Effect of Ethanol Ingestion in The Pregnant Albino Rat on The Development of Pyramidal Neurons

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Abstract: The current study shows the effect of low dose of diluted ethyl alcohol (0.5 ml of 33% ethyl alcohol) on cerebral cortex neurons in albino rat pups. The pups were divided into five groups A, B, C, D and E, each of 15 animals. Neuronal loss, oedema, pyknotic cells, vacuolation, neurocyte chromatolysis and dilated blood vessels were observed in cerebral cortex of the treated pups. The intensity of Nissl granules were reduced in the treated groups. In conclusion, the present study showed that alcohol ingestion by pregnant dams at low dose lead to pathological alterations in the pups in addition to retardation of pyramidal neurons in the treated groups depending on the duration of alcohol exposure. So, the most affected group was group B.
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1. Introduction

Alcohol is now widely recognized as a neuroteratogen (Thomas *et al*., 1998; Maier and West, 2003; Sandra; Michael, 2003; Ohrtman, 2006; Moutard et al., 2012 and Allam and Abdul-Hamid, 2013). Some women who drink heavily during the pregnancy their children may be affected with alcohol-related deficits, such as neuroanatomical malformations (Clarren *et al*., 1978; Alex and Feldmann, 2012), cognitive dysfunction (Coles *et al*., 1991), or other behavioral disorders (Coles *et al*., 1985; Ernhart *et al*., 1985 and Thanabhorn, 2006).

Animal research indicated a multifactorial mechanism of the teratogenicity of alcohol resulting from nutrient deficiencies, fetal hypoxia alterations in enzyme activities and cell function (Hankin *et al.*, 2000). Ethanol may also impair lactational performance, affecting mammary gland function and pups growth (Ludena *et al*., 1983). Moreover, chronic alcohol administration to the lactating rats also affects suckling-induced prolactin release (Tavares *et al*., 1999; Luisa *et al*., 2001; Paintner et al., 2012).

The cerebral cortex is characterized by the presence of the pyramidal cells and axons provide the principal output of the cortex (Hellwig, 2000). In mammalian cortex , there was inside out gradient of cortical migration where the new, late-produced cortical neurons were able to move toward the surface post the layers of already migrated cells (Aboitiz, 1999). In the developmental mammalian cerebral cortex there are two early waves and other late wave of cell migration before the establishment of the cortical plate (future cerebral cortex).

Motor neuron dysfunction due to spinal cord injury is a disastrous complication in humans (Sakurai *et al*., 2000). Impaired reflex behavior has been described in patients with spastic gait resulting from spinal cord injury (Sinkjaer *et al*., 1995). Also, it was believed that the primary cause of the spinal muscular atrophy is motorneuron degeneration, while muscle weakness and atrophy occur secondarily (Williams *et al*., 1999).

The appearance and the maturation of a number of sensorimotor reflexes are components of the mature motor repertoire of the animal, and the expression of these can be correlated with the development and maturation of the nervous system (Cassidy *et al*., 1994). Smart and Dobbing (1971) studied the effect of early nutritional deprivation on reflex ontogeny and recorded that the development of physical features and reflexes was significantly retarded in the malnourished rats. Chronic mild protein restriction for tow generation also caused delays in the appearance of certain reflexes in nestling rats (Cowley and Griesel, 1963).

The present study aiming to investigate the effect of alcohol administration on the histological structure of both cerebral cortex and spinal cord at D7 and D14 as well as the development of sensorimotor reflexes in the rat pups of different groups between D2 and D25.

2. Materials and methods

Experimental Animals

The present study was carried out on 100 albino rats (*Rattus novregicus*), 75 mature virgin females weighing 120-160 g and 25 mature males. Daily examination of vaginal smear of each virgin female was carried out to determine the estrous cycle. Mating was induced by housing proestrous females with male in ratio of 2 females with one male overnight. In the next morning, the presence of sperm in vaginal smear determined the zero day of gestation. At birth, each mother was housed with its

pups in large cage kept in a ventilated room at constant temperature on a 12:12 hr light/dark cycle. Saturated rodents pellet diet manufactured by the Egyptian Company for Oil and Soap as well as some vegetables as a source of vitamins *ad libitum*.

Drug used

Ethyl alcohol was purchased from Reideld-de-Haun Company (Germany) at purity of 99.9%. Alcohol was diluted and orally administrated to pregnant females by gastric intubation daily from D7 of gestation as pure ethanol in a dose of 0.5 ml of 33% ethyl alcohol, which is equivalent to 700 mg/kg. This dose is low if compared to the doses used by Maier and West (2001), which were 2.5, 4.5 & 3.6 g/kg/day, or to the dose (6.6 g/kg/day) used by Thomas *et al*. (1998).

Animal grouping

The pups of rats were divided into five groups as follows:

-Group A: normal pups (control).

-Group B: the mothers of these pups were given alcohol from D7 of gestation till D21 after birth.

-Group C: the mothers of these pups were given alcohol from D7 of gestation but alcohol administration was stopped at birth and the mothers were given the chance to lactate their pups to postnatal D21.

-Group D: pups of treated mothers were transferred at birth to live and lactate with normal surrogate mothers to postnatal D21.

-Group E: the pups of normal mothers were transferred at birth to be lactated by treated surrogate mother for 21 days.

Toluidine blue stain:

The prepared serial sections of cerebrum at D 21 were de-waxed then transferred to 95% alcohol; the slides were put in alcoholic colophonium solution for 5 minute (10g colophonium in 105ml 95% alcohol. The slides were then transferred to two changes of 95% alcohol each for 3 minutes, followed by staining in toluidine blue 0.1% for 30 seconds, and then were differentiated in a mixture for 10% analene & 95% alcohol. Clearing in different changes of Cajput oil, and finally mounting in Canada balsam.

Light microscopy study

Cerebrum were immediately cut into small pieces of 5 mm^3 and fixed in 20% neutral buffered formalin for 24 hours. The tissues were washed to remove the excess of fixative and then dehydrated in ascending grades of ethyl alcohol 70, 80, 90 and 95% for 45 minutes each, then in two changes of absolute ethyl alcohol for 30 minutes each. This was followed by clearing in two changes of xylene for 30 minutes each. The tissues were then impregnated with paraplast plus (three changes) at 60ºC for three hours and then embedded in paraplast plus. Sections (4 to 5 µm) were prepared with a microtome, de-waxed, hydrated and stained in Mayer's haemalum solution for 3 min. The sections were stained in Eosin for one min, washed in tap water and dehydrated in ethanol as described above. Haematoxylin and Eosin stained sections were prepared according to the method of Mallory (1988).

Electron microscopy study

At D21 after birth, small pieces of cerebellum of rat pups of groups A and B were immediately fixed in 3% glutaraldehyde, rinsed in the cacodylate buffer then followed by postfixation in 1% osmium tetroxide. Specimens were then dehydrated in a series of alcohols and cleared in propylene oxide and finally embedded in Epon epoxy resin. The blocks were then trimmed and sectioned with glass knives on an ultramicrotome. Semethin sections were stained with toluidine blue and examined by light microscope to select the appropriate area for thin sections. Ultrathin sections (60 nm) were cut on the same ultramicrotome and stained with uranyl acetate and lead citrate. The stained sections were examined on Joel CX 100 transmission electron microscope operated at an accelerating voltage of 60 kV.

3. Results

Histoarchitecture of cerebral cortex

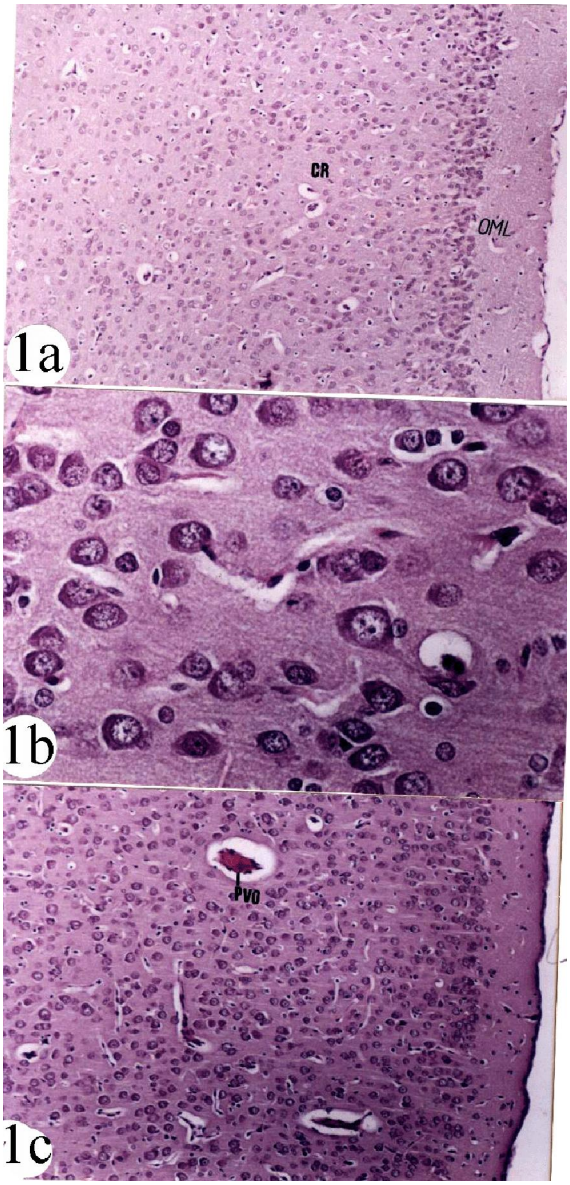
At D21, the outer molecular layer was defined (Fig.1a). The apical dendrites were perpendicular to the pial surface in both normal and treated groups (Figs. 1b, c, d, e & f). At D21, the normal and treated pups showed no obvious lamination in the cortical plate except the outer molecular layer which was sharply defined (Figs. 1a & b). The normal cells of the cerebral cortex at D21 had spherical or pyramidal perikaryons whose nuclei were large, also the neurons arranged in a regular pattern (Figs. 1a & c). Generally the cerebral neurons appeared more developed toward the white matter (Fig. 1a).

Nissl granules of the normal cells of cerebral cortex appeared as compact bodies in the form of flakes and granules. These granules arranged around the nucleus and especially at the proximal parts of the dendrites. The intensity of the Nissl granules in the cytoplasm was variable and increased with the age progress at D21 (Figs. 2a). At D21, the normal pyramidal cells were well stained (Fig. 2a) and moderately stained in groups D and C (Figs. 2d & c), while faintly stained in groups B and E (Figs. 2b & e).

Ultrastructural Studies

At D 21, in the present normal newborns, the ultrastructural study of the external granular layer cells showed the elongated shape of these cells. The

perikaryon of these cells have large elongated nucleus occupied all the entirsoma except a narrow strip of cytoplasm. The chromatin condensation was distributed uniformly in the nucleus (Fig. 3a). In treated group B, these cells are swelling and elongated in shape and their nuclei take the shape of the entirsoma. In addition, the chromatin condensation was low and concentrated adjacent to the nuclear membrane (Fig. 3b).



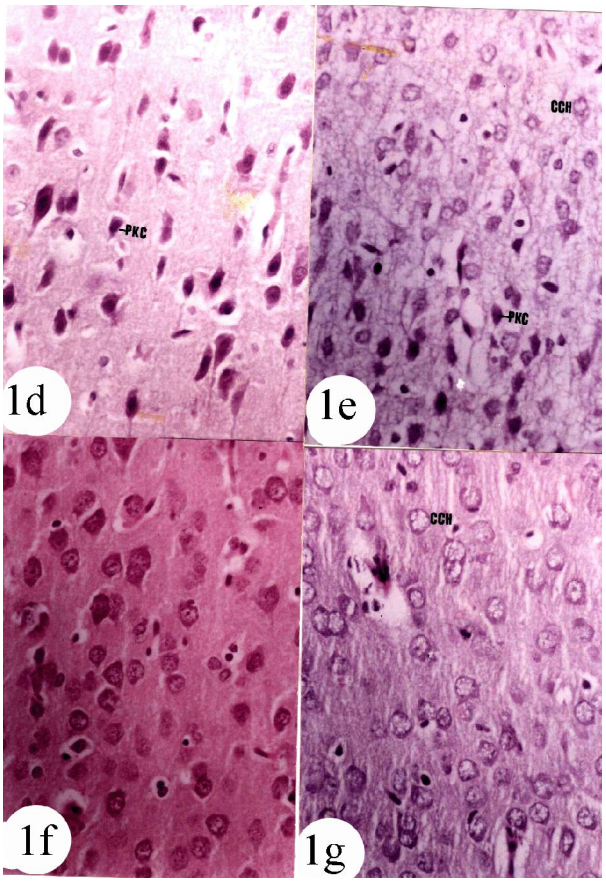


Figure 1. Sagittal sections in the cerebral cortex at D21 show Pyramidal cells (PYC), neurocyte chromatolysis (CCH), Pericellular oedema (PCO), Pyknotic cell (PKC), Vaculation (V), Outer molecular layer (OML). Ggroup A (a, x100 & b, x400). Group B (c, x400). Group C (d, x400). Group D (e, x400). Group E (f, x400) (H & E).

Several pathological cases were observed from the investigation of many sections in the pups of each treated group. In treated group B, perivascular oedema and pyknotic cells detected but by high level also vaculation and neurocyte chromatolysis were observed by moderate level and dilated blood vessel was present (Figs. 1c, 2b& 3b). In group C, at D21, vaculation was recorded by high level, perivascular oedema and pyknotic cells were detected by moderate level, while neurocyte chromatolysis were observed by low level (Fig. 1d). Similar results were observed at D21 but with less severity with presences of dilated blood vessels (Fig. 1c). In group D, there were moderate neurocyte chromatolysis at D21 but by low level (Figs. 1e& 3d). Group E revealed perivascular oedema and vaculation by low level at D7 (Fig. 1f), while at D21, perivascular oedema (low) and neurocyte chromatolysis (moderate) were observed (Fig. 1a, c & d).

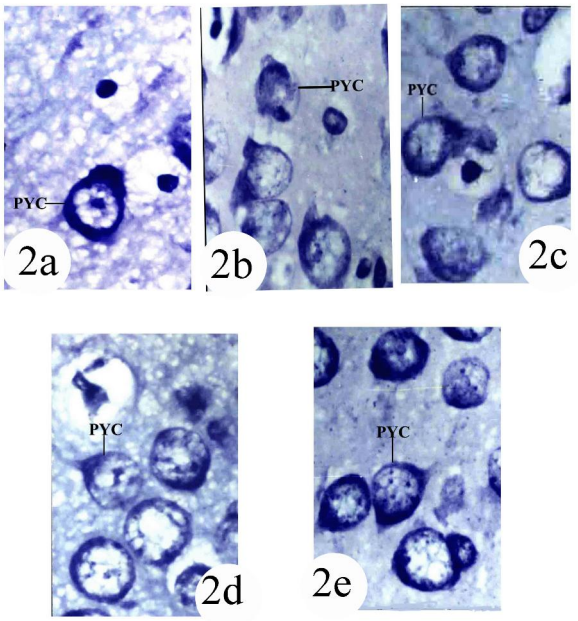


Figure 2. Sagittal sections in the cerebral cortex at D21 show the distribution of Nissl granules in Pyramidal cells (PYC). Group A (a). Group B (b) Group C (c). Group D (d). Group E (e). (Toluidine-blue stain, x1000).

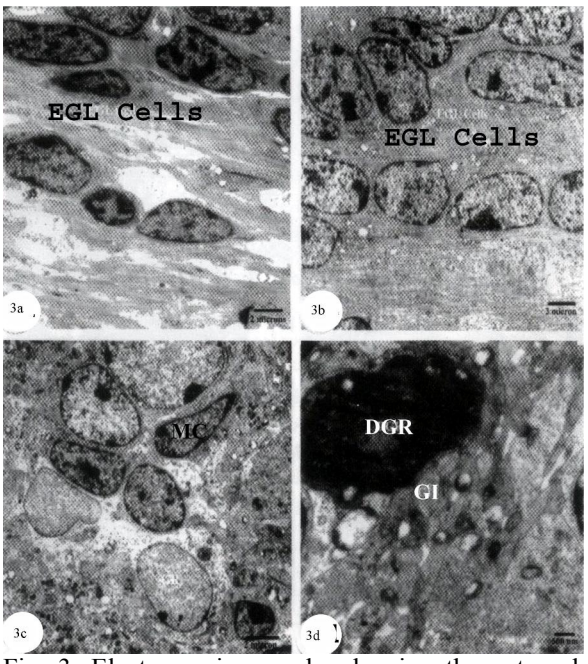


Fig. 3: Electron micrographs showing the external granular layer neurons (EGL cells) in normal pups at D21, (a, x1000& b, x3000). Notice the completely degenerated neurons cell (DGR) in treated pups at D21 (d, x2000).

4. Discussion

Group A is the normal group. Group B, C, D & E are the treated groups. Alcohol and its product acetaldehyde not only pass readily through placenta due to its solubility in water and lipids but also caused ethanol-associated placenta-toxicity, which resulted in reduction of nutrition providing to the developing fetus as recorded by Luke (1990). The alcohol passes through mother's milk to here pups (Luisa et al., 2001). In addition, alcohol leads to bad lactation because it induced inhibition of oxytocin resulted in reduction of milk ejection and consequently leads to malnutrition (Nathaniel et al., 1986). Therefore, the pups of group B suffer from exposure to alcohol with its product and malnutrition pre and postnatal.

In group C, the pups suffer from prenatal alcohol exposure that leads to nutrition deprivation. In addition, postnatal malnutrition regarded to sudden ethanol withdrawals for the mothers resulted in alteration of maternal behavior (Nathaniel et al., 1986). Some mothers in this group eat some of their pups or only eat the HL of them when they were able to walk that may be to prevent them from moving to obtain milk and that indicates the abnormalities in the mother behavior. The pups of group D exposed to alcohol with its product intrauterine, which leads to malnutrition. In group E, the pups will suffer from exposure to alcohol with its product and bad postnatal lactation (malnutrition).

In the normal pups of the present study, the fur appeared at D 9 and delayed in the treated groups. Sampson et al. (1997) reported that alcohol causes growth retardation. Groups B& C suffered from similar worth condition, so the fur appeared at D 12-13. The fur appeared at D 11-12 in groups D& E, retarded than in normal group and earlier than in group B &C. This resulted from different conditions exposure of alcohol and malnutrition during development. It was obvious that the above results in agreement with the symptoms of FAS which mentioned by Wells et al. (2012).

The pups of treated dams suffered from prenatal alcohol exposure, which resulted in loss of body weight. The mean body weight of them at birth was 4.1±0.42 g that was very highly significant compared to 6.5±0.07 g in the normal pups. Nathaniel et al. (1986) and Luisaet al. (2001) recoded this reduction in weights of pups because their mothers ingested alcohol during pregnancy. Intrauterine alcohol exposures undermine the primary function of the placenta so it leads to growth deficiency to the developing fetus, which is a symptom of FAS (Sampson et al., 1997). The intrauterine malnutrition to the developing fetuses caused by alcohol prevents

normal growth (Luke, 1999). The most dangerous effect of alcohol on embryos is intrauterine because fetuses lack the enzymes required to break down the substances once they have entred the blood supply (Adlard and Dobbing, 1971). The change in weights was regularly increased with age in normal group while in the treated groups, the change in weights was slow except in pups of group D that fostered from normal mothers. In groups B& E, the alcohol affects on the function of mammary gland thus, it leads to impair lactation (Ludena et al., 1983 and Luisa et al., 2001). So, there were nutritional deprivations. The treated pups take longer time to attach to the nipple and they are incapable of exerting adequate pressure also they have a reduced number of rapid rhythmic sucks per minute of suckling (Chen et al., 1982). Luisa et al. (2001) found that maternal alcohol consumption resulted in reduction of organs weights of rat pups. The mechanism of the teratogenicity of alcohol resulting from nutrient deficiencies, fetal hypoxia alterations in enzyme activities and cell function (Zajac & Abel, 1992). Also, Ledig et al. (1991) mentioned that paternal alcohol exposure leads to reduction in body weight of the offspring.

The present study showed that, the typical normal pyramidal neurons appeared with their apical dendrites project toward the pial surface. In treated groups, the density of cerebral cortex cells was low because the alcohol interferes with the neuronal differentiation, interrupts cells migration and increases the rate of cells death. These results are in agreement with and Daniel *et al*. (1996), Maier and West (2003) and O'Leary et al. (2012) who mentioned that ethanol- induced neuronal loss and consequently abnormal behaviors due to neuronal deficits. Goodlett *et al*. (1992) reported that spatial memory deficits have been correlated with loss of pyramidal cells in the cerebral cortex. In addition, Resnickoff *et al*. (1993) reported that alcohol interfere with the activity of growth factors, which regulate cell proliferation and survival. Loss of normal growth factor signaling prevents normal growth and development. Moreover, Miller (1996) recorded that alcohol can alter the speed of cell division.

The most striking features of brain damage in treated groups revealed by oedema, pyknotic cells, vaculation, neurocyte chromatolysis and dilated blood vessel which were detected at all investigation stages. The severity of neurocyte chromatolysis and vaculation decreased with age progress. The above pathological cases reflect the brain injures which caused by ethanol (Iqbal *et al*., 2004). Moreover, Daniel *et al*. (1996) recorded that alcohol caused brain damage. These changes because pups exposed to malnutrition which resulted from bad behavior of mother postnataly due to alcohol deprivation (Nathaniel *et al*., 1986). The severity of brain damage decreases with age progress because the pups may develop an antagonistic mechanism to the alcohol effect. At D14 no pathological cases were detected in cerebral cortex, which indicates the improvement of the pups in group D when stopped to ingest alcohol and lived in normal condition. The present neurocyte chromatolysis was detected by high level due to chronic alcohol exposure, which leads to cells loss as observed by Sandra and Michael (2003).

In the present treated groups, there were motorneurons appeared pyknotic at D7. They were detected in group B but by a low rate in group C & D because of ethanol withdrawal and improvement in the developmental condition. The improvements in group D is better than group C. These results are in accordance with West *et al*. (2001), Maier and West (2003) and Sandra & Michael (2003) who reported that alcohol ingestion by pregnant dams caused cells loss especially in the central nervous system (CNS) of embryos during the development. Also, exposure to ethanol reduced motorneurons excitability and decreased motorneurons ability to generate repetitive action potential firing, without significantly changing of motorneurons resting membrane potential (Cheng *et al*., 1999). Also, it effects on the pattern of neuronal firing, which vary considerably in different areas of the CNS. Therefore, in comparing with the existing literature, ethanol induced dysfunction of neuronal electrical activity in the mammalian spinal cord (Crews *et al*., 1996).

The intensity of Nissl granules in the neurons refered to the high metabolic activity of these neurons (Stevens and Lowe, 1997). In normal pups, the intensity of Nissl granules in the pyramidal cells was high at all investigation stages, if compared with other treated groups. Alcohol impair cell metabolism (Hu *et al*., 1995) and leads to protein deficiency (Luke, 1990) so the intensity of Nissl granules is low in the treated groups. These evidences are in agreement with Stevens and Lowe (1997) who found the human pups with high metabolic activety. Heaton *et al.* (2000) mentioned that alcohol causes disturbance in the metabolism and leads to cell dysfunction.

In conclusion, the present study showed that alcohol ingestion by pregnant dams at low dose leads to pathological alterations in the pups in addition to the retardation in the development of pyramidal neurons in cerebrum.

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