthesis of brain 5-HT whereas withdrawal may cause the opposite effects. The excitotoxin tryptophan metabolite quinolinic acid may play a role in the behavioral manifestation of the alcohol withdrawal syndrome. Alteration in tryptophan metabolism alters the ratio of tryptophan metabolites, i.e., neuroprotective (kynurenic acid) and neurotoxic metabolites (quinolinic acid) that have clinical significance. Most importantly, the increase in quinolinic acid has major role in inducing alcohol

withdrawal seizures. The TDO activity should be directly targeted to develop new strategies in the development of pharmacological interventions for alcohol dependence.

**Acknowledgement:** We thank Higher Education Commission for the financial support.

## AUTHORS' CONTRIBUTION

SB: Study design, write-up, and proof reading. IA: Data collection, data analysis. WN: Data interpretation, literature search.

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Submitted: 13 February, 2018

Revised: 18 March, 2019

Accepted: 24 April, 2019

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