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Original Article

Melatonin alleviates valproic acid-induced neural tube defects by modulating Src/PI3K/ERK signaling and oxidative stress

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Running title: Melatonin alleviates VPA-induced neural tube defects
Abstract

Neural tube defects (NTDs) represent a developmental disorder of the nervous system that can lead to significant disability in children and impose substantial social burdens. Valproic acid (VPA), a widely prescribed first-line antiepileptic drug for epilepsy and various neurological conditions, has been associated with a fourfold increase in the risk of NTDs when used during pregnancy. Consequently, urgent efforts are required to identify innovative prevention and treatment approaches for VPA-induced NTDs. Studies have demonstrated that the disruption in the delicate balance between cell proliferation and apoptosis is a crucial factor contributing to NTDs induced by VPA. Encouragingly, our current data reveal that melatonin (MT) significantly inhibits apoptosis while promoting the restoration of neuroepithelial cell proliferation impaired by VPA. Moreover, further investigations demonstrate that MT substantially reduces the incidence of neural tube malformations resulting from VPA exposure, primarily by suppressing apoptosis through the modulation of intracellular reactive oxygen species levels. In addition, the Src/PI3K/ERK signaling pathway appears to play a pivotal role in VPA-induced NTDs, with significant inhibition observed in the affected samples. Notably, MT treatment successfully reinstates Src/PI3K/ERK signaling, thereby offering a potential underlying mechanism for the protective effects of MT against VPA-induced NTDs. In summary, our current study substantiates the considerable protective potential of MT in mitigating VPA-triggered NTDs, thereby offering valuable strategies for the clinical management of VPA-related birth defects.

Key words: melatonin, valproic acid, neural tube defects, reactive oxygen species
Introduction

Neural tube defects (NTDs) are the second most common birth defect in the world, and their incidence is second only to congenital heart disease[1, 2]. NTDs are serious birth defects of the central nervous system caused by neural tube closure malformations in the early stage of embryonic development, which results in an enormous social and economic burden[3, 4]. NTDs are polygenic genetic diseases that occur under the influence of genetic factors, environmental factors, chemicals, drugs and other factors, although the etiology is not completely clear[5, 6]. The detailed molecular mechanism underlying NTDs remains to be further elucidated[7, 8].

It has been reported that in the United States, approximately 7.6 million to 12.7 million women suffer from epilepsy each year, and nearly 25,000 of them are pregnant[9-11]. Valproic acid (VPA) is a clinical antiepileptic drug and is often used to treat mental disorders such as mania and bipolar disorder, although administering VPA in early pregnancy can lead to a fourfold increase in the incidence of fetal NTD[12-14]. Given that VPA stands as the most efficacious antiepileptic drug presently accessible, it becomes imperative to diligently observe preventive measures against NTDs induced by VPA [15]. Among the mechanisms through which VPA can impede normal neural tube development, encompassing the disruption of oxidative stress, DNA hypomethylation, histone deacetylation, and alteration of tetrahydrofolic acid content, the perturbation of oxidative stress assumes a central and pivotal role[16, 17]. VPA induces a surplus generation of reactive oxygen species (ROS) within neuroepithelial cells, triggering apoptosis and consequently impeding the timely closure of the neural tube. This disruption primarily manifests as malformation with exposed brain tissue. This imperative lies in the exploration of efficacious prophylactic interventions aimed at mitigating fetal NTDs resulting from VPA administration.

Melatonin (N-acetyl-5-methoxytryptamine, MT) is an indoleamine synthesized mainly in the pineal gland and was originally found to have physiological and neuroendocrine functions as a diurnal hormone.[19, 20] Meanwhile, MT is a clinically reliable antioxidant with a strong scavenging effect on free radicals[21]. The mechanism underlying MT's resistance to free radicals and oxidative stress involves its direct elimination of free radicals and their byproducts by upregulating the expression of antioxidant enzymes, restraining the activation of catalase, and preserving mitochondrial homeostasis[22, 23].

Given the pathogenesis of fetal NTD induced by VPA and MT’s inherent antioxidant capabilities, we postulate that MT exerts a safeguarding influence against the adverse repercussions of VPA administration on NTD occurrence in the clinical context.

In the current investigation, we employed an animal model of VPA-induced rodent NTD and an in vitro cell model utilizing HT-22 cells to explore the beneficial protective influence of MT against VPA-induced NTD. This comprehensive study delves into both the in vivo and in vitro mechanisms underlying the effects of MT. The findings elucidate that MT exerts a corrective influence on the expression of antioxidant genes by modulating the ERK and src signaling pathways, thereby ameliorating the heightened fetal oxidative stress resulting from maternal VPA administration. Consequently, this intervention effectively diminishes the occurrence of NTD. These
current research outcomes offer novel insights for the clinical prevention and treatment of VPA-induced NTD, holding promising potential in the realm of translational medicine.

Materials and Methods

Reagents and antibodies

VPA (P4543), melatonin (M5250), and DCFH-DA (D6883) were ordered from Sigma Chemical (St Louis, USA). Primary antibodies against src (#2109), p-src (#12432), ERK (#68303), p-ERK (#4370), PI3K (#4249), and p-PI3K (#17366) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA); antibodies against PH3 (ab177218), Ki67 (ab16667) and BCL-2 (ab182858) were ordered from Abcam (Branford, USA); and GAPDH (60004-1-Ig) was purchased from Proteintech Group (Wuhan, China). FAST DiO (D3898) was purchased from Thermo Fisher Scientific (Waltham, USA). DMEM (11960044) and fetal bovine serum (10099141) were ordered from Thermo Fisher Scientific (Waltham, USA). The ROS Assay Kit was purchased from Beyotime (Shanghai, China).

In vivo mouse experiments

All 6- to 7-week-old SPF male and female CD-1 mice were raised in the Environmental Barrier Animal Laboratory of Experimental Animal Center of Shanxi Medical University with animal licence number: SYXK (Jin) 2019-0007. All animal experiments were examined and approved by the Ethics Committee of Shanxi Medical University. All animal procedures strictly followed the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

The mice were maintained on a 12-hour light/dark cycle (lights on from 8:00 am–8:00 pm), with water and food provided ad libitum. Pregnant female mice were obtained by mating with fertile males of the same strain (day 0.5 is the day of the vaginal plug). On day 7.5 of pregnancy (E7.5), VPA was intraperitoneally injected only once at a dose of 400 mg/kg to establish the NTD embryo model. From E7.5 evening, pregnant mice were given 10 or 20 mg/kg of body weight MT by daily i.p. injection until being killed (VPA+MT10 group and VPA+MT20 group). Mice in the control and VPA groups were injected with normal saline in the same manner. The brains of embryos were collected for further analysis.

Analysis of gross morphology

The number of implanted embryos and viable fetuses were carefully examined on E10.5, and the incidence of viable fetuses was calculated as a percentage of the number of implantation sites. Viable fetuses were then screened for NTDs with an M205 FA stereomicroscope (Leica, Wetzlar, Germany). The incidence of NTDs was calculated as a percentage of viable fetuses.
**In vitro cell culture and treatment**

HT-22 (immortalized hippocampal neuron cells) were obtained from ATCC (Rockville, USA), a surrogate of neuroepithelial cells, and cultured in DMEM containing 10% FBS.

To explore the protective effect of MT on the toxic effects of VPA, HT-22 cells were cotreated with 10 mM VPA and 20 μM MT for 48 h. Cultured cells were harvested for further analysis.

**EdU labelling and immunofluorescence staining**

Cell proliferation was also analysed using a Cell-Light EdU Kit (RiboBio, Guangzhou, China) following the manufacturer’s instructions. Immunofluorescence signals were visualized and imaged using an immunofluorescence microscope (Nikon, Tokyo, Japan). ImageJ was adopted to quantify the proliferative cells in different random optical fields.

Immunofluorescence staining of PH3. After being fixed in 4% neutral formaldehyde overnight and embedded in paraffin, fetal brains were cut into 4 μm sections, deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in water. Then, the sections were subjected to antigen retrieval in pH 6.0 sodium citrate buffer and blocked with 10% donkey serum at 37°C for 1 h. Next, the sections were incubated with a primary antibody against PH3 overnight at 4°C and a fluorescent-conjugated secondary antibody for 40 min at room temperature and counterstained with DAPI (C1002, Shanghai, China) (1 μg/mL in PBS) for 10 min. Images were taken by an ECLIPSE Ti2 fluorescence microscope. ImageJ was adopted to quantify the positive signals.

**TUNEL assay**

The TUNEL staining assay was performed as described previously. An In Situ Cell Death Detection kit, POD (Roche, USA) was used to assess apoptosis following the manufacturer’s instructions. HT-22 cell crawling plates or E10.5 embryo paraffin sections were washed three times in PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, and then incubated in 3% H$_2$O$_2$ in methanol in the dark for 20 min. After washing three times with PBS, the samples were incubated in a mixture of TdT and dUTP (1:9) from the TUNEL kit at 37°C for 1 h, followed by incubation with converter-POD at 37°C for 30 min. Finally, the samples were counterstained with DAPI for nuclear staining. TUNEL-positive cells were observed under the ECLIPSE Ti2 fluorescence microscope.

**Measurement of ROS**

Intracellular ROS levels were detected by an ROS Assay Kit (D6883; Sigma) according to the manufacturer’s instructions. Briefly, cells were stained with DCFH-DA (10 μM) and incubated in a cell incubator at 37°C for 20 min after washing three times with serum-free cell culture medium to fully remove the DCFH-DA that did not enter the cells. Cell fluorescence was then detected by using the ECLIPSE Ti2 fluorescence microscope as described by our group[24].
Western blot analysis

Western blotting was carried out as described previously[25]. Briefly, total protein of the fetal brain samples was extracted on ice with RIPA lysis buffer and diluted to a uniform concentration. Ten micrograms of protein was loaded on 10% SDS-PAGE for separation and transferred to PVDF membranes, which were blocked with 5% skim milk and hybridized with primary antibody. Then, the membrane was incubated in 5% nonfat milk containing HRP-conjugated secondary antibody (1:5000) for 1 h. Signals were detected by an ECL kit (Millipore, Massachusetts, USA). Experiments were repeated at least three times. ImageJ was used to detect the gray value of all protein pictures. GAPDH was used as a housekeeping control.

Real-time PCR

Real-time PCR was performed as previously described. Briefly, total RNA was isolated from E10.5 embryos using a TRIzol reagent kit (15596026, Invitrogen, Waltham, USA); a PrimeScript reverse transcriptase reagent kit (R223-01, Vazyme, Nanjing, China) was used to reverse-transcribe RNA into cDNA. Real-time PCR was performed by using a SYBR Premix Ex Taq kit (Q711-02, Vazyme, Nanjing, China) on the QuantStudio 5 Real-Time System (ABI, Waltham, USA). The reaction conditions were as following: 95°C for 30 s; 95°C for 10 s, 60°C for 30 s, 40 cycles; and then the default melting curve acquisition program. The data were normalized against the levels of Rpl7 expression. The sequences of primers used in this study are listed in Table 1.

Flow cytometry

Flow cytometry was used to analyse the effects of VPA and MT on cell apoptosis with an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (eBioscience, San Diego, CA, USA). After treatment with VPA and MT, HT-22 cells were collected and then centrifuged at 400 g for 5 min. Cells were grouped into three groups as follows: (1) control group, (2) VPA group, and (3) VPA + MT group. Cells were subsequently incubated with 5 μL of annexin V-FITC and 10 μL of propidium iodide (Sigma-Aldrich, St. Louis, USA) staining solution for 15 min at room temperature in the dark, and cell apoptosis in each group was detected by flow cytometry (BD Biosciences, San Jose, USA).

HE and immunohistochemistry analysis

Immunohistochemistry (IHC) was performed as described previously[26]. Briefly, fetal brains were fixed in 4% neutral formaldehyde overnight and embedded in paraffin. Sections (4 μm) were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in water. An HE staining kit was used to describe the morphology. After antigen retrieval, endogenous horseradish peroxidase (HRP) inactivation and blocking, the sections were incubated with the indicated primary antibody. Then, a DAB Horseradish Peroxidase Color Development Kit was used to detect the positive signals according to the manufacturer’s protocol (Zhongshan Golden Bridge, Beijing, China). The sections were counterstained with hematoxylin.
Statistical analysis
For mouse studies, at least three mice were included in each group. Statistical analysis
was performed using GraphPad Prism 6.0 software. Two datasets were analysed by
Student’s t test. Nonnormally distributed data were analysed by the Kruskal-Wallis test.
The \(2^{-\Delta\Delta C_t}\) method was used to analyse the PCR results in all experiments. Quantified
data are expressed as the mean ± SEM. A \(P\) value < 0.05 was considered statistically
significant.

Results
MT reduces the incidence of VPA-induced abnormal neural tube closure
To investigate the preventive effect of MT on the embryonic toxicology of VPA, an
NTD mouse embryonic model was established by intraperitoneal injection of 400
mg/kg VPA in CD1 mice, which was comparable to previous studies[27, 28]. VPA-
treated embryos displayed obvious growth retardation and malformations along with a
small and hypoplastic brain vesicle by stereomicroscope observation (Figure 1A).
Transverse sections were performed on the brain vesicles of fetal mice in each group
(Figure 1B), and HE staining showed that the fetal forebrain failed to close in the VPA
treatment group, while the fetal brain vesicles closed completely in the VPA+MT group
(Figure 1C). The total incidence of NTDs in this VPA model was up to 53.7%, while
the incidence of NTDs was 14% in the VPA and 20 mg/kg MT cotreatment group
(Table and Figure 1D). These data suggested that neural tube closure was perturbed in
VPA-treated mouse embryonic tissue, and the incidence of NTD could be significantly
reduced by MT (P<0.05).

MT blocks the inhibition of neuroepithelial cell proliferation in the mouse forebrain
causd by VPA
During the development of the mouse embryonic neural tube, cell proliferation is the
basic developmental process to ensure the normal development of the neural tube[29,
30]. The proliferation of neuroepithelial cells in the E10.5 forebrain from all the groups
was detected by PH3 and ki67 assays. Compared with the control group, the PH3
immunofluorescence signal decreased significantly in the VPA group, while the PH3
signal increased significantly in the VPA+MT group compared with the VPA group
(Figure 2A). A histogram of the quantitative results of the number of PH3-positive cells
is shown in Figure 2B. Western blotting was used to measure the levels of PH3 protein
in each group, which was consistent with the immunofluorescence results (Figure 2C).
A quantitative histogram was also used to quantify the results of Western blotting
(Figure 2D). Moreover, Ki67, a classical marker of cell proliferation, was also used to
detect the proliferation of anterior neuroepithelial cells in each group by
immunohistochemistry. The data showed that compared with the control group, the
positive ki67 signal in the VPA group was reduced, while in the VPA and MT
cotreatment group, the positive ki67 signal basically recovered to the level of the
MT alleviates the overapoptosis of neuroepithelial cells in the mouse forebrain caused by VPA

During the development of the mouse embryonic neural tube, excessive apoptosis is also an important mechanism of neural tube defects[30]. Therefore, the TUNEL assay was used to reveal the level of DNA damage in embryonic neuroepithelial cells. The results showed that the TUNEL signal was significantly increased in the VPA group compared with the control group, while the TUNEL signal was reduced to the control level after MT cotreatment (Figure 3A). The quantitative results of TUNEL are shown in a histogram in Figure 3B. This suggests that the reduction in excessive DNA damage caused by VPA may be one of the reasons why MT can reduce the incidence of neural tube damage. The ratio of Bcl-2/Bax is an important indicator reflecting the level of intracellular apoptosis. Therefore, we detected the protein expression levels of Bcl-2 and Bax in each group and calculated their ratio. The results showed that compared with that in the control group, the apoptosis level of fetal forebrain neuroepithelial cells in the VPA group was significantly increased, while that in the MT cotreatment group was restored (Figure 3C). The quantitative results of Bcl-2 and Bax are shown in a histogram in Figure 3D. The Bcl-2/Bax ratio is shown in a histogram in Figure 3E. These results suggest that MT may play a protective role by inhibiting excessive apoptosis in VPA-treated fetal brain vesicles.

MT reverses VPA-induced abnormalities in proliferation and apoptosis of HT22 cells

Furthermore, we investigated the recovery effect of MT on the VPA-induced imbalance of cell proliferation and apoptosis in vitro in the neuronal cell line HT-22. We conducted EdU staining on the cells in each group, and the results showed that, compared with the control group, VPA could significantly reduce the number of EdU-positive cells, and MT cotreatment could effectively save the proliferation decline caused by VPA (Figure 4A). A histogram of the quantitative analysis is shown in Figure 4B. In addition, TUNEL staining of each group of cells was also examined, and it was found that, consistent with the in vivo results, VPA significantly induced apoptosis of HT-22 cells, while MT could save the excessive apoptosis caused by VPA (Figure 4C). The histogram of quantitative analysis is shown in Figure 4D. It was necessary to detect the apoptosis level of HT-22 cells in each group by flow cytometry. The results showed that, compared with the control group, VPA induced a large number of apoptotic cells, while MT cotreatment reduced the number of apoptotic cells (Figures 4E-H). All in, those in vitro cell results above further corroborate the in vivo findings.

MT can alleviate excessive ROS induced by VPA by restoring antioxidant enzyme
expression in HT-22 cells

Studies have shown that excessive ROS is an important mechanism of neural tube defects induced by VPA\cite{16}, and excessive ROS production can inhibit cell proliferation and promote cell apoptosis\cite{31, 32}. However, MT is a highly effective antioxidant and free radical scavenger\cite{33, 34}. We therefore examined the changes in VPA-induced ROS activity following MT treatment of cultured HT-22 cells for 48 hr. The results showed that compared with the control group, the VPA group had a higher ROS signal, while MT significantly inhibited the production of ROS (Figure 5A-5D). To further analyse how MT prevents excessive ROS production in the fetal brain, the gene transcription levels of key antioxidant enzymes in vivo were examined to evaluate the mechanism by which MT reduces ROS. The data showed that compared with the control group, the expression levels of Sod1, Sod2 and gpx1 were downregulated after VPA treatment, while the expression levels of Sod1, Sod2 and gpx1 were recovered in the VPA+MT group. These results indicate that MT inhibits the production of excess ROS caused by VPA by regulating the expression of antioxidant enzymes in vivo.

The Src-PI3K-ERK pathway may be involved in the preventive therapeutic mechanism of MT against VPA-induced NTD

To elucidate the mechanism by which MT regulates the VPA-triggered imbalance of cell proliferation and apoptosis in the fetal brain, we explored the possible effects of MT on the Src-PI3K-ERK signal transduction pathway. In an in vivo mouse embryo model, the phosphorylation of src was inhibited by VPA treatment, and MT cotreatment rescued the phosphorylation of Src, PI3K and ERK, showing similar expression regulation patterns (Figures 6A-D). In the in vitro cell model, the data showed that MT had rescue effects on p-Src, p-ERK and P-PI3K inhibition induced by VPA to varying degrees (Figures 6E-H), which was mostly consistent with the results of the in vivo embryo model. In conclusion, the Src-PI3K-ERK signaling pathway may mediate the imbalance of proliferation and apoptosis caused by VPA, while MT may play a role in blocking the occurrence of embryonic neural tube defects by maintaining the Src-PI3K-ERK signaling pathway.

Discussion

The global prevalence of epilepsy affects an estimated 30 million women, with approximately one-third falling within the childbearing age group\cite{35}. Despite VPA’s recognized teratogenic risks, it remains extensively prescribed for pregnant women with mental disorders. Given that employing VPA in treating epileptic conditions during pregnancy is often an unavoidable necessity\cite{28}, there arises a pressing imperative to innovate and implement preventive approaches that counteract the teratogenic potential of VPA.

The present investigation revealed that MT effectively mitigates the occurrence of neural tube malformation in a VPA-induced mouse embryonic model. The underlying
mechanism appears to involve the inhibition of ROS overproduction and the restoration of the PI3K and ERK signaling pathways, culminating in the reduction of neural tube malformation incidence.

While considerable efforts have been directed towards comprehending the neurodevelopmental toxicity mechanism of VPA, a comprehensive understanding of its intricate pathogenesis remains elusive. Nevertheless, a growing body of evidence underscores that a pivotal mechanism contributing to VPA-induced NTDs is the perturbation of redox balance and the subsequent generation of excessive ROS. Emerging research suggests that mitigating redox disruptions could potentially lead to a decline in the prevalence of NTDs. Resveratrol and vitamin E could rescue VPA-induced teratogenicity. The antecedent study data align harmoniously with our current findings, which exemplify that VPA indeed prompts fetal neural tube cells to generate excessive ROS and concurrently inhibits the expression of crucial antioxidant enzymes such as SOD1, SOD2, and GPX1, corroborated by both in vivo animal experiments and in vitro cell experiments.

Melatonin, a hormone synthesized by the mammalian pineal gland, exerts diverse physiological regulatory functions and possesses notable neuroprotective properties. Its capacity to stimulate the proliferation and differentiation of neural stem cells underscores its protective potential against neonatal brain injury induced by hypoxia. Melatonin has demonstrated its protective potential against neural tube defects induced by lipopolysaccharide and diabetic neural tube lesions in mice. Notably, its favorable lipid-water distribution coefficient facilitates its efficient cellular entry through the cell membrane, enabling the exertion of its potent antioxidant activity. In the realm of natural pregnancy, melatonin plays a pivotal role, as plasma melatonin levels in pregnant women rise significantly by 200-300% during the initial 20 weeks of gestation, appearing to be crucial for successful pregnancy outcomes. Furthermore, the remarkable ability of melatonin to traverse the placenta and cross the blood–brain barrier into the central nervous system positions it as a potential therapeutic agent for preventing VPA-induced NTDs in pregnant women. Although it is not clear whether MT has a protective effect against VPA-induced NTD until now, our subsequent investigations have shed light on this matter. Encouragingly, our recent findings demonstrate that melatonin effectively diminishes the incidence of VPA-induced NTD by reinstating the expression of essential antioxidant enzymes, namely, SOD1, SOD2, and GPX1, and thus counteracting the deleterious overproduction of ROS.

Melatonin therapy is widely acknowledged for its favorable safety profile. Notably, toxicity assessments of melatonin on mouse embryonic development revealed no discernible adverse effects. Moreover, in a separate investigation, high doses of melatonin administered during pregnancy had no detrimental impact on fetal abnormalities or maternal health. Consistent with these findings, our current study demonstrated that the in vivo administration of 20 mg/kg melatonin had no discernable effect on the incidence of NTDs.
It has been proposed that the intricate orchestration of neural stem cell processes—proliferation, migration, differentiation, and apoptosis—is a key determinant in neural tube formation\cite{46}. Studies have substantiated that neural tube disorders can arise from diminished proliferation and heightened apoptosis of neuroepithelial cells within the developing neural tube\cite{47,48}. VPA exposure has been linked to fetal neural tube DNA damage and leads to downstream alterations, including cell cycle arrest and apoptosis\cite{49}. Our findings suggest that melatonin supplementation fosters neuroepithelial cell proliferation and reduces cell apoptosis in VPA-treated fetal brains at E10.5. The results derived from our in vitro HT-22 cell model align with those observed in vivo. An increasing body of evidence supports the notion that oxidative stress can trigger apoptosis\cite{50,51}, potentially leading to an insufficient number of cells participating in the folding and fusion of neural tube walls. In this study, melatonin effectively curbed excessive apoptosis of neuroepithelial cells in VPA-treated mice and HT-22 cells in vitro, consequently mitigating the incidence of neural tube malformations. In addition, melatonin treatment significantly elevated the Bcl-2/Bax ratio, decreased TUNEL expression and reduced apoptosis incidence. Notably, melatonin also upregulated the expression of PH3, KI67 and edU, thus reinstating neuroepithelial cell proliferation. These results collectively highlight melatonin's antioxidant properties and its role in counteracting apoptosis, aligning consistently with prior research findings\cite{52}.

The Src/PI3K/ERK signaling pathway has been intricately associated with cell proliferation\cite{53}. Inhibition of phosphorylated ERK1/2/MAPK, known for its role in cell proliferation, has been linked to hyperthermia-induced NTDs\cite{54}. Moreover, considering the pivotal role of PI3K/Akt in embryonic development\cite{55}, its downregulated expression appears to hold significance in the context of VPA-induced neural tube defects. Drawing inspiration from the aforementioned literature, our study sought to elucidate the molecular mechanism underlying MT treatment for VPA-induced neural tube malformations. Our results revealed that VPA treatment profoundly suppressed the activation of Src/ERK/PI3K phosphorylation in fetal brain tissue and HT-22 cells. Remarkably, concurrent MT cotreatment significantly restored the levels of Src/ERK/PI3K phosphorylation, suggesting its potential involvement in the amelioration of VPA-induced neural tube malformations by MT. It should be noted that p-PI3K in HT-22 cell lines treated with VPA and VPA+MT showed no significant difference (figure 6H), although the results of our in vivo experiments in mice (figure 6D) showed that P-PI3K levels were significantly restored after MT treatment compared to VPA treatment, so this difference between the in vitro and in vivo data may not affect the main conclusion of this paper, and the mechanism of the inconsistency needs further research in future studies.

In conclusion, our current study provides compelling evidence that MT therapy during pregnancy holds promise in averting VPA-induced neural tube disorders by effectively modulating the Src/PI3K/ERK signaling pathway and mitigating oxidative stress.
Nonetheless, it is essential to acknowledge that further research is warranted to extend these findings to human applications.

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Figure and legends
Figure 1. MT reduces the incidence of VPA-induced abnormal neural tube closure. (A) Representative images showing the morphological developmental defects of the brain in the VPA-treated group. (B) Comparison of fetal brain vesicle morphology and section direction in the control group, VPA group and VPA+MT group. (C) HE staining of fetal brain vesicles in the control group, VPA group and VPA+MT group. (D) The total incidence of NTDs in the control group, VPA group and VPA+MT group. Each group included at least three mice. All data are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. MT blocks the inhibition of neuroepithelial cell proliferation in the mouse forebrain caused by VPA. (A) A representative image showing PH3 immunofluorescence in fetal brain vesicles in the control group, VPA group and VPA+MT group. (B) The quantification of PH3-positive cells in each group of Figure 1A. (C) The level of PH3 protein in fetal brain vesicles in the control group, VPA group and VPA+MT group by Western blotting. (D) Quantitative histogram of grayscale values in each group of Figure 2C. (E) The expression of Ki67 in fetal brain vesicles in the control group, VPA group and VPA+MT group by immunohistochemistry. (F) The quantitative results of the Ki67-positive cell number in each group of Figure 2E. Each group included at least three mice. All data are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 3. MT alleviates the overapoptosis of neuroepithelial cells in the mouse forebrain caused by VPA. (A) TUNEL staining of fetal brain vesicles in the control
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657 group, VPA group and VPA+MT group. (B) The quantitative results of TUNEL in
658 each group of Figure 3A. (C) The protein expression levels of Bcl-2 and Bax in fetal
659 brain vesicles in the control group, VPA group and VPA+MT group. (D) The
660 quantitative results of Bcl-2 and Bax in each group of Figure 3C. (E) The Bcl-2/Bax
661 ratio in each group of Figure 3C. Each group included at least three mice. All data are
662 expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

663

Figure 4. MT reverses VPA-induced abnormalities in proliferation and apoptosis
664 of HT22 cells  (A) EdU staining of HT22 cells in the control group, VPA group and
665 VPA+MT group. (B) The quantitative results of EdU in each group of Figure 4A. (C)
666 TUNEL staining of HT22 cells in the control group, VPA group and VPA+MT group.
667 (D) The quantitative results of TUNEL in each group of Figure 4C. (E–G)
668 Representative image of cell apoptosis in HT22 cells treated with control, VPA and
669 VPA+MT by flow cytometry. (H) Quantitative plots of apoptosis rates in each group
670 are shown in Figure 4E–G. Each experiment was repeated three times. All data are
671 expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

672

Figure 5. MT can reverse the abnormal proliferation and apoptosis of HT-22 cells
673 caused by excessive ROS induced by VPA  (A–C) Representative image of ROS
674 staining in HT22 cells treated with control, VPA and VPA+MT. (B) The quantitative
675 results of ROS in each group of Figure 5A–C. (E) The relative mRNA expression of
676 Sod1. (F) The relative mRNA expression of Sod2. (G) The relative mRNA expression
of *Gpx1*. Each experiment was repeated three times. All data are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

**Figure 6** The Src-PI3K-ERK pathway may be involved in the preventive therapeutic mechanism of MT against VPA-induced NTD. (A) Western blot analysis of Src/p-Src, PI3K/p-PI3K and ERK/p-ERK levels in fetal brain vesicles in the control group, VPA group and VPA+MT group. (B) Quantitative analysis of Src and p-Src/Src in each group in Figure 5A. (C) Quantitative analysis of PI3K and p-PI3K/PI3K in each group in Figure 5A. (D) Quantitative analysis of ERK and p-ERK/ERK in each group in Figure 5A. (E) Western blot analysis of Src/p-Src, PI3K/p-PI3K and ERK/p-ERK levels in HT22 cells in the control group, VPA group and VPA+MT group. (F) Quantitative analysis of Src and p-Src/Src in each group in Figure 5E. (G) Quantitative analysis of PI3K and p-PI3K/PI3K in each group in Figure 5E. (H) Quantitative analysis of ERK and p-ERK/ERK in each group in Figure 5E. Each experiment was repeated three times. All data are expressed as the mean ± SEM. *P<0.05, * *P<0.01, ***P<0.001.
**A** PH3  DAPI  Merge

Con

VPA

VPA+MT

**B**

Ph3+ cell number

Control  VPA  VPA+MT

---

**C**

Con  VPA  VPA+MT

PH3

15 kDa

GAPDH

37 kDa

**D**

Relative expression of PH3

Control  VPA  VPA+MT

---

**E**

Con

VPA

VPA+MT

**F**

Ki67+ cell number

Control  VPA  VPA+MT

---

141x171mm (300 x 300 DPI)
155x122mm (300 x 300 DPI)
179x90mm (300 x 300 DPI)
Table 1. Sequence of primers for real-time qPCR

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<tr>
<th>Genes</th>
<th>Forward primers (5'→3')</th>
<th>Reverse primers (5'→3')</th>
<th>Size (bp)</th>
<th>Accession Number</th>
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<td>Gpx1</td>
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Highlights

The findings in present study elucidate that MT exerts a corrective influence on the expression of antioxidant genes by modulating the Src/PI3K/ERK signaling pathways, thereby ameliorating the heightened fetal oxidative stress resulting from maternal VPA administration:

1. Melatonin (MT) can reduce the incidence of neural tube defects induced by VPA.
2. MT can inhibit the apoptosis and restore proliferation of neuroepithelial cells disrupted by VPA.
3. MT reduce the concentration of intracellular reactive oxygen species in neural tube malformations caused by VPA.
4. Src/PI3K/ERK signaling pathway was involved in the protective effects of MT on NTD induced by VPA.