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Effect of Maternal Morphine Sulfate Exposure on Neuronal Plasticity of Dentate Gyrus in Balb/c Mice Offspring

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Abstract: This study carried out to evaluate the effects of maternal morphine exposure during gestational and lactation period on the neuronal cells of dentate gyrus in 18 and 32 days Balb/c mice offspring. In this experimental study 10 female mice were randomly allocated into cases and controls. In experimental group, animals were received morphine sulfate 10 mg/kg/body weight intraperitoneally during 7 days before mating, gestational period (GD0-21), 18 and 32 days after delivery. The control animals were received an equivalent volume normal saline. Cerebrum of six infant for each group were removed and stained with cresyl violet and monoclonal anti-neuronal nuclei (NeuN) antibody. Quantitative computer-assisted morphometric study was done on dentate gyrus of hippocampus. In the P18 mice , the numbers of granular cells in dentate gyrus medial blade and dentate gyrus lateral blade significantly reduced from 171.45±4.2 and 174.51±3.1 cells in control group to 153.32±2.8 and 151.23±3.2 cells in 10000 μm^2 area of granular layer in treated group ($p<0.001$). In P32 mice the numbers of granular cells in mb and lb of dentate gyrus significantly decreased from 155.31±4.1 and 153.77±3.4 in control group to 138.33±4.5 and 135.13±4.3 in treated group, respectively ($p<0.001$). The granular layer thickness in mb and lb area of dentate gyrus significantly reduced in treated mice in compared to controls in P18 and P32 mice ($p<0.05$). This study revealed that morphine administration before, during pregnancy and lactation period causes neuronal cells loss of dentate gyrus in 18 and 32 days old infant mice.

Key words: Morphine sulfate, dentate gyrus, granular cells, prenatal exposure, Balb/c mouse

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INTRODUCTION

The number of opiate users has risen in worldwide particularly in young people (United Nations Office on Drugs and Crime, 2006; Nestler, 2004). It is estimated that 1 in 1000 people, have been exposed to opiates in early life in the United States (Zagon and MacLaughlin, 1992). Morphine (C17 H19 O3 N) is one of the strongest known analgesic compounds (Zhang *et al*., 2008) and as one of the addictive drugs leads to increase cause of death, morbidity and lost productivity (Nestler, 2004).

Several studies have shown that infants of opioid dependent mothers had several behavioral abnormalities including hyperactivity, lower Mental Development Index and Lower Motor Development Index (NIDA, 1996; Ornoy *et al*., 1996; Wilson *et al*., 1979).

In the other hands, several investigations are founded that morphine has toxic effects on neurons in brain and spinal cord in animal model (Mao *et al*., 2002; Atici *et al*., 2004; Turchan-Cholewo *et al*., 2006; Bekheet *et al*., 2010; Ghafari *et al*., 2011).

Also, prenatal morphine exposure impairs learning and memory in juvenile rats (Yang *et al*., 2003, 2006). In the other hand, synaptic plasticity in the hippocampus is critical for learning and memory processes (Morris *et al*., 1986; Morris 1989; Silva *et al*., 1992).

Hippocampus is implicated in the control of several brain functions such as memory and learning and represents a neuronal structure with a high degree of functional and cellular complexity.

Also, several researches have shown that the process of neurogenesis, including cell proliferation, survival, migration and differentiation continues in the dentate gyrus well into adulthood in rodents, non human primates as well as humans (Jackson-Guilford *et al*., 2000; Gould *et al*., 2000; Gould and Gross, 2002; Cameron and Gould, 1994; Gould and Tanapat, 1997). Several studies have shown the toxic effects of morphine on neuronal

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cells in different parts of CNS ether in adult or fetal period (Mao *et al*., 2002; Svensson *et al*., 2008; Seatriz and Hammer, 1993; Mei *et al*., 2009; Emeterio *et al*., 2006; Eisch *et al*., 2000; Niu *et al*., 2009).

Several studies have shown that opiates reduce hippocampal neurons (Svensson *et al*., 2008), sematosensory neurons of 6 day old (Seatriz and Hammer, 1993), neurons in layer II/III in lateral secondary visual cortex of rats (Mei *et al*., 2009), inhibits neurogenesis in the adult rat hippocampus (Eisch *et al*., 2000) and loss of GABA-containing neurons in the Dentate Gyrus (DG) area of rat offespring (Hauser *et al*., 1994).

Regarding to the high prevalence of opioid abuse in worldwide especially in young adult and rare study about the effect of morphine sulphate on neuronal development of dentate gyrus, the present study was carried out to clarify the neurotoxic effects of prenatal morphine sulphate administration on neuronal density of dentate gyrus in mice offspring.

MATERIALS AND METHODS

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

Experimental 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 h alternating light/dark cycle, 20-22°C temperature. Dry food pellets and water were provided *ad libitum*.

Drug: Each vial contained 1 mL of morphine sulphate (Darou Pakhsh Co., Iran) dissolved in 3.3 mL sterile saline solution (0.85%) to give 10 mg morphine sulphate dose intraperitoneally injected into mice.

Treated groups: After 2 weeks of acclimation to the diet and the environment, females were randomly divided into control and treated groups. Ten female mice in treated group received 10 mg kg^{-1} b.wt. of morphine sulphate intraperitoneally (IP) during 7 days before mating, gestational period (GD 0-21) 18 days after delivery in experimental group I and 32 days after delivery in experimental group II.

Twelve female mice in control groups received an equivalent volume normal saline intraperitoneally (IP) during 7 days before mating, gestational period (GD0-21) 18 days after delivery in experimental group 3 and 32 days after delivery in experimental group IV.

After parturition, in each group, six postnatal days 18 and 32 (P18, P32) were randomly selected and were scarified after anesthesia. The brain was exposed and fixed by immersion into the fixative solutions (10% neutral-buffered formalin). After techniques processing, brains sectioned at 6 micrometer thickness using a microtome (Microm HM 325 Germeny). The coronal sections (serial sections of anterior to posterior cerebrum) serially were selected according to anatomical landmarks corresponding to bregma -1.055 to -3.30 mm of the hippocampal formation with an interval of 24 μm between every two consecutive sections. The sections were used for immunohistochemistry. Adjacent sections stained with cresyl violet for morphometrical examination.

Immunohistochemistry: Immunocytochemical labeling to detect the neuronal marker was performed by monoclonal anti-neuronal nuclei (NeuN) antibody (Millipore corporation Billerica, MA 0 1821 USA) on 5 μm thick hippocampal coronal sections.

In brief, deparaffinized sections were preincubated with citrate buffer and were washed for 9 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and treated with 0.3% hydrogen peroxide in 0.01 M PBS including 10% methanol. The brain sections were preincubated with blocking reagent and washed in 0.01 M PBS. Then, brain sections were incubated with anti-NeuN antibody (1:100) in a humidified chamber for 1 h at room temperature.

After rinse in 0.01 MPBS, the sections were incubated with the biotinylated secondary for 10 min and then with Streptavidin HRP and rinsed in PBS. Immunoreactivity was visualized using DAB (chromogen reagent) for 30 min at room temperature. Subsequently, the tissue specimen were counterstained with Mayer's hematoxylin and mounted with entellan (Merck, USA).

Morphometric techniques: For histomorphometric study, the sections were observed under the light microscope.

In each postnatal mouse, ten similar sections of anterior to posterior of dentate gyrus were selected and images by Olympus BX 51 microscope and DP12 digital camera attached to OLYSIA autobioreport software (Olympus Optical, Co. LTD, Tokyo, Japan). The thickness of layers of dentate gyrus included moleculare layer (mo), granule cell layer (gc) and polymorph layer (pr) were obtained from 200X magnification (Fig. 1). The granular cells density evaluated through counting numbers of neuron per 10000 μm^2 area of both dentate gyrus medial blade (DGmb) and dentate gyrus lateral blade (DGlb) in 1000X magnification.

Statistical analysis: The significance of difference between the means value for the obtained data was calculated according to the Student's t-test using SPSS 11.5 software. A significance level of 0.05 was predetermined for all statistical analyses.

RESULTS

Granular cells density: In the P18 mice the numbers of NeuN positive neurons in Dentate Gyrus medial blade (DGmb) and Dentate Gyrus lateral blade (DGlb) significantly decreased from 171.45±4.2 and 174.51±3.1 cells in control group to 153.32±2.8 and 151.23±3.2 cells in 10000 μm^2 area of granular layer in treated group ($p<0.001$) (Fig. 1-5).

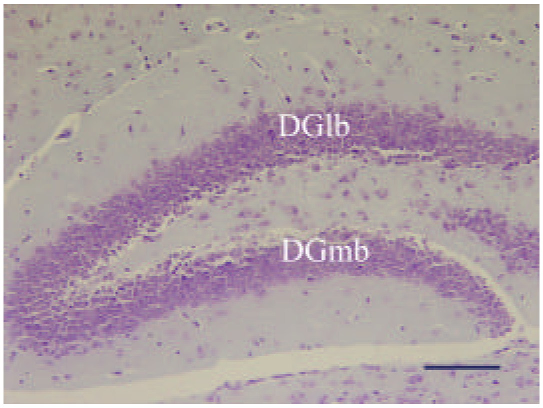

Fig. 1: Overview of dentate gyrus areas used for quantitative measurements from Balb/c mice (P18) control animal. Coronal sections stained with cresyl violet. Quantification areas are: DGmb, dentate gyrus medial blade; DGlb, dentate gyrus lateral blade (×20 magnification, Scale bar: 100 μm)

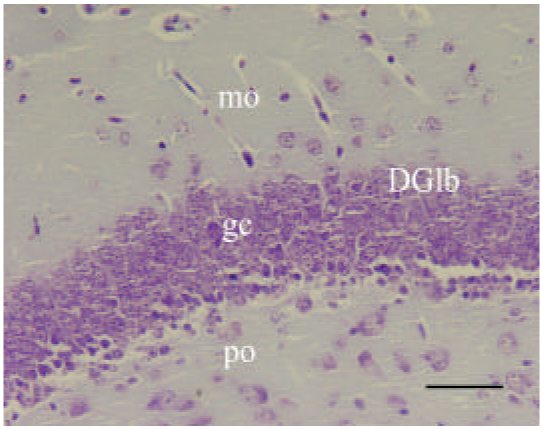

Fig. 2: Histological section of dentate gyrus in Balb/c mice (P18) control animal. Dentate gyrus lateral blade (DGlb) stained with cresyl violet (layers including: molecular layer (mo), granule cell layer (gc) and polymorph layer (po), ×400 magnification, Scale bar: 50 μm)

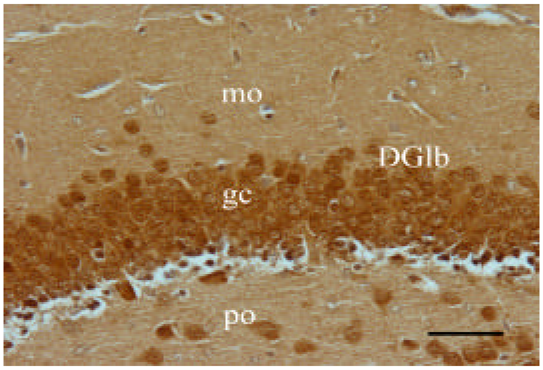

Fig. 3: Immunohistochemical staining for granular cells of dentate gyrus in Balb/c mice (P18) control animal. Neu N Immunopositive granular cells in granule cell layer of dentate gyrus lateral blade (DGlb). (layers including: molecular layer (mo), granule cell layer (gc) and polymorph layer (po), ×400 magnification, Scale bar: 50 μm)

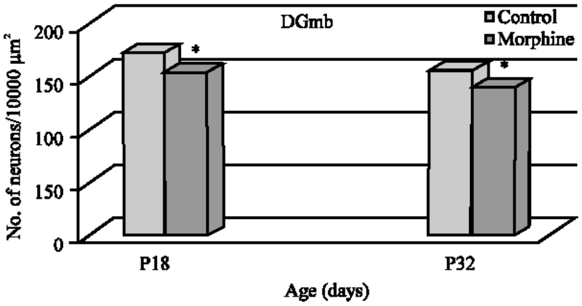

Fig. 4: The mean number of granular cells in dentate gyrus medial blade in P18 and P32 mice of control and morphine sulphate treated mothers. The cells were expressed as the number of granule cells per 10000 μm^2, (results are Means±SEM, *Compared with control animals $p<0.001$, $n = 6$)

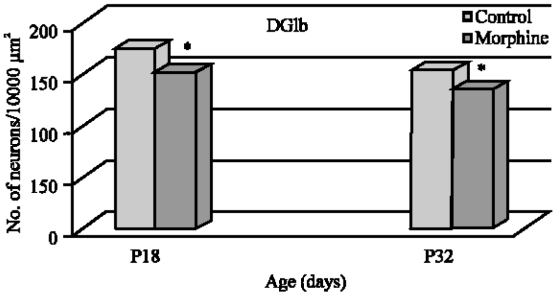

Fig. 5: The mean number of granular cells in dentate gyrus lateral blade in P18 and P32 mice of control and morphine sulphate treated mothers. The cells were expressed as the number of granule cells per 10000 μm^2, (results are Means±SEM, *Compared with control animals $p<0.001$, $n = 6$)

Table 1: Thickness of the various layers of dentate gyrus in postnatal day (P18, P32) of morphine sulphate and control mothers

		P18		P32	
	Hippocampal formation	Control	Morphine	Control	Morphine
DGmb	Moleculare layer (mo)	83.52±2.3	90.42±3.0*	92.12±2.2	107.28±3.8*
	Granule cell layer (gc)	64.50±1.4	55.16±1.7*	68.35±0.9	61.38±1.1*
	Polymorph layer (po)	45.67±4.2	36.02±3.3*	51.70±2.4	42.92±1.5*
DGlb	Moleculare layer (mo)	98.85±2.2	109.80±4.1*	112.18±2.1	125.54±2.4*
	Granule cell layer (gc)	67.49±1.6	59.08±1.2*	70.97±1.4	63.20±2.3*
	Polymorph layer (po)	41.38±1.8	36.40±1.4*	47.02±1.3	41.07±2.1*

Results are expressed as Mean±SEM of the mean (*compared with control group, $p<0.05$, n = 6)

In P32 mice the numbers of NeuN positive granular neurons in mb and lb of dentate gyrus significantly decreased from 155.31±4.1 and 153.77±3.4 cells in control group to 138.33±4.5 and 135.13±4.3 cells in treated group ($p<0.001$) (Fig. 1-5).

Thickness of dentate gyrus layers: The thickness of mb and lb of dentate gyrus layers in P18 and P32 mice in cases and controls offspring mice is depicted in Table 1.

The results revealed a significant reduction in the granular layer thickness in mb of dentate gyrus of treated mice (55.16±1.7, 61.38±1.1) in compared to controls (64.50±1.4, 68.35±0.9) in P18 and P32 mice ($p<0.05$), respectively.

Also, the mean thickness of granular layer significantly reduced in the treated group (59.08±1.2, 63.20±2.3) in comparison with control group (67.49±1.6, 70.97±1.4) in lb of dentate gyrus in the postnatal 18 and 32 day mice ($p<0.05$), respectively (Table 1).

DISCUSSION

The current study found that morphine sulfate administration before, during pregnancy cause granular cells loss and reduction of dentate gyrus layer in 18 and 32 days old infant mice. Also our study showed that toxic effect of morphine continues on granular cells of hippocampus even after lactation period.

Several studies have shown the toxic effects of morphine on neuronal cells in different parts of CNS either in adult or fetal period (Mao *et al*., 2002; Svensson *et al*., 2008; Seatriz and Hammer, 1993; Mei *et al*., 2009; Emeterio *et al*., 2006; Eisch *et al*., 2000; Niu *et al*., 2009).

Another study (Mao *et al*., 2002) reported that morphine induces apoptosis in rat's spinal cord neurons.

Besides, other study has shown that morphine reduces number of hippocampal neurons and concluded that morphine induces apoptosis in hippocampal neuronal cells of mice fetus in *in-vitro* model (Svensson *et al*., 2008).

Several studies have reported that morphine reduces the number of neurons in sematosensory of 6 day old rats an d in layer II/III in lateral secondary visual cortex of rats (Seatriz and Hammer, 1993; Mei *et al*., 2009).

Furthermore, Emeterio *et al*. (2006) by using Double-immunofluorescence staining for the neuronal marker Neu-N and active caspase-3 and TUNEL assay combined with immunocytochemistry for the glial marker GFAP have shown that chronic morphine administration induces apoptosis in neuronal and glial cells.

Bekheet *et al*. (2010) showed that morphine exposure (during gestational period) orally reduces both cortical thickness and the numbers of neurons in the developing fetal frontal cerebral cortex.

Also, it is found that the long-term administration of morphine sulphate in dams significantly reduces the Purkinje cells numbers of mice offespring (Ghafari *et al*., 2011).

Indeed, the two studies have shown the toxic effects of morphine exposure on neuronal cells in dentate gyrus in the adult rat and rats offspring (Eisch *et al*., 2000; Niu *et al*., 2009).

Niu *et al*. (2009) study have reported that prenatal morphine exposure impairs the juvenile offspring's dentate synaptic plasticity and spatial memory. They concluded that decreased GABAergic inhibition may play a role in these effects.

Furthermore, long-term exposure to opiates inhibits neurogenesis in the adult rat hippocampus. Also, chronic, but not acute, morphine decreases the number of BrdUrd-positive cells in the subgranular zone of the dentate gyrus (Eisch *et al*., 2000).

Regarding to the effects of morphine on central nervous system, several following possible mechanisms can be considered neuronal cell loss in morphine treated animals can be due to Apoptosis or necrosis (Hauser *et al*., 1994). Morphine increase both Ca^{2+} and production of carbonyl oxidation. Ca^{2+} and carbonyl oxidation produce neuronal apoptosis or necrosis (Hauser *et al*., 1998).

Also, morphine increased Bax and Caspase-3 and reduced Bcl in rats which are indicator of apoptosis in neurons (Mao *et al*., 2002).

Furthermore, DNA synthesis blocking in neuroblasts can be established by morphine which this precursors arrest the neuronal cell proliferation during embryonic period. Also, this study showed that morphine (*in vitro*) arrests the genesis of mouse cerebellar granule neuron precursor and subsequently neuronal death (Hauser *et al*., 2000).

Besides, several investigations have shown that acute opioids exposure can arrested proliferation, differentiation and survival of neuroblasts and astroglia (Hauser *et al*., 1987; Lorber *et al*., 1990; Schmahl *et al*., 1989; Hammer *et al*., 1989; Zagon and MacLaughlin, 1987).

Neurotoxic effects of opioids can be induced by NMDAR-Caspase pathway. NMDA receptors are suggested to play a critical role in morphine-induced apoptosis in the superficial spinal cord dorsal horn of tolerant rats (Mao *et al*., 2002).

Also, prolonged morphine administration induces up-regulation of proapoptotic proteins caspase-3 and Bax as well as down-regulation of antiapoptotic protein Bcl-2. The general caspase inhibitor and caspase-3-specific inhibitor prevent morphine neurotoxicity (Mao *et al*., 2002).

Furthermore, mitochondrial damage (Cheng, *et al*., 2003; Pretorius and Bornman, 2005; Jacob, 2007) and reduction of calbindin protein as a neuroprotective agent in neurons can be considered as possible mechanism for the neuronal cells loss in hippocampus (Garcia *et al*., 1996).

Indeed, it was suggested that opioids block the neuronal activity, causing the neurons to receive internal signals to commit suicide (apoptosis) (Farber and Onley, 2003).

CONCLUSION

This study determined that morphine administration before and during gestational and lactation period causes the neuronal cells loss and reduction of the granular layer thickness of dentate gyrus in 18 and 32 days infant mice. Also, it can be conclude that the neurotoxic effect of morphine will be continued even with disconnect of exposure.

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