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Ethanol exposure induces cerebellar neuronal loss in rats

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SUMMARY

Ethanol exposure causes cerebellar dysfunction and cerebellar ataxia. Cerebellar Purkinje cells damage has not been explained as a constant finding in studies addressing the alcoholic brain nor in experimental studies. The present study aimed to find out the changes of cerebellar Purkinje cells in adult rats.

Adult rats were divided into control (C) and ethanol treated (E) groups eight animal each. The rats in group E were exposed to ethanol 1g/kg body weight for three months. Moderate ethanol intake produces significant reduction in the count of Purkinje cells in the anterior lobe of cerebellum with irregular shrunken outline losing their characteristic pyriform shape. Eosinophilic swelling seen adjacent to Purkinje cell bodies. Purkinje cells were observed without a prominent nucleolus or well defined nuclear membrane. Pyknosis of Purkinje cells was also observed.

Key words: Ethanol – Cerebellum – Purkinje cell – Neurodegeneration – Histology

INTRODUCTION

The cerebellum is a part of hindbrain area, situated at the base of the rear side of the brain. The cerebellum plays an important role to maintain balance and posture. It coordinates different muscle groups to produce smooth body movements and equilibrium, and it helps to learn new motoric performance. It may also be involved in some cognitive functions. Cerebellar damage produces symptoms of frequent fall, incoordination, poor movement, difficulty with walk, and poor ability to learn motor performance (Jung, 2015).

Ethanol is used mostly as psychoactive drugs in the world. Because of low cost, availability and easy access, contribute to high ethanol consumption among adolescents. Consequently, alcohol misuse during early life can result in long-lasting health problems, including central nervous system (CNS) impairments. Indeed, a reduced sensitivity to the ethanol-induced motor impairments among adolescents has been observed, which may contribute to heavy drinking as well as the early development of alcohol dependence (Silva et al., 2018).

Excessive alcohol exposure results in cerebellar ataxia and alterations in hand movements, speed when striking a target, impaired postural stability and balance, and slower attenuated foot tapping (Norman et al,. 2009). In addition, the developing cerebellum is particularly vulnerable to the toxic effects of alcohol. Children with fetal alcohol spectrum disorder (FASD) show many symptoms associated specifically with cerebellar deficits (West et al., 1993). Children and adolescents with a history of prenatal alcohol exposure display a reduction in cerebellar volume and a decrease in the size of the vermis (Ramo et al., 2002). The cerebellar cortex neuron function is tightly controlled by GABAergic inhibitory inputs provided by specialized interneurons located in the molecular layers and granule layers. GABA receptor-dependent neurotransmission alteration has been implicated in underlying ethanol-induced impairment of cerebellar function (Jaatinen et al., 2008). These highly spe-

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cialized GABAergic neurons provide powerful inhibitory input to deep cerebellar nuclei neurons, regulating their activity.

GABA releasing is induced by ethanol not only in Purkinje cells and also in molecular layer cells and granule cells. Ethanol exposure increases GABA release at molecular layer interneuron-to-Purkinje cell synapses and also at reciprocal synapses between molecular layer interneurons. In granule cells, ethanol exposure both potentiates tonic currents mediated by extrasynaptic GABA receptors and also increases the frequency of spontaneous inhibitory postsynaptic currents mediated by synaptic GABA receptors. Currently, there are two distinct models on how ethanol produces these effects. In one model, ethanol primarily acts by directly potentiating extra-synaptic GABA receptors, including a population that excites granule cell axons and stimulates glutamate release onto Golgi cells. In the other model, ethanol acts indirectly by increasing spontaneous Golgi cell firing via inhibition of the Na+/K+ ATPase, a quinidine-sensitive K+ channel, and neuronal nitric oxide synthase (Luo, 2015).

The reduction in weight and volume of cerebral cortex, hippocampus and cerebellum regions has been found among alcoholics (Harper, 2009). Moreover, it is well documented that ethanol is more deleterious to the brain during adolescence, probably because many CNS structures are under maturation, with changes ranging from molecular components to brain weight (Toga et al., 2006). Few epidemiological studies have shown a marked change in the ethanol consumption profile in females, with early consumption during adolescence, often in a heavy binge-drinking manner (Elliott and Bower, 2008). However, few studies have addressed the long-lasting impact on brain structures morphology and function of chronic exposure to high ethanol doses in adolescent female subjects. It is demonstrated that adult female rats administered chronically with ethanol (6.5 g/kg/day) during adolescence displayed long term emotional and memory deficits associated with morphological and molecular alterations in the hippocampus (Oliveira et al., 2015).

No loss of Purkinje cells was observed when rat pups were exposed to ethanol during the 7th and 8th postnatal days or at any of the later exposure times. However, loss of Purkinje cells was seen when exposure to ethanol was between the 4th and 7th postnatal days (Hamre et al., 1993). In all cerebellar measurements, the chronic alcoholics did not differ significantly from the non- alcoholic controls, suggesting that chronic alcohol consumption per se does not necessarily damage human cerebellar tissue (Baker et al., 1999). The present study was undertaken to find out the occurrence of histomorphometric changes in the adult rat cerebellum following moderate ethanol consumption.

MATERIALS AND METHODS

Sixteen Wistar rats including equal number of male and female rats of an average weight of 200 g and an average age of 120 days were used in this study. Animals were kept individually in plastic cages in noise free, air conditioned animal house with temperature maintained at 75°F and on a light dark cycle of 12:12 hours. Humidity was maintained with a minimum of 50%. Rats were fed on diet pellets, tap water ad libitum and treated with utmost humane care. The experimental protocol was approved by the Institute Animal Ethics Committee and the procedures were performed according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India). After one week of acclimatization, rats were randomly divided (with the help of Random Allocation Software Version 1.0, May 2004) into 2 groups namely:

Group C (Control group, 8 animals) received the normal saline water (Sodium chloride) for 3 months.

Group E (Experimental group, 8 animals) received 10% ethanol v/v in plain water ad libitum for 3 months. On average, each rat of group E consumed 10 ml (equivalent to 1g/kg body weight) of the ethanol solution daily. The treatments were carried out through oral feeding gavage. Their weights were recorded daily.

After 3 months, the animals were anaesthetized with pentobarbitone (i.p) and an intracardiac perfusion of normal saline followed by 10% formol saline was performed. The brains of both groups of animals were dissected out and blotted. The cerebellum was separated from the cerebral hemisphere and brain stem and weighed. After sagittal sectioning, the right half of the cerebellum of all animals was processed for routine paraffin embedding.

Histological study

Formalin-fixed whole brain was carefully dissected to isolate the cerebellum. Cerebellums were stained with haematoxylin eosin (H&E) staining, Periodic acid Schiff (PAS) staining and Bielschowsky's silver staining according to John D Bancroft Theory and Practice of Histological Techniques. Illustrative images from the experimental groups were obtained with a digital camera (Moticam 2500, USA) attached to a microscope (Nikon, Eclipse 50i, USA).

RESULTS

The cerebellar cortex of the ethanol-fed rats showed a loss of the normal cellular architecture of Purkinje cells. Representative photomicrographs of cerebellum of control groups were presented in Fig. 1a, Fig. 2a, Fig. 3a and Fig. 4a, showing three layers forming the cerebellar cortex - ML

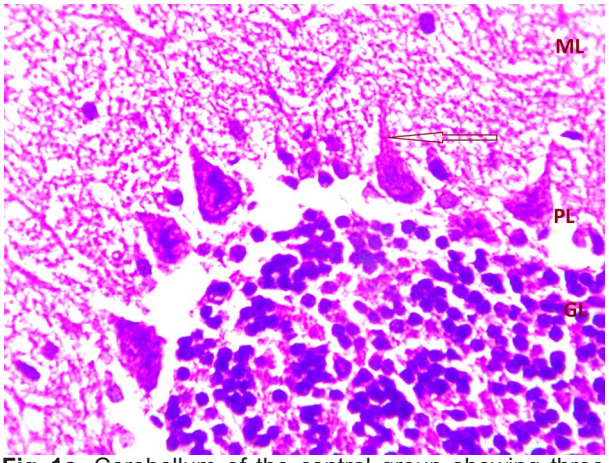

Fig 1a. Cerebellum of the control group showing three layers forming the cerebellar cortex. ML (molecular layer) PL (Purkinje layer) GL (granular layer). The outer molecular layer contains fewer small scattered cells. The Purkinje cell layer contains Purkinje cells, which have a pyriform shaped cell body with their dendrites towards the molecular layer (arrow). Haematoxylin & Eosin stain, x 400.

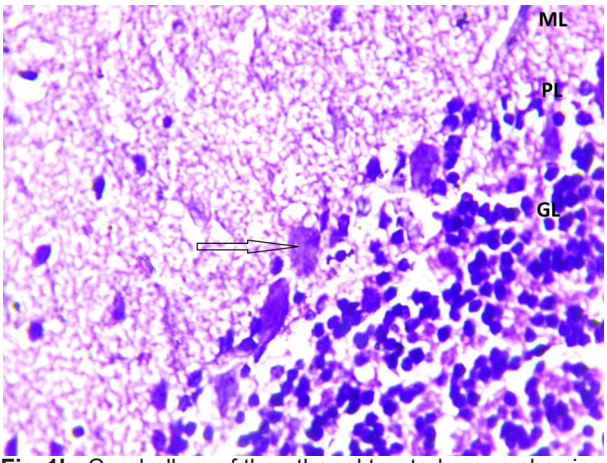

Fig 1b. Cerebellum of the ethanol treated group showing three layers forming the cerebellar cortex - ML (molecular layer) PL (Purkinje layer) GL (granular layer) with irregular shrunken outline losing their characteristic pyriform shape (arrow) in Purkinje cell layer. Haematoxylin & Eosin stain, x 400.

(molecular layer), PL (Purkinje layer) and GL (granular layer). The outer molecular layer contains fewer small scattered cells. The Purkinje cell layer contains Purkinje cells, which have a pyriform-shaped cell body with their dendrites towards the molecular layer. The granular layer contains granule cells and Golgi cells, and the axons of pyramidal cells.

Photomicrograph of cerebellum of the ethanol-treated group were presented in Fig. 1b, Fig. 2b, Fig. 3b and Fig. 4b, showing three layers forming the cerebellar cortex - ML (molecular layer) PL (Purkinje layer) and GL (granular layer) with acute neurodegeneration of Purkinje cells.

Eosinophilic swelling was adjacent to Purkinje cell bodies. Some vacuolation appeared between the cells of Purkinje cell layer. Many Purkinje cells were observed without a prominent nucleolus or well defined nuclear membrane. Pyknosis of Purkinje cells was also observed.

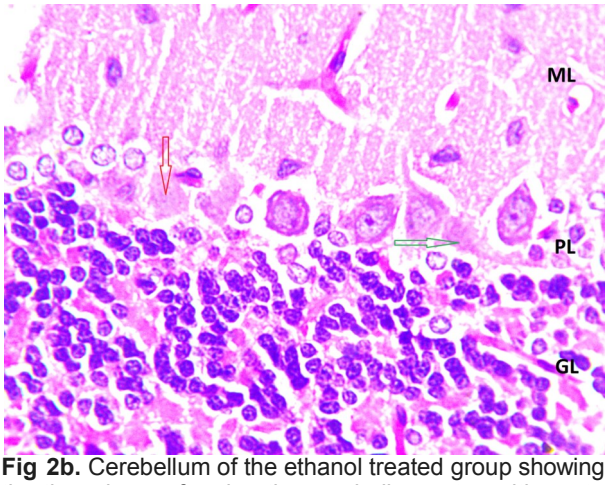

Fig 2b. Cerebellum of the ethanol treated group showing the three layers forming the cerebellar cortex with acute neurodegeneration of Purkinje cells. Eosinophilic swelling adjacent to Purkinje cell bodies (green arrow). Purkinje cells were observed without a prominent nucleolus or well defined nuclear membrane (red arrow). Pyknosis of Purkinje cells was also observed. PAS stain, x 400.

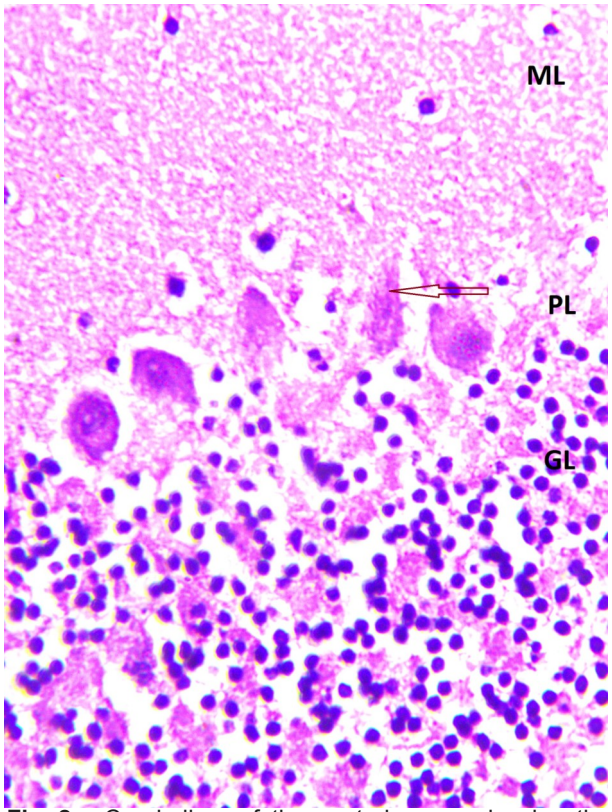

Fig 2a. Cerebellum of the control group showing the three layers forming the cerebellar cortex. The outer molecular layer contains fewer small scattered cells. The Purkinje cell layer contains Purkinje cells, which have a pyriform shaped cell body with their dendrites towards the molecular layer (arrow). PAS stain, x 400.

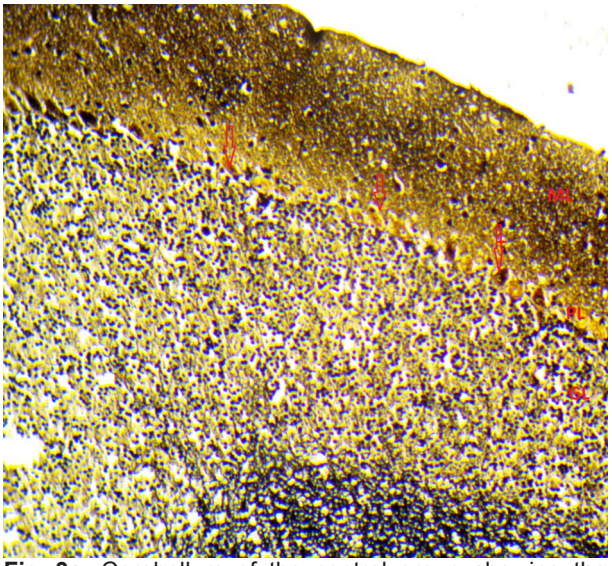

Fig 3a. Cerebellum of the control group showing the three layers forming the cerebellar cortex. The outer molecular layer contains fewer small scattered cells. The Purkinje cell layer contains Purkinje cells, which have a pyriform shaped cell body with their dendrites towards the molecular layer (arrow). Bielchowsky silver stain, x 100.

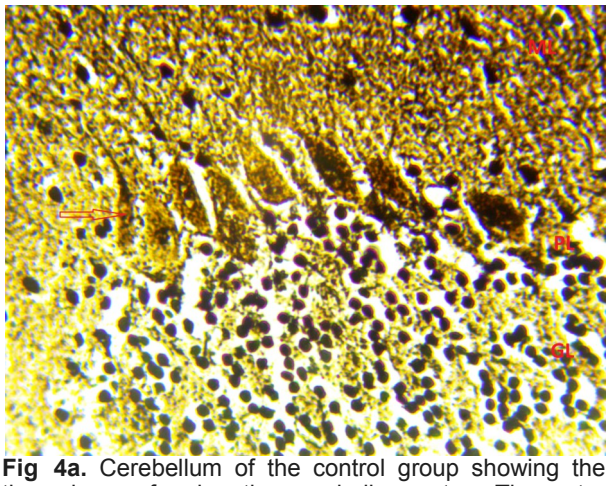

Fig 4a. Cerebellum of the control group showing the three layers forming the cerebellar cortex. The outer molecular layer contains fewer small scattered cells. The Purkinje cell layer contains Purkinje cells, which have a pyriform shaped cell body with their dendrites towards the molecular layer (arrow). Bielchowsky silver stain, x 400.

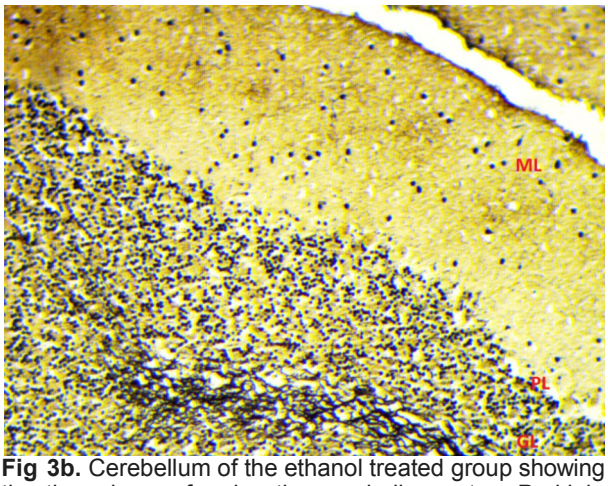

Fig 3b. Cerebellum of the ethanol treated group showing the three layers forming the cerebellar cortex. Purkinje layer showing marked decrease in number of Purkinje cells. Purkinje cells occur sparsely and Purkinje cell linear density was greatly reduced. Bielchowsky Silver stain, x 100.

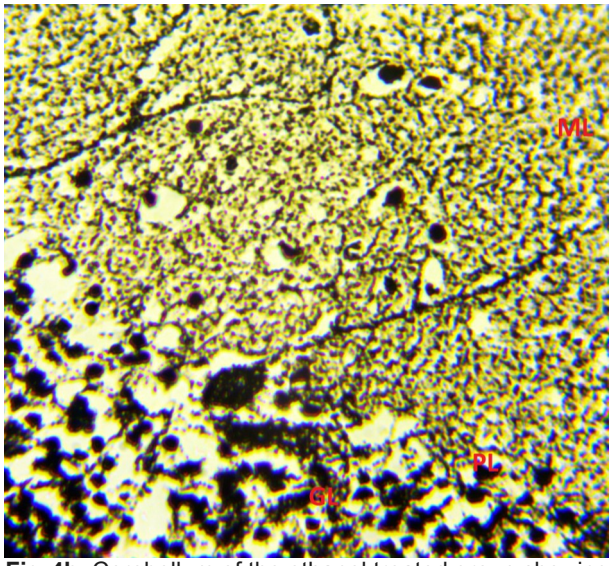

Fig 4b. Cerebellum of the ethanol treated group showing the three layers forming the cerebellar cortex with acute neurodegeneration of Purkinje cells. Bielchowsky silver stain, x 400. .

DISCUSSION

The present study demonstrated that consumption of moderate dose of ethanol for 3 months decreased the count of Purkinje cells in most of the lobules of the cerebellar vermis. In a previous study, it was observed that there was 20-25% reduction in total number of Purkinje cells per midline section in rats exposed to ethanol for 20 weeks (Walker et al., 1981). Chronic intake of ethanol alters cell membrane fluidity by complex neurochemical adaptive changes, and neurochemical alteration of the cell is important for ethanol dependence (Edwards and Peters, 1994). Purkinje cells use GABA as a neurotransmitter. Ligand gated inhibitory GABA receptors act as an important target for the action of ethanol. Whole cell voltage clamp recordings show dose dependent increases in miniature inhibitory postsynaptic currents from Purkinje neurons in cerebellar slices by ethanol (Ming et al., 2006). Cerebellar Purkinje cells are GABAergic neurons, and consist of the single cerebellar cortex output neurons that project to the vestibular nuclei and deep cerebellar nuclei which, in turn, form the connections with the thalamus and brainstem and are involved in motor control (Kayakabe et al., 2014). Moderate doses of alcohol cause motor incoordination by potentiation of

GABAergic activity (Bowen et al., 2015). However, chronic exposure to ethanol is accompanied by changes in structure and function of GABA-A receptors, including their down regulation by increased endocytosis, and thus reducing phasic and tonic inhibition by GABAergic actions, producing motor impairments (Kayakabe et al., 2014).

In a post-mortem histological study, the Wernicke's encephalopathy group was found to have a significant 29% lower Purkinje cell count relative to controls, while the alcoholic group had a non-significant 10% lower cell count (Philips et al., 1990). Animals exposed to alcohol via artificial rearing during the period of the brain growth spurt displayed a comparatively greater deficit in hind limb coordination as compared to suckle control animals (Meyer et al., 1990). The basic mechanisms underlying these ethanol-induced functional alterations and the related neuropathology in the cerebellum have mostly been clarified only recently. These mechanisms include: (i) excitotoxicity; (ii) dietary factors, especially thiamine depletion; (iii) glial abnormalities; (iv) changes in growth factors; (v) apoptotic mechanisms; (vi) oxidative stress; and (vii) compromised energy production (Jaatinen et al., 2008). Damage to the cerebellar structures (i.e., in the Purkinje cells or in the cerebellar connections with other brain areas) promotes impairment in motor tasks, such as increases in the slip numbers and reduced latency to fall in the beam walking test and rotarod, respectively (Oleas et al., 2013). On the other hand, previous studies have demonstrated that chronic ethanol exposure produces changes in the development of climbing fibers, leading to poor innervation of Purkinje cells, resulting in significant ataxia and incoordination (Pierce et al., 2011). Moreover, Pascual et al. (2007) demonstrated that heavy drinking during adolescence/peri-adolescence increases the production of inflammatory and apoptotic mediators in the brain, which leads to the impairment of motor functions, such as poor performance in the rotarod and beam walking tests. Such mechanisms are poorly understood; however, there is a consensus that structural changes in the cerebellar circuitry occur, as well as communication between the cerebellum and other brain areas (Stanley et al., 2005; Chanraud et al., 2007; Rozas et al., 2007).

In the current study, structural modification was observed in the cerebellum, with marked cerebellar atrophy, although with no changes in cerebellar mass. In the protocol by Huang et al. (2012) in which ethanol (3.75 g/kg) was administered intraperitoneally in mice during peri-adolescence (postnatal day 25-70) or adulthood (postnatal day 180-225), the authors reported that in early adolescence ethanol did not alter the cerebellar mass, thus corroborating our findings. On the other hand, previous studies addressing cerebellar morphometry have revealed diminished size following chronic ethanol administration (Chanraud et al., 2007). Binge drinking during the past 3 months by 16-19 years old subjects induced smaller bilateral cerebellar gray and white matter volumes (Lisdahl et al., 2013). Such alcohol cerebellar effects on histological findings were attributed to the action of alcohol on GABAergic neurons and glutamatergic granule cells (Valenzuela et al., 2010), affecting Purkinje neuron plasticity (Pierce et al.,2011) and upregulating inflammatory mediators, which lead to neuronal death, atrophy and or reduced synaptic refinement (Pascual et al., 2007).

In the present study, microscopic evaluation showed that alcohol exposure alters cellular density in Purkinje layers. Moreover, the present data demonstrated that early chronic ethanol exposure reduces the number of neuronal cells in the cerebellum. Similar results have already been described by using the same ethanol protocol in other brain areas, such as the motor cortex (Teixeira et al., 2014) and hippocampus (Oliveira et al., 2015). Subsequent ultrastructural studies of Purkinje dendrites showed dilation of the extensive smooth endoplasmic reticulum (SER) which preceded and accompanied dendritic regression. The component of the SER that was most affected by ethanol was the sarco/endoplasmic reticulum Ca (2+) ATPase pump (SERCA) responsible for resequestration of calcium into the SER. Ethanol-induced decreases in SERCA pump levels, similar to the finding of SER dilation, preceded and occurred concomitantly with dendritic regression. Ethanol-induced ER stress within the SER of Purkinje dendrites was proposed as an underlying cause of dendritic regression (Dlugos, 2015).

In our results we found a decrease in neuronal density, showing that at this dose and time of exposure to ethanol in adult can trigger neuronal degeneration (Teixeira et al., 2014; Oliveira et al., 2015). While we observe this decrease of mature neurons in the cerebellum, we find an increase in the total number of non-neuronal cells, which we believe to be glial cells. Glial cells, especially microglia and astrocytes, are considered the initial effector cells of the ethanol-mediated immune response, responsible for secreting inflammatory mediators and neurotoxic factors (Henriques et al., 2018). In this work, we investigated whether there was neurodegeneration in the cerebellum, but other studies may better elucidate the ethanol-mediated glial response in adolescence. All this together reflected in a poor performance on motor task.

Our results provide new evidence that chronic exposure to moderate ethanol doses promotes motor damage related to cerebellar dysfunction in rats. In Purkinje cells the toxicity is related to alcohol-induced mitochondrial damage with cytochrome release from mitochondria, caspase 3 activation and apoptosis of targeted neuronal cells. The exact mechanisms involved in the observed

ethanol-induced motor impairments should be investigated in future research, but the present histological data revealed that the current ethanol administration protocol induced atrophy and neuronal loss in the cerebellum of rats.

CONCLUSION

Moreover, the present data demonstrated that early chronic ethanol exposure reduce the number of neuronal cells in the cerebellum. The dose of 1 g alcohol/kg body weight used in this study is closer to a chronic exposure considering that it is taken every day ad libitum.

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