DOES INTAKE OF ETHANOL AND KHAT (CATHA EDULIS FORSK) AFFECT THE TOTAL NUMBER OF PURKINJE NEURONS IN EARLY POSTNATAL RATS?

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Summary

The leaves of khat (Catha edulis) are found to have stimulating and pleasurable effect. The stimulating and insomnial effect is counteracted by ethanol intake. The concomitant use of khat and ethanol ingestion is increasing at an alarming rate, in this country. To study the effect of ethanol and khat on total number of Purkinje neurons and volume of cerebellar cortex, animals of postnatal day 6 were used. They were grouped into control, ethanol, khat and combination of khat and ethanol treated animals each group containing 5 pups. They were deeply anesthetized and sacrificed after 30 days of treatment by perfusion with phosphate buffered formaldehyde. The Cavalier principle and physical dissector methods were used to estimate the volume of cerebellar cortex and numerical density of Purkinje neurons, respectively. The total number of Purkinje neurons was computed from these estimates. It was found that the volume of cerebellar cortex as well as the total number of Purkinje neurons of the ethanol treated animals were significantly less than that of the controls and khat treated animals. No statistically significant difference was observed between the controls and khat treated animals. The numerical density and volume fraction of Purkinje neurons of ethanol treated animals were found to be significantly greater than those of control or khat treated animals. In addition, the numerical density and volume fraction of Purkinje neurons were greater in the khat treated rats than in their corresponding controls, but no statistically significant difference was observed. In conclusion, the study depicted that PND 6 is an extremely vulnerable period during which the rat cerebellar Purkinje neurons are particularly susceptible to the effect of high dose of ethanol. Treatment of khat does not significantly change the above mentioned parameters. On the other hand, combination of khat and ethanol killed all the animals.

Key words: Purkinje neurons, Khat, Ethanol, volume, Numerical density & volume fraction

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Introduction

Ethanol intake by pregnant women has deleterious effects on the developing fetus. These effects are manifested as fetal alcohol syndrome (1). This is characterized by specific facial features (short palpebral fissures, epicanthic folds, lower nasal bridge, short nose, indistinct philtrum, thin lip and flat midface), growth retardation, microcephaly and mental retardation (2). It is also associated with a wide range damage of the central nervous system. Primarily it is the cerebellum which is affected by prenatal ethanol exposure. This is manifested by the gait disturbance, problems with fine motor tasks, tremor, ataxia and delay in motor development (3).

In man the 'brain growth spurt', a period during which the brain is particularly vulnerable to environmental influences, begins at about midgestation (2nd trimester) and peaks around birth (4). In rats, it peaks at about postnatal days 6-8 (4). Thus, prenatal ethanol exposure during PND 6-8 of pups and midgestation period of pregnant women causes mental retardation, psychomotor dysfunction (5) and cerebellar shrinkage. Cerebellar shrinkage is associated with the dendritic atrophy, loss of Purkinje neurons density (6). Purkinje neurons are known to be sensitive to the acute and chronic ethanol exposure (7).

Khat (Catha edulis Forsk) is an evergreen shrub of the Celastraceae family. When chewed, the leaves of the khat release many substances into saliva, including a number of alkaloids (8). One of these alkaloids, cathinone, is thought to be responsible for production of sympathomimetic effect and CNS stimulation analogous to the effects of amphetamine (9). These effects include elevated blood pressure, anorexia, insomnia, alertness, elevated mood and loquacity (10). Chronic khat chewing results in unpleasant effect of cognitive defects and psychosis associated with severe neurological illness and loss of motor coordination (11). It also causes abnormalities deep in the white matter of both cerebral hemispheres and marked cortical atrophy (12). However, the effect of khat use in the cerebellum specifically on the total number of Purkinje neurons and volume of cerebellar cortex remains uncertain. The main objective of the present study is, therefore, to evaluate the effect of ethanol and khat on the total number of Purkinje neurons and volume of cerebellar cortex.

Materials and Methods

Plant material preparation

Khat leaves grown in Gelemso (Ethiopia) were purchased from a local market in Addis Ababa. Methods developed by Connor et al. (13) and Makonnen (14) were employed for the extraction process. The leaves were finely chopped with knife, weighed by electronic digital balance and placed in an Erlenmeyer flask containing organic solvents diethyl ether (Whitehouse Industrial Estate, Reagent Chemical Services Ltd., Cheshire) and chloroform (BDH Chemicals Ltd) in a 3:1 ratio. Enough volume of volatile solvent was added in such a way that it covered the minced plant material in the flask. The flask was closed by flask stopper and the contents were continuously stirred using magnetic stirrer for 24 hours. The extractant was decanted, filtered by Whatman No.1 filter paper, and concentrated using a Rota-vapor under low pressure. The concentrated extractant was then poured on a petridish and subject to a vacuum until the organic solvents were completely evaporated. The dry

residue was weighed to calculate the total yield, which was found to be 0.73 %. The resulting residue was kept covered and refrigerated until used. On the day of experimentation, khat extract was reconstituted with 2% Tween 80 in distilled water to dissolve cathinone. The dose was expressed in terms of dry weight of extract per body weight.

Animal preparation

Pregnant white Wistar rats were obtained from the Animal House the Faculty of Medicine, Addis Ababa University and were housed in a standard plastic cage on straw bedding in a temperature controlled room (21 ± 1^0C) maintained at 12/12 hrs light/ dark cycle. They were fed on pellets and given drinking water *ad libitum*. The rats were checked everyday to determine whether they gave birth or not. The day of birth for any group of pups was assigned as postnatal day (PND) 0.

Animal treatment

In this work pups having age of PND 6 were used. These animals were further categorized randomly into control, ethanol treated, khat treated and combination of ethanol and khat treated groups each group containing 5 pups.
On each day of the experiment, all the animals were taken from their cage. They were weighed using Swiss Quality electronic digital balance with 0.01 precision, since weight of animals was necessary to determine the dose of drugs (khat and ethanol) and vehicle. Test substances as well as the vehicle were administered into the stomach through a blunted feeding needle for one month. The ethanol groups received 3ml/100 gm body weight of 20% ethanol and their control group received the same amount of vehicle (distilled water) according to their weight. The khat groups received 20mg/100 gm of body weight khat suspension and their control received vehicle (2% Tween 80 in distilled water) based on their body weight. The combination group received 20mg/100 gm body weight of khat first followed by 20% of ethanol (3ml/100 gm of body weight) after an hour, and their control received only vehicle (2% Tween 80 in distilled water).

Animal perfusion, brain dissection and fixation

The brain of rat is small and soft in the fresh state. To avoid physical injury it was preferably fixed by perfusion before exposing it from the skull.
Prior to all steps of perfusion, the perfusate solution 4% formaldehyde in 0.1M phosphate buffered saline at PH of 7.3 was prepared. The animals were deeply anesthetized with diethyl ether. This was achieved by putting the animals in a tight dessector having cotton soaked with diethyl ether. The rats were then placed in supine position on an operating board and their limbs were stretched using pins. The thoracic cavity was opened by parasagittal skin incision. The ribs were reflected and held laterally. An 18-gauge needle, which was attached to a clear plastic tube connected to the syringe with perfusate, was inserted into the left ventricle and tied with artery forceps. To ease perfusion, the whole tube connections had been freed of air bubbles prior to connection. The right atrium was then opened with a pair of scissors to allow the blood and the fixative leave the body during perfusion. By applying the gravity method, the rats were perfused with about 14% of total body weight of perfusate solution for 10-15 minutes until the fluid that comes out of the rat became clear and free of blood (15).

After perfusion, the pins were detached from operating board and the rat was placed in a prone position. The skin located on the head region was incised and reflected posterolaterally, and held with pins to expose the skull. The skull was cut coronally at the level of lamina cribrosa and then sagittally until it reached to lambda by a small bone cutter. Continuously, the skull was cut sagittally from lambda to the foramen magnum. The dissected portion of the skull was reflected laterally to expose the brain as a whole. Finally, the brain was separated from spinal cord at the level of foramen magnum using a pair of scissors. Immediately thereafter, the entire brain was weighed (Adam Equipment electronic digital balance of 0.01 precision), immersed in fixative and kept in the refrigerator for 18 hours.

Tissue sampling

After fixation, the brain as a whole and the cerebellum separately were weighed. The cerebellum was then cut according to stereological, multistage fractionator rules (16). This process incorporates four stages. In the first stage, the cerebellum was cut sequentially into 2mm thickness parasagittally with which six slices were obtained. These slices were arranged in sequence and a random sampling accomplished by a lottery system, was used to select any three slices. The chance of each slice for being selected was ½ (f1=2). In the second step, the selected slices of tissues were further sectioned in an approximate area of 4 mm2 (2mm x 2mm) and nine small squared stripes were obtained, three from each slice. A random sampling procedure with a lottery system was also used here and three stripes were selected out of nine with a probability of 1⁄3 (f2= 3). The chosen tissues in the latter step were processed for routine paraffin procedure. By taking such 3 blocks of tissues from each animal, a total of 15 blocks per groups were therefore collected and sectioned with a thickness of 6 µm (17). About 400 sections were obtained from each block. For the purpose of stereological analysis, every 20th section of the tissues was collected in random fashion. The probability of any tissue to be selected was 1 /20(f1=20).

Tissue processing

Tissue processing after fixation for routine histological section preparation encompasses four basic procedures, namely dehydration, clearing, impregnation and staining. Tissues were dehydrated with alcohol in an order of increasing concentration (Ethyl alcohol absolute 99.7 %, El Nasr Pharmaceutical Chemicals, Egypt), cleared with xylene (BDH Laboratory supplies Poole BH15 1TD, England), impregnated and embedded in paraffin wax (Paraffin wax m.pt. 58-600C, Dongnam petrochemical MFG. Co. Ltd, Korea).

Each tissue block was sectioned on Zeiss Microtome (Carl Zeiss Zunch AG, West Germany) and collected into egg albumin coated microscopic slide. Just after sectioning, the slides were put in a 60^0C oven for 8 hours to fix the tissue firmly on the slide. Subsequently, sections were deparraffinized and cleared with two changes of xylene, for 5 minutes each, and hydrated with alcohol in an order of decreasing concentration (absolute alcohol, 90%, 70% and 50% of alcohol for 5 minutes each). The sectioned tissues were then stained with toluidine blue for 20 minutes and washed under running tap water to avoid over staining. Stained tissues were then dehydrated and cleared in a reverse direction to maintain the quality of stain. Tissues were, then, mounted in pertex (medite GmbH, Wollenweberstrasse12, D-31303 Burgdorf, Germany) and cover slipped.

Stereology

Stereology deals with a body of mathematical methods for the exploration of three-dimensional spaces when only two-dimensional sections through solid bodies or their projections are available (18). This mathematical method is reliable and unbiased to meet the goal of this investigation. It was done for the estimation of volume of cerebellar cortex, numerical density, total number and volume fraction of Purkinje neurons.

Estimation of volume of cerebellar cortex

The most commonly used stereological method for estimating reference volume is the Cavalier method (18). It was done by using exhaustive series of sectioned tissue in a fixed distance, T units. In the present work, the volume of cerebellar cortex was estimated by taking every 20th section of the tissue block sectioned at 6 m thickness. The sampling distance between every two successive sections was therefore 120 m. The serially sectioned representative sample of the tissues was photographed. A transparent calibrated point- counting grid (Grid P2) (from Agar Scientific Ltd.), was superimposed onto the picture. The points overlie on the cerebellar cortex were counted. The area, which was represented by each point, was estimated by using point grid having known area associated with each point (a/p). Then, the volume of cerebellar cortex was estimated from the following formula (16).

$$\mathbf{V_{cc} = T.a/p.\ \sum P_i}$$

Where Vcc: Volume of cerebellar cortex
T: Fixed distance between parallel sections, in this case 120μm
a/p: Area associated with each point
Pi : Number of points landing within the cerebellar cortex transect on the i^{th} section

Estimation of numerical density of Purkinje neurons

The numerical density of neurons was estimated by the dissector method. The dissector represents the ultimate minimalist approach to a three- dimensional probe. It consists of a pair of serial sections, a "reference" section and an adjacent section a "look-up" section, with known distance "h". According to the dissector method, neurons to be counted are those that appear in the reference section and not on the look-up section. This counting rule is unbiased estimator of numerical density (18). To apply this principle in the present study randomly selected area of cerebellar cortex from the reference section was photographed. A similar area of cerebellar cortex was also photographed from the look-up section. On the photographs the transparent point- counting grid (Grid F2 from Agar Scientific Ltd.) was randomly thrown on the reference section and after that it was superimposed on the same area of look-up section. Then, neurons within the grid and those intercepted by the right vertical and top grid bars (acceptance line) were included in the count but those intercepted by the left vertical and bottom bar (forbidden line) were not counted. Counting was, therefore, made of the total number of Purkinje neurons appearing in the micrograph from the "reference" section, but not appearing in the corresponding micrographs of the "look-up" sections (16).
Then, the numerical density in any regions was calculated using the formula established by (18).

$$N_A = \frac{1}{a/f.\ h} \cdot \frac{\sum Q^-}{\sum P}$$

Where, N_A: Numerical density of neurons
Q- : Number of profiles seen on the reference section not on the look-up section
a/f: Area associated with each frame
h : Distance between sections
P: Number of frame associated points hitting the tissue

Estimation of total number of Purkinje neurons

Estimation of the total number of Purkinje neurons in cerebellum was calculated after estimation of the number of neurons per unit volume (numerical density) and volume of cerebellar cortex. Thus, the total number of neurons was calculated by the following mathematical equation (18).

$$N_n = N_A \cdot V_{cc}$$

Where Nn : Total number of neurons
N A : Numerical density of Purkinje neurons
Vcc : Volume of cerebellar cortex

Estimation of volume fraction of Purkinje neurons

The volume fraction of Purkinje neurons was estimated by throwing the grid (Grid P2 from Agar Scientific Ltd.) on the micrograph randomly and followed by counting points hitting the nerve cells separately and other parts of the tissue in general on the photograph. The volume fraction was then calculated using the following formula (18).

$$Vv\ (neuron, cerebellum) = \sum P(neuron) / \sum P\ (cerebellar\ cortex)$$

Where Vv: Volume fraction of Purkinje neurons
P (neuron) : Number of grid points falling within the image of Purkinje neurons
P (cerebellar cortex): Number of points falling within the image of cerebellar cortex.

Statistical Analysis

The data were analyzed by one way analysis of variance (ANOVA). Post hoc tests were carried out where appropriate using Scheffe test. All statistical analysis was carried out using version 11.5 statistical packages for social sciences (SPSS).

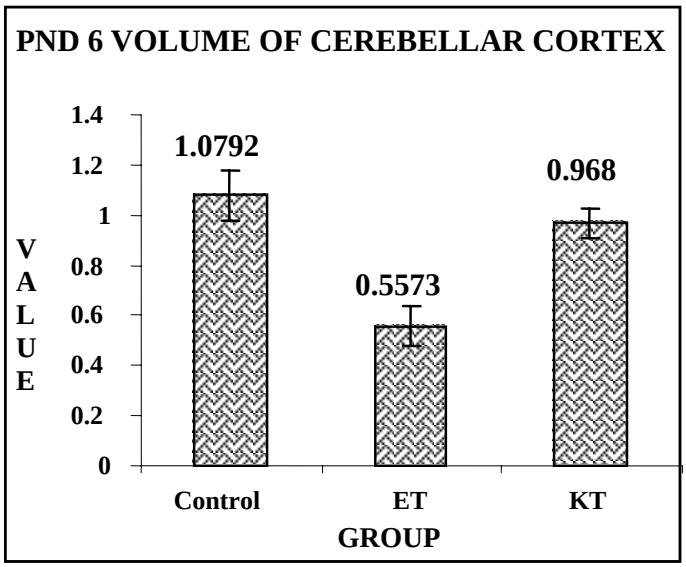
Results

After two days of treatment of combination of khat and ethanol all the animals died. Thus, no further study was carried out on the estimation of the volume of cerebellar cortex, numerical density, total number and volume fraction of Purkinje neurons.

Estimation of volume of cerebellar cortex

The volume of cerebellar cortex of the control, ethanol and khat treated animals is indicated in figure 1. The volume of cerebellar cortex was estimated to be 1.0793 mm3 in the control, 0.5573 mm3 in the ethanol treated and 0.9680 mm3 in khat treated animals. Hence, the volume of the cortex was significantly smaller in ET than the control group by 48.36% ($P<0.01$). The volume of cerebellar cortex of khat treated animals was appeared to be less by 10.30% as compared with the control group though not statistically significant. In addition, the volume of the cortex in the ethanol treated animals was significantly reduced by 42.43% from those of KT group ($P<0.01$).

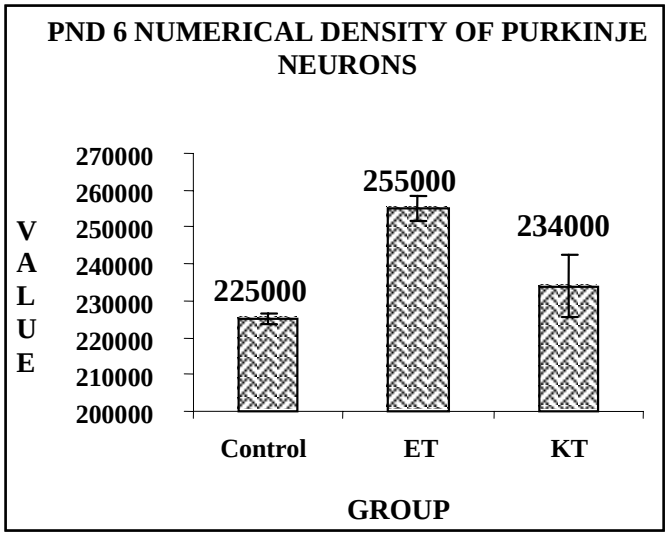
Figure 1: Volume of cerebellar cortex (mm^3) of rats treated with ethanol, and khat and their age matched controls after 30 days of treatment for PND 6.



Estimation of numerical density of Purkinje neurons

The numerical density of Purkinje neurons is shown in figure 2. The numerical density of Purkinje neurons was 225000/ mm3 in the control, 255000/ mm3 in the ethanol treated groups and 234000/ mm3 in khat treated groups. This indicated that the numerical density of Purkinje neurons was significantly greater in the ethanol treated animals than those of control and khat treated groups by 11.76% and 8.24%, respectively ($P< 0.05$). Furthermore, the numerical density of these neurons appeared to be less in the control than khat treated groups by 3.85%, although it was not statistically significant.

Figure 2: Numerical Density of Purkinje neurons (per mm^3) of rats treated with ethanol, and khat and their age matched controls after 30 days of treatment for PND 6.



Estimation of total number of Purkinje neurons

The total number of Purkinje neurons of cerebellum is shown in figure 3. The total number of Purkinje neurons was estimated to be 237859.2 in the control, 147279.6 in the ethanol treated and 223430.4 in the khat treated animals. This showed that the total number of Purkinje neurons was less in the ethanol treated animals by 38.08% and the khat treated animals by 6.07% as compared with the control groups. The difference was statistically significant between the control and ET ($P<0.01$), but not between control and KT. In addition, the total number of Purkinje neurons was significantly greater in the khat treated animals than ethanol treated animals by 34.08% ($P<0.05$). For multiple comparisons, the data were analyzed by the Post hoc analysis using Scheffe test. This indicated significant difference on the total number of Purkinje neurons of ET from those of the age matched control and KT groups ($P<0.01$).

Estimation of volume fraction of Purkinje neurons

The volume fraction of Purkinje neurons is shown in figure 4.The volume fraction of ethanol treated animals and khat treated animals was greater than the control group by 11.43% and 5.34%, respectively. The difference was statistically significant only in the ethanol treated groups ($P<0.05$). In addition, the volume fraction of ethanol treated animals was greater than khat treated groups by 6.43%, although it was not statistically significant.

Figure 3: Total number of Purkinje neurons of rats treated with ethanol and khat and their age matched controls after 30 days of treatment for PND 6.

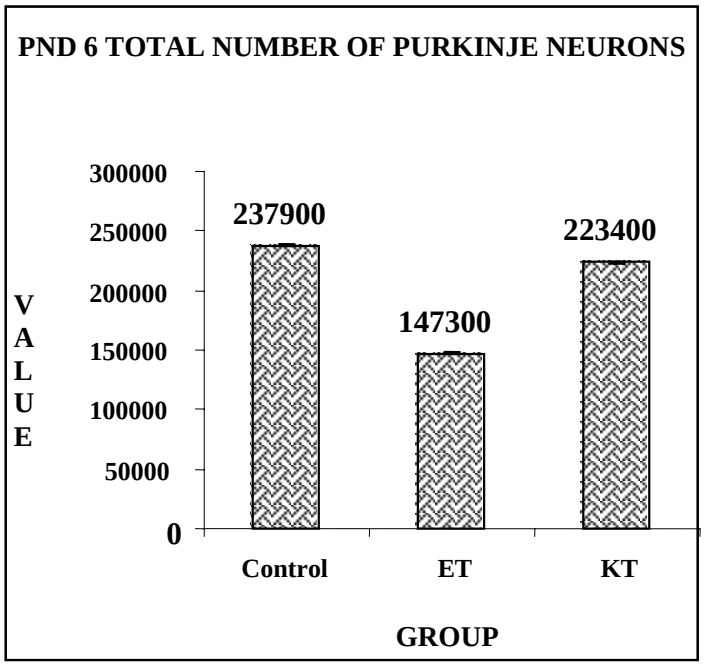
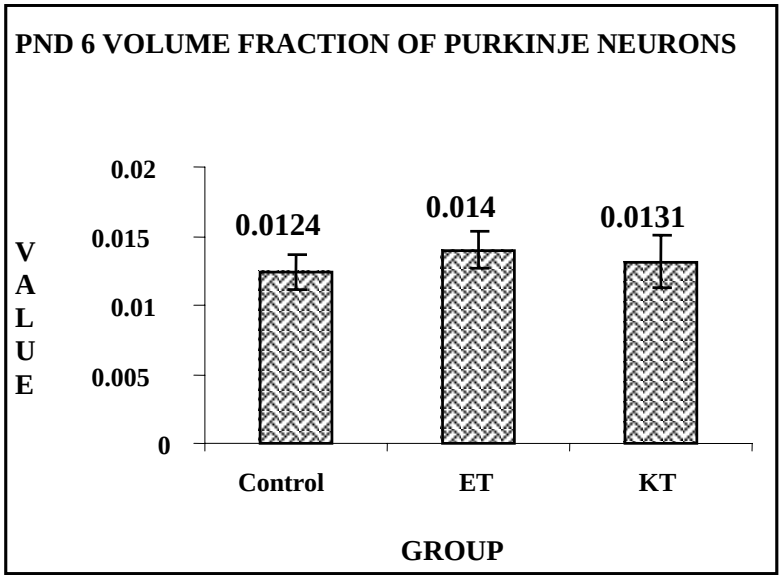


Figure 4: Volume fraction of Purkinje neurons rats treated with ethanol and khat and their age matched controls after 30 days of treatment for PND 6.



Discussion

In the present study, the volume of cerebellar cortex was found to be decrease in both khat and ethanol treated animals though the difference in the case of khat treated animals was not significant. The reduction in the volume of the cortex might be associated with the embryonic development of brain as PND 6 in rats or second trimester in human fetus is the crucial period of brain development. Thus, it appears that exposure of animals or pregnant women large concentration of ethanol for sometime might severely affects the brain development and cause shrinkage of cerebellum (19). The severity of cerebellar shrinkage is largely dependent on the exposure time and age of individuals (20). It is also thought that khat might not affect non-neuronal and other neuronal cells of cerebellar cortex and as a result the cortex did not shrink and the volume was not significantly reduced the volume. This, however, is largely dependent on the time of exposure.

Numerical density (neural package density) in the ethanol treated group was greater than the rest of the experimental groups as well as their age matched controls. The numerical density difference among various groups in the present study and other related studies is difficult to interpret because the density can be influenced both by a change in number and size of neurons and/or by a change in the volume of cerebellar cortex (21). The explanation usually offered for the marked increment of numerical density in the ethanol treated animals is that ethanol exposure during early life or during a period of synaptogenesis (a period at which development takes place) delays the maturation of the dendritic arborization (branching) of neurons and cause a deficit in the cortical volume (22). This results in the formation of package of neurons in a smaller volume of tissue thus giving an increased numerical density of Purkinje neurons in ethanol treated group which is in agreement with the findings of Mitra and Mukherjee (23). In addition, the numerical density can be explained in terms of number and size of neurons. Ethanol and khat consumption during early life causes significant decrement of Purkinje neurons size, which might contribute for significant increment of neural package density. This means that, as the size of neurons gets smaller, they became crowded in a smaller volume of the cortex; consequently, the number of neurons included in the counting frame is increased. Same, however, this was not observed in khat treated rats, though khat decreased the neuron size. It is most likely that, khat might not delay dendritic arborization or did not significantly decrease the volume of cerebellar cortex, hence did not significantly decrease numerical density.

The results of the numerical density in all age groups were supported by the estimation of volume fraction of Purkinje neurons in the cerebellar cortex that indicated the relative proportion of neurons to that of other non- Purkinje neurons as well as non- neuronal cells in the cortex. The relative proportion of Purkinje neurons of cerebellum of the ethanol administered rats was greater than both khat treated and control groups. It could be attributed to the shrinkage of cerebellar cortex due to ethanol intake. As the cortex significantly shrunken, neurons became crowded in the smaller volume and this contributes for significant increment of volume fraction. Unlike to ethanol treated animals, the volume fraction of khat treated animals did not significantly increase as compared with the age matched control. This might be explained in terms of the insignificant shrinkage of the cortex by khat. Even though neuronal loss largely depends on the duration of exposure and level of blood alcohol concentration (24) only the time of exposure was determined in the present study. The observation that all alcohol treated rats were intoxicated for a few hours could demonstrate the high blood alcohol level. The administration of 3 ml/100 gm body weight of 20% ethanol induced marked loss of Purkinje neurons.

This suggests that Purkinje neurons are particularly vulnerable to ethanol and its metabolite (acetaldehyde) at the early life (PND 6). Though Purkinje neurons are not affected by khat, chronic administration might affect them as khat contains mutagenic and carcinogenic components like polyphenol (25). This effect might be exacerbated by concomitant administration of khat and ethanol. Hence, ethanol drinking after khat chewing for a longer time might severely affect neurons.

In the present work, the total number of Purkinje neurons in the ethanol treated animals was in agreement with Miki et al., (17). This signifies that ethanol treatment during early life decreases the total number of Purkinje neurons. However, Pentney and Dlugos (22) stated that alcohol does not reduce the total number of Purkinje cells of cerebellum rather decreases the total number of synapses on dendritic arbors of these neurons, and hence impairs the brain by damaging the ability of neurons to communicate with others.

The neural death can be induced by excess activity of certain neurotransmitters, including glutamate. This phenomenon, called excitotoxicity, may contribute to alcohol related damage to the developing brain (26). Under certain conditions, when glutamate interacts with the N- methyl- D- aspartate (NMDA) receptor, it causes calcium to flow into the signal receiving neurons (27). This calcium influx is a powerful regulator of the activity and function of a neuron. In the fetus, the calcium influx generated at the NMDA receptor is an important signal in neuron development and synapse formation (28). Excessive activation of NMDA glutamate receptor, however, can lead to dangerously high calcium accumulation inside the neuron and consequently leads to cell death (27).

In conclusion, the exposure of ethanol during early life (PND 6) markedly affects the Purkinje neurons. The vulnerability of these neurons during this critical period leads to significant deficit in the volume of cerebellar cortex, numerical density, total number and volume fraction of Purkinje neurons of cerebellum. Khat, however, didn't significantly affect the Purkinje neurons. Thus, the effect of ethanol and khat in neuronal and non- neuronal cells of other regions of CNS and other neurons of cerebellum needs further study.

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