

Effect Of Diazepam On The Prenatal Development Of Neuropil Of The Purkinje Cell Layer Of The Cerebellar Cortex Of The Rats Embryo (Electron Microscope Study)

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Abstract

Diazepam is one of the most widely used sedative hypnotic agents, and many women take this drug during pregnancy.

The purpose of this study was to investigate the effects of diazepam on the development of the neuropil of the Purkinje cell layer of the cerebellar cortex.

Twenty female pregnant rats were used in this study. The rats were divided into two groups, the first group (5 rats) was used as control group and the second group (15 rats) were given 10 mg/kg/day of diazepam orally and sacrificed at 15th, 20th, 25th days of pregnancy. It was difficult to differentiate between the dendrites and the axonal knobs at 15th day of pregnancy. As age advanced, the dendrites were swollen with absence of cytoplasmic organelles or contained few free ribosomes and immature mitochondria. The observed axonal knobs contained ill-defined synaptic vesicles. Immature axo-dendritic synapses were recognized on the 15th and 20th days of pregnancy, while on the 25th days of pregnancy symmetrical axo-dendritic synapses were recognized. From this study, it appears that the exposure to diazepam causes marked changes on prenatal development of neuropil of the Purkinje cell layer of the cerebellar cortex.

Aim of the work

The aim of the work was to study the effects of diazepam on the development of the neuropil of Purkinje cell layer of the cerebellar cortex.

Introduction

Diazepam is the most important sedative hypnotic drug used, it is given orally, intramuscular and intravenous, and it is absorbed very rapidly into the blood following oral or intramuscular administration and concentrated in the nervous tissues. The therapeutic dose is 5 mg twice daily (Katzung 2001). It is metabolized in the liver and excreted via the kidneys (Chouinard G. 1999 and Avallone *et al* 2002). It causes sedation and hypnosis, it is also used in anesthesia, convulsion and relaxation of the muscles (Walsh and Schweitzer 1999 and Musavi & Kakkar 2003).

Recently, the morphology of the cerebellar neurons and their intercortical connections is being intensively studied. The cerebellar cortex has a limited number

of neuron types and a stereotyped pattern of architectural organization which is characterized by its structural uniformity in the whole extension of the cortical mantle (Cormack 2001). The cerebellar cortex is composed of three layers; the molecular cell layer, Purkinje cell layer and granular layer (Gartner and Hiatt 2000)

The germinal zone, which surrounds the 4th ventricle, gives rise to the cells of Purkinje, Golgi, and the cerebellar glia (William 1995). The other neurons of the cerebellar cortex such as the star cells, basket cells, granular cells, and other glial elements are derived from the original germinal cells in the rhombic heights (Altman, 1972).

Purkinje cell layer: the Purkinje cells are large Golgi type I neurons. They are

flask-shaped and are arranged in a single layer in a plane transverse to the folium, the dendrites of these cells are seen to pass into the molecular layer where they undergo profuse branching. The primary and secondary branches are smooth and subsequent branches are covered by short, thick dendritic spines. It has been shown that the spines form synaptic contacts with the parallel fibers derived from the granule cell axons. (Cormack 2001). At the base of the Purkinje cell, the axon arises and passes through the granular layer to enter the white matter. On entering the white matter, the axon acquires a myelin sheath and it terminates by snapping with cells of one of the intracerebellar nuclei. Collateral branches of the Purkinje axon make synaptic contacts with the dendrites of basket and stellate cells of the granular layer in the same area or in distant folia. A few of the Purkinje cell axons pass directly to end in the vestibular nuclei of the brain stem. (Snell 1997)

Neuropil was a term given to the areas of the central nervous system neurons that most of the synaptic junctions occurred (Peters, 1970). Between the nests of the cell stomata in the developing Purkinje cell layer, there are areas of cell processes in various stages of differentiation which are identified as dendrites. The dendrites are not very numerous and contain mitochondria, free ribosomes, some elements of the rough and smooth endoplasmic reticulum, neurofilaments and microtubules (Doulazmi, 2002) Large dendritic profiles more rich in organelles are identified as dendritic trunks, while smaller dendritic profiles having fewer organelles are considered as dendritic tips. A second component of the neuropil is the dendritic spine which is recognized along the course of some dendrites. The dendritic spine has a short slender stem with expanded tip. Some of these spines might contain spine apparatus (Valsecchi *et al* 1993). A third component of the neuropil consists of numerous elements of varying size containing numerous vesicles 300-600 Å in diameter. They are axonal knobs which contain round and/or flat synaptic vesicles (.Schaal *et al* 1985).

The axo-dendritic synapses are classified into:

1-Gray's type I (symmetrical synapse): It has a wide synaptic cleft and shows a prominent post-synaptic thickening.

2- Gray's type II (asymmetrical synapse): it has a narrow cleft without postsynaptic thickening

The cerebellar syndrome is the most neurological alteration frequently resulting from diazepam. This has raised the idea of the existence of a marked vulnerability of the cerebellum to the action of diazepam. For this reason, in the present work, we analyzed the pathological effect of diazepam on the neuropil of the Purkinje cell layer of the cerebellar cortex of the rat embryo (William 1995).

Material and Methods

Twenty female pregnant rats were used in this study. They were divided into two groups. Rat of the first group (5 rats) was used as a control group. Rats of this group were given 0.1 c.c. physiological saline orally by gastric tube. The rats of the second group (15 rats) were given diazepam in a dose equal to 10 mg/kg/day orally by gastric tube.

Both groups were kept in metal cages with proper ventilation and humidity. All the rats were sacrificed on the 15th, 20th, and 25th days of treatment respectively. The cerebella were extracted from the embryo and cut into slices 1 µm thick. The slices of the cerebella of both control and treated groups were fixed in 1% glutaraldehyde and osmic tetroxide. Then the specimens were prepared for the electron microscope study.

Results

1- Control Group:

On the 15th day of treatment, the neuropil of the Purkinje cell layer contained small dendritic profiles and profiles of axonal knobs. The dendritic profiles had a relatively clear dendroplasm. Profiles of axonal knobs were seen with ill-defined synaptic vesicles. Immature axo-dendritic contacts were observed but without marked synaptic thickening (Fig.1).

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On the 20th day of treatment, the neuropil of the Purkinje cell layer contained dendrites, dendritic spines and axonal knobs. The dendritic profiles contained mitochondria and neurofilaments (Fig. 3). The dendritic spines were recognized at this age, (Fig.3), Axonal knobs containing synaptic vesicles were clearly identified. Both symmetrical and asymmetrical axo-dendritic synapses were recognized (Fig. 2 and 3)

On the 25th day of treatment, the neuropil of the Purkinje cell layer contained dendrites, dendritic spines and axonal knobs. The primary dendrites of the Purkinje cell neurons arose from the parent cell body. The dendrites exhibited the same organelles that were found in the soma cytoplasm (Fig. 4). Longitudinal sections of the secondary and terminal parts of these dendrites contained only mitochondria, neurofilaments (Fig.5), and micro tubules (Fig. 6). Dendritic spines were recognized along the course of some of the dendrites. The spines had a short slender stem with expanded tip (Figs. 5, 6, 7 & 9), some of these spine might show spine apparatus (Fig. 6 & 9). The axonal knobs could be identified in the neuropil of the Purkinje cell layer.

2- Treated group:

In the neuropil of the treated Purkinje cell layer on the 15th day of treatment, it was difficult to differentiate between the axons and dendrites. The axons and dendrites separated from each other by wide cleft-like spaces. The axo-dendritic contacts were easily identified (Fig. 10).

On the 20th day of treatment, the neuropil showed swollen dendritic profiles with absence of cytoplasmic organelles. The dendrites and axonal terminals were separated by cleft-like synaptic spaces. Dendritic spines were not clearly identified. Axonal knobs were observed with ill-defined synaptic vesicles. Immature axo-dendritic synapses were observed without synaptic thickening (Fig. 11)

On the 25th day of treatment, the dendritic profiles were swollen and empty or contained few free ribosomes and immature mitochondria. Neurofilaments and microtubules were not identified. Dendritic

spines could not be observed. The axonal knobs were observed containing ill-defined synaptic vesicles. Rounded and flat vesicles were not identified. Axo-dendritic synapses were recognized as symmetrical synaptic thickening. Asymmetrical axo-dendritic synapses were not observed (Fig. 12).

(Fig.1) Electron micrograph of the neuropil of Purkinje cell layer of the cerebellum of 15-days control rat. The dendritic profiles (**d**) have a relatively clear dendroplasm. The axonal knobs (**a**) are with ill-defined synaptic vesicles. (X 80000)

(Fig-2) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 20-days control rat. It shows dendrites of Purkinje cells (**d**). There are symmetrical (**S**) and asymmetrical (**S+**) types of axo-dendritic synapses, dendritic spine (**P**) was noticed (X64000)

(Fig.3) Electron micrograph of the neuropil of Purkinje cell layer of the cerebellum of 20-days control rat. It shows large dendrite (**d**) containing mitochondria (**m**) and neurofilaments (**n**). Dendritic spines (**P**) are present. Axonal knobs (**a**) containing synaptic vesicles are clearly identified. Both symmetrical (**S**) and asymmetrical (**S+**) axo-dendritic synapses are present. (X80000)

(Fig.4) Electron micrograph of the Purkinje cell layer of the cerebellum of 25-days control rat. It shows a portion of the Purkinje cell nucleus (**N**) and its cytoplasm (**C**). The dendrites (**d**) also seen which are the similar to the soma cytoplasm. Notice the presence of axonal knobs (**a**). (X 32000)

(Fig. 5) Electron micrograph of the neuropil of the Purkinje cell of the cerebellum of 25-days control rat shows large dendrite (**d**) having neurofilaments (**n**) and mitochondria (**m**). Dendritic Spine (**P**) with spine apparatus is seen. Axonal knobs with synaptic vesicles (**a**) are seen. Axo-

dendritic synapses of symmetrical (S) and asymmetrical (S+) types are present. (X160000)

(Fig 6) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 25-days control rat. It shows Purkinje cell dendrites (**d**) containing mitochondria (**m**), neurofilaments (**n**) and microtubules (**t**).The dendritic spine arises from the dendrite (**p**). (X 100000)

(Fig-7) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 25-days control rat. It shows dendrites with the presence of a dendritic spine (P), spine apparatus making synapse with an axonal knob (a) by symmetrical type. The axonal knobs (a) contain round synaptic vesicle and dense - core vesicle. (X80000)

(Fig.8) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 25-days control rat. It shows dendrite (**d**) surrounded by axonal knobs (**a**) with round synaptic vesicle (**r**) and flat synaptic vesicle (**f**) (X80000)

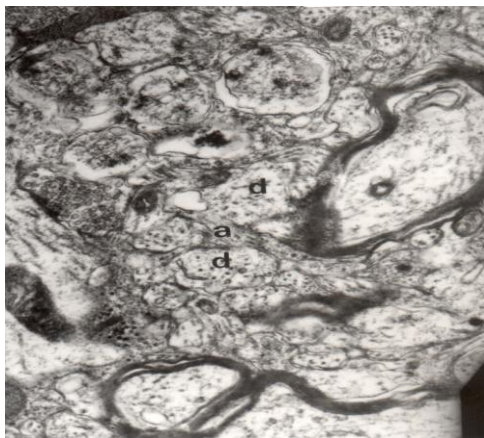
(Fig.9) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 25-days control rat. It

shows the presence of dendrite (**d**). The dendritic spines contact with axonal knobs by symmetrical (S) and asymmetrical (S+) types of synapses. The axonal knobs contain round (**r**) synaptic vesicles and dense core vesicles (**v**). (X80000)

(Fig. 10) Electron micrograph neuropil of the Purkinje cell layer of the cerebellum of 15-days rats treated with diazepam It shows the profiles of terminal axon (**a**) and dendrite (**d**) without any organelles. (X 80000)

(Fig.11) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 20-days rats treated with diazepam. It shows swollen dendritic processes (**d**) with absence of cytoplasmic organelles. Axonal knobs (**a**) contain few ill-defined synaptic vesicles. (X 80000)

(Fig.12) Electron micrograph of the neuropil of the Purkinje cell layer of the cerebellum of 25-days rats treated with diazepam. It shows empty dendritic processes (**d**) and axonal knobs (**a**) with ill-defined synaptic vesicles. (X 80000)

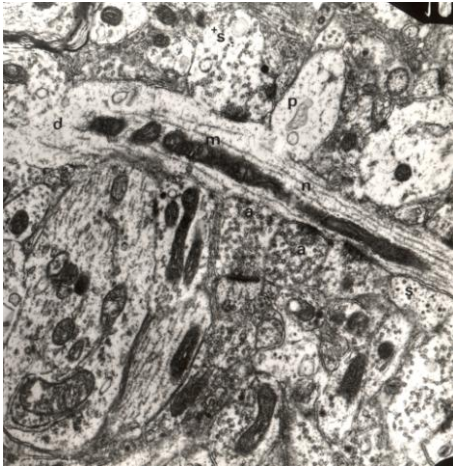


(Fig. 1)

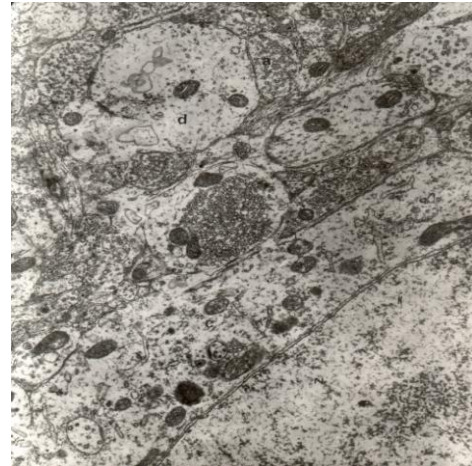


(Fig. 2)

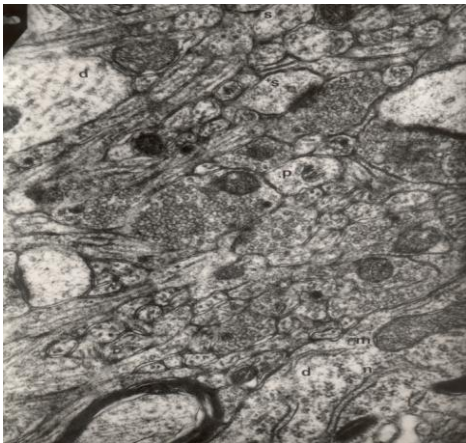
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(Fig. 3)



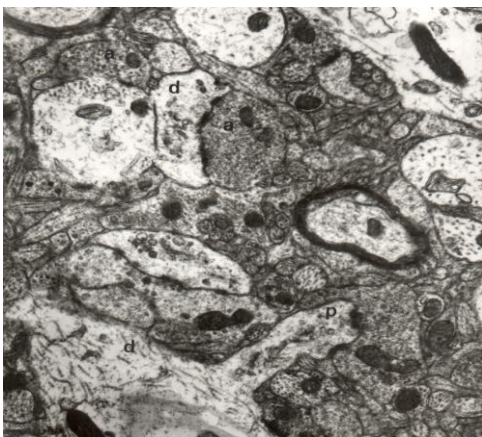
(Fig. 4)



(Fig. 5)



(Fig. 6)



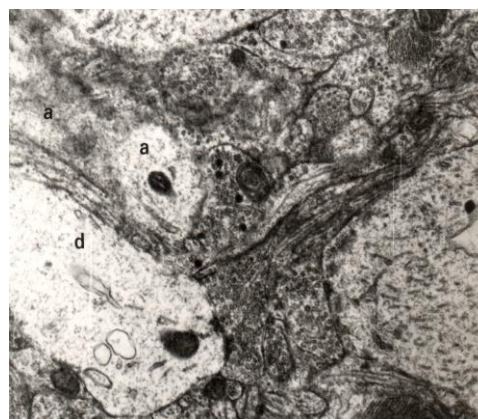
(Fig. 7)



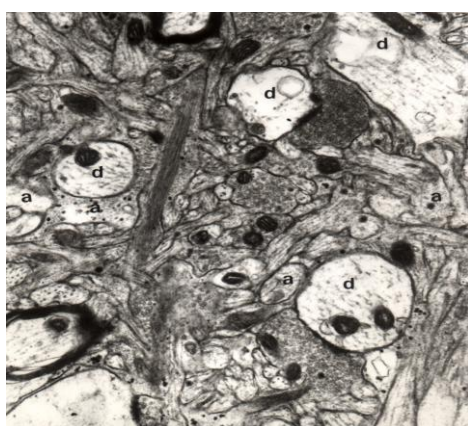
(Fig. 8)



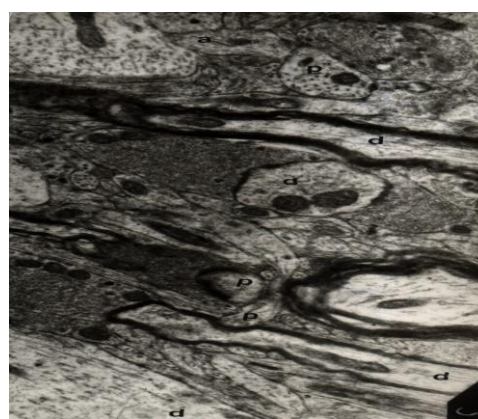
(Fig. 9)



(Fig. 10)



(Fig. 11)



(Fig. 12)

Discussion

The cerebellum controls the equilibrium and is particularly affected by many hypnotics like diazepam. It is known that all the drugs used by human are able to produce tolerance; diazepam is one of the drugs which cause many changes on the cerebellum. The pathological changes following exposure to diazepam occurred more frequently in undifferentiated cells or during their process of differentiation. Also, the adverse action of diazepam was dependent on the neuronal age. Thus, our present study is to observe the possible effects of diazepam in different stages of cerebellar development of rat embryo.

The present data indicate that on the 15th day of treatment, it was difficult to differentiate between the dendrites and the axonal knobs. As age advanced, the dendrites were swollen with absence of the cytoplasmic organelles or contained few free ribosomes and immature mitochondria.

The axonal knobs were observed containing ill-defined synaptic vesicles. Also on the 15th and 20th days of pregnancy, immature axo-dendritic synapses were observed, while on the 25th days of pregnancy, axo-dendritic synapses of symmetrical type were recognized. These observations may be related to the accumulation of drug and the blood level of diazepam. The data are compatible with the results obtained from Gallager & Mallorga (1980), Westmoreland & Bass (1971), Vorhees *et al* (1990) and Deluka *et al* (2002).

The findings of the present work confirm those of many workers who showed that the most striking changes in the structure of the rat cerebellum after administration of diazepam were observed in the synaptic buttons mainly in the Purkinje cell layer of the cerebellar cortex. The synapses affected were predominantly of symmetrical and asymmetrical types of

axo-dendritic synapses with decreased number of the synaptic vesicle hypertrophy of the axonal terminal. Origuchi *et al* (1975), Desor *et al* (1978), Mennini *et al* (1984) and Schwartz *et al* (1985) showed that the Purkinje cell layer of the cerebellar cortex of the rat treated with diazepam showed numerous degeneration dendrites within the neuropil in the form of swollen dendritic profiles with clear dendroplasm or degenerated cytoplasmic organelles especially mitochondria. Early axonal injuries were also present, which contained ill-defined synaptic vesicles.

The results of this study on the effects of prenatal exposure to diazepam indicate that on the 20th day of treatment, the neuropil showed swollen dendritic profiles with absence of cytoplasmic organelles. The dendrites and axonal terminals were separated by cleft-like synaptic spaces. Dendritic spines were not clearly identified. Axonal knobs were observed with ill-defined synaptic vesicles. Immature axo-dendritic synapses were observed without synaptic thickening. The results are compatible with behavioral observations of Dam (1972), Klotz (1979), Vaccarino *et al* (1983), Takeichi (1983) and Hevers & Luddens (2002) who stated that the neuropil of Purkinje cell layer of the mouse cerebellum after prenatal and postnatal diazepam administration showed degenerative synaptic terminals and depletion of synaptic vesicles. Immature axo-somatic and axo-dendritic contacts were recognized as demonstrated by the electron microscope.

Our study showed that the histopathological alterations on the neuropil of the Purkinje cell layer of the cerebellar cortex of the mice due to effect of diazepam was in the form of deformed swollen dendritic profiles with clear dendroplasm or contained deformed and degenerated cytoplasmic organelles especially mitochondria with absence of other cytoplasmic organelles especially neurofilaments and microtubules. The axonal buttons were observed containing ill-defined synaptic vesicles with immature axo-dendritic contacts, but without synaptic thickening. These findings may be due to inhibition of

neuronal growth factors. The result are in agreement with the result of Candy & Martin (1979), Rotter & Frosthalm (1988), Marquez *et al* (1994) and Musavi &Kakkar (2000) who studied the effect of diazepam on the neuropil of the Purkinje cell layer of the cerebellar cortex of the rat. They found degenerated swollen dendritic profiles with absence of the cytoplasmic organelles or contained few ribosomes and immature or degenerated mitochondria. Other cytoplasmic organelles of the dendroplasm as neurofilaments, microtubules, and endoplasmic reticulum were absent. Deformed axonal terminals with ill-defined synaptic vesicles were recognized

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تأثير عقار – الديازيبام على نمو محتويات النسيج العصبي (نيوروبل)
لطبقة خلايا بركنجي لقشرة المخ في أجنة الجرذان دراسة بالميكروسكوب
الإلكتروني

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إستخدم في هذا البحث عشرون أنثى فأر حامل و قد تم تقسيم الجرذان إلى مجموعتين' المجموعة الأولى خمسة جرذان استخدمت كمجموعة ضابطة و المجموعة الثانية (١٥) جرذ' و قد تم إعطاء كل جرذ في المجموعة الثانية ما يوازي ١٠ ملجم لكل كيلو جرام من عقار الديازيبام يوميا و قد تم استخدام الميكروسكوب الإلكتروني لفحص النسيج العصبي لطبقة خلايا (بركنج) لقشرة المخ في أجنة الجرذان. و قد وجد أنه من الصعب التفريق بين الغصينات و المحاور العصبية بعد ١٥ يوم من فترة الحضانة' و لكن مع تقدم عمر الجنين إلى ٢٠ و ٢٥ يوم في فترة الحضانة وجد تأخر لنمو الغصينات و قلة الجسيمات بها و تأخر نمو المحاور العصبية و وجود فقاعات غير واضحة المعالم و اختفاء الالتصاقات غير المتماثلة مع تأخر ظهور الالتصاقات المتماثلة. و من هذه الدراسة تبين أنه يجب أن تتحاشى الأمهات الحوامل إستخدم المهدئات طوال فترة الحمل و خاصة في الشهور الأولى.