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Effect of intrauterine morphine sulfate exposure on cerebellar histomorphological changes in neonatal mice

Soraya Ghafari[1], Danial Roshandel[2], Mohammad Jafar Golalipour[3]

[1]Department of Anatomical Sciences, Golestan University of Medical Sciences, Gorgan, [2]Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, [3]Department of Anatomical Sciences, Gorgan Congenital Malformations Research Center, Golestan University of Medical Sciences, Gorgan, Iran

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Abstract

Neurotoxic effects of morphine sulfate in adult cerebellar cortex and neonatal cerebral cortex have been studied in animal models. This study was done to determine the neurotoxic effects of prenatal morphine exposure on the histomorphological changes of cerebellar cortical layer and Purkinje cells in mice neonates. In this experimental study 30 female mice were randomly allocated into cases and controls. In the case group, animals received morphine sulfate 10 mg/kg/body weight intraperitoneally for 7 days. After mating, dams received morphine sulfate 10 mg/kg/body weight intraperitoneally for 20 days of gestation. Animals in the control group received normal saline. On the day of delivery (P0), the cerebella of six neonates for each group were removed and stained with cresyl violet. Quantitative computer-assisted morphometric study was done on the cortical layer of the cerebellum. Morphine exposure caused a non-significant increase in fetal weight in the case group. Purkinje cells in cases were decreased in comparison with controls ($p < 0.05$). Histomorphometric examination revealed that the thickness of Purkinje and internal granular layers of the cerebellar cortex decreased in the morphine-exposed group ($p < 0.05$). This study revealed that morphine administration before and during pregnancy can cause Purkinje cell loss and reduction of thickness of the Purkinje and internal granular layer of the cerebellar cortex and size of Purkinje cells in neonatal mice.

Key words: *morphine sulfate, cerebellum, cortex, Purkinje cells, mouse.*

Introduction

Morphine (C17 H19 O3 N) is one of the 40 alkaloids present in opium from *Papaver somniferum* and it is one of the strongest known analgesic compounds [44].

The prevalence of opioid abuse is high worldwide, especially in young people. As one of the addictive drugs, morphine is an increasing cause of death, morbidity and lost productivity in society [25].

Recently, researchers have reported that morphine may play important roles other than analgesia,

Communicating author:
Dr. Mohammad Jafar Golalipour, Gorgan Congenital Malformations Research Center, Golestan University of Medical Sciences, Gorgan, Iran, P.O. Box: 49175-1141, phone & fax + 98(171)4425165, 4425660, e-mail: mjgolalipour@yahoo.com

such as suppression of immune responses [8,31] and modulation of tumour cell proliferation [4]. Morphine has been suggested to modulate cell death/survival in neurons of the central nervous system [44].

Several studies have shown that opioid abuse may affect the embryos of pregnant women. In this regard, it has been shown that opioid administration during pregnancy can cause delay in embryonic development, preterm labour, fetus death, chromosomal anomalies, neural tube defects and reduced birth weight [10,23,24,27,30,41].

In addition, a study has shown that morphine neonatal abstinence was common in the infants of opioid-dependent mothers [6]. It was also found that these children had several behavioural abnormalities including hyperactivity, lower mental development index, and lower motor development index [24,27,41].

Mao *et al*. (2002) have shown that high dosage of morphine caused neurotoxicity in neurons in an animal mode [22]. Furthermore, it is reported that chronic administration of morphine can induce apoptosis in brain and spinal cord [1]. Elsewhere, morphine increased neurotoxicity on human neurons in an *in-vitro* model [40].

Sadraie *et al.* (2008) reported that oral administration of morphine sulphate decreased the number of neurons and the thickness of cortex of cerebrum in mice neonates [32].

Recently, Bekheet *et al.* (2010) showed that oral administration of morphine sulphate significantly reduced the number and diameter of Purkinje cells and thickness of both molecular and granular layers of adult rat cerebellum [3].

Regarding the high prevalence of opioid abuse worldwide, especially in young adults, and scarcity of studies on the effect of morphine sulphate on development of cerebellum, the present study was carried out to clarify the neurotoxic effects of prenatal morphine sulphate administration on the histomorphological changes of the cerebellar cortical layer and Purkinje cells in one-day-old mice neonates.

Material and methods

This experimental study was performed at the Gorgan Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran. Guidelines on the care and use of laboratory animals were followed and approval of the ethics committee of Golestan University of Medical Sciences was obtained before the study.

Animals

Balb/c mice, weighing 28-30 g (8-9 weeks old) were used in this study. The animals were maintained in a climate-controlled room under a 12-hour alternating light/dark cycle, 20°C to 22°C temperature, and 50% to 55.5% relative humidity. Dry food pellets and water were provided *ad libitum*.

Drugs

Each vial contained 1 ml of morphine sulphate (Darou Pakhsh CO. Iran) dissolved in 3.3 ml sterile saline solution (0.9%) to give a 10 mg morphine sulphate dose intraperitoneally injected into mice.

Animal groups and treatment

After 2 weeks of acclimation to the diet and the environment, females were randomly divided into control and treated groups. Fifteen female mice in the treated group received 10 mg/kg/body weight of morphine sulphate intraperitoneally (IP) for 7 days. The control group (15 mice) received an equivalent volume of normal saline. After one week, 2 females were caged overnight with a male of the same strain. The presence of a vaginal plug the following morning confirmed that mating had taken place and was designated as gestational day (GD) 0. Animals in the treated group continuously received morphine sulfate (10 mg/kg/body weight) daily from GD0 to 20 by IP injection. Control animals received normal saline daily from GD0 to 20 by IP injection.

After parturition, in each group, six neonatal mice (the day of birth was defined as postnatal day 0 = P0) were randomly selected and were killed quickly with chloroform anaesthesia. The brain was exposed by partial removal of the skin and skull and then fixed by immersion into the fixative solutions (10% neutral-buffered formalin). Then samples were dehydrated in a gradual series of ethanol, embedded in paraffin wax and sectioned at 6 μm thickness using a microtome (Microm HM 325, Germany). The coronal sections (serial sections of anterior to posterior cerebellum) were serially collected with an interval of 24 μm between every two consecutive sections. The sections were stained with cresyl violet for morphometric examination.

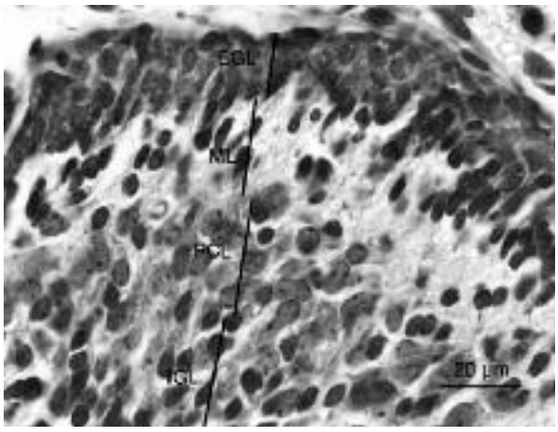

Fig. 1. Photomicrograph of coronal section of cerebellar cortex of neonatal mice (P0) in control group. Purkinje cells are recognizable by their larger soma and nuclear size. Coronal section stained with cresyl violet. EGL – external granule cell layer, ML – molecular layer, PCL – Purkinje cell layer, IGL – inner granule cell layer (100× objective).

Table I. Average body weight, brain weight, brain to body weight ratio in newborn Balb/c mice (P0)

Variable	Control (6)	Morphine (6)
Body weight	1.50 ± 0.08	1.72 ± 0.12
Brain weight	0.13 ± 0.003	0.13 ± 0.003
Brain to body weight ratio	0.087 ± 0.004	0.077 ± 0.007
Relative brain weight	8.76 ± 0.4	7.77 ± 0.7

Results are expressed as mean ± SE of the mean

Morphometric techniques

In each sample, six similar sections of anterior lobes (I-V) of cerebellum were selected and images of three separate fields per section in the left side were captured by an Olympus BX 51 microscope and DP12 digital camera attached to OLYSIA autobioreport software (Olympus Optical, Co. LTD, Tokyo, Japan).

The morphometric analysis of the cerebellar cortex including thickness of the external granular cell layer (EGL), molecular layer (ML), Purkinje cell layer (PCL, PCs are in multiple rows in P0) and internal granular cell layer (IGL) was performed at low magnification (40× objective) (Fig. 1).

The morphometric analysis of the Purkinje cells including the number, diameter and soma area of Purkinje cells, which are easily recognizable by their larger soma and nuclear size and by their short thick apical dendrite, was performed at high magnification (100× objective). The characteristics of Purkinje cells in this study were measured carefully due to the standard of capacity of OLYSIA software. In our study, during measurement of area of Purkinje cells the auto figures magnified to 120× for exact measurements.

An appropriate grid was applied on the image and the number of Purkinje cells was counted in 50 000 µm^2 areas in the Purkinje cell layer. Also the diameter and the relative area of the cell body were calculated by the software.

The results are expressed as the mean thickness of layers, diameter (micrometres), soma area (square micrometres) and densities of Purkinje cells (the number of Purkinje cells/50 000 µm^2 area of Purkinje cell layer) in each group.

Statistical analysis

The mean values for the obtained data are calculated and represented in tables as mean ± standard error of the mean. The significance of the difference between the means was calculated according to Student's *t* test and Mann-Whitney U test using SPSS 11.5 software. A significance level of 0.05 was predetermined for all statistical analyses.

Results

Morphine sulphate treatment non-significantly increased the body weight of mice neonates as compared to the controls, but the brain weight was similar to treated and control groups (Table I).

The mean thickness of the cerebellar cortex layer decreased in the treated group in comparison with the control group ($p < 0.05$).

Morphine caused a non-significant increase in the thickness of EGL and ML, whereas it caused a marked significant reduction in the Purkinje cell layer and inner granular layer when compared to the control group ($p < 0.05$) (Table II).

Purkinje cell density: the numbers of PCs decreased from 1099.50 cells in the control group to 901.25 cells in 50 000 µm^2 area of the pc layer in the treated group in P0 mice ($p < 0.001$).

Table II. Thickness of the various layers of cerebellar cortex (μm) in newborn Balb/c mice (P0)

Layer	Control (P0)	Morphine (P0)	*p* value
EGL	37.6 ± 2.33	41.2 ± 0.91	> 0.05
ML	23.44 ± 1.61	26.52 ± 1.47	> 0.05
PCL	81.99 ± 3.25	62.51 ± 4.78	0.004
IGL	145.51 ± 3.51	126.7 ± 8.1	0.04
TCL	288.56 ± 5.72	256.93 ± 10.23	0.01

EGL – external granule cell layer, ML – molecular layer, PCL – Purkinje cell layer, IGL – inner granule cell layer, TCL – total cerebellar cortex layer
Results are expressed as mean ± SE of the mean

Table III. Quantitative characteristics of Purkinje cells of cerebellum in control and morphine newborn Balb/c mice (P0)

Characteristics of PC	Control (6)	Morphine (6)	*p* value
PC density (PC Number/50 000 μm^2 area of PCL)	1099.50 ± 38.75	901.25 ± 12.52	< 0.001*
Area of PC (μm^2)	52.78 ± 1.5	44.72 ± 0.89	0.001**
Perimeter of PC (μm)	30.1 ± 1	25.75 ± 0.7	0.001**
Diameter of PC (μm)	6.54 ± 0.11	5.91 ± 0.12	0.04**

**Mann-Whitney U test, **Student's t test*
Results are expressed as mean ± SE of the mean

The results revealed a significant reduction in the diameter of Purkinje cells in treated mice in comparison with the controls (p = 0.04).

In addition, the mean area of the Purkinje cells in the control group was larger (52.78 ± 1.5 μm^2) than the treated group (44.72 ± 0.89 μm^2) (Table III).

Discussion

This study revealed that morphine sulfate administration before and during pregnancy can cause Purkinje cell loss, and decrease of the Purkinje layer, internal granular layer and size of Purkinje cells in neonatal mice. Our findings are in agreement with two previous studies [3,32].

Sadraie *et al*. in 2008 reported that morphine exposure (0.1 mg/ml on the 21st day of gestation orally) reduced both cortical thickness and the numbers of neurons in the developing fetal frontal cerebral cortex and the thickness of the cortical palate [32].

Bekheet *et al.* [3] reported that the long-term administration of morphine sulphate at a dose level of 5 mg/kg body weight day after days 10, 20 and 30 in adult rats caused a significant reduction in the diameter of Purkinje cells and in the thickness of both molecular and granular layers. Also in treated groups, size and numbers of the Purkinje cells were decreased and these cells lost their specific shaped appearance. They concluded that morphine sulphate may induce cell death or necrosis in the rat cerebellum and modulate the neurotransmitter system [3].

Also, in our study the diameter, size and area of Purkinje cells decreased in treated animals as compared with controls. This finding is similar to Bekheet's study [3].

Several possible mechanisms have been postulated regarding the effect of morphine on the central nervous system. Hauser *et al.* reported that morphine with apoptosis and or necrosis can cause neurotoxicity in Purkinje cells [14]. Also another study has shown that morphological alterations of astrocytes due to morphine increase both Ca^{2+} and production of carbonyl oxidation which subsequently promote apoptosis and/or necrosis in neurons [15]. In another study, Hauser *et al.* have shown that morphine blocks the proliferation of neuroblasts in the molecular layer by preventing DNA synthesis [16]. On the other hand, a study reported that opioids affect granular cells of mice cerebellum [17]. Also increase of neurons in rat

neonates due to opioid antagonist substances has been shown by Zagon and McLaughlin [42].

Furthermore, several studies have shown that acute opioid exposure blocks the proliferation, differentiation and survival of neuroblasts and astroglia of cerebellum [13,18,21,33,42]. Also Oehmichen *et al.* reported decrease of Purkinje cells in chronic morphine users [26].

The involvement of opioids in cerebellar growth regulation has been revealed by experimentally perturbing the endogenous opioid system. Endogenous opioid peptides and receptors are widely expressed by developing cerebellar cells [19,35,38,43,45].

Although heroin and morphine preferentially activate μ opioid receptors, at high concentrations they can activate δ and κ receptors [12,29].

Continuous opioid receptor blockade accelerates cerebellar growth in postnatal rats, while overstimulating opioid receptors, as occurs with opiate drugs, retards cerebellar growth [18,37,42].

Some studies have shown that morphine as well as heroin can cause reduced proliferation, differentiation and increase of Purkinje cell death in the cerebellum [14,33,35,39].

Also a study showed that neurotoxic effects of opioids can be induced by the NMDAR-caspase pathway [22].

Furthermore, during brain development, block of endogenous opioids induces development and maturation of nerve cells in brain but morphine can cause restriction of proliferation, maturation and development of neural cells in brain [34].

Also several studies have shown that mitochondrial damage rules out the possibility of cell death [5,20,28]. Ultrastructural changes including swelling of the mitochondria, which appeared rounded and lacked their elongated shape with degeneration of their cristae and the internal membranes ending in their transformation into vacuoles with electron-transparent contents and with the emergence of membranous inclusions and accumulation of fine granular materials, destroyed RER and SER cisterns appearing as small fragmented rods with fall in the number of ribosomes, intensified chromatolysis and shrinkage of Golgi apparatus and the nucleus and uneven appearance of the contour of the nucleus, and degenerated nuclear envelop with increased number and size of the nuclear pores and accumulation of lipids and glycogen in the cytoplasm in Purkinje cell type have been reported by previous studies [3,16].

In our study morphine induced a significant reduction in the thickness of the Purkinje layer as well as the internal granular layer and increase in the thickness of the external granular and molecular layers in all treated mice. Bekheet *et al.* in 2010 reported that morphine significantly decreased the diameter of Purkinje cells and thickness of molecular and granular layers in all treated animals [3]. Also, Demeri *et al.* reported that morphine induced a significant decrease in the molecular and granular layer thickness of the rat cerebellum and lowered Purkinje cell numbers in unit length of the Purkinje cell layer [7]. Furthermore, Atta showed that morphine decreased the number and size of Purkinje cell and the size of granular cells [2]. Our results showing a decrease of the thickness of the Purkinje layer as well as the granular layer is similar to other studies [2,3,7]. We observed a non-significant increase of molecular layer thickness, in spite of proliferation and migration of basket and stellate cells in the molecular layer initiated after 7 postnatal days [36]. This minor increase of molecular layer thickness may be due to either the time of administration of morphine during the pregnancy period or reduction of Purkinje cells and layer in our study. Further study is required to survey the type of cells and layers of cerebellum in different postnatal periods after administration of morphine in dams.

Also, a possible mechanism of histological changes may involve significant reduction of calbindin protein as a neuroprotective agent in neurons [11]. Also, previously Farber and Onley [9] suggested that opioids block the neuronal activity, causing the nerve cells to receive internal signals to commit suicide (apoptosis).

Conclusion

This study revealed that morphine administration before and during pregnancy can cause Purkinje cell loss and decrease of thickness of Purkinje and internal granular layers of the cerebellar cortex and size of Purkinje cells in neonatal mice.

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