European Review for Medical and Pharmacological Sciences 2022; 26: 198-203

Effects of chronic intranasal dantrolene on nasal mucosa morphology in mice

B. JIANG[1,2], Y. SHI[1,3], M.B. ABOU[1], L. XU[4], G. LIANG[1], H. WEI[1]

[1]Department of Anesthesiology and Critical Care, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[2]Department of Anaesthesiology, Peking University People's Hospital, Beijing, China
[3]Department of Anesthesiology, Children's Hospital of Fudan University, Shanghai, China
[4]Department of Pathology, Nanjing Medical University, Nanjing, China

Abstract. – OBJECTIVE: We have previously shown that the intranasal administration of dantrolene ameliorated cognitive dysfunction in the 5XFAD mouse model of Alzheimer's disease. This study examines the morphology of the nasal mucosa after 10 months of intranasal dantrolene in 5XFAD mice.

MATERIALS AND METHODS: 5XFAD mice were either treated with intranasal dantrolene (5 mg/kg, 3 times/wk) from 2 months to 12 months of age or given no treatment at all. The mice were euthanatized at 12 months of age and the snouts were processed for histological examination. The morphology of the nasal mucosa was assessed and compared between the two groups.

RESULTS: There were no significant differences in the thickness of the olfactory epithelium or the proportion of the thickness of the glandular layer to the wall of mucosa and submucosa in the nasal passages.

CONCLUSIONS: Long-term intranasal administration of dantrolene did not significantly change the nasal mucosa morphology in 5XFAD mice.

Key Words:
Alzheimer's disease, Blood-brain barrier, Dantrolene, Histology, Intranasal administration, olfaction, Side effects, Toxicity.

Abbreviations

AD: Alzheimer's disease; IN-DAN: intranasal dantrolene treatment group; CON: control group; H&E: hematoxylin and eosin; RyRs: ryanodine receptors.

Introduction

Dantrolene, an FDA approved drug to treat muscle spasms and malignant hyperthermia, has been shown to be cyto-protective[1] and organ protection[2] in various disease models, especially models of neurodegenerative diseases[3-6]. Further studies have suggested dantrolene as a promising drug for the treatment of Alzheimer's disease (AD)[5,7-9], which is a leading cause of death and the most common form of dementia worldwide[10]. However, the poor penetration of dantrolene into the CNS[6,11] and liver toxicity[12] have previously impeded the chronic oral administration of dantrolene[8,13]. Recently, however, we have demonstrated in mice that the intranasal administration of dantrolene increased the concentration and duration of dantrolene in the brain[13] compared to the oral approach and caused no significant liver toxicity nor impaired olfaction or motor function[8]. However, the concern that the long-term use of intranasal dantrolene may damage the nasal mucosa must be addressed since the mucosa is not only the first defense of the respiratory system but also the site of the first cranial nerve, the olfactory nerve. This study provides evidence that the chronic administration of intranasal dantrolene does not cause morphologic damage to the nasal olfactory mucosa in AD transgenic mice.

Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. Male and female 5XFAD transgenic mice (Line Tg6799, B6SJL-Tg (APPSwFlLon, PSEN1*M-146L*L286V)6799Vas/Mmjax) from the Jackson Laboratory (Bar Harbor, ME) were bred and used in this study, which were the same mice

Corresponding Author: Huafeng Wei, MD, Ph.D; e-mail: huafeng.wei@pennmedicine.upenn.edu

used in our previous study[8]. These mice express mutant human PSEN1 and APP transgenes and recapitulate AD-related phenotypes[14]. Mice were housed at controlled room temperature with a 12-hour light-dark cycle in the animal facility of the University of Pennsylvania. Food and water were available ad libitum. Transgenic and non-transgenic offspring were genotyped by PCR of tail DNA.

Drug Administration

Dantrolene (Sigma-Aldrich, St. Louis, MO, USA) was diluted to a final concentration of 5 mg/ml in the same vehicle (125 mg mannitol, 25 mg polysorbate 80.4 mg povidone K12 in 5 ml of sterile water, and pH adjusted to 10.3) as RYANODEX® (dantrolene sodium, Eagle Pharmaceuticals, Inc., NJ, USA). Mice were randomly divided into two experimental groups at one month of age. For the intranasal group (IN-DAN, n = 4) dantrolene was administered intranasally with a dose of 5 mg/kg, 3 times per week starting at 2 months and ending at 12 months of age. For the control group (CON, n = 4), no treatment was given. During intranasal dantrolene administration, the mice were held upright and fixed by the scruff of their necks. The total dose, which was subdivided into several drops, approximately 5 µl total, was delivered coinciding with inspiration in alternating nostrils with a 10-second pause between each drop. During the pauses, the head of the mouse was restrained in a tilted back position to prevent the drug from leaking out of the nostrils.

Euthanasia and Nasal Cavity Histology

At 12 months of age, the mice were anesthetized via a nose cone with 4% isoflurane and euthanized by transcardial perfusion with phosphate-buffered saline and ensuing exsanguination. The whole nose was dissected and post-fixed in 4% paraformaldehyde overnight at 4°C prior to paraffin embedding for sectioning. Nasal transverse sections (10 µm) located immediately posterior to the incisors[15] were imaged for olfactory mucosa histological assessment using standard hematoxylin and eosin (H&E) staining[16]. In brief, the paraffin slides were deparaffinized and hydrated. Then, the samples were placed in the hematoxylin solution for 3 min, washed under running tap water for at least 5 min, and immersed in working eosin Y solution for 2 min, successively. Finally, the samples were dehydrated, cleared with xylene, and cover-slipped with Permount. To evaluate the effects of intranasal dantrolene on gross nasal structure, an Olympus BX51W1 microscope equipped with a Q Imaging Retiga 2000R digital camera and iVision imaging software (BioVision Technologies, Exton, PA, USA) was used to image all the sections. Sections were evaluated by a pathologist who was blinded to the groups and recorded as normal or mild changes, moderate degenerative or proliferative lesions, or severe lesions with extensive damage to the tissue architecture. To further evaluate the histological scale of inflammation and edematous changes in the nasal cavity, both the thickness of the olfactory epithelium and the thickness of glandular layer of the epithelium (relative thickness of the glandular layer to the thickness of the entire wall consisting of mucosa and submucosa), analogous to the Reid index in chronic bronchitis[17], were measured. These measurements were all focused on the olfactory epithelium from the dorsal meatus of the mouse nasal passage[15]. The thickness was quantified as the average of three measurements in randomly determined nasal sections per mouse.

Statistical Analysis

The unpaired *t*-test was performed to analyze the data with Graph Pad Prism 8.0 as described in the pertinent figure legend. Data are reported as means with 95% confidence intervals (CI). The significance level was set at 95% ($p< 0.05$).

Results

Figure 1 illustrates the anatomy[18] of the mouse nasal passage in the level 1 nasal cavity sections of mice from both groups. Morphometric analyses of the olfactory epithelium from the dorsal meatus of the nasal passage were employed to compare the histological changes between the two groups (Figure 2). No significant difference was determined in the thicknesses of the olfactory epithelium, between CON 40.1 (24.2, 56.0) µm and IN-DAN 34.9 (20.6, 49.2) µm groups, (t=0.77, p=0.47). These results suggest that there was no evident loss or necrosis of epithelial cells in the IN-DAN group when compared to the control group, which was consistent with the gross observation showing the organized polarity of the epithelium.

There was no significant difference between groups in the glandular proportion of the nasal epithelium between CON, 51.5% (44.1, 58.9), and

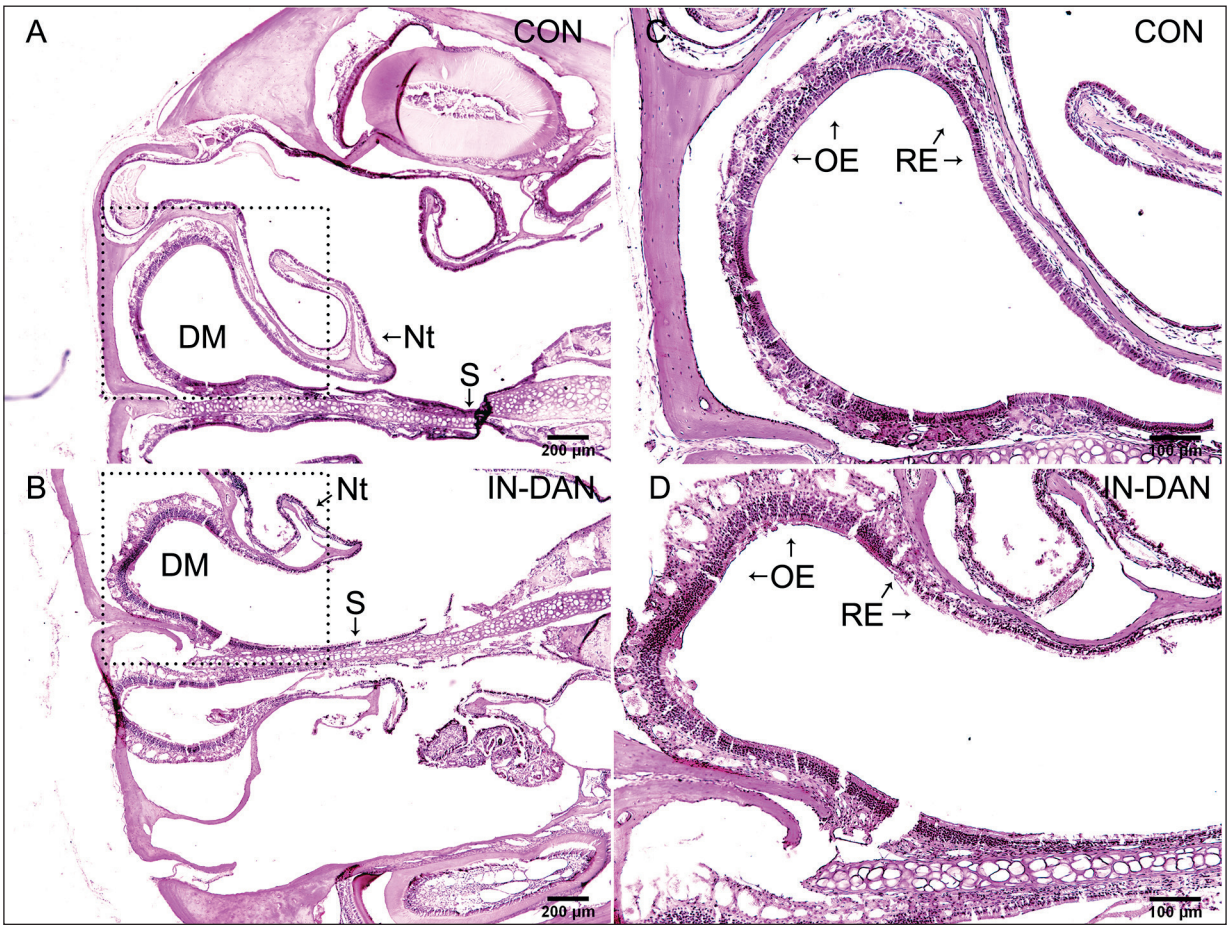

Figure 1. Representative H&E-stained slide images of the nasal cavity from control and intranasal dantrolene treatment groups in 5XFAD mice. Coronal sections of representative mouse nasal passages[15] from the control group (**A,** CON, n = 4) and the intranasal dantrolene treatment group (**B,** IN-DAN, n = 4), stained with Hematoxylin and Eosin, illustrating the nasal anatomy[18]. Higher magnification focused on the dorsal meatus (DM) as indicated in A and B (dashed blocks) are presented in **C** (CON) and **D** (IN-DAN), respectively. Nt, nasoturbinate; S, nasal septum; OE, olfactory epithelium; RE, respiratory epithelium. In CON, nothing was administrated *via* the intranasal route. Intranasal administration of dantrolene was administered 3 times per week from 2 months to 12 months of age in IN-DAN. Scale bars: 200 µm (**A, B**); 100 µm **(C, D)**.

IN-DAN, 51.5% (45.1, 57.8), (t=0.02, p=0.99). This result further demonstrated that long-term intranasal dantrolene did not cause significant chronic inflammation of the nasal olfactory mucosa in 5XFAD mice (Figure 2).

Discussion

This study demonstrated that chronic intranasal dantrolene treatment did not lead to significant gross histological changes in the nasal tissue when compared to the control group and provided evidence to support the tolerable long-term use of intranasal dantrolene. The pathological examination revealed no significant impairment of the nasal mucosa, indicating relative safety after chronic use of intranasal dantrolene for up to 10 months.

Dantrolene, as an antagonist of ryanodine receptors (RyRs), inhibits the excessive and abnormal activation of RyRs and restores the intracellular Ca^{2+} homeostasis[1], has been a promising repurposed drug to treat varieties of neurodegenerative diseases[3-6], especially AD[5,8,9]. On the other hand, its limited ability to penetrate the CNS[6,11] and concern about its dose-dependent side effects[12], has hampered clinical studies. Our previous studies have demonstrated that the intranasal administration of dantrolene could circumvent the blood-brain barrier to maintain an effective neuroprotection level in the brain while poten-

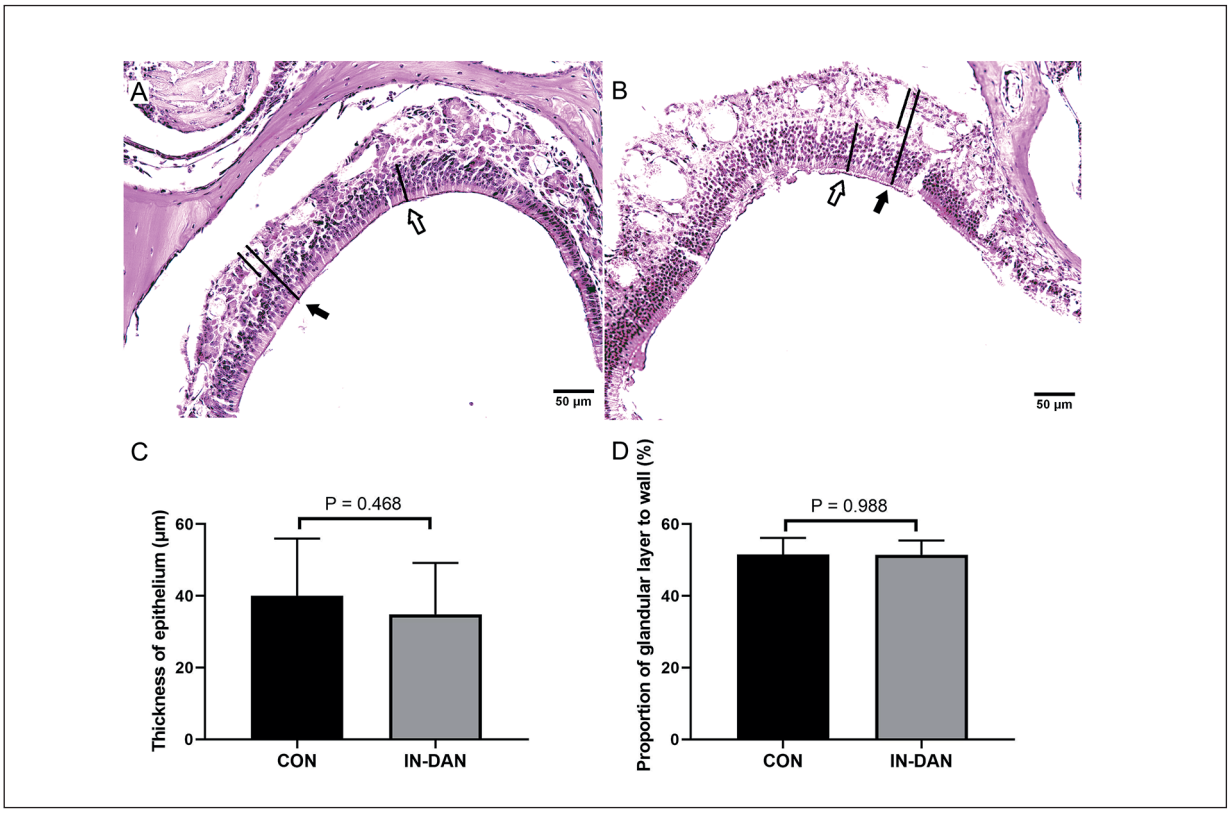

Figure 2. Intranasal dantrolene treatment did not change the nasal mucosal membrane thickness. Representative Hematoxylin and Eosin stained images **(A)** control group (CON); **(B)** intranasal dantrolene treatment group (IN-DAN)) are presented to illustrate the morphometric measurements. The thickness of the epithelium (hollow arrows in A and B) and the glandular proportion (a Reid index[17] measure of the relative thickness of the glandular layer to the thickness of the entire wall consisting of mucosa and submucosa; solid arrows in A and B) were examined as an indication of inflammation. The measurements were focused on the olfactory epithelium from the dorsal meatus of the mouse nasal passages[15]. **(C)** No significant differences were detected either in terms of the thickness of the epithelium (40.1 (24.2, 56.0) µm *vs.* 34.9 (20.6, 49.2) µm, t=0.77, p=0.47) or (**D**) the glandular proportion (51.5 (44.1, 58.9) % *vs.* 51.5 (45.1, 57.8) %, t=0.02, p=0.99), between CON and IN-DAN, respectively. The thickness was quantified as the average of three measurements in a randomly determined nasal section per mouse (N = 4 in each group). The unpaired t-test was used to analyze the data by GraphPad Prism 8.0. Data are presented as means with 95% CI. Scale bars: 50 µm (**A** and **B**).

tially reducing systemic exposure and therefore the side effects of dantrolene[8]. Furthermore, we found that this noninvasive approach could also significantly increase both the peak concentration and duration of dantrolene in the brain[13]. Last, but not least, intranasal dantrolene nearly abolishes memory loss even after the onset of cognitive dysfunction in 5XFAD mice[8].

For the chronic use of intranasal dantrolene to be considered for AD therapy in patients, the side effects need to be thoroughly examined. We have previously investigated the systemic side effects and found no significant liver toxicity nor impaired olfaction or motor function[8] after long-term intranasal dantrolene. However, damage to the delivery site needed to be further examined. In this study, we investigated the morphology of the nasal mucosa in 5XFAD mice after about 10 months of intranasal administration of dantrolene and demonstrated no significant nasal mucosal damage. We employed morphometric measurements of the thickness of the olfactory epithelium and the glandular proportion from the dorsal meatus of the mouse nasal passage to reflect the degree of change in inflammation[17]. The glandular proportion was derived from the Reid index, which was previously confirmed to be a valid instrument to evaluate chronic inflammation in the respiratory tract[17]. As a relative thickness, the glandular proportion may attenuate any bias ensuing from the random measurements in the sections. The histopathologic changes of degeneration are generally regarded as the major component of the lesions in the olfactory region of the nasal cavity

following an insult by a toxicant[19]. In our study, no significant difference was found in terms of either the thickness of the olfactory epithelium or the glandular proportion, which suggested intranasal dantrolene administration is unlikely to cause moderate or severe inflammatory changes. These results are consistent with our previous studies that intranasal dantrolene did not impair olfaction in either 5XFAD or C57BL/6 mice[8,13]. This study boosts the confidence needed to adopt intranasal administration of dantrolene for the treatment of various neurodegenerative diseases, although further studies are needed to confirm the conclusion.

This study has several limitations: (1) A small sample of animals were examined, running the potential risk of false-negative results; (2) The present results were derived from mice and may not be directly translatable to humans; (3) This study is limited to a less comprehensive histological analysis, which only focused on the morphometric analyses of the olfactory epithelium from the level 1 section[15]. Further studies examining both the respiratory and olfactory epithelia from the three transverse tissue levels[15] may provide more robust evidence.

Conclusions

The chronic intranasal administration of dantrolene did not cause significant inflammatory changes in the olfactory epithelium from the dorsal meatus of the mouse nasal passages in 5XFAD mice.

Conflict of Interest

Drs. Huafeng Wei and Ge Liang are listed as inventors of a US provisional patent application entitled "Intranasal Administration of Dantrolene for Treatment of Alzheimer's Disease" filed on June 28, 2019 (Serial number 62/868,820) by the University of Pennsylvania Trustee. The provisional patent application is also part of the research collaboration agreement between the University of Pennsylvania and Eagle Pharmaceuticals, Inc., which produces and sells a new formula of dantrolene (Ryanodex) for the treatment of malignant hyperthermia. Dantrolene used in this study was purchased from Sigma Company, USA. Other authors declare that they have no conflict of interest.

Acknowledgements

We appreciate the editing of the manuscript by Rovert Vera at the Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, PA 19104.

Ethics Approval and Consent to Participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Availability of Data and Materials

Further data and material can be accessed by contacting the last author.

Funding

This work was supported by grants to HW from the National Institute of General Medical Sciences (R01GM084979), the National Institution Aging (R01AG061447, 3R01AG061447-03S1).

Authors' Contribution

Huafeng Wei conceived the idea. Bailin Jiang, Yun Shi, Matan Ben Abou, and Ge Liang collected the data. Bailin Jiang and Lu Xu analyzed the data. Bailin Jiang and Huafeng Wei wrote the manuscript. All authors contributed to the writing of this manuscript. All authors have read and approved the manuscript.

References

1) Inan S, Wei H. The cytoprotective effects of dantrolene: a ryanodine receptor antagonist. Anesth Analg 2010; 111: 1400-1410.
2) Boys JA, Toledo AH, Anaya-Prado R, Lopez-Neblina F, Toledo-Pereyra LH. Effects of dantrolene on ischemia-reperfusion injury in animal models: a review of outcomes in heart, brain, liver, and kidney. J Investig Med 2010; 58: 875-882.
3) Chen X, Wu J, Lvovskaya S, Herndon E, Supnet C, Bezprozvanny I. Dantrolene is neuroprotective in Huntington's disease transgenic mouse model. Mol Neurodegener 2011; 6: 81.
4) Staats KA, Van Rillaer M, Scheveneels W, Verbesselt R, Van Damme P, Robberecht W, Van Den Bosch L. Dantrolene is neuroprotective in vitro, but does not affect survival in SOD1($G^{93}A$) mice. Neuroscience 2012; 220: 26-31.
5) Oulès B, Del Prete D, Greco B, Zhang X, Lauritzen I, Sevalle J, Moreno S, Paterlini-Bréchot P, Trebak M, Checler F, Benfenati F, Chami M. Ryanodine receptor blockade reduces amyloid-β load and memory impairments in Tg2576 mouse model of Alzheimer disease. J Neurosci 2012; 32: 11820-11834.
6) Chen X, Tang TS, Tu H, Nelson O, Pook M, Hammer R, Nukina N, Bezprozvanny I. Deranged calcium signaling and neurodegeneration in spinocerebellar ataxia type 3. J Neurosci 2008; 28: 12713-12724.
7) Lacampagne A, Liu X, Reiken S, Bussiere R, Meli AC, Lauritzen I, Teich AF, Zalk R, Saint N, Arancio O, Bauer C, Duprat F, Briggs CA, Chakroborty S, Stutzmann GE, Shelanski ML, Checler F, Chami

M, Marks AR. Post-translational remodeling of ryanodine receptor induces calcium leak leading to Alzheimer's disease-like pathologies and cognitive deficits. Acta Neuropathol 2017; 134: 749-767.

8) Shi Y, Zhang L, Gao X, Zhang J, Ben Abou M, Liang G, Meng Q, Hepner A, Eckenhoff MF, Wei H. Intranasal Dantrolene as a Disease-Modifying Drug in Alzheimer 5XFAD Mice. J Alzheimers Dis 2020; 76: 1375-1389.
9) Chakroborty S, Briggs C, Miller MB, Goussakov I, Schneider C, Kim J, Wicks J, Richardson JC, Conklin V, Cameransi BG, Stutzmann GE. Stabilizing ER Ca2+ channel function as an early preventative strategy for Alzheimer's disease. PLoS One 2012; 7: e52056.
10) Rice DP, Fillit HM, Max W, Knopman DS, Lloyd JR, Duttagupta S. Prevalence, costs, and treatment of Alzheimer's disease and related dementia: a managed care perspective. Am J Manag Care 2001; 7: 809-818.
11) Wuis EW, Rijntjes NV, Van der Kleijn E. Whole-body autoradiography of 14C-dantrolene in the marmoset monkey. Pharmacol Toxicol 1989; 64: 156-158.
12) Chan CH. Dantrolene sodium and hepatic injury. Neurology 1990; 40: 1427-1432.
13) Wang J, Shi Y, Yu S, Wang Y, Meng Q, Liang G, Eckenhoff MF, Wei H. Intranasal administration of dantrolene increased brain concentration and duration. PLoS One 2020; 15: e 0229156.
14) Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, Van Eldik L, Berry R, Vassar R. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci 2006; 26: 10129-10140.
15) Kittel B, Ruehl-Fehlert C, Morawietz G, Klapwijk J, Elwell MR, Lenz B, O'Sullivan MG, Roth DR, Wadsworth PF. Revised guides for organ sampling and trimming in rats and mice--Part 2. A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol 2004; 55: 413-431.
16) Cardiff RD, Miller CH, Munn RJ. Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harb Protoc 2014; 2014: 655-658.
17) Karger B, Fracasso T, Brinkmann B, Bajanowski T. Evaluation of the Reid index in infants and cases of SIDS. Int J Legal Med 2004; 118: 221-223.
18) Coppola DM, Craven BA, Seeger J, Weiler E. The effects of naris occlusion on mouse nasal turbinate development. J Exp Biol 2014; 217: 2044-2052.
19) Hardisty JF, Garman RH, Harkema JR, Lomax LG, Morgan KT. Histopathology of nasal olfactory mucosa from selected inhalation toxicity studies conducted with volatile chemicals. Toxicol Pathol 1999; 27: 618-627.