METTL3-Mediated m6A Modification is Involved in Neural Tube Defects via Modulating Wnt/ β -Catenin Signaling Pathway

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Keywords: Neural tube defects, M6A modification, METTL3, S-adenosylmethionine, Ethionine, Apoptosis

Posted Date: June 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-609334/v1>

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Abstract

Neural tube defects (NTDs) remain one of the most life-threatening birth defects affecting infants. Most patients with a NTDs eventually develop lifelong disability, which cause significant morbidity and mortality and seriously reduce the quality of life. Therefore, identifiable of novel pathogenic strategies for NTDs patients is urgently required. Increasing evidence indicates that METTL3-mediated m6A modification is present in many physiopathological processes of cell apoptosis and survival. Our results demonstrate that SAM play not only a compensatory role, SAM can also lead to m6A modification changes in neural tube development and regulation. This research provides a good theoretical basis for further research on folic acid deficiency leading to NTDs, reveals the important role of SAM in the development of NTDs, and provides new clue for clinical researchers and clinical work.

Introduction

Neural tube defects (NTDs) are increasingly prevalent condition which mainly include anencephaly, spina bifida and encephalocele. The incidence of NTDs is 0.5-2/1000, especially in Shanxi Province of China, where the incidence is as high as 13.9/1000 (Z et al., 2006). The pathogenesis of NTD involves both genetic and environmental factors. Many results suggest that folate deficiency is a risk factor for NTDs (Rogner et al., 2000). At present, folic acid has become a new treatment for NTDs, but it cannot prevent all types of NTDs (JR et al., 2015). Therefore, there is an urgent need to develop a new intervention to prevent and treat NTDs. Recently, it has been reported that exogenous folic acid may prevent NTDs by regulating epigenetic modification and/or cell proliferation (2015; AJ et al., 2013; ND and AJ, 2014). However, the research on its mechanisms was not deep enough.

Ethionine is a natural compound, and it is an S-ethyl analog of methionine with a small change in structure (PATTERSON et al., 1964; Zhang et al., 2020b). It competes with methionine in combination with methyladenosyltransferase (MAT), leading to a reduction in S-adenosylmethionine (SAM) during the synthesis of DNAs, RNAs and proteins (Dunlevy et al., 2006). Some studies have found ethionine can cause NTDs by blocking the methionine cycle (Leung et al., 2017; Zhang et al., 2020a). However, research on ethionine-induced NTDs is currently limited to ethionine can induce NTDs, leading to a one-carbon unit metabolic disorder, and the SAM level and SAM/SAH ratio are reduced. The specific pathogenesis of NTDs caused by SAM decline induced epigenetic modification remain elusive.

M6A is the most common and highly conserved RNA modification in eukaryotic cells, and affects the stability, alternative splicing, nuclear export and translation efficiency of mRNA metabolism (C et al., 2017b). Increasing evidence indicates that m6A modifications are present in many physiopathological processes of cell apoptosis and survival (J et al., 2019a). In recent years, m6A modification has attracted great interest in the field of embryonic development. It is known that METTL3 is one of the key methylase in the m6A modification (C et al., 2017b). Several teams have recently confirmed METTL3-mediated m6A modification is essential in mammalian embryonic development (J et al., 2019b; X et al., 2020). However, the complex mechanism of epigenetic of NTDs have yet to be determined.

Neural tube is regulated by precise programming among various developmental regulation-related signaling pathways during the closure process (DP et al., 2017; IE et al., 2003). Studies have shown that Wnt/ β -catenin signaling pathway is one of key signaling pathways, which affect the development of the neural layer and the failure of neural tube closure (Z et al., 2018). Wnt/ β -catenin signaling regulates embryo development in all aspects of embryonic pattern and cell size to regulation of cell movement and tissue polarity (C, 2012; R et al., 2015). Wnt/ β -catenin signaling has been shown to play an important role in maintaining self-renewal and regulating NSC differentiation (C et al., 2019).

Previous research also found that folic acid supplementation protected against PM2.5 cardiac development toxicity by Regulating Wnt/ β -catenin signal pathways (C et al., 2017a). However, the regulatory mechanism by which the Wnt/ β -catenin signaling pathway affects neural tube closure is unclear.

Herein, our previous studies demonstrated that m6A modification reduced by knocking down METTL3 or treating with ethionine, and METTL3-mediated m6A was necessary for the balance between cell proliferation and apoptosis. Our report provided the theoretical evidence of the NTDs arising from failure of m6A modifications.

Materials And Methods

Animals

All C57BL/6 mice (9–10 weeks, 19–25g) procedures were approved from the Animal Laboratory Center of Shanxi Medical University, Taiyuan, People's Republic of China. The procedure was in accordance with the "Guidelines for the Use of Nursing Animals" issued by the National Institutes of Health (NIH, 8th Edition, 2011). The experimental protocol was approved by the Experimental Animal Management Committee of Shanxi Medical University. The mice were maintained on a 12-hour light/dark cycle (lights on from 8:00 am–8:00 pm). On day 7.5 of pregnancy (E7.5), ethionine (Sigma-Aldrich, USA) was intraperitoneally injected only once at a dose of 500 mg/kg to establish the NTDs embryo model. And SAM (MedChemExpress, USA) was intraperitoneally injected only once at a dose of 30 mg/kg. The same dose was intraperitoneally injected to the pregnant mice for control group.

Cell culture and treatments

Immortalized hippocampal neuron cell (HT-22), maintained in DMEM (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA). All cells were incubated in atmosphere with 5% CO₂ at 37°C. The cells were treated with 20 mmol/L ethionine, 2mmol/L SAM for 48 hours. The siRNA-METTL3 plasmid was synthesized by Sangon Biotech (Shanghai, China), and transfected into the cells using Lipofectamine RNAiMax (Santa Cruz) according to the manufacturer's instruction. The sequence of siMETTL3 was forward: 5'-GCUGCACUUCAGACGAAUUTT-3', reversed: 5'-AAUUCGUCUGAAGUGCAGCTT-3', and that of the negative control (siCon) was forward: 5'-UUCUCCGAACGUGUCACGUTT-3', reversed: 5'-ACGUGACACGUUCGGAGAATT-3'.

Enzyme-Linked Immunosorbent Assay for SAM and SAH

The concentrations of SAM and SAH in embryonic tissue were determined by enzyme-linked immunosorbent assay (ELISA; Elabscience®, wuhan, China).

m6A RNA Methylation Quantification

The m6A RNA Methylation Quantification Kit (Abcam, Massachusetts, US) uses colorimetry to quantify the m6A modification in the total RNA of the sample. Total RNA was isolated from embryonic brain tissues and its concentration were detected., Binded negative control, positive control and sample RNA to assay wells with Binding Solution for 90 min at 37°C according to the instructions. After the capture antibody was incubated at room temperature for 60 min, the detection antibody and enhancement solution were added and incubated for 30 min respectively. The Developer Solution were added to each well and incubated for 10 min away from light at room temperature. When the color in positive control wells turned medium blue, added Stop Solution and detected the absorbance at 450 nm within 10 min.

TUNEL staining assay

Apoptosis was assessed using an In Situ Cell Death Detection kit, POD (Roche, USA). The E10.5 embryos paraffin sections was removed, incubated and cells were permeated at 37°C for 20 minutes. Then the TUNEL staining assay was performed as described previously (Zhang et al., 2020a).

Immunofluorescence analysis

The sections were identified by the standard immunofluorescence staining. The following primary antibodies were used: rabbit anti-Cyclin D1 (1:200; ab16663; Abcam, Massachusetts, US), rabbit anti- β -catenin (1:100, Cell Signaling Technology), PCNA (1:200, Santa Cruz Biotechnology, sc-56), TCF-4 (1:100, Santa Cruz Biotechnology, sc-166699), and secondary antibodies used as Goat Anti-rabbit IgG H&L (Invitrogen, A11011). Nuclei were counterstained with DAPI (Sigma-Aldrich). Images were captured with using a fluorescence microscope (Nikon, Tokyo, Japan).

Protein isolation and Western blot

Total protein was extracted from cells or embryo brain tissues by RIPA lysis buffer (Solarbio, Beijing, China) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, USA) and 10 mM PMSF (Solarbio, Beijing, China), and protein was assayed by the BCA protein assay kit (Thermo Scientific). 20 μ g proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Which were blocked with 5% skim milk in PBST (PBS with 0.05% Tween-20) for 1 h at room temperature. The transferred proteins were reacted with primary antibody overnight at 4°C and then labeled with secondary antibody for 1 h at room temperature. The primary antibodies used were antibody against β -catenin (1:1000, Cell Signaling Technology), TCF-4 (1:100, Santa Cruz Biotechnology, sc-166699), phosphor-GSK-3 β (Ser9) (1:1000, Cell Signaling Technology, D85E12), axin-2 (1:1000, Abcam, ab32197), C-myc (1:1000, Abcam, ab32072), Cyclin D1 (1:500, Abcam, ab16663), PCNA (1:200, Santa Cruz Biotechnology, sc-56), BCL-2 (1:1000, Abcam, ab182858), Cleaved Caspase-3 (Asp175) (1:1000, Cell Signaling Technology, 5A1E), rabbit anti- β -Tublin (1:1000; Abcam, Massachusetts, US) and secondary antibodies were goat anti-rabbit IgG (1:3000; ZB-2301; ZSGB-BIO, Beijing, China), and goat anti-mouse IgG (1:3000; ZB-2301; ZSGB-BIO, Beijing, China), and β -Tublin was used as a housekeeping control. The protein complexes were visualized using an enhanced chemiluminescent (ECL) blot detection system (ChemiDoc™ Imaging Systems, BIO-RAD, USA) following the manufacture's instruction.

Flow cytometric analysis of cell apoptosis and cell cycle

The Annexin V-FITC/PI apoptosis detection kit was used for the apoptosis assay (KeyGEN BioTECH, Nanjing, China). The cell cycle detection kit (KeyGEN, Suzhou, China) was used for the cell cycle analysis. Then the cell apoptosis and cell cycle assay were performed as described previously (Zhang et al., 2020b). Experiments were performed three times for each group. Both data analyze with FlowJo 7.6 software.

EDU analysis of cell proliferation

Cell proliferation was also estimated using Cell-Light Edu Apollo DNA in vitro Kit (RiboBio, Guangzhou, China). Proliferative cells were visualized and imaged using a Zeiss LSM 510 META Laser Scanning Confocal Microscopy (Nikon, Tokyo, Japan). Proliferative cells were counted in different optical fields (magnification $\times$ 200) selected in a random manner and analyzed by the software of Image J.

Bromodeoxyuridine analysis of cell proliferation

Cells were inoculated in a 24-well plate. Treated with SiMETTL3 and SiNC for 24 hours, washed 3 times with PBS for 5 minutes each time and placed them on a shaker with slight shaking. Added 1ml 4% polyoxymethylene at room

temperature for 30min. Then added 2mol/L HCl in 37°C for 10min, mixed in 1ml 0.1% TritonX-100 at room temperature for 5min; washed 3 times with PBS for 10min each time; used 10% goat serum to close at 37°C for 1h. Added the diluted Bromodeoxyuridine antibody overnight at 4°C. The next day, after 2h incubation at room temperature, washed 3 times with PBS, added the corresponding secondary antibody at 37°C for 2h. Washed 3 times with PBS. Then added appropriate DAPI to the wells at room temperature for 8 min. Washed 3 times with PBS for 10min and observed under a microscope.

AO-EB analysis of cell apoptosis

Detection of apoptosis by AO-EB double staining (Solarbio, Beijing, China) was also performed. The cells were cultured in the 24-well plate; 24 h after with ethionine and SAM treatment, the residual medium and the non-adherent cells were removed by washing with PBS and adding fresh PBS to the cells. A volume (20 ml) of working solution per millilitre of PBS was added (according to the dosage, mixing AO solution and EB solution into the working volume at a 1:1 ratio). After incubation for 3 mins at room temperature, cells were observed using a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, CA) and the data was presented as mean $\pm$ SEM. Statistical differences were performed by Student's *t*-test for two group comparisons and one-way ANOVA for more than two group comparisons. Moreover, in one-way ANOVA analyses, LSD *t*-test were used to estimate the significance of the results. Differences were considered statistically significant when the $*p<0.05$, $**p<0.01$ or $***p<0.001$.

3. Results

3.1. SAM prevents neural tube defects formation

Ethionine is a natural compound, which is an S-ethyl analog of methionine (Dunlevy et al., 2006). In previous studies, we proved that 500 mg/kg of ethionine caused the highest incidence of NTD (54.8%) (Zhang et al., 2020a). Based on these theoretical foundations, ethionine and SAM were intraperitoneally injected to establish the NTDs embryo model in C57BL/6 mouse on E7.5. Results found that the incidence of NTDs was 49.3%. The incidence of NTDs was 25.6% in ethionine and SAM treatment group (Table 1), suggesting that ethionine causes NTDs via reducing the metabolic level of SAM. We found that a full appearance of the structural characteristics was observed in the control embryos (Fig. 1A). Ethionine treated embryos displayed an obvious growth retardation and malformations along with a small and hypoplastic brain vesicle by Stereomicroscope (Figs. 1B-D). Simultaneously, we also extracted the pregnant mice embryonic tissue, we found that there had a reduced abundance of SAM in mouse embryonic tissue analyzed (Fig. 1E). Moreover, SAH abundance was significantly altered in NTDs embryonic tissue compared control group (Fig. 1F), and with a consequent reduction in the SAM/SAH ratio (Fig. 1G). In contrast, the levels of SAM and SAM/SAH ratio were increased significantly in ethionine and SAM treatment group (Fig. 1E, G), results showed that neural tube closure was perturbed in ethionine-induced mouse embryonic tissue. These findings suggested that ethionine induced NTDs by blocking the generation of SAM.

Table 1
Embryonic phenotypes of mice treated with ethionine and SAM

	Pregnant mice (n)	Embryos (n)	Normal n (%)	Resorption n (%)	Growth retardation n (%)	NTDs n (%)	Other malformation n (%)
SAM	36	223	207(93.0)	6(2.7)	10(4.3)	0(0)	0(0)
Ethionine	38	261	51(19.5)	31(11.8)	36(13.7)	128(49.3)	11 ^a 4 ^b (5.7)
Ethionine + SAM	37	246	142(58.1)	21(8.1)	15(6.1)	63(25.6)	3 ^a 2 ^b (2.1)
^a craniofacial malformation ^b polydactyly							

3.2. Ethionine induces apoptosis and inhibits proliferation in E10.5 embryos

To determine whether the ethionine-induced NTDs could be caused through an imbalance with cell proliferation and apoptosis, we detected protein expression level of proliferation-related indicators-PCNA using immunofluorescence. We found that ethionine-induced embryos exhibited a reduction in the density of PCNA-positive (Fig. 2A). Western blotting result found an obvious decreased in the level of PCNA in ethionine treatment group (Fig. 2B). On the contrary, the expression of PCNA was markedly increased in the ethionine and SAM-treated group compared with the ethionine-treated group (Fig. 2B). Suggesting that ethionine-induced NTDs could be due to reduced proliferation or increased mitotic progression. In addition to this, we also observed cell apoptosis in ethionine-induced NTDs embryo, we detected the ratio of TUNEL-positive cells on serial histological sections. There was an obvious increase in TUNEL-positive cell numbers in ethionine-induced embryos compared with normal group (Fig. 3A). Moreover, we also evaluated that there was a significant up-regulation and down-regulation of Cleaved Caspase-3 and BCL-2 protein, respectively in ethionine-induced embryos compared with the normal embryos (Fig. 3B). These results showed that ethionine induced apoptosis in the embryos treated with ethionine. Excessive apoptosis and reduced proliferation are manifested in the ethionine-induced NTDs embryos, however, apoptosis decreased and cell proliferation increased after SAM supplementation. Suggesting that these situations may be causal events in ethionine-induced NTDs. Thus, subsequent studies were performed in how does ethionine cause cell proliferation and apoptosis imbalance? To determine the causal relationship between reduced proliferation/enhanced apoptosis and NTD formation.

3.3. Sam Activates M6a Modification Through Mettl3

Firstly, we detected the expression of *Mettl3*, *Mettl14*, *Zc3h13*, *Rbm15*, *Kiaa1429*, *Fto* and *Alkbh5* as shown in Fig. 4A which are essential for m6A RNA modification in embryonic brain tissue. The results found that compared with the normal group, the mRNA levels of methylases *Mettl3*, *Mettl14*, *Zc3h13*, *Rbm15*, *Kiaa1429* were decreased, and the expression of demethylase *Fto*, *Alkbh5* were increased in NTDs group ($P < 0.05$), compared with ethionine-induced NTDs group, there had a Significant rise for the mRNA levels of methylases *Mettl3*, *Mettl14*, *Zc3h13*, *Rbm15*, *Kiaa1429* were increased, and the expression of demethylase *Fto*, *Alkbh5* were obviously decreased in ethionine combined with SAM group. qRT-PCR analysis observed that the m6A RNA methylases were inhibited, and the m6A RNA demethylases were specifically activated (Fig. 4A). Secondly, we selected m6A RNA methylation quantification kit to detect m6A RNA methylation levels in normal embryos, ethionine-induced embryos, and ethionine and SAM-treated embryos by ELISA. We found that the m6A modification was significantly reduced in the ethionine-treated group compared with the normal group, but after the SAM supplementation, the m6A modification was obviously increased, indicating that

methylation reactions could potentially be compromised in ethionine-induced embryos (Fig. 4B). METTL3 is first identified component of the methyltransferase complex and it is a key factor for m6A modification, METTL3 has effects on cell division in mammals (Y et al., 2014).

To further examine whether ethionine suppressed the expression of METTL3, we synthesized siRNA-METTL3 plasmid and transfected into the cells using Lipofectamine 2000. We tested the transfection efficiency by flow cytometry, and showed that the transfection rate was as high as 87.4%, indicating that both SiNC and SiMETTL3 were successfully transfected into the cells (Figure. 4C). Next, SiRNA-METTL3 transfected cell (SiMETTL3 group) with abundance of METTL3 mRNA was significantly decreased compared with cells transfected with SiNC-METTL3 (SiNC group) (Figure. 4D). Western blot analysis also showed that METTL3 was knocked down in the SiMETTL3 group compared to the SiNC group (Figure. 4E). The expression of METTL3 both at protein and mRNA level were consistent, confirming that METTL3 successful knocked down in cells. Three replicate experiments were performed with three samples in each group. Simultaneously, we tested whether METTL3 knocked down would change the m6A modification. As expected, the m6A level was dramatically reduced on METTL3 knockdown (Fig. 4F). These findings indicated that SAM might activate m6A modification by deleting the *Mettl3* gene.

3.4 SAM activates Wnt/ β -catenin signaling pathway by deleting *Mettl3* gene

To understand whether ethionine inhibits the Wnt/ β -catenin signaling pathway, and how ethionine affects neural tube development, we performed Western blot to test whether ethionine has a bad effect on Wnt/ β -catenin signaling in E10.5 embryos brain tissue. Results showed that the expression level of Axin-2 was up-regulated, in contrary, the expression levels of β -catenin, TCF-4, and p-GSK-3 β were significantly down-regulated in ethionine-induced embryos compared with the normal embryos (Fig. 5A). Subsequently, we also detected the protein expression levels of CyclinD1, C-myc, the target genes related to downstream proliferation of the Wnt/ β -catenin signaling pathway, we found that there was a significant drop in ethionine-induced embryos compared with normal embryos (Fig. 5B). In addition to this, we used immunofluorescence analysis to locate β -catenin, TCF-4, CyclinD1, and P53 proteins in serial paraffin sections, we found the same experimental results as Western blot (Fig. 5C). Which suggested that SAM supplementation successfully reversed the inhibitory effect of ethionine on Wnt/ β -catenin signaling pathway (Figs. 5A-C). Together, these in vivo data support a model in which Wnt/ β -catenin signaling is involved in promoting neural tube development. Moreover, to test whether the suppression of Wnt/ β -catenin signaling could be a consequence of METTL3-mediated m6A modification. We also performed Western blot analysis to detect the expression levels of Wnt/ β -catenin signaling in control or METTL3 knockdown cells. Western blot analysis showed that the expression levels of β -catenin, CyclinD1, C-myc, and p-GSK-3 β were significantly down-regulated in SiMETTL3 group compared with SiNC group (Fig. 5D). Suggesting that ethionine had inhibited Wnt/ β -catenin signaling pathway, caused changes in the expression of downstream proteins and thus resulted in imbalance between cell proliferation and apoptosis, participated in NTDs development.

3.5. Sam Reverses Balance Between With Proliferation And Apoptosis

Disrupting the proper balance of proliferation and apoptosis will lead to the occurrence of diseases. Ethionine has been found to induce excessive apoptosis of NTDs embryonic tissues. To test whether SAM supplement or METTL3 deletion would direct suppressed proliferation and induced apoptosis. Firstly, to investigate the relationships between SAM and cell biological, we treated HT-22 cells with ethionine and SAM. We used flow cytometry to detect cell cycle, and the results showed that the intervention of ethionine cause aggregation in G1 phase, and cell decline in S phase,

that is, cell accumulate in G1 phase and can't transform to S phase, inhibiting the cell from entering the process of mitosis. After ethionine combined with SAM treatment, cells in G1 phase decreased and S phase increased (Fig. 6A). Figure 6B showed that the ratio of EDU-positive cells had a conspicuous reduction in ethionine group compared with control group, and there was a significant rise in ethionine + SAM group compared with ethionine group, indicating that SAM can promote the decline of cell proliferation caused by ethionine. Finally, this study also used Western blot to detect the protein expression level of PCNA, an indicator of cell proliferation. Consistent with these findings, the protein levels of PCNA were found to be significantly lower in ethionine group than in ethionine + SAM group (Fig. 6C).

Cell proliferation and apoptosis are equally important. Flow cytometry analysis found that the combined early (lower right quadrant) apoptosis rates were rise significantly in the ethionine group compared with cells in control group, and cell apoptosis rates was reduced in the ethionine + SAM group compared with ethionine group (Fig. 7A). This work also used AO/EB staining to detect cell apoptosis, and we found that the ethionine group can induce cell apoptosis (0.1186 ± 0.009862 vs 0.8547 ± 0.0132 , $p < 0.001$), in contrast, cell apoptosis rates was reduced in the ethionine + SAM group compared with ethionine group (0.3437 ± 0.006241 vs 0.8547 ± 0.0132 , $p < 0.001$) (Fig. 7B). Next, we evaluated whether SAM was necessary for regulation of BCL-2 and Cleaved Caspase-3 by Western blot. As shown in Fig. 7C, a significant upregulation of Cleaved Caspase-3 proteins was detected after ethionine treatment. In contrast, the expression of the anti-apoptotic protein BCL-2 was reduced. Simultaneously, all results indicated that SAM supplement reversed these events. The above results indicated that SAM reversed balance between with proliferation and apoptosis.

3.6. Knockdown of METTL3 represses cell proliferation and increases cell apoptosis

To elucidate the functional roles of m6A modification in NTDs, flow cytometric analysis was performed to analyze the biological role of METTL3 in cell cycle distribution. The cell cycle results showed that the population of cells in the G1 phase was significantly increased after knockdown of METTL3. In contrast, the cell quantity in the S phase was reduced in the SiMETTL3 group compared with the SiNC group (Fig. 8A). Moreover, we examined the effect of knockdown of METTL3 on cell proliferation and apoptosis by flow cytometry and Brdu. It was also found that the SiMETTL3 group cells showed high rate of early and late apoptosis compared with cells in the SiNC group (Fig. 8B). In addition to this, Fig. 8C showed that the ratio of Brdu-positive cells was significantly decreased in SiMETTL3 group compared with SiNC group, suggesting that the downregulation of METTL3 significantly promoted cell apoptosis formation and represses cell proliferation.

4. Discussion

NTDs are the most common and most severe human birth defects, mainly due to the failure of neural tube closure during embryonic development, leading to severe neurological consequences and even fatalities (AJ et al., 2013; CA et al., 1997). The generation, proliferation, differentiation, and migration of NSCs and the resulting morphological changes are the cytological basis of neural tube development, and any abnormalities in this process can cause neural tube defects (F et al., 2013; Yu et al., 2017). The overall objective of the current study was to understand the complicated effects of m6A on neural tube closure, which might be the foundation for resolving the pathogenesis of NTDs. Our study has confirmed that SAM play not only a compensatory role, and SAM can also lead to m6A modification changes in neural tube development and regulation. Our data also indicated that METTL3 may induce apoptosis and inhibit proliferation by activating Wnt/ β -catenin signaling pathway, which provides new clue for clinical researchers and clinical work.

With this purpose, we first examined the effects of ethionine pretreatment on pregnant mice resulting in reduced levels of SAM metabolism on embryonic neuroepithelial cell proliferation and apoptosis. It was found that after pretreatment with ethionine, TUNEL-positive cells increased significantly, whereas PCNA-positive cells, which are indicators of proliferation decreased. The activation of caspase-3 is one of the most important events in the process of apoptosis, so the detection of cleaved caspase-3 is a common method in the study of apoptosis. Subsequently, we also extracted E10.5 embryonic brain tissues and tested the effects of ethionine on proliferation and apoptosis using Western blotting. The same experimental results were found, and the results showed that ethionine can induce cells Reduced proliferative capacity and excessive apoptosis.

SAM is a provider of methyl donors in the body. After pretreatment with ethionine in this study, SAM metabolism levels will decrease. Increasing evidence shows that transcription factors and epigenetic modifications jointly regulate and act on gene transcription and participate in the development of diseases (J et al., 2013; X et al., 2014). Research report that m6A modifications are involved in various biological processes including cell fate determination, embryonic development, and cell cycle control (Horiuchi et al., 2013; Liu et al., 2014). Increasing evidence indicates that m6A modification plays an important role in mammals, and that the METTL3 mutation is lethal to both mammalian and plant embryos (M et al., 2018). Some studies have used Si-METTL3 to interfere with oocytes, and it was found that the reduction of METTL3 can inhibit the translation efficiency of mRNA and destroy the degradation of mRNA, further indicating that the reversible m6A modification has a role in mammalian oocyte maturation and preimplantation embryo development important role (X et al., 2020). In this study, to explore the roles of METTL3 in the m6A modification, we synthesized siRNA-METTL3 plasmid and transfected into the cells, results found METTL3 deletion reduced m6A modification, as well as inhibited cell proliferation and promoted apoptosis.

The Wnt/ β -catenin signaling pathway is one of the key pathways for many gene expressions that regulate cell proliferation, differentiation, and survival. The previous research group underwent low-folate culture and performed expression profiling. As a result, they also found that the Wnt/ β -catenin signaling pathway is involved in folic acid metabolism. Subsequently, at the embryonic tissue and cell level, we used Western blotting and immunofluorescence experiments. As a result, we found that ethionine inhibits the activation of the Wnt/ β -catenin signaling pathway, leading to changes in the expression of classic proteins and downstream target genes, which is reflected as the expression of upstream classical protein Axin-2 was up-regulated. On the contrary, the expression levels of β -catenin, TCF-4, and p-GSK-3 β protein were significantly decreased. After supplementation with SAM, this phenomenon of suppressing activation was also changed, and the Wnt/ β -catenin signaling pathway was activated, and downstream target genes played a regulatory role. These results indicate that ethionine affects histone methylation and m6A RNA methylation modification by reducing SAM metabolism, inhibits the activation of Wnt/ β -catenin signaling pathway, regulates the expression of downstream target genes, and participates in the occurrence and development of NTDs.

However, ethionine inhibits the activation of the Wnt/ β -catenin signaling pathway, regulates the expression of downstream target genes, affects the biological characteristics of cells, and through which biological changes affect the neural tube closure? This is also the most important scientific issue. Based on this, combined with the first part at the embryonic level, ethionine has been found to cause excessive apoptosis and decreased proliferation of neuroepithelial cells. At the cell level, we give ethionine and SAM pretreatment to observe cell proliferation, Apoptosis and differentiation levels, we used flow cytometry, Western blotting, EDU and other experimental methods, and found that after the treatment with ethionine, the same over-apoptosis, proliferation inhibition, and blocked differentiation occurred at the cell level. In addition, based on the importance of the METTL3 gene, after we silenced the METTL3 gene, we observed changes in the cell phenotype and found that the level of apoptosis increased and the level of proliferation decreased. These results indicate that the deletion of ethionine and METTL3 genes can lead to imbalance in apoptosis and proliferation, and participate in the development of NTDs.

In conclusion, our results demonstrate that SAM play not only a compensatory role, it can also lead to m6A modification changes in neural tube development and regulation. We first found that ethionine affected m6A modification by reducing SAM metabolism; second, we found that changes of m6A modification inhibited Wnt/ β -catenin signaling pathway; Finally, we found that target genes downstream of the Wnt/ β -catenin signaling regulate cell biological changes, causing imbalances in proliferation, apoptosis, and differentiation. From the perspective of disease prevention and treatment, this research provides a good theoretical basis for further research on folic acid deficiency leading to NTDs, reveals the important role of SAM in the development of NTDs, and provides new research for clinical researchers and clinical work, ideas and new clues.

Abbreviations

NTDs: Neural tube defects; METTL3: methyltransferases methyltransferase-like 3; METTL14: methyltransferase-like 14; FTO: fat-mass and obesity-associated protein; ALKBH5: alkylation repair homolog protein 5; TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling; DMEM: Dulbecco's Modified Eagle's Medium;

Declarations

Author contributions

LZ and RC wrote the manuscript. AK and XW helped in revising the manuscript. LZ, DL and YS analyzed the data. JZ contributed to the manuscript for literature research. JX, JX and BN revised and approved the manuscript. All authors read and approved the manuscript for submission.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We thank all the mice which contributed to this study.

Availability of data and materials

All the data generated or analyzed during this study are included in this article.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. 81671462), the Beijing Municipal Natural Science Foundation (No.7182025), Natural Science Foundation of Health Commission of Shanxi Provincial (2020090), and School-level Doctoral Foundation of Shanxi Medical University (XD2005-SD2004).

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Figures

Figure 1

Ethionine induced neural tube defects by damaging the methionine cycle. (A-D) Ethionine-induced anencephaly in C57BL/6 mouse embryos at E10.5. Scale bars, 500 μ m. (E) The abundance of S-adenosylmethionine (SAM) was significantly lower in E10.5 NTDs embryos than in wild-types ($*p < 0.05$, F and G) S-adenosylhomocysteine concentration (F, SAH) was elevated, and the SAM/SAH ratio (G) was reduced in ethionine-induced embryos compared with normal embryos ($*p < 0.01$, significantly differs from ethionine-induced embryos). However, the levels of SAM were increased significantly after treating with 2 mM SAM. (F) SAH and the SAM/SAH ratio (G) was reduced in ethionine-induced mouse embryos compared with normal embryos ($**p < 0.01$), which is significantly different from Control. Similarly, after giving 2 mM SAM compensation, the levels of SAH and SAM/SAH ratio were obviously increased ($*p < 0.05$). $n=8$ samples per genotype for each tissue.

Figure 2

Ethionine inhibited cell proliferation in E10.5 embryos. (A) PCNA positive cell (green) of E10.5 NTDs embryos and normal embryos by immunofluorescence. Cell nuclei were stained with DAPI (blue). Scale bars, 500 μ m. (B) Western blot detected the expression profiles of PCNA in embryonic tissue treated with ethionine and SAM. β -actin levels were also evaluated to confirm equal loading (n = 3). ***P < 0.001 compared with WT; **P < 0.01 compared with ethionine+SAM.

Figure 3

Ethionine induced excessive cell apoptosis. (A) The cell apoptosis were examined by TUNEL assay (green) in WT, ethionine, SAM, ethionine+SAM E10.5 embryos. Cell nuclei were stained with DAPI (blue). Scale bars, 500 μ m. (B) BCL-2, Cleaved Caspase-3 protein levels in WT, ethionine, SAM, ethionine+SAM embryos brain in E10.5 were evaluated via Western blot and quantified; β -actin levels were also evaluated to confirm equal loading (n=3). Each experiment was carried out in triplicates ***P< 0.01 vs. control group.

Figure 4

SAM activated m6A modification through METTL3. (A) qRT-PCR was used to detect mRNA level of N6-methyladenosine RNA methylase and demethylase in normal and NTDs embryo brain tissue at E10.5. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs control. (B) ELISA was used to detect m6A levels in normal and NTDs embryo brain tissue at E10.5. *** $P < 0.001$ and **** $P < 0.0001$ vs control. (C) Flow cytometry was used to detect the effect of SiRNA transfection, and SiNC was used as a negative control. (D) Western blot analysis detected the METTL3 protein level in the SiMETTL3 and SiNC groups was evaluated, and the expression of β -Tubulin was used as the loading control. (E) METTL3 gene expression as assessed by RT-qPCR analysis in HT-22 cells. Mean $\pm$ SEM * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.005$ vs. The β -actin gene was used as a control. (F) ELISA was used to detect m6A levels in SiMETTL3 and SiNC cells. **** $P < 0.0001$ vs control.

Figure 5

SAM activated Wnt/β-catenin signaling pathway by deleting *Mettl3* gene. (A) Axin-2, β-catenin, TCF-4 and p-GSK-3β (A) and C-myc, Cyclin D1 (B) in E9.5 embryos. Bar graphs for protein abundance were quantitative data from three independent experiments. (C) Representative micrographs of immunofluorescent staining for β-catenin (green), TCF-4 (green), and Cyclin D1 (red) to determine whether SAM activated Wnt/β-catenin signaling pathway. Nuclei stained blue with DAPI. (D) β-catenin, p-GSK-3β, C-myc, and Cyclin D1 in HT-22 cells treated with SiMETTL3 and SiNC. Bar graphs for protein abundance were quantitative data from three independent experiments. *indicated significant differences ($P < 0.05$) compared to the other groups in one-way ANOVA followed by Tukey tests.

Figure 6

SAM inhibited cell apoptosis of HT-22 cells. (A) The cell apoptosis in the HT-22 cells treated with ethionine and SAM was analyzed by flow cytometry. (B) Cell apoptosis was detected by AO-EB double staining and quantified. The late apoptosis cells with bright red fluorescence in ethionine-treated groups were significantly increased compared with that in the control group and ethionine+SAM group. Scale bar: 100 μ m. (C) BCL-2 and Cleaved Caspase-3 in HT-22 cells treated with ethionine and SAM. Bar graphs for protein abundance were quantitative data from three independent experiments.

Figure 7

SAM promoted cell proliferation of HT-22 cells. (A) The cell cycle distribution in the HT-22 cells treated with ethionine and SAM was analyzed by flow cytometry. (B) The effect of SAM and ethionine on cell proliferation was detected using Cell-Light Edu Apollo DNA in vitro Kit and was quantified. Images of immunofluorescence cell staining against the EDU (shown in green), and the nuclei were counterstained using DAPI (shown in blue). Scale bar: 500 μ m (C) PCNA protein levels in the HT-22 cells treated with ethionine and SAM were evaluated by western blot analysis (n=3). β -Tubulin was used as the loading control.

Figure 8

Effects of METTL3 on proliferation and apoptosis of HT-22 cells. (A) HT-22 cells were treated with wild type (SiNC) and METTL3 knock-out (SiMETTL3), and cell cycle distribution was analyzed by flow cytometry. The percentages of cells in cell cycle phases were calculated from three independent experiments. (B) Apoptosis in HT-22 cells was measured and analyzed by flow cytometry. The combined early and late apoptosis rates were upregulated significantly in the SiMETTL3 group compared with those in the SiNC group. (C) The BrdU-positive cell of HT-22 cells from SiMETTL3 was lower than that from SiNC. *** $P < 0.001$ vs SiNC group.