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Maternal Oral Consumption of Morphine Increases Bax/Bcl-2 Ratio and Caspase 3 Activity During Early Neural System Development in Rat Embryos

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Abstract Maternal morphine consumption has been shown to result in physical and neurobehavioral defects in fetus and offspring, but the underlying molecular mechanisms of these defects remain unclear. Regarding the critical role of apoptosis in normal development of central nervous system, the present study was designed to investigate the effect of intrauterine morphine exposure on programmed cell death of neuroblasts during the early development of neural system. Pregnant Wistar rats received morphine sulfate through drinking water at the concentration of 0.01 mg/ml (20 ml water per day for each rat) from the first day of gestation to the time of sampling. Control groups received tap water. Control and morphine-treated pregnant rats, each in five separated groups, were killed on gestational days 9.5 to 13.5, and the embryos were taken out, fixed, and embedded in paraffin. Immunohistochemical assay was used to reveal the protein expression of Bax, Bcl2, and the activation of caspase 3. The results showed a significant increase in Bax immunoreactivity in all of the mentioned embryonic days (E9.5 to E13.5) and a significant decrease in Bcl-2 immunoreactivity at days E10.5 and E12.5 in morphine-treated groups compared with control. Data analysis revealed that Bax/Bcl2 ratio was increased in all of the morphine-exposed groups. Consistent with these results, immunostaining of cleaved caspase 3 showed a significant increase at days E11.5 to E13.5. These findings suggest that morphine exposure during the first embryonic days may enhance the susceptibility of neuroblasts to apoptosis by upregulating the ratio of Bax to Bcl-2 protein expression and increasing downstream caspase-3 activity. The increased probability of neuroblast apoptosis may be the cause of morphine-induced defects in the central nervous system development and its structural and neurobehavioral consequences.

Keywords Morphine · Prenatal exposure · Bax · Bcl-2 · Caspase 3 · Early neurulation

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Introduction

Morphine is one of the most common opiates used for medicinal (analgesics) and recreational (euphorics) purposes. Pregnant women are of higher importance among morphine abusers, since it has been demonstrated that morphine can easily transfer across the placenta, and its retention in placenta prolongs fetal exposure to morphine (Kopecky et al. 1999). Furthermore, the existence of opioid receptors of μ, κ, and δ types has been confirmed in fetal tissues and placenta villi (Ahmed et al. 1989; Leslie et al. 1998; Ray and Wadhwa 1999).

Human and animal studies show that prenatal exposure to opioids can increase both fetal or neonatal mortality or lead to a variety of short- and long-term physical and neurobehavioral consequences, yet the exact molecular mechanisms underlying these defects remain to be elucidated (Fujinaga and Mazze 1988; Hunt et al. 2008; Malanga and Kosofsky 1999; Vucinovic et al. 2008). Decreased birth weight, delayed embryonic development, and brain growth retardation are among the consequences of maternal morphine consumption (Raye et al. 1977;

Sobrian 1977; Zagon and McLaughlin 1977). Behavioral abnormalities such as hyperactivity, lower Mental Development Index, and lower Psychomotor Development Index in children of opioid-addicted mothers (Hans and Jeremy 2001; Weissman et al. 1999) may be due to defects in the development of central nervous system. We previously demonstrated that neural tube development and its closure would be delayed by prenatal oral morphine consumption (Nasiraei-Moghadam et al. 2005). In a recent study, it has been also shown that oral morphine consumption just during the second week of gestational period can significantly decrease the cortical thickness and the number of neurons in frontal cerebral cortex of rat fetus (Sadraie et al. 2008).

Impaired long-term potentiation and memory consolidation is another important consequence of prenatal exposure to morphine that is demonstrated in chick and rat (Che et al. 2005; Sarkaki et al. 2008). Recently, it has been shown that memory deficits in mice exposed to heroin during embryonic days 9–18 is due to the increased probability of apoptosis in hippocampus of offspring (Wang and Han 2009). A growing number of studies show that morphine can induce apoptosis in different situations (Hsiao et al. 2009; Li et al. 2009; Lin et al. 2009). In vitro studies have revealed that morphine can induce apoptosis in cultured neurons and microglia in a caspase-3-dependent manner (Hu et al. 2002; Svensson et al. 2008). Tolerance to the analgesic effect of morphine has been correlated to neuronal apoptosis in the superficial spinal cord dorsal horn (Mao et al. 2002). But more recently, it has been also shown that chronic application of morphine may down-regulate the proapoptotic factor Bax levels in cultured human and rat neurons and protect them from cell death (Chen et al. 2008; Cui et al. 2008).

Apoptotic cell death is a crucial physiological process that regulates the normal development of nervous system (Oppenheim 1991), but at the other end is involved in brain cell death accompanying aging (Muradian and Schachtschabel 2001), and is a pathologic feature of neurodegenerative diseases such as Alzheimer's disease and other inflammatory conditions of central nervous system such as ischemia and infections (Friedlander 2003; Lorz and Mehmet 2009). It has been revealed that, during the early organogenesis in normal rat embryonic tissue, Bcl-2 family proteins are involved in regulating apoptosis, and the balance between the important proapoptotic member, Bax, and the antiapoptotic member, Bcl-2, plays a crucial role in the occurrence of embryonic cell apoptosis (Sun et al. 2002). In addition, caspase 3 is one of the most important effector proteases in which its role in developmental and pathological cell death in fetal tissues has been approved (Mooney and Miller 2001; Weingärtner et al. 2008; Xiao and Zhang 2008).

Therefore, in this study, we assessed the effect of prenatal morphine exposure on the expression of apoptosis regulatory proteins during the early neurulation.

Materials and Methods

Animals, Experimental Groups, and Morphine Administration

Female Wistar rats, weighing 250–300 g, were obtained from Pasture Institute (Tehran, Iran). The female rats were housed with male Wistar rats overnight for approximately 12 h. The day after mating was considered as embryonic day 0.5 (E0.5). Observation of a sperm-positive vaginal plug confirmed the occurrence of pregnancy. The pregnant rats were separated and housed six per cage in a temperature/humidity-controlled facility, with 12-h dark/12-h light cycle and available ad lib food and water.

The pregnant rats randomly were assigned to one of two experimental groups (30 pregnant rats per group, each group then would be divided to five subgroups). One group received tap water as control, and the other received morphine sulfate in tap water. Maternal morphine administration was conducted as described previously (Nasiraei-Moghadam et al. 2005). Briefly, morphine sulfate (Temad, Iran) was dissolved at the concentration of 0.01 mg/ml in tap water, and approximately 20 ml of water was allotted to each rat. Morphine administration and control treatment were started from day E0.5 and continued daily to the time of sampling (days E9.5, E10.5, E11.5, E12.5, and E13.5, respectively, for five mentioned subgroups).

All experiments were conducted in accordance with standard ethical guidelines and approved by the local ethical committee (Neuroscience Research Center, Shahid Beheshti University of Medical Sciences).

Tissue Sampling

On each of the five mentioned days (E9.5, E10.5, E11.5, E12.5, and E13.5), the pregnant animals of the corresponding subgroups were anesthetized with chloroform; the uterus was externalized, and embryos were obtained by Caesarian section. For tissue slide preparation, embryos were washed with saline, fixed in 10% neutral buffered formalin for 24 h, and embedded in paraffin. Sections of the embryonic tissue (3–4-μm thickness) were prepared serially using a microtome rotary apparatus (Cut5062, Germany). Four sections were selected from each eight sections. The first section was stained by hematoxylin and eosin (H&E), and subsequent sections were immunostained as will be described. The sections were mounted on glass slides and studied by Digi3 software under light microscopy (Labomed USA) after appropriate staining.

H&E Staining

Morphology of the different slices was monitored using hematoxylin and eosin staining. H&E staining was done according to the common method described by Gamble and Wilson (2002).

Immunohistochemistry

Immunohistochemistry for Bax, Bcl-2, and cleaved caspase 3 were carried out on formalin-fixed, paraffin-embedded sections and according to the manufacturer's instructions provided for each antibody. Sections were deparaffinized and rehydrated. Antigen retrieval was carried out by microwaving in citrate buffer (pH 6) for 1 and 2×5 min for Bax and Bcl-2, respectively. Cleaved caspase 3 antigen unmasking was performed by microwaving in EDTA/Tris-buffered saline Tween-20 (TBST) for 10 min. The sections were quenched with 3% hydrogen peroxide (H_2O_2) in absolute methanol and blocked with 10% normal goat serum (NGS) + 1% bovine serum albumin in phosphate-buffered saline (PBS) for Bax and Bcl-2 and with 5% NGS+ in TBST for cleaved caspase 3. Primary antibodies were applied overnight at 4°C. These were either: Bax rabbit polyclonal antibody (abcam, 1/100), Bcl-2 rabbit polyclonal antibody (abcam, 1/100), and cleaved caspase 3 rabbit monoclonal antibody (Cell Signaling, 1:100). The sections were washed and then incubated with a ready-to-use antirabbit secondary antibody from Dako (EnVision Plus®), and color reaction was developed using diaminobenzidine as the chromagen. The slides were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted with Permount mounting medium (Entellan®, MERK).

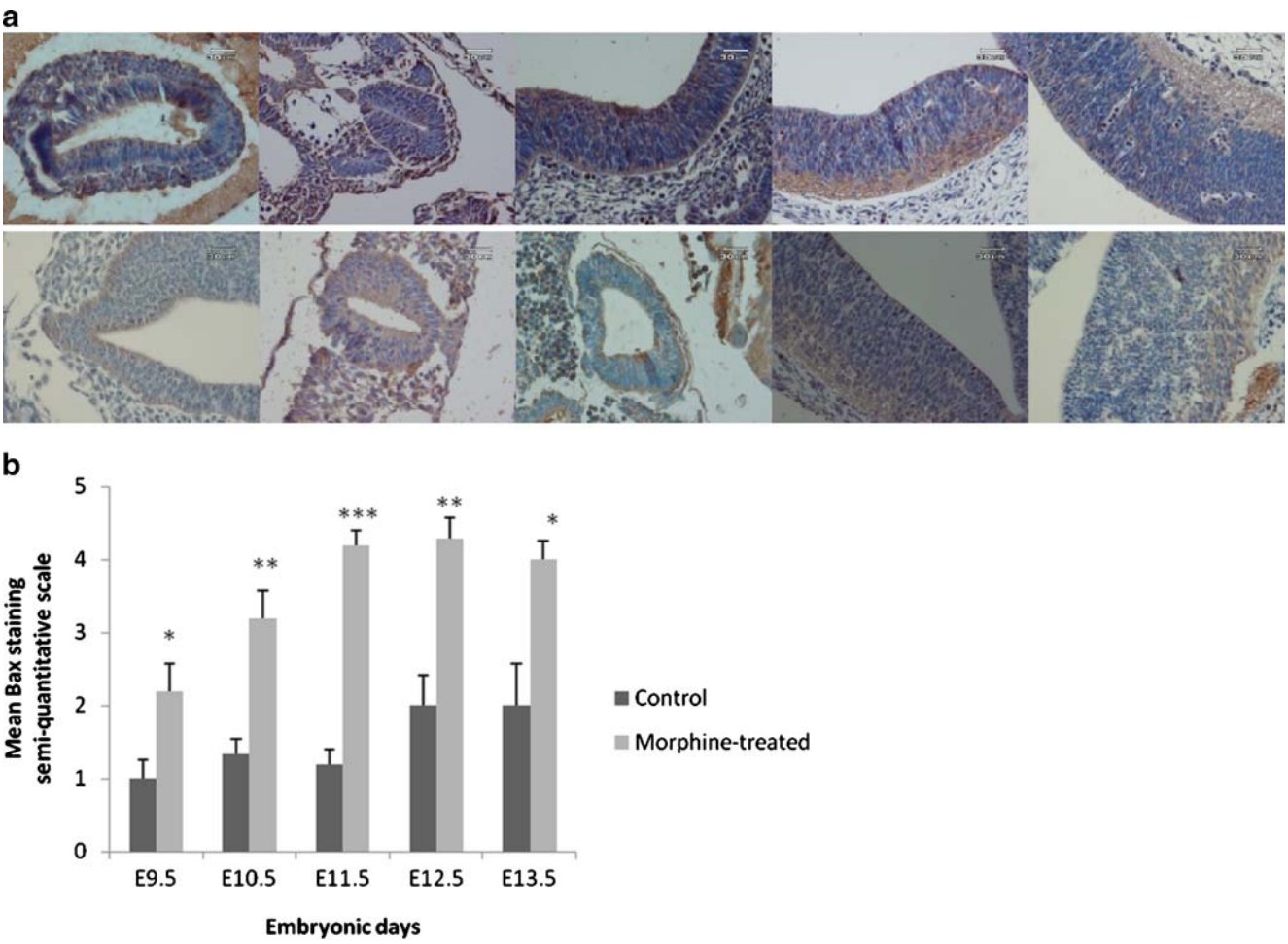

Figure 1 Effect of maternal morphine consumption on Bax protein expression in developing neural system of rat embryo. **a** Immunostaining of Bax in morphine-treated (*top panels*) and control (*bottom panels*) groups at different embryonic days (from *left* to *right*: E9.5, E10.5, E11.5, E12.5, and E13.5). **b** Semiquantitative data obtained from histological assay. Values are represented as mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with control

Sections serving as negative controls were incubated with the primary antibody diluents, PBS or TBST, instead of the primary antiserum.

Scoring and Statistical Analysis

The degree of Bax, Bcl-2, and caspase 3 immunostaining was assessed semiquantitatively by two blinded pathologist using a six-point scale where 0 = no staining and 5 = maximal staining within the experiment. The degree of immunostaining was scored for five high-power field (×40 objective) per section, and the average score was calculated for each section (Watson et al. 2001). Scoring was accomplished considering the presence of a section as negative control on each individual slide.

The ratio of Bax to Bcl-2 immunostaining was determined at each of the embryonic days of control and morphine-treated groups by using the mean scales of Bax and Bcl-2 in the same animal.

All of the data are presented as means ± SEM and analyzed by *t* tests or two-way analysis of variance as appropriate using the SPSS program. $P<0.05$ was considered statistically significant.

Results

Effects of Morphine on Bax and Bcl-2 Protein Levels

To determine whether morphine leads to changes in Bcl-2 family protein levels in the neural tube of developing rat embryo, we examined the Bcl-2 and Bax protein immunostaining. As shown in Fig. 1, Bax protein was minimally detected in different embryonic days in control animals.

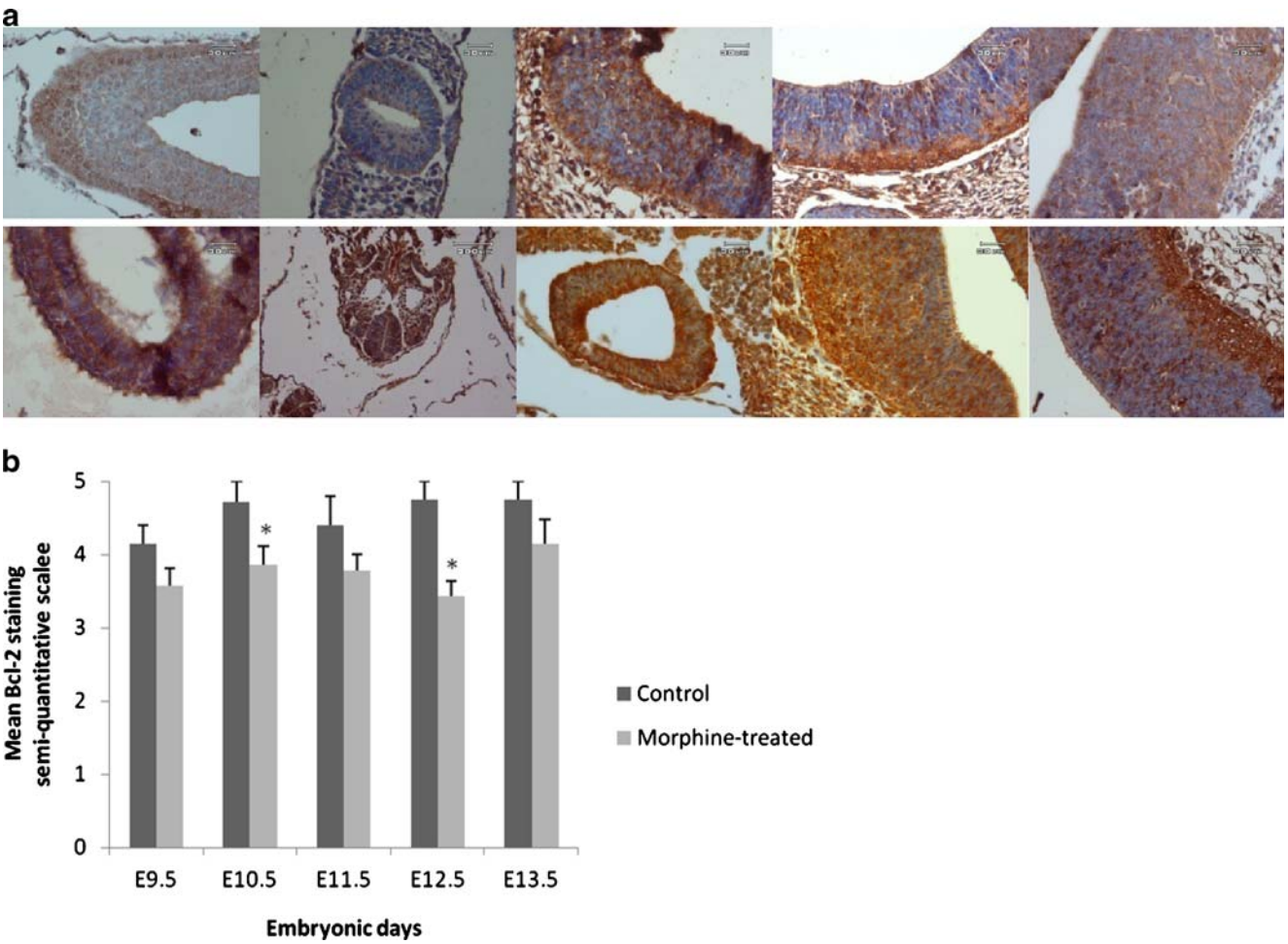

Figure 2 Effect of maternal morphine consumption on Bcl-2 protein expression in developing neural system of rat embryo. **a** Immunostaining of Bcl-2 in morphine-treated (*top panels*) and control (*bottom panels*) groups at different embryonic days (from *left* to *right*: E9.5, E10.5, E11.5, E12.5, and E13.5). **b** Semiquantitative data obtained from histological assay. Values are represented as mean ± SEM. *$P<$ 0.05, compared with control

However, in morphine-treated animals, Bax immunostaining significantly increased in all of the considered embryonic days ($F_{1,\ 43}$=108.004, P<0.001). In contrast with Bax, Bcl-2 staining showed a vigorous expression of this antiapoptotic protein in all of the five examined embryonic days in control group, and this refers to the constitutive expression of Bcl-2 protein in normal conditions, as shown in Fig. 2. Morphine exposure slightly decreased Bcl-2 immunostaining, and this decrement was statistically significant at E10.5 and E12.5 days (Fig. 2, $F_{1,59}$=18.691, P<0.05). The Bax to Bcl-2 ratio was calculated for each embryonic tissue as explained above. In control tissues, this ratio was constant over different embryonic days. But as shown in Fig. 3, morphine treatment significantly increased this ratio ($F_{1,\ 38}$=74.169, P<0.001). In addition, there was a significant main effect of the embryonic days on increment of Bax/Bcl-2 ratio in morphine-treated groups ($F_{4,\ 38}$=3.976, P<0.01), as raising the ratio at E11.5, E12.5, and E13.5 are significantly more than the first day of early neural system development, E9.5.

Effects of Morphine on Activated Caspase 3 Immunostaining

To confirm whether the increase of Bax/Bcl-2 ratio does lead to the activation of apoptotic cascade, we analyzed the immunostaining of cleaved caspase 3 in all of the experimental groups. Rabbit monoclonal antibody used in this study detects cleaved caspase 3, the large active fragment that is derived from inactive full-length caspase 3. As shown in Fig. 4, the degree of cleaved caspase 3 immunostaining was low and rather constant through different embryonic days of control groups. But as expected according to the raised Bax/Bcl-2 ratio in morphine-treated groups, active caspase 3 immunoreactivity significantly increased as a result of morphine consumption ($F_{1,\ 59}$= 46.572, P<0.001) at embryonic days E11.5 to E13.5.

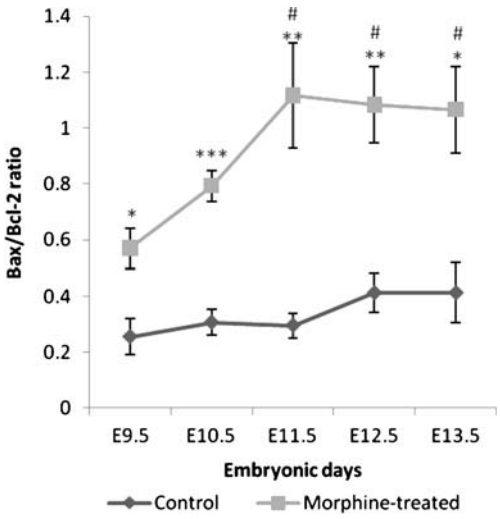

Figure 3 Effect of maternal morphine consumption on the ratio of Bax to Bcl-2 protein expression in developing neural system of rat embryo. Values are represented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, compared with control. $^{\#}P$<0.05, compared with E9.5

Discussion

There is a growing body of evidence that implicates that the intrauterine exposure to a variety of drugs including prescribed agents or drugs of abuse is associated with an increased risk of developmental abnormalities and long-term physiological, psychological, and behavioral defects (Berman et al. 2008; Chiriboga et al. 2007; Kyle 2006). Neurobehavioral and neuropsychological difficulties in offspring exposed to opioids such as morphine and heroin as a result of maternal drug abuse have been approved, but the causal effects, mechanisms of injury, and the molecular pathways have not been well documented (Hunt et al. 2008; Vucinovic et al. 2008).

We previously showed that maternal oral consumption of morphine at the dose of 0.01 mg/ml and approximately daily amount of 20 ml for each pregnant rat could significantly reduce the weight and length of the embryo and delay the closure of neural tube. The dose of 0.01 mg/ml morphine more effective than those of 0.1 and 0.05 mg/ml delayed the development of neural tube and even disrupted the neuro-ectoderm layer morphologically (Nasiraei-Moghadam et al. 2005). In another histomorphometric evaluation of developing rat fetus, it has been revealed that the thickness of cortical plate and the number of neurons in the frontal cerebral cortex would be decreased by prenatal exposure to morphine (Sadraie et al. 2008).

In the present study, we immunohistochemically investigated the molecular response of neuroblast cells during neurulation to morphine exposure from the beginning of embryogenesis. The results showed that the expression of apoptosis regulatory proteins, Bax and Bcl-2, would be altered by morphine (Figs. 1 and 2). Morphine changed the Bax/Bcl-2 ratio in favor of apoptosis in embryonic days E9.5 to E13.5 (Fig. 3) that may be considered as early neurulation stage of rat embryo. We showed that the cleavage of caspase 3 and activity of this effector of programmed cell death was increased by morphine, consistent with the elevation of Bax/Bcl2 ratio (Fig. 4).

Normal apoptosis occurs in the central nervous system from the embryonic stages through senescence (Sun et al. 2002; White and Barone 2001). Formation of the neural tube, the precursor of the entire central nervous system, through the process of neurulation is a critical event in embryogenesis. During nervous system development, programmed cell death in conjunction with cell proliferation is critical in pattern formation and shaping a flat sheet

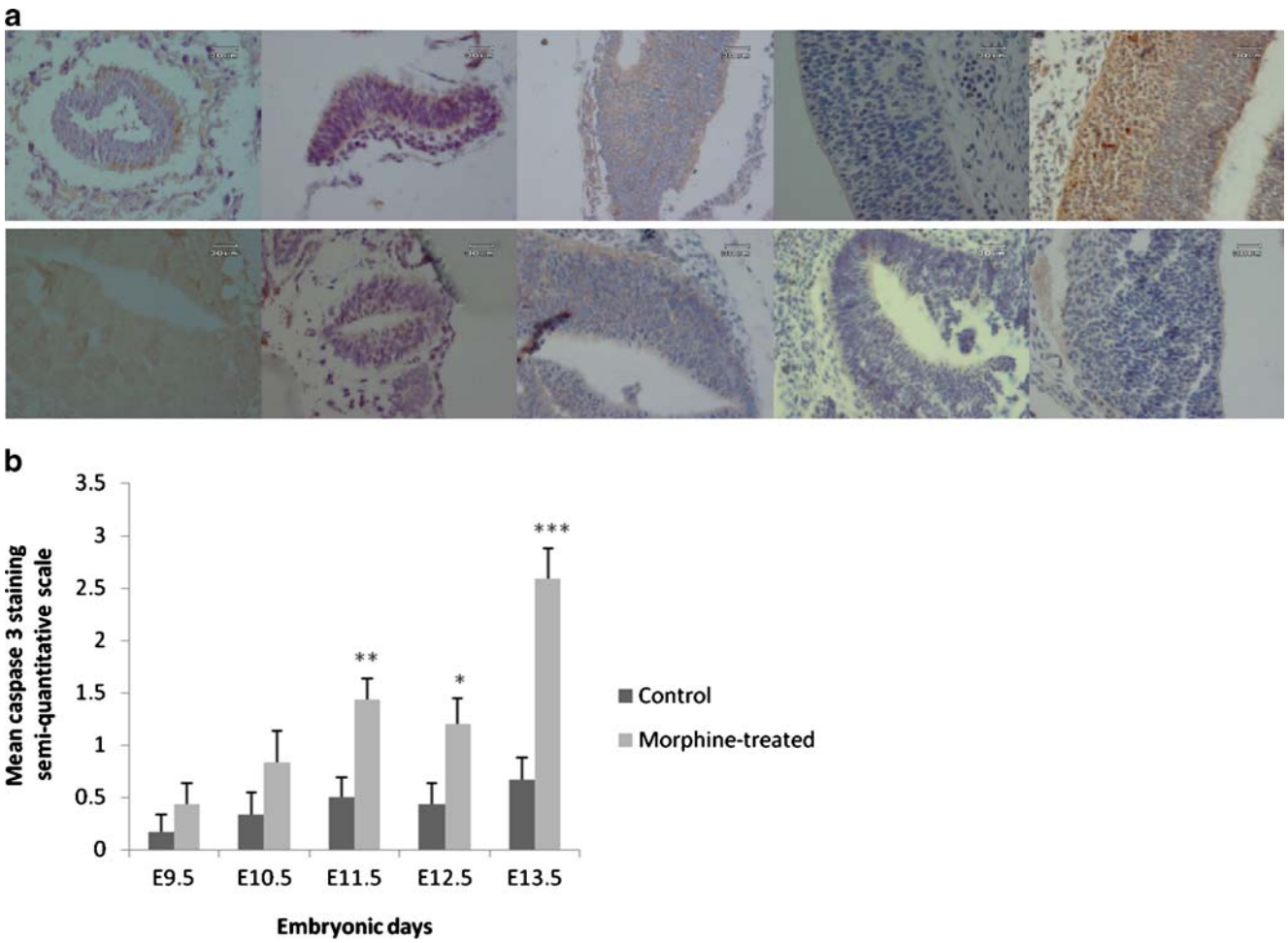

Figure 4 Effect of maternal morphine consumption on caspase 3 activity in developing neural system of rat embryo. **a** Immunostaining of cleaved caspase 3 in morphine-treated (*top panels*) and control (*bottom panels*) groups at different embryonic days (from *left* to *right*: E9.5, E10.5, E11.5, E12.5, and E13.5). **b** Semiquantitative data obtained from histological assay. Values are represented as mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with control

of cells into the neural tube (Copp 2005; Stern 2002). These processes are mediated by genetic and epigenetic signaling and lead to diverse and segmented structures of the nervous system (Barrow et al. 2000; Nott and Riccio 2009). It has been estimated that approximately half of all neurons produced during fetal development will die, some during the fetal period in mainly proliferative zones and some in postmitotic cells (Blaschke et al. 1996). Therefore, both neural proliferation and programmed cell death determine the cell number of a specified neural structure (de la Rosa and de Pablo 2000).

It has been approved that disturbances in nonrandomly occurring and tightly controlled spatiotemporal pattern of programmed cell death during development can lead to some neurological diseases. Down syndrome is one example of such defects in which, due to the increased apoptosis in early development, brain cell number is decreased, and adult individuals usually exhibit an Alzheimer's like dementia (Seidl et al. 2001). In addition to genetic causes, perturbations in developmental apoptosis may be due to neurotoxicant exposure. It has been shown that nicotine exposure, beginning on embryonic day 9.5, evokes neuroepithelium cell death during neurulation of embryonic rat brain (Roy et al. 1998). Other investigators suggest that prenatal exposure to ethanol can affect the early postnatal expression of death-related proteins in the rat cortex (Mooney and Miller 2001). So it is no wonder that the increased apoptosis of neuronal cells play a key role in learning and other behavioral disabilities resulting from maternal abuse of such drugs.

Naturally occurring developmental cell death largely exhibits morphological features of apoptosis (Sun et al. 2002). A key factor determining cell death or survival following apoptotic signals is the relative expression of Bax

and Bcl-2 proteins. The interactions between these proapoptotic and antiapoptotic proteins regulate the release of cytochrome c and propagation of apoptotic cascade (Deveraux et al. 2001; Korsmeyer et al. 1993; Oltvai et al. 1993). Caspase 3 is considered to be the main executioner caspase involved in apoptotic cell death (Kumar 2007). The role of these apoptotic proteins in adjusting the number of neural precursors and postmitotic neurons during the development of nervous system has been established (Krajewska et al. 2002; Mooney and Miller 2000; Pompeiano et al. 2000; Sun et al. 2002), but it is noticeable that activation of caspase 3 alone may not provide a reliable evidence for the occurrence of apoptosis (Racke et al. 2002).

Therefore, regarding the role of increased Bax/Bcl-2 ratio in initiating apoptotic cell death and activated caspase 3 as a requirement for apoptosis, the results of the present study emphasize that prenatal morphine exposure may increase susceptibility to apoptosis at day E11.5 to E13.5.

Such intracellular events and increased probability of cell death at the earliest stage of neural system development may be the basis of long-term deleterious effects of maternal morphine consumption on offsprings. Mechanisms of a similar kind have been proposed for some of the other drugs of abuse. Cocaine-induced neuronal defects and abnormal development are correlated to upregulation of the Bax/Bcl-2 ratio, activation of caspase 3, caspase 8, and caspase 9, and expression of apoptotic nuclei in fetal rat brain (Xiao and Zhang 2008). Other investigators have revealed that fetal alcohol exposure can lead to increased expression of cyclin D1, premature S-phase entry, disjointed DNA synthesis, and apoptotic events during early neurulation of cultured mouse embryos. They proposed this abnormal cell cycle regulation as a mechanism of growth deficits and neurological abnormalities induced by alcohol (Anthony et al. 2008).

Some studies introduce apoptosis as a requirement for neurulation and show that inhibition of caspase activity may prevent neural tube closure (Selçuki et al. 2008; Weil et al. 1997). While it has been also shown that excessive apoptosis is associated with failure of neural tube closure (Copp 2005; Ikeda et al. 2001). We previously reported that morphine exposure would delay the closure of neural tube. According to the results of this study, at embryonic day 9.5, the time of neural tube closure, we merely observed a significant increase in Bax/Bcl-2 ratio without any considerable change in caspase 3 activity. Therefore, it seems that delayed neural tube closure in morphine-exposed embryos is not due to excessive apoptosis and just the early biochemical changes involved in apoptotic cell death may be the causal effects. In a recent study, using genetic and pharmacologic models for inhibition of apoptosis showed that normally occurring apoptosis is not required for neural tube closure in the mouse embryo (Massa et al. 2009). But the disturbing role of enhanced apoptosis as well as related molecular context remains to be more investigated.

In addition to neural tube defects, increased apoptosis during early neurulation probably leads to decreased neural counts in affected areas. Some investigators have demonstrated that prenatal treatment with morphine decreases the number of neurons in ventrolateral caudate putamen and nucleus accumbens of neonates and also in cortex of developing fetal brain (Sadraie et al. 2008; Tempel et al. 1995).

Since prolonged intrauterine morphine exposure may cause fetal and placental vasoconstriction (Collins et al. 2005), it seems that detrimental effects of morphine are partly due to provoked oxidative stress in placental tissue. It has been demonstrated that hypoxia can stimulate apoptosis in placental tissues, and increased level of placental apoptosis may lead to fetal growth restriction (Chen and Dai 2008; Gude et al. 2000; Myatt and Cui 2004). Transfer of morphine across the placenta and direct effect on developing embryo would be the more important part of its detrimental role, however.

In conclusion, our study has demonstrated that maternal consumption of morphine may alter the expression of apoptosis regulatory proteins during early neural development. We showed that, due to intrauterine exposure to morphine from the beginning of the embryogenesis, the relative expression of Bax and Bcl-2 would be changed in favor of apoptosis. The finding of increased caspase 3 activity re-enforced the conclusion that morphine may trigger the molecular cascade leading to apoptosis in developing neural tube. Considering the important role of apoptosis in normal nervous system development, it is likely that the increased susceptibility to apoptosis play a key role in morphine-induced embryonic and fetal developmental abnormalities. Ultimately, such defects may be the cause of long-term neurobehavioral defects in offspring of morphine-treated mothers.

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