Identification of Morphine Accumulation in the Rat Embryo Central Nervous System: A C14-Morphine Administration Study

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Original Article

ABSTRACT

Background: Previous studies have shown that morphine consumption during pregnancy may cause delay or defect of embryo development or abnormal nervous system function in the human and animal models. In the present study, the highest density of morphine accumulation in the central nervous system of rat embryos was evaluated using C14-morphine.

Methods: Female Wistar rats (W 170-200 g) used and were crossed with male rats and coupling time was recorded (Embryonic day 0-E0). Experimental groups received 0.05 mg/ml of C14-morphine in drinking water daily. On the 10th and 17th days of pregnancy, pregnant rats were anesthetized and the embryos with these uterus and placenta were surgically removed and were fixed in formalin 10% for 4 week. Then the embryos were processed, sectioned in 25 μm and 5 μm thicknesses, fixed on the glasses for further evaluations. The sectioned in 25, the glasses were fixed on the Blanc black and white film for 6 h. Then, the films were appeared and their negatives were prepared. The sectioned in five staining hematoxylin and eosin by light microscope and MOTIC software.

Results: Our results indicated that the highest C14-morphine accumulation was observed in the vesicles and the ventricular choroid plexus (CP) of (E17) embryos, whereas, in the (E10) embryos. Highest concentration was observed in the brain vesicles and the ventricular CP. In addition, this study showed the surface area of lateral, 3rd and 4th ventricular CP in the experimental groups were increased in compared to control groups.

Conclusions: Our results indicated that effects of morphine on reduction of embryos brain development may be due to the highest accumulation of C14-morphine in the CP and brain vesicles.

Keywords: C14-morphine, choroid plexus, embryo development, rat, vesicle brain

INTRODUCTION

Addiction and opiate dependency in humans is growing on. According to the previous studies addictive drugs consumer is

exposed to the risks of opiates. Mothers are one of the most important narcotic consumers that addiction side-effects will involve them and the next generation. Studies have shown that natural development of placental layers may be delayed in morphine-dependent pregnant mice.[1,2] The fetus of mothers taking drugs is also vulnerable. Many motion and behavioral problems have been reported among newborn children born to opiate-addicted mothers.[1,3] Expansion of drug consumers as well as increased abnormalities in addicted mothers' fetuses show the necessity of research in the field of addictive drugs function in the body of organisms. Previous studies have proved that opiates due to delay in development of placenta and embryo, but there is no research where the maximum density of drugs is. To conduct this research and determine the site of morphine concentration a labeled substance is required, so C14-morphine was used for this study. Based on previous studies oral administration of morphine can pass the placental barrier and have destructive effects on development of defects in fetal visual system and cerebral cavities in rats and also cerebellum in mice.[4-6] Central nervous system (CNS) is a body organizer and any defects in its development and function may cause disorder in other systems. Several studies have indicated the presence of opioid receptors on placental villi and nerve cells. The effects of morphine are mediated by mu, kappa, and delta opioid receptors. The activation of these receptors leads to reduction in cyclic adenosine monophosphate, increment of potassium ions exit and decrease the calcium ions entry to the cell and calcium reduction prevent from development.[7-9] Stimulation of opioid receptors with morphine causes placental vasoconstriction and resulting in reduced blood-flow through the fetus and hypoxia.[10-12] Opiate drugs administration during pregnancy leads to retardation of growth and development, decreased birth weight, and neural defects such as Spina bifida and Meningocele.[13-15] On the other hand, delay in development of cavities and central canal of the CNS leads to dysfunction of choroid plexus (CP) and impaired cerebrospinal fluid (CSF) secretion and thus, can cause abnormalities such as hydrocephalus.[16,17] CP is an important source of nutrition for nerve cells. The main functions of CP are to provide blood for brain cells and also absorption and secretion of CSF by Ependyma cells.[18,19] Ependyma cells with abundant blood cells and vascular network form the blood-brain barrier. They are also thought to act as neural stem cells in spinal cord injuries.[20,21] Regarding to microscopic studies the structure of CP has evolved in mammals, for example there are lots of villi on epithelial cells (ependyma cells), which increase the surface of absorption and secretion. Studies also have demonstrated that blood supply in CP of rats is 10 times more than cerebral cortex[17,21] and any disruption in absorption and secretion of CP cells and also the secretion of CSF causes malformation of nervous system.[15,16] Opiate drugs abuse in society is very important and the abnormalities resulting from addiction are not only subject pregnant mother it but also offspring will be involved. Furthermore, pregnant mother is very sensitive to external factors such as morphine and destructive effects of addiction during pregnancy have been associated with fetal development retardation.[15] Healthy fetus should have healthy organizer system (brain), which is required for a healthy mother. Regarding to these important issues were mentioned above, the present research was conducted. In this study, a labeled substance (radioactive morphine) was used to determine the site of highest density absorption of C14-morphine. Highest density absorption of C14-morphine was evaluated in two stages of fetal development. In the first group, the development of cerebral cavities' blood vessels in 10-day-old Wistar rat embryos and in the second group the development of cerebral cavities' blood plexus in 17-day-old Wistar rat embryos were investigated.

METHODS

In this research, female Wistar rat with average weight equal to 170-200 g was used. Every two rats were located in the same cage and the environmental temperature was 24°C ± 1°C with natural light period (12 h light/dark). Sufficient food and water were available for rats during experiment. Rats maintained in animal house in Baqyiatallah Medical University. Animal experiments carried out according to ethical issue.

In this study, radio labeled morphine sulfate (C14) from Pasture Institute Tehran, Iran, was used. The rats divided into two groups. For each groups six rats were used. A total of 24 female rats in dual

groups copulated with an adult male rat. After making sure about pregnancy (with the observation of vaginal plug and existence of sperm in vagina), they were separated from male rats the next morning and kept in the same dual-groups. Thereafter (0 day of pregnancy), experimental group received a daily dose of 0.05 mg/ml (5 mg radioactive morphine in 1000 ml potable water from city pipeline for six rats). The amount of consumed morphine in 10 ml water for every 100 g of rat's weight was computed but attempts were made to make available the amount which the animal needed. In 10th and 17th days of pregnancy the rats were anesthetized by Chloroform and the embryos and uteruses separated from the mother rats and transmitted to the 10% formalin solution for one week and changed the solution. After this phase, embryos separated from the uterus's end meter and then the embryos were put in the tissue processing machine and were prepared for the molding. For molding the embryos' heads were separated from the trunks and were put in the paraffin. Then the sectioning phases of the blocks were carried out by microtome, the sections were provided in two types of section included sagittal section (for embryos 10-day) and frontal section (for embryos 17-day) in the thickness of 5 μm and 25 μm thicknesses as serial and then fixed on the glasses for further evaluations. The 25 μm sections were stocked on wooden sheets (30 cm long and 8 cm wide). The slides were covered by photographic strips (black and white photography) and incubated on dark room for 3 days. After this stage, they were transferred to photographic archives to prepared negative. These films were assayed after emergence. At first photos were prepared with black and white negative and then in order to have better and clearer interpretation (position of C14-morphine) photos with color negative was prepared. Photograph of sections by microscope Equipped with camera (Dino Capture, 50×). The prepared slides from 5 μm sections were stained by hematoxylin and eosin (H and E) method. The samples were examined by light microscope and the surface area of lateral, 3rd and 4th ventricular CP.

In the experimental and control groups were measured by MOTIC software.[22]

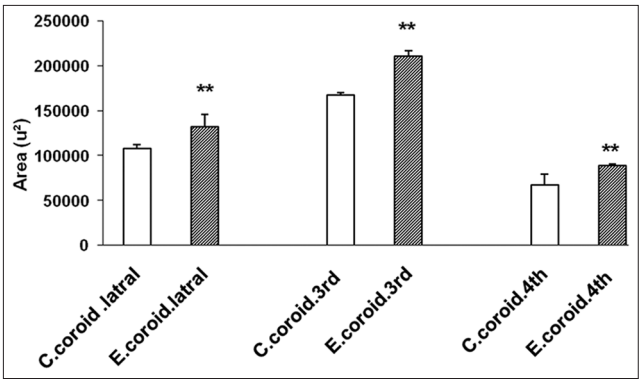
Statistical analysis

Results were reported as mean ± SEM. Differences between group means were calculated by a one-way analysis of variance (ANOVA) and *post-hoc* Duncan test using used the SPSS computer program (version 11.0). Statistical significance between two measurements was determined by the two-tailed unpaired sample *T*-Test. Result were considered statistically significant when $P < 0.05$.

The CP ventricles areas of embryos brain were compared in experimental and control groups. Tissues were measured by MOTIC software. The used system includes of microscope that is connected to a computer and monitor by a software and is able to take a photo from slides. Subsequently, a number of cells on each layer were counted and compared with the experimental groups.[1]

RESULTS

Morphological results of this study on brain tissue of 10-day-old of rats' embryos by using C14-morphine in 25 μm sections, the highest density absorption of C14-morphine on opioid receptors of blood cells endothelium membrane in brain vesicle in experimental groups indicated [Figure 1]. Study of the 5 μm sections prepared to H and E method showed that CP surface of hindbrain in 10 day embryos that their mothers had consumption of morphine in during pregnancy (experimental group) significantly increased in compared to control groups [Figure 2 and Graph 1]. Furthermore, assessment of



Graph 1: Comparison of oral morphine effect on of the choroid plexus (CP) ventricles brain embryos. The effect of oral morphine consumption in the CP 4th ventricle area in 10-day of pregnant rats and the CP lateral and 3rd ventricles area in 17-day of pregnant rats. The data have stated as mean ± SEM. The numbers of samples in each group have been 6. (**$P < 0.01$ are the indexes of the signification of lateral, 3rd and 4th CP in experimental [E] in comparison with control [C] group)

sections with 25 µm thickness of 17-day-old of rats embryos brain tissues by using C14-morphine the highest absorption of labeled morphine on opioid receptors of blood cells endothelium membrane in blood plexus of brain ventricles in experimental groups indicated [Figure 3]. The results achieved from the comparison are 5 µm sections prepared to H and E staining indicated that CP surface of Midbrain and Forebrain in experimental groups significantly increased in compared to control groups [Figures 4-6 and Graph 1].

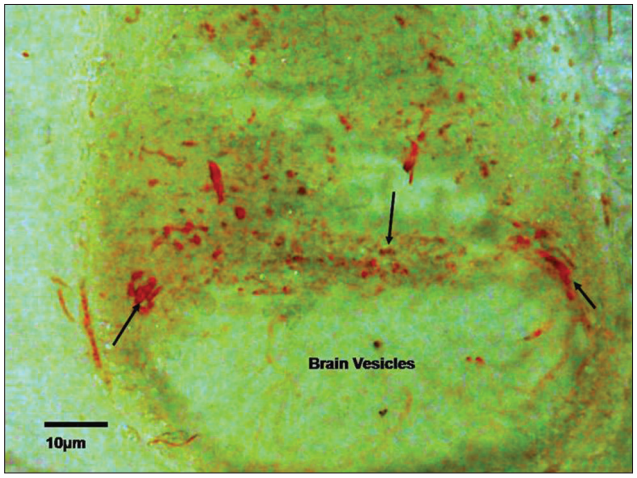


Figure 1: The effect of oral C14-morphine consumption in blood tissue of the vesicles brain embryos. The effect of oral C14-morphine consumption on 10 day old of the brain vesicles embryos. Microscopic observations indicated that the highest density absorption C14-morphine on blood vascular of brain vesicles. (Imaged by Dino Capture, ×50)

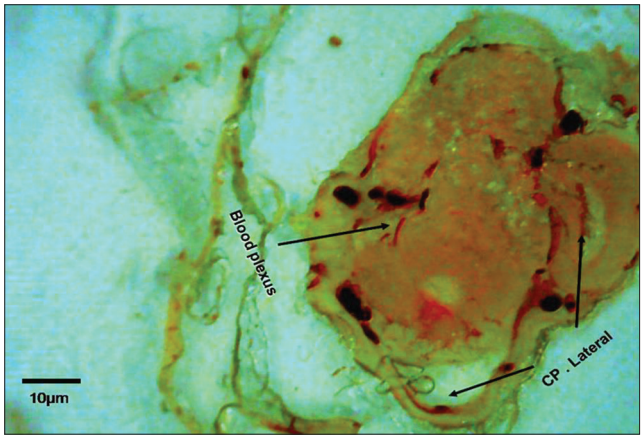


Figure 3: The effect of oral C14-morphine consumption in blood tissue of the brain choroid plexus (CP) embryos. The effect of oral C14-morphine consumption on 17 day old of the CP of (lateral ventricle [CP lateral], blood plexus) embryos. Microscopic observations indicated that the highest density absorption C14-morphine on blood vascular of the CP brain. (Imaged by Dino Capture, ×50)

DISCUSSION

Previous studies have confirmed the presence of mu, kappa, and sigma opioid receptors on the membrane of nerve cells and on the placental

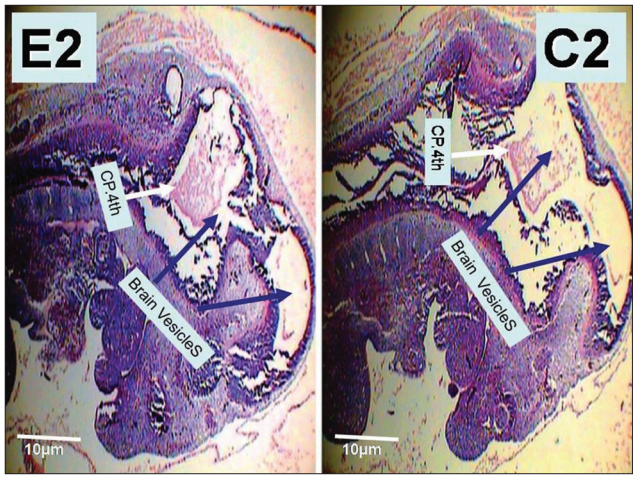


Figure 2: The effect of oral morphine consumption in choroid plexus (CP) of the brain ventricle 4th embryos. Comparison of oral morphine effect on of the experimental group (E2) and control group (C2) on 10 day old of the brain vesicles embryos. Sagital section and H and E, staining (×40). Consider to the changes of areas on CP of 4th ventricle

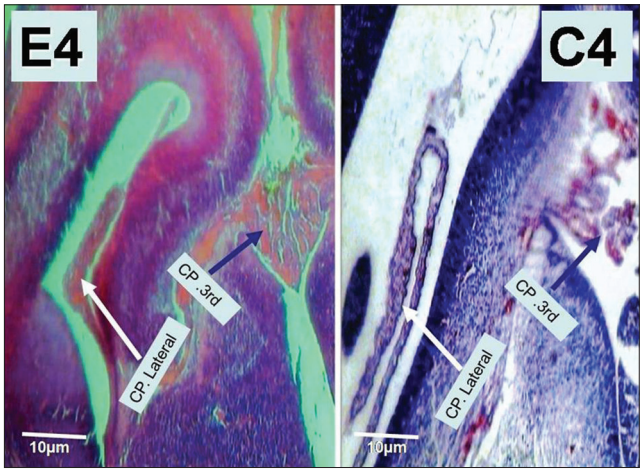


Figure 4: The effect of oral morphine consumption in choroid plexus (CP) of the brain ventricle lateral embryos. Comparison of oral morphine effect on of the experimental group (E4) and control group (C4) on 17 day old of the CP ventricles embryos. Frontal section and H and E, staining (×100). Consider to the changes of areas on CP 3rd ventricle and CP lateral ventricle

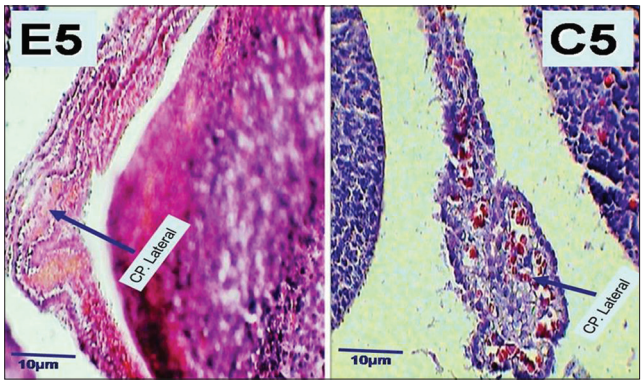


Figure 5: The effect of oral morphine consumption in choroid plexus (CP) of the brain ventricle lateral embryos. Comparison of oral morphine effect on of the experimental group (E5) and control group (C5) on 17 day old of the CP lateral ventricle embryos. Frontal section and H and E, staining (×400). Consider to the changes of areas on CP of lateral ventricle

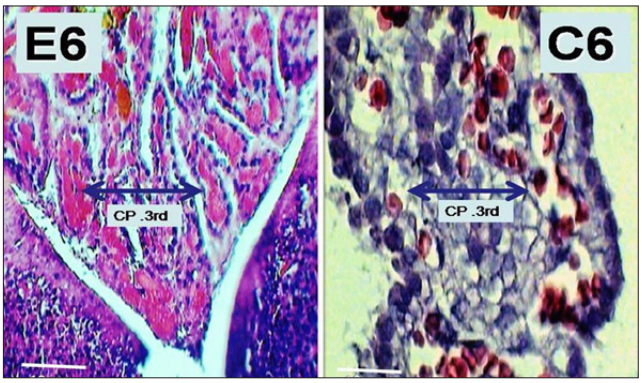


Figure 6: The effect of oral morphine consumption in choroid plexus (CP) of the brain ventricle 3rd embryos. Comparison of oral morphine effect on of the experimental group (E6) and control group (C6) on 17 day old of the CP 3rd ventricle embryos. Frontal section and H and E, staining (×1000). Consider to the changes of on CP 3rd ventricle

villi.[8,9,12] The present study, aimed at determining the site of highest density absorption of morphine on the brain tissues with the thickness of 25 µm in 10 day embryos by a C14-morphine [Figure 1]. It was shown that the highest density site of morphine was around cerebral cavities, especially, places with more blood cells. Using the present findings, it can be estimated that the density of protein opioid receptors (mu, kappa, and sigma) is higher on the vascular endothelial membrane of blood cells; thus, morphine density is also higher in these places. As a result, destructive effects of morphine such as neural abnormalities of the infants born to addicted mothers are noticeable.[23,24] Considering that cerebral cavities of rat embryo develop on the 10th day of pregnancy and according to the findings of this study, the dense place of the morphine was around vesicle brain on blood cells, this investigation made an attempt to complete these findings. Therefore, 5 µm sections were made of the cerebral cavities of a ten-day-old rat embryo and were microscopically examined using H and E staining. These results indicated the increased blood plexus surface of the hindbrain in the brain tissues of 10-day embryos in the experimental group compared with the control one [Graph 1]. These results were in line with the findings of the C14-morphine that the highest density of the morphine is on the opioid receptors of blood vessels. Then, it can be concluded that, when the highest density of morphine is on blood cells, it can lead to nutritional deficiency of nerve cells via the dysfunction of blood cells; consequently, this may make a delay in the normal function and development of nerve cells and create neural defects in the CNS of the embryos of addicted mothers.[15,23,24] One of the important neural disorders is changes in the secretion of CSF. Since, the main source of CSF secretion is supplied by CP, any disorder in the normal function in terms of increase or decrease of CSF causes irreparable defects.[16-18] Various factors such as drugs cause disorders in the secretive function of CP. Previous studies have demonstrated that opiates (morphine) have an inhibitory effect on the development of CP.[14,15,23] Many investigations have been carried out on the destructive effect of opiate on the development of nervous system; however, the site of highest density of these materials on the nervous system has not been identified.[3-6] The C14-morphine was used in this investigation to demonstrate the place with the highest density absorption of the morphine on the CP of the brain tissues with 5 µm thickness in a 17-day-old rat embryo. The density of C14-morphine was more observable around ventricles, especially on the blood plexus. So, it can be concluded that the density of opioid receptors is higher on these parts, i.e. on the blood cells of the CP; thus, these parts are the sites of C14-morphine density and the abnormalities caused by C14-morphine happen more in these places [Figure 2]. Moreover, 5 µm sections were made of the brain tissues of 17-day rat embryos using the H and E staining method. The studies demonstrated that CP surfaces of the forebrain and midbrain were higher in the brain tissues of the 17-day embryos in the experimental group compared

with the control one[4-6] and [Graph 1]. Since, CP is responsible for nutrient supply, cell regeneration and detoxification of brain cells and the main function of CP is to excrete, absorb and secrete CFS to cerebral ventricles and most of these actions take place in the ependyma cells of the CP,[17,20,21] any kind of disorder in the normal function of CP (secretion and synthesis of CFS) leads to neural abnormalities. While CFS secretion to cerebral ventricles increases, the surface of ventricles increases, and cerebral cortex thickness decreases; as a result, the person may suffer from hydrocephalus.[13-15] According to the previous studies, morphine causes defects in the normal development of cerebral cavities; this defect can be observed as a decrease in the surface of cerebral cavities, which may indicate the decrease in the secretion of CSF.[5,23] On the other hand, based on the findings of the present study using both methods of detection via labeled morphine and also H and E staining method, the place with the highest density of morphine was blood cells. Therefore, the side-effects caused by morphine function are more observed in this part. Increase in the surface of CP is an abnormality along with the decrease of the CSF which leads to the decrease in the surface of cerebral cavities, i.e., considering that the highest density of (C14) morphine is on the opioid receptors of blood vessels, morphine may impair the function of blood cells and ependymal cells; as a result, although, the blood plexus is wider and filled with blood, the secretion of CSF decreases since the secretive function of the CP has a disorder. Previous studies have indicated that morphine may delay the normal development of cerebral cavities and this delay can be observed as a decrease in the surface of cavities.[5,24,25] The findings of this study using the labeled substance showed that, when the number of opioid receptors was high on the CP, the destructive effects and disorders of morphine on the normal function of the cells in CP raised. Morphine disorder in the secretion and synthesis functions of CFS leads to the decrease in the CSF and consequently, the decrease in the surface of ventricles. According to the conducted studies, morphine causes cell proliferation. The increase in the surface of CP can be attributed to the effect of morphine since it induces cell proliferation of CP (ependyma cells); based on this stimulation, the number of cells abnormally increases with shortening the interphase stage.[13,24,26] Morphine administration may release stress hormones like corticosterone; while being exposed to morphine, corticosterone function may increase blood pressure and fill the CP with blood in rats.[14,27,28] The results of this study probably confirmed the findings of the previous studies that morphine increased the surface and blood in the CP by affecting the opioid receptors of the blood plexus cells and by impairing their secretion and synthesis functions.[16,21,29] Due to its small molecular size and high solubility in fat, morphine can easily pass through the placental barrier and reach the embryo. Furthermore, opioid receptors on the placental villi and vessels have been identified.[7-9] The stimulation of these receptors by morphine may lead to vasoconstriction and reduced blood supply to the embryo;[10,11] when blood supply to the embryo is reduced, the CNS is the most sensitive system to the abnormality risk caused by morphine effect.[30,31]

CONCLUSIONS

It can be concluded that as the choroids plexus function in development and nutrition of brain cells is important, its normal function deficiency leads to abnormalities of nervous system cells. The highest density of morphine was observed on endothelial membrane of blood cells of nervous system cerebral cavities and choroids plexus in addicted mothers' 10 and 17-day-old rat embryos. More research and finding an effective dose of morphine in normal function (synthesis and secretion) of CSF in choroids plexus can be a way for treatment of hydrocephalus patients.

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