Oxidative stress and apoptotic index modifications in the hippocampus of rat pups born to mothers exposed to buprenorphine during lactation

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ABSTRACT

Background: The effect of opioids administration during lactation on nervous system has not fully understood. The aim of this study was to evaluate the buprenorphine (BUP) impact on oxidative stress indexes and apoptotic gene expression in the hippocampus of neonates exposed to this drug through breastfeeding.

Methods: Lactating female rats were subcutaneously injected with BUP (1 or 0.5 mg/kg). After 28 days, the pups were anesthetized, then their hippocampus were obtained for measurement of oxidative stress parameters [glutathione (GSH), thiobarbituric acid reactive substances (TBARS), total antioxidant capacity (TAC) and superoxide dismutase (SOD)] and gene expression of apoptotic indices (Bcl2, Bax and caspase 3).

Results: This study showed that BUP (0.5 and 1 mg/kg) could not markedly change oxidative stress indexes levels and apoptotic markers expression in the hippocampus of pups versus controls.

Conclusion: This study did not find BUP effect on the apoptosis and oxidative stress indices in the hippocampus of pups born to mothers exposed to this drug during lactation.

1. Introduction

Opioid maintenance treatment (OMT) with buprenorphine has been suggested for women during pregnancy and lactation [1,2]. However, the developmental brain of neonates may be vulnerable to exogenous opioids [3]. OMT for pregnant women may be no threat for neonates, as suggested for women during pregnancy and lactation [1,2]. However, the molecular mechanisms involved in the brain damage induced by BUP have not fully understood. Therefore, this investigation aimed to find the BUP effect on apoptotic and oxidative stress indices in the hippocampus of pups exposed to this drug during breastfeeding.

have a high risk of cognitive and behavioral dysfunction versus the non-exposed children [8-11]. In addition, experimental studies have indicated that BUP impaired cognitive function in prenatally exposed rat pups [3,12,13]. Hippocampus is the central region for regulating cognitive function with the high expression of µ-opioid receptors. During activation of µ receptors by opioids, the overgeneration of reactive oxygen species (ROS) generation and apoptosis may be induced in the hippocampus, leading to the disruption of molecular signaling implicated in cognitive function. However, the molecular mechanisms involved in the brain damage induced by BUP have not fully understood.

Therefore, this investigation aimed to find the BUP effect on apoptotic and oxidative stress markers [Caspase 3, Bax, Bcl2, glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), and total antioxidant capacity (TAC)] in the hippocampus of pups exposed to this drug during breastfeeding.

Keywords:
Buprenorphine
Lactation
Apoptosis
Oxidative stress
Brain
Rat
Pup
2. Materials and methods

2.1. Animals

Here, 24 female Wistar rats (260–280 gr) were randomly selected. Animals were kept under standard conditions with 25 ± 2 °C and a cycle of light-dark (12 h). Before the experiment, the animals were housed in polypropylene cages for 3 days to adapt to the new environment and conditions and freely used standard water and food (Khorasan Young Company, Iran). Males and females are kept together for mating. After vaginal smear examination confirmed the plug formation, the male was then separated from the female and returned to another cage.

3. Experimental procedure

After parturition, 12 lactating rats (n = 4) and 18 pups (n = 6) were separated into 3 groups: two treated BUP groups which were exposed to 0.5 and 1 mg/kg of this drug and one control group which received olive oil (0.5 cc/rat). Olive oil was used as a vehicle for BUP. The animals were administrated subcutaneously daily for 28 days. Previous animal studies have shown that subcutaneous injection of BUP was sufficient to enable the drug to readily enter the systemic circulation and milk, at levels that exceed the threshold for analysis. Then six pups randomly were selected from each group for our experiment. The pups were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg), and their hippocampus were removed. for biochemical and molecular assessment.

4. Oxidative stress parameters measurement

4.1. Malondialdehyde assay

The evaluation of MDA was performed by Nalondi Kit (Navand Salamat Company, Iran). The thiobarbituric acid in this kit was used to evaluate lipid peroxidation. The procedure was as follows: a) Prepare a solution containing a sample or standard (200 ml) with the working solution (800 ml). b) put the sample at 95 °C for 45 min. c) After the sample was cooled at 15 min-3000 rpm, a centrifuge was performed. The reaction between MDA and thiobarbituric acid, producing a red fluorescent at 550 wavelengths.

4.2. Glutathione assay

Nargol kit (Navand Salamat Company, Iran) was used to assess GSH levels. The sample (20 μl) was mixed with DTNB and glutathione reductase and incubated for 30 s. Then a cofactor solution was added. Conversion of reduced glutathione to glutathione oxidizes hydrogen peroxide to water, then glutathione reductase converts glutathione oxidize to reduced glutathione and it’s performed at 412 nm.

4.3. Total antioxidant capacity

TAC was evaluated according to the Naxifer Total Antioxidant Assay Kit TAC (Navand Salamat Company, Iran).

4.4. Superoxide dismutase activity

Nasdox kit (Navand Salamat Company, Iran) was used to measure SOD activity by pyrogallol autoxidation. Pyrogallol (C₆H₆OH)₃) is an organic compound that is sensitive to oxygen. The solution was incubated for 5 min and measured at 405 nm.

4.5. Evaluation mRNA expression

4.5.1. The extraction of RNA

Pars Tous nucleic acid extraction kit was used to extract RNA. Briefly, five gram of the tissue was placed in to RL solution and incubated for 5 min at normal temperature. Then, 150 μl of chloroform was put in to the mixture and vortexed (15 s)-then incubated (3 min at 25 °C). The centrifuge of sample was performed at 13,000 rpm at 4 °C for 12 min. Then, the liquid phase (400 μl) was injected in to a new tube and 400 μl of ethanol was added and transferred to the column. Then, the mixture was centrifuged at 13,000 rpm for 1 min. PW solution was used for washing the filter tube (one minute- 13,000 rpm). At last, RNA was washed DEPS.

The purity and concentrations of the extracted RNAs were checked by measuring the UV absorption at 260 / 280 nm using the NanoDrop spectrophotometer. The acceptable purity for RNAs was 260/280 ~ 2. The agarose gel electrophoresis 2 % was used to investigate the quality of the purified RNAs.

4.5.2. cDNAs synthesis

The cDNAs were fabricated using Yekta Tajhiz Azma cDNA synthesis kit according to the protocol.

4.5.3. The quantitative - PCR (qRT-PCR)

Synthesized cDNAs were used as the next model of the RT-qPCR experiment. To quantify the relative mRNA level of each gene, SYBR green-based real-time PCR was performed on a Stratagene mx3000p Real-Time PCR using Real-time PCR Master Mix (SYBR Green). Approximately 50 ng of 1 μl cDNA samples from each forward and reverse primer (10 μM) and 10 μl (SYBR Green) were used (final PCR reaction volume-20 μl).

RT-qPCR temperature process:
- Denaturation: 40 cycles - 95 °C - 45 sd.
- Annealing, 61 °C - 45 s.
- Expansion, 72 °C - 45 s.

All steps were performed in duplicate. In each run performed a cDNA-free reaction as a negative control, and as an internal control for normalization, the gapdh housekeeping gene was used. For confirming of the specificity of the primer pair and purity of real-time PCR products were used melting curve analysis.

“Actin F: AAGATCTGTACGGAGCTTG, Actin R: CAG-CACGTGGTGGACATAGG, GAPDH F: AGGCGCTGTGT- TAACTTCTG, GAPDH R: CCCACTTGATTTFGGAGAG, Bax F: TGCTTCAGGTTTCTATCCA, Bax R: GACACTGGTCACGTCTTGTG, Bcl2 F: GATAACGGAGGCTGTTGAGG, Bcl2 R: CCA- GAGGAGGAGGTAGGAG, Caspase3 F: GTTTGAGCCTGACAGA- GACAT, Caspase2 R: GGCGACATCATCCACACATAC.”

4.6. Statistical analysis

The data was analyzed by Using Stat 3.0 software. Using a single-sample, Kolmogorov-Smirnov test was done to find the normality of data. ANOVA and post-Tukey tests were used to compare all groups. P below 0.05 was considered as significant.

5. Result

5.1. Oxidative stress parameters

During lactation, subcutaneous injection (0.5 and 1 mg/ kg) of BUP compared with significant changes in oxidative stress index in the hippocampus of neonates versus controls. In addition, the significant change was not found in the SOD activity and the levels of GSH, MDA, and TAC in the hippocampus of neonates of the two BUP treated groups (Table 1).

5.2. The gene expression of Bcl2, Bax, and Caspase - 3

During lactation, treatment with BUP (0.5 and 1 mg/kg) did not
significantly change the expression of Bcl-2, Bax, and Caspase – 3 in the neonatal hippocampus versus the control group. The expression of these apoptotic markers were not significantly differed between BUP 0.5 and 1 groups (Figs. 1–2).

6. Discussion

There are several unknowns regarding to the impact of various amounts of BUP on the developing brain until now. The relationship between the exertion of maternal BUP and other opioids through milk and the bioavailability of this drug in infants and their toxic effects have not been fully understood. During opioid maintenance therapy, breastfeeding was not allowed in several countries for years. However, several studies have indicated that methadon exertion through breast milk caused little effect on the infant in recent years [14,15]. To our knowledge, information related to the safety of BUP therapy during lactation has been lacking.

In this study, subcutaneous injection (0.5 and 1 mg/kg) of BUP to mothers during lactation did not cause significant changes in oxidative stress parameters in the hippocampus of neonates compared with the control group. In addition, treatment with BUP (0.5 and 1 mg/kg) could not significantly change the expressions of Bcl-2, Bax, and Caspase – 3 in the neonatal hippocampus versus the control group.

Although the breastfed rats were exposed to BUP at the doses of 0.5 and 1 mg/kg showed that the BUP treatment during lactation did not cause significant changes in oxidative stress parameters in the hippocampus of neonates compared with the control group. In addition, treatment with BUP (0.5 and 1 mg/kg) could not significantly change the expressions of Bcl-2, Bax, and Caspase – 3 in the neonatal hippocampus versus the control group.

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Table 1

| Groups            | GSH   | TAC   | SOD   | MDA   |
|-------------------|-------|-------|-------|-------|
| Control           | 21.24 | 4.94  | 5.00  | 12.22 |
| Buprenorphine 0.5 | 1.42  | 0.43  | 0.18  | 0.48  |
| kg                | 23.15 | 5.79  | 4.95  | 14.54 |
| Buprenorphine 1   | 1.19  | 0.56  | 0.14  | 0.73  |
| mg/kg             | 19.83 | 5.41  | 5.26  | 12.21 |
|                   | 1.13  | 0.69  | 0.36  | 0.820 |

| F   | P   | F   | P   | F   | P   |
|-----|-----|-----|-----|-----|-----|
| 1.75| 0.207| 0.91| 0.43| 3.69| 0.049|

Fig. 1. Bax and Bcl2 mRNA expressions in neonatal rat hippocampus. Abbreviations: C: control, BUP 0.5: Buprenorphine 05 mg/kg, BUP 1: Buprenorphine 1 mg/kg.

Fig. 2. Caspase 3 mRNA expressions in neonatal rat hippocampus. Abbreviations: C: control, BUP 0.5: Buprenorphine 05 mg/kg, BUP 1: Buprenorphine 1 mg/kg.

complete clearance [16].

However, a few studies conducted in this subject indicated that BUP maintenance therapy causes no side effects in the breastfed infants, the exception a mild abstinence syndrome [17–20].

The impact of opioids on the developing central nervous system (CNS) has been evaluated in several studies. However, there are contradictory findings regarding the effect of opioids on CNS. In this regard, Grecco et al., 2021, indicated physical and neurobehavioral dysfunction in neonates which exposed to methadone and oxycodone during fetal and breastfeeding stages [21]. Other experimental studies indicated that prenatal mice exposure to cocaine (30 mg/kg) produced damage in the neocortical layers, gliogenesis, and defective neuritic outgrowth and bundling. However, methadone (40 mg/kg) did not cause damage in brain development [22].

The investigation related to the opioids effects on the developmental brain in humen is difficult because of various susceptibilities to opioid drugs induced by different doses, timing, and route of exposure and genetic background. A systematic and meta-analysis study on 26 cohort studies conducted on children 3–18 years exposed to opioids, indicated
cognitive dysfunction in children 3–6 years and no reported for those aged 7–18 years [23]. Gower et al., 2014 indicated no adverse effects in infants exposed to BUP through breast milk 4 weeks after birth [24].

BUP could cause cognitive dysfunction by inducing inflammation and oxidative stress in the hippocampus of an adult rats exposed to 1 mg/kg of this drug for 4 weeks [25].

Over expression of apoptotic genes are the important mechanism related to the opioids effects on cognitive functions [26]. The inhibitory impact of opioids on μ receptors may play a main role learning and memory dysfunction [27]. Activation of μ receptors by opioids induces ROS production that affects important signaling pathways involved in cognitive function. Hippocampus is an important region involved in the regulation of memory function that affected by oxidative stress and inflammation [27]. The prooxidant impact of BUP has been reported in the liver of rats [28]. Furthermore, numerous evidence showed the association between opioid drugs, oxidative stress and memory dysfunction [29,30]. In this regard, Cai et al. (2016) indicated that morphine activated μ receptors in hippocampal neurons, leading to ROS production, and decreasing in cognitive function in rats [31]. However, our findings indicated no change in the oxidative stress and apoptotic parameters in the hippocampus of neonates which received BUP through breast milk. Our previous experimental study also did not find a change in the oxidative stress parameters in the heart tissue of pups born to the mother which exposed to BUP during lactation [32].

It should be considered that a small amount of BUP is excreted in milk and is also transmitted through the placenta [33]. This study has main limitations as follows: We did not measure the concentration of BUP exertion through milk and BUP concentration in the plasma of neonates. In humans, opioid maintenance therapy is usually initiated during gestation. In this study, we only focused on the toxicity index and applied the selected doses based on the previous articles that have been used for their relevance to human exposure. However, it is necessary to evaluate the pharmacological profile in future studies. In addition, the cognitive function was not considered, however; previous studies have already addressed this issue. Numerous investigations found that infants born to mothers under OMT have a high risk of cognitive and behavioral dysfunction versus the non-exposed children. In addition, experimental studies have indicated that BUP impaired cognitive function in prenatally exposed rat pups. Hippocampus is the central region for regulating cognitive function with the high expression of μ-opioid receptors. During activation of μ receptors by opioids, the over-generation of ROS generation and apoptosis may be induced in the hippocampus, leading to the disruption of molecular signaling implicated in cognitive function. However, the molecular mechanisms involved in the brain damage induced by BUP have yet to be fully understood.

The oxidative and apoptotic markers were not assessed in other brain regions such as the cortex, or even regions related to the motor activities. Although, the hypothalamus is a reasonable tissue to start with in addressing the issues related to cognitive function, but it would be reasonable to evaluate the more specific effects on the other brain regions. Therefore, we could not confirm the safety of this drug in using human infants born to mothers under BUP therapy during lactation.

7. Conclusion

The results of this study showed no effect of BUP at doses 0.5 and 1 mg/kg on the oxidative stress parameters and apoptotic gene expression in the hippocampus of neonates exposed to opioids during breastfeeding. However, we suggest to design further human and animal studies to evaluate the BUP effects on cognitive function in infants. This study can not strongly confirm the BUP safety during lactation due to the major limitations in this study.

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CRediT authorship contribution statement

Saeed Samarghandian, Vahid Noferesti and Shahnaz Rajabi: Conceptualization, Methodology, Software Saeed Samarghandian, Vahid Noferesti and Shahnaz Rajabi: Data curation, Writing- Original draft preparation. Saeed Samarghandian, Vahid Noferesti and Shahnaz Rajabi: Visualization, Investigation. Tahereh Farkhondeh: Supervision. Tahereh Farkhondeh: Software, Validation. Saeed Samarghandian, Michael Aschner, Tahereh Farkhondeh: Writing- Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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None.

Ethics approval and consent to participate

This study was approved by the ethics committee of National Institutes for Medical Research Development (NIMAD), Iran according to the ARRIVE guidelines. Approval number: IR.NIMAD.REC.1398.144.

Human and animal rights

All animal research procedures followed were in accordance with the standards of Guide for the US National Research Council’s and Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

The standard for reporting

ARRIVE guidelines and methodology were followed.

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