Alcohol Exposure Mediated Purkinje Cell Loss in Developed Albino Rats

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Abstract: The normal (group A) Purkinje cells were arranged in a single row to form Purkinje cell layer, while in the treated groups they appeared in more than one row. The ultrastructural studies of normal Purkinje cells at D14 showed their large pear-shaped perikaryon with large round nucleus. The treated groups showed loss of Purkinje cells represented by neurocyte chromatolysis, or vacuolation. In addition, alcohol affects the dendritic tree of Purkinje cells so it was reduced in groups B & C but was moderate in size in groups D & E. The normal (group A) Purkinje cells were deeply stained; this reflects the high amount of Nissl granules if compared with other treated groups due to the high metabolic activity of the normal pups. In group B, some of the detected neurons were degenerated. In group C, most of neurons were pale at D14, while they were moderately-stained in groups D & E. After birth the pups were divided into five groups A, B, C, D and E, each of 15 animals. On days 7 and 21 after birth, 5 of the pups from each group were sacrificed to study the effect of alcohol at different periods and conditions. Group A: normal pups (control). Group B: the mothers were given alcohol from D 7 of gestation till the D21 after birth. Group C: the pregnant females 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 for 21 days. Group D: the pups of treated mothers were transferred at birth to be lactated by normal surrogate mothers for 21 days. Group E: the pups of normal mothers were transferred at birth to be lactated by females that were given alcohol every day (treated surrogate mother) for 21 days.


Key words: Ethanol – cerebellum – Purkinje cells – pups – histopathology – ultrastructure

1. Introduction

Animal research indicated a multifactorial mechanism of the teratogenicity of alcohol resulting from nutrient deficiencies, fetal hypoxia alterations in enzyme activities and cell functions (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).

The effect of low doses of ethanol (0.5 ml of 33% ethyl alcohol) on the histology and ultrastructure of the cerebellar cortex neurons in rat pups. After birth the pups were divided into five groups A, B, C, D and E, each of 15 animals. On days 7 and 21 after birth, 5 of the pups from each group were sacrificed to study the effect of alcohol at different periods and conditions. Group A: normal pups (control). Group B: the mothers were given alcohol from D 7 of gestation till the D21 after birth. Group C: the pregnant females 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 for 21 days. Group D: the pups of treated mothers were transferred at birth to be lactated by normal surrogate mothers for 21 days. Group E: the pups of normal mothers were transferred at birth to be lactated by females that were given alcohol every day (treated surrogate mother) for 21 days.

The present results confirmed that ethanol during pregnancy has adverse effects on the neurogenesis of cerebellar cortex. These effects extended to the postnatal stages.

The cerebellum has received a great deal of attention from neuroscientists in order to understand its basic organization, neural circuitry and development (Altman and Das, 1970; Bondok et al., 1991; Mohammed et al., 1997; Ahmed, 2004; Allam et al., 2013).

The objective of this study was to determine the effect of low dose of ethyl alcohol on the neurons in the rat pups at D14. This is monitored by histological and ultrastructural studies on control and treated pups.

2. Materials and Methods

1. Experimental Animals:

The current study was carried out on 60 albino rats (Rattus novergicus), 45 mature virgin females weighing 150-180 g and 15 mature males.

Daily examination of vaginal smear of each virgin female was carried out to determine the estrous cycle. Mating was induced by housing 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 a 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 and water were provided ad libitum.

2- Drug used:

Pure ethanol was purchased from Reideld-de-Haun Company (Germany) at purity of 100%. Alcohol was diluted and orally administrated to non-anesthetized pregnant females by gastric intubation every day from the D7 of gestation 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) which was used by Thomas et al. (1998).

3. Animal grouping: (Fig. 1)

The pups of rats were divided into five groups as the following:

- 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 received alcohol from D7 of gestation and still fostered their pups
- Group D: the pups of treated mothers were transferred at birth to be lactated by untreated normal mothers (normal surrogate mothers) for 21 days.
- Group E: the pups of normal mothers were transferred at birth to be lactated by females that were given alcohol every day (treated surrogate mother) for 21 days.

4. Histological preparations:

For the histological preparations, the pups of control and treated pups. After birth the pups were divided into five groups A, B, C, D and E, each of 15 animals. On days 7 and 21 after birth, 5 of the pups from each group were sacrificed to study the effect of alcohol at different periods and conditions. The cerebellum was 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. Semithin sections were then stained with toluidine blue and examined by light microscope to select the appropriate area for ultrathin 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.

5. Ultrastructural preparations:

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. Semithin sections were then stained with toluidine blue and examined by light microscope to select the appropriate area for ultrathin 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

1. Toluidine-Blue Stain: (Figure 1)

The density of the Nissl granules was increased with time in the normal pups. The Nissl granules were distributed in the cytoplasm around the nucleus and in the proximal parts of the dendrites. At D14, in group A, Purkinje cells were deeply-stained (Fig. 1a), while in groups D & E they were moderately stained (Figs. 1d & e) and pale-stained in groups B & C (Figs. 1b & c). At D14, the normal Purkinje cells were intensely-stained with clear apical dendrites (Fig. 4a). In group B, some degenerated Purkinje cells were observed (Fig. 1b).
Fig. 1: Sagittal sections in the cerebellar cortex at D14 in the experimental groups A (a), B (b), C (c), D (d) and E (e) showing the distribution of Nissl granules in Purkinje cells (PC). The arrow refers to the apical dendrites. (Toluidine blue stain, x1000)

2. Golgi-Copssch Method: (Fig. 2)

At D14, the Purkinje cells in all groups appeared possessing one or two main apical dendrites. The dendrites arise as a main process from the upper end of the cell body, then branches and extends through the molecular layer. This main dendrite repeatedly branch to form a large and fully formed dendritic arborization. The arborizations are formed from lateral processes that emerged from the main dendrite. The Purkinje cells arborizations that could be traced in the molecular layer were well-developed in group A (Fig. 2a) but moderately developed in most cells of groups D & E (Figs. 2d & e). In addition, the arborizations nearly disappeared in most cells of groups B & C especially in group B. The main dendrites were still present and extended in the molecular layer. In groups B & C, the Purkinje cells bodies lost their pear-shaped and became more elongated and flattened (Figs. 2b & c).

4. Ultrastructural Studies: (Fig. 3)

The ultrastructural study of the normal Purkinje cells at D14 showed that such cells have pear-shaped body and large nuclei. The nuclei mainly contain euchromatin. In addition, there were numerous mitochondria distributed homogeneously in the cytoplasm. The endoplasmic reticulum cisternae and ribosomes were well represented in Purkinje cells cytoplasm especially near the nucleus (Figs. 3a and b). In group B, degenerated Purkinje cells were recorded. In addition, in the treated group B, degeneration was recorded in Purkinje cells where one or more vacuoles were observed in the cytoplasm of these neurons and this reflects the cell loss. These cells appeared as electron-dense, shrunken and dark. They may be engulfed by several glial processes (Figs. 3c and d).

Fig. 2: Sagittal sections in the cerebellar cortex at D21 in the experimental groups A (a), B (b), C (c), D (d) and E (e) showing the Purkinje cell (PC) dendrites. (Golgi-Coppsch stain, x200)

Fig. 3: Electron micrographs showing the pear-shaped cell body (soma) of Purkinje cells (PC) from normal pups at D21, the nucleus (N) and electronlucent cytoplasmic profiles contain mitochondria (M) and rough endoplasmic reticulum (RER). (a, x1000& b, x3000). Notice the completely degenerated Purkinje cell (DPC) in treated pups at D14 cell which engulfed by several glial processes (GI) (c, x3000). Another degenerated Purkinje cell with number of vacuoles (V) (d, x2000).
4. Discussion

The present results confirmed that ethanol induces neurohistological malformations in the cerebellar cortex cells; retarded maturation and differentiation of neurons in addition to neuronal loss. The external granular layer still represented at D14 by one row of cells or absent in some regions. The disappearance of this layer was attributed to the migration of its cells, which in turn increased in the width during the resorption of the external granular layer (Bondok et al., 1991).

The external granular layer was the external germinal layer which generates the granular cells then the cells start to migrate through the Purkinje cell layer at postnatal D14 to form the internal granular layer (Marcelo and Fahad, 2002). Therefore, the internal granular layer will be affected by the aberration in the external granular layer. This layer was variable in its thickness between groups at D14. The pre- and post-natal chronic alcohol administration delays the proliferations of the cells of this layer (Saleh et al., 1993). At D14 this layer was thin due to low rate of cell division resulted from prenatal alcohol exposure and bad maternal behaviour (Miller, 1996). At D 14, this layer was thick in groups B & C and thin in groups D and E due to the retardation in cells migration of this layer as described by Marcelo and Fahad (2002) and Iqbal et al. (2004).

In the present study, the internal granular layer of the treated groups was undifferentiated from the white matter until D14. Saleh et al. (1995) reported that the granular layer was still undemarcated from the white matter until D10 in alcohol-exposed rat pups. The present electron microscopical study of the treated groups showed degenerated cells in the granular layer due to alcohol exposure. This result is in accordance with Behav and Hoffmann (1997) and Heaton et al. (2000) who reported that alcohol may induce apoptosis in granular cells of cerebellum. Moreover, Tavares and Paula-Barbosa (1986) count the number of granular cells per unit volume in both the normal and ethanol treated rats and found that alcohol exposure increase the granular cell loss. The width of this layer in the treated groups was narrow which reflects the brain restriction and microcephaly (Luke, 1990; Sulik, 2005).

In normal rat pups, the molecular layer increased in thickness at D14. The molecular layer width was depending on the number of its neurons and size of Purkinje cell arborization (Bondok et al. 1991; Ahmed, 2004). Therefore, the size of the molecular layer at D14 was narrow in groups B and C and moderate in groups D and E. West et al. (2001) reported that alcohol mediates cells loss especially Purkinje cells. Griffin et al. (1977) recorded that malnutrition induces aberration in Purkinje cells dendrites. These results confirmed that the width of molecular was narrow in treated groups. The present reduction in the width of molecular layer in the treated groups reflected the microcephaly, which was caused by the action of alcohol (Luke, 1990; Maier et al. 1997; Sulik, 2005).

At D14, Purkinje cells are arranged in one row in the present normal pups. These cells are arranged to form Purkinje cell layer at D14 at the junction between the molecular and internal granular layer; this result is in agreement with Bondok et al. (1991) and Saleh et al. (1993). Altaman and Winfree (1977) explained the arrangement in a single row due to the pressure exerted on the growing Purkinje cells from below by the expanding granular layer and the barrier formed above these cells by the pile of the parallel fibres.

In the present normal pups, at D14, the main dendrite of Purkinje cells arises from the upper end of the perikaryon and extends through the molecular layer and repeatedly branch to form large dendritic arborizations. Similar results were recorded in humans by Krause and Cutts (1994) and in rats by Bahgat et al. (2005).

The present study showed that Purkinje cells were arranged in more than one row in the treated groups at D7 & at D21 due to the late migration of these cells. These results are in agreement with Saleh et al. (1993). Thomas et al. (1998) reported that alcohol-induced Purkinje cells loss depends on the duration of alcohol exposure. Therefore, there is variability in the rate of Purkinje cells loss in these treated groups where the high number was in group B which detected by light and electron microscopical studies. The ultrastructural study showed some degenerated Purkinje cells and others with vacuoles, which may result from degenerated cell organelles and leads to cell loss. These results were explained by the action of alcohol which induces free radicals, oxidative stress and mitochondrial dysfunction (Bredensen, 1996 a & b and Heaton et al., 2000). In addition, alcohol metabolism in the cytoplasm leads to acidosis, which affect on cell functions (El-Raghy, 1993). In groups C, D and E Purkinje cells loss took place due to prenatal alcohol exposure and this loss increased postnataly due to malnutrition (Griffin et al., 1977), which resulted from bad maternal behaviour. In group D, Purkinje cells loss resulted from prenatal alcohol ingestion but this loss stopped due to postnatal normal developmental condition. In group E, Purkinje cells loss occurred postnatal because of chronic alcohol administration during the lactation. The above result was supported by detection of pyknotic Purkinje cells in groups B, C &
The high loss of Purkinje cells was represented by wide distances between Purkinje cells. These results go parallel with the results of Daniel et al. (1996) who mentioned that there was loss of Purkinje cells in primates following weekly exposure to ethanol during gestation. On the other hand, the arborizations of Purkinje cells in groups B & C, were reduced in most cells. In addition, malnutrition reduced the size of arborizations (Griffin et al., 1977). Alcohol has indirect effect on cell dendrites as it impairs the placenta and the mammary glands functions, so it leads to fetus malnutrition (Luke, 1990 and Luisa et al, 2001).

The intensity of Nissl granules in the neurons refers to the high metabolic activity of these neurons (Stevens and Lowe, 1997). The present study showed normal intensity of Nissl granules in the normal Purkinje cells. These evidences are in agreement with Stevens and Lowe (1997) who found the pups of humans with high metabolic activity. In group B, many neurons were degenerated. In group C, most of neurons were pale-stained, while moderately-stained in groups D & E. According to the present results, it could be suggested that, in treated pups the amount of Nissl granules were less than that in the normal ones. This result is in agreement with Heaton et al. (2000) who mentioned that alcohol causes disturbance in the metabolism and leads to cell dysfunction. Alcohol impaired cell metabolism (Hu et al., 1995) and led to protein deficiency (Luke, 1990) so the intensity of Nissl granules was low in the treated groups.

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