Dear Editor in Chief

Thank you for your quick response and revision of our manuscript entitled “Maternal Sciatic Nerve Administered Bupivacaine Induces Hippocampal Cell Apoptosis in Offspring”. We have carefully studied reviewers’ comments and made corrections that we hope will meet their expectations. The revised portions of the text are marked in Blue throughout the paper. The main corrections in the paper and our responses to your comments are as follows:

Reviewer 1:

Question 1: A) Please explain the rationale for looking for hippocampal apoptosis at 30 days postnatal in rats exposed to bupivacaine on day 15 of gestation. B) Peak expression of activated caspase 3 occurs hours after an insult and is quite low days after the insult. I would not expect activated caspase 3 expression at 30 days of age to result from an insult more than 5 weeks earlier, and I don't know what this single time point means. To attribute the increased activated caspase 3 to the maternal exposure during pregnancy would require study at multiple other time points.

Response: We greatly appreciate reviewers' detailed attention. A) About 0.5% to 2% of pregnant women will suffer non-obstetric surgery (1) and most of these procedures (up to 73%) must be completed under general anesthesia (2). Increasing reports suggested that many non-obstetric surgeries can be safely performed in the late pregnancy (3-5). However, increasing evidence indicates that most general anesthetics are harmful for neuronal development and cause cognitive deficits in offspring (3). Local anesthetic, is widely used for spinal and epidural anesthesia,
peripheral nerve blockade, sympathetic nerve block and postoperative analgesia in clinical patients, especially in pregnant patients through providing excellent sensory anesthesia (6). Because our goal was to evaluate the Neurotoxicity effect of a single dose of maternal bupivacaine use (as a local anesthetic) on offspring, we chose day 15 for injection which is about equal to late pregnancy in humans.

B) Based on some previous studies (7-9) for investigation of long-term apoptosis, day 30 was selected. In this regard some previous studies have shown that an insult in gestation can lead to long-term apoptosis in offspring. Gonzalez-Maciel and et al investigated the effect of prenatal exposure to oxcarbazepine on hippocampal apoptosis in rat offspring. Their results showed that prenatal exposure to oxcarbazepine during days 7-15 gestation increases hippocampal apoptosis in rat offspring. Long-term apoptosis started on day 7 after birth and continued until day 30 after birth (7). In addition Benbrahim-tallaa and et al investigated the role of fetal androgen disruption on alterations of Sertoli cell activity in the long-term testicular germ cell death. Pregnant rats were administered flutamide by daily gavage from day 10 of gestation (GD 10) up to the day before delivery (GD 21 or 22). The results showed that fetal androgen disruption causes a long-term apoptosis in testicular germ cells in adult male rat offspring at day 90 postnatal (8).

Question 2: The references cited for accumulation and retention of bupivacaine in fetal tissues after maternal administration also show short term (hours) results, with declining levels in fetal tissues at the end of their study time points. These studies do not provide evidence that bupivacaine persists in postnatal animals weeks after intrauterine exposure.
Response: Thanks for reviewers' constructive points. Based on our knowledge, there is no published evidence on the long term accumulation and retention of bupivacaine in fetal tissues after maternal administration. Based on previous studies on long term effect of maternal drug administration in offspring (7, 8), this study aimed to investigate the possible long-term effect of maternal bupivacaine use on offspring hippocampal apoptosis. Due to the increase in apoptosis in the offspring of bupivacaine group compared to control group, it is possible that bupivacaine remained in the fetal tissue and postnatal animals for a long time. But according to reviewers' comment, proof of this claim requires further studies that we will certainly address in future studies.

Question 3: If caspase 3 has been activated in neurons for this prolonged time period since exposure, there should be evidence of considerable hippocampal neuron loss. This should be easily demonstrated by histological examination. If this delayed or prolonged caspase 3 activation is hypothesized to be in other non-neuron cell types, this could be better demonstrated by immunohistochemistry than by Western blots.

Response: Our goal in this study was to investigate the effects of maternal bupivacaine use on offspring hippocampal cell apoptosis, regardless of the type of apoptotic cells. Because caspase 3 cleavage is considered as a marker of apoptosis activation (10, 11), we evaluated apoptosis through the cleavage of caspase 3 by western blot. Thanks for this interesting suggestion, the effect of maternal bupivacaine use on hippocampal neuron loss and other non-neuron cells will consider in future studies.
Question 4: The use of ketamine to anesthetize the pregnant rats complicates interpretation of any results as being due solely to exposure to bupivacaine. Ketamine is highly neurotoxic to the developing brain, and while all groups were exposed to ketamine, the neurotoxic effects of ketamine have been shown to be worse when ketamine is combined with a number of other drugs. 

Response: Thanks for the accurate reviewers' point. In our studies, the rats of the control group were also anesthetized with ketamine and hippocampal cell apoptosis was not observed in this group. According to previous studies, we have used ketamine as an anesthetic (12). However, in future studies, we will consider this subtle point of the referee.

Question 5: Why was only one pup from each litter used in this study? The other pups could have been used for other time points or other types of assessments. 

Response: To reduce data variation and increase the reproducibility of study through biological samples, we treated different mothers and took one pup from each mother. Thanks for reviewers' attention to ethics, we used other puppies for other studies.

Question 6: No data is provided on the pups used in this experiment. Were they similar in birth weight, growth and behavior to their littermates? 

Response: Thanks for reviewers' kind reminding. We added the sentence in the method section and highlighted it in the revised manuscript.

Question 7: Increased activated caspase 3 five weeks after a single maternal local anesthetic exposure would require the presence of an on-going or delayed insult. Was there any evidence of seizures or other neurologic abnormalities that might explain delayed apoptosis? 

Response: We greatly appreciate reviewers' detailed attention. Based on our knowledge, there is no published evidence that epilepsy or other neurological disorders affect delayed apoptosis also our goal was not to study the effect of neurological disorders on delayed apoptosis. According to our experimental procedure, the results showed that maternal bupivacaine use could lead to an increase in hippocampal cell apoptosis in pups at 30 days of age. We agree with you, the reasons of delayed apoptosis need to further investigation and we will certainly address in future studies.

Reviewer 2:

The major finding of this manuscript is that use of bupivacaine in pregnancy may result in substantial placental transfer, to induce neurotoxicity in the offspring. The authors have used an animal model of Wistar rats to assess this adverse effect of bupivacaine on the hippocampus of the offspring. They also propose a mechanism for the same, based on inhibition of Akt activation. This manuscript could contribute to further understanding of the side effects of local anesthetics, in this case, bupivacaine, on the neonatal brain. Future research in this area could focus on the adverse effect of maternal bupivacaine on the fetus. But to replicate the same in humans would probably require substantially long periods of follow-up.

Thanks very much for reviewers' attention. According to the reviewer's constructive comment, we will consider this point in future studies.
The methodology seems consistent with a well-conducted animal trial. The authors seem to have followed almost all the protocols in designing this study. The only thing that authors could possibly have done is, maybe design a model where the exposure to bupivacaine would have occurred during labor/delivery. This might have allowed them to measure fetal blood pH just before delivery from the umbilical cord. As we know, numerous investigators, using different animal models, have studied the effects of fetal acidemia on local anesthetic distribution across the placenta, demonstrating fetal accumulation as the fetal pH decreases. This could have allowed them to factor the influence of fetal pH on eventual neurotoxicity from bupivacaine.

Thanks for this useful comment to improve our revised manuscript. We agree with you and we will consider this point in future studies.

10. Ghasemzadeh MR, Amin B, Mehri S, Mirnajafi-Zadeh SJ, Hosseinzadeh H. Effect of alcoholic extract of aerial parts of Rosmarinus officinalis L. on pain, inflammation and apoptosis induced by
