Okadaic acid exposure induced neural tube defects in chicken (Gallus gallus) embryos

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Research

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Abstract

Background

Okadaic acid (OA) is an important liposoluble shellfish toxin distributed worldwide, and mainly responsible for diarrheic shellfish poisoning (DSP) in human beings. It has a variety of toxicities, including cytotoxicity, embryonic toxicity, neurotoxicity, and even genotoxicity. The embryotoxicity of OA is due to it can cross the placental barrier, which was proven in mice. However, there is no direct evidence of its developmental toxicity in human offspring. The chicken (*Gallus gallus*) embryo is a classic animal model for the studies of early vertebrate embryogenesis and late organogenesis due to its multiple advantages, such as convenience for observation, similarity to mammalian embryo, easy accessibility, and manipulation, etc.

Results

OA exposure could cause NTDs and inhibit the neuronal differentiation. Immunofluorescent staining demonstrated that OA exposure promoted cell proliferation and inhibit cell apoptosis on the developing neural tube. Besides, the down-regulation of *Nrf2* and increases in ROS content and SOD activity in the OA-exposed chicken embryos indicated that OA could result in the generation of oxidative stress in early chick embryos. The inhibition of *BMP4* and *Shh* expression in the dorsal neural tube suggested that OA could also affect the formation of dorsolateral hinge points. The expression of *LBP*, *JUN*, *FOS*, and *CCL4* in Toll-like receptor signaling pathway was significantly increased in the OA-exposed embryos, suggesting that the NTDs induced by OA might be associated with Toll-like receptor signaling pathway.

Conclusion

OA exposure can induce NTDs in chick embryos and increase the incidences of embryo mortality and malformation. Oxidative stress in early chick embryos may be subsequently responsible for the formation of NTDs. OA exposure can affect cell proliferation and apoptosis. Toll-like receptor signaling pathway may be responsible for the NTDs induced by OA.

Introduction

As one of important marine toxins, OA is mainly responsible for diarrhetic shellfish poisoning (DSP) in human beings [1, 2]. OA usually accumulates in the tissues of filter-feeding bivalves, and eventually pose a great threaten to human health through consumption of contaminated shellfish [3]. Obviously, OA has become a serious concern for shellfish industry and public health since it is one of the most frequent and globally distributed marine biotoxins [2, 4].

Previous studies have proved that OA is a potent and specific inhibitor of serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) [5–7]. Studies show that OA has a variety of toxic effects, including cytotoxicity, carcinogenicity, neurotoxicity, as well as embryotoxicity [1, 8]. OA is able to induce cell apoptosis in multiple human cell lines, such as TR14, NT2-N and SHSY5Y cells [9, 10], malignant glioma cells [11], and HepaRG cells [12]. Interestingly, the nervous system is reported to be more sensitive to OA than other systems, though OA is not considered as a classical neurotoxin previously [9]. It has been demonstrated that OA can induce spatial memory impairment and neurodegeneration [13] and cause hippocampal cell loss in rats [14]. Due to its neurotoxicity, OA is judged to be an emerging tool for the studies on Alzheimer's disease [15, 16].

In addition, OA is embryotoxic potential, and can delay the development of embryos and increase the incidence of malformation and mortality in frog *Xenopus laevis* [17], fish *Oryzias latipes* [18] and *Gallus gallus* (chicken) [19]. Moreover, the embryotoxicity of OA is due to it can cross the placental barrier, which was proven in mice [20]. The content of OA in fetal tissues is even higher than that of adults, indicating the accumulation of OA in fetal tissue that cause even more damage to fetus than adults [2, 4]. So far, however, there has been no direct evidence about OA's prenatal developmental toxicity on human beings [1].

As an important morphogenetic event in embryonic development, neurulation takes place in the early stage of chordate embryogenesis, and eventually a closed neural tube is formed [21, 22]. The failure of neural tube closure will cause a group of common and severe malformations called neural tube defects (NTDs). As the second most prevalent malformations [22], NTDs affect more than 300,000 newborns worldwide each year [23]. The frequency of NTDs in pregnancies is about 1 per 1000. When NTDs occurs in the head, anencephaly, hydrocephalus, encephalocele, it is frequently associated with other malformations. If NTDs is presented in the trunk, there is a greater chance of the occurrence of congenital defects such as spina bifida [24, 25]. Therefore, based on the potential embryotoxicity of OA, it is of significant to evaluate the effect of OA exposure on embryonic neurogenesis and try to prevent them.

The chicken (*Gallus gallus*) embryo is an excellent animal model and has been extensively used for the studies of early vertebrate embryogenesis and late organogenesis [26]. As an *in vivo* experiment, the chicken embryo model has many advantages, such as convenience for observation, similarity to mammalian embryo, easy accessibility, and manipulation [27]. In this study, we employed the chick embryo as a model to explore the effects of OA exposure on embryonic neurogenesis and underlying mechanisms involved.

Materials And Methods

Chemicals

Okadaic acid (Purity≥95% by HPLC, Zaoyan, China) was dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 1 mM. The stock concentration of OA was then diluted with phosphate-buffered saline (PBS) to concentration of 100 nM.

Chick embryos

Fertilized chicken eggs were purchased from an avian farm of South China Agricultural University in Guangzhou, China. For early stage of chick embryos, early chick (EC) culture [28] was employed, in which a filter paper carrier was used to hold the early blastoderm when vitelline membrane was under tension, and the embryo grew on a substratum of agar-albumen. The agar-albumen medium was prepared as described in our previous study [19]. The HH1 chick embryos were incubated with PBS (control) or the culture media containing different concentrations of OA in an incubator (37 ℃ and 70% humidity) (Boxun, Shanghai, China) until the embryos developed to the desired stage (HH10). For the late stage of chick embryos, the eggs that have been pre-incubated for 1.5 days were administrated with the same volume of PBS or OA through pre-windowed small hole, and then incubated in an incubator (37℃ and 70% humidity) for further 3 days. The holes were sealed with UV-irradiated transparent tape to avoid dehydration and contamination.

Immunofluorescent staining

The HH 10 chick embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4℃. The primary antibodies, including (NF, 1:200, Life Technologies, USA), Tuj1 (1:200, Neuromics, USA), (1:200, DSHB, USA), Pax7 (1:400, DSHB, USA), Pax6 (1:200, DSHB, USA), pHIS3 (1:400, Santa Cruz, USA) and c-Caspase3 (1:400, Cell Signaling Technology, USA), were employed in the immunofluorescent staining of whole-mount embryos. Briefly, the fixed HH 10 chick embryos were incubated with the primary antibody at 4℃ overnight on a shaker and then washed carefully in PBST (0.1% tween-20). Next, the embryos were washed with PBT for 5 min, then blocked in blocking buffer for 6 h. Subsequently, they were incubated with a related Alexa Fluor® 488 or 555 labelled secondary antibodies (1:1000, Invitrogen, USA) at 4℃ overnight on a shaker. Finally, all the chick embryos were counterstained with DAPI (1:1000, Invitrogen, USA) at room temperature for 40 min. After photographed, the stained embryos were embedded in a solution of 7.5% gelatin-15% sucrose (w/v) and stored at -80°C. Whereafter, the embedded embryo was sectioned at a thickness of 12 μm using a freezing microtome (Leica CM1900, Germany).

In situ hybridization

In situ hybridization of whole-mount chick embryo was carried out according to the method described previously [29]. Briefly, HH 10 chick embryos were fixed in 4% paraformaldehyde overnight at 4℃, washed twice with PTW (dissolve 0.1% tween-20 in PBS) for 5 min each time, and in a graded series of methanol (25%, 50%, 75%, 100%) for 5 min, respectively. After rehydrated in methanol (75%, 50%, 25%) and PTW for 5 min, respectively, the embryos were incubated in hybridization buffer for 5 h. Subsequently, antisense probes were added to the cultures, and the embryo were incubated overnight at 65℃. Digoxigenin-labeled antisense probes were generated to specifically detect mRNA levels of bone morphogenetic protein 4 (*BMP4*) and Sonic hedgehog (*Shh*). The primer sequences used in probes are summarized in Table 1. After hybridization, the embryos were washed by using post-hybridization buffer and TBST (dissolve 0.1% tween -20 in TBS) twice for 30 min at 65℃, respectively. After blocked with a blocking reagent (Roche, Switzerland) for 5 h, the embryos were incubated with anti-DIG (digoxigenin)

antibody (1:1000, Roche, Switzerland) overnight at 4℃ on a shaker. Finally, the embryos were incubated in BCIP/NBT chromogen solution (Sigma, USA) at room temperature for staining. The stained embryos were pictured and sectioned at a thickness of 16 μm using a freezing microtome (Leica CM1900, Germany). Image-Pro Plus 7.0 (IPP 7.0) was employed to calculate the area of the target region (labelled with probes).

Photography

After immunofluorescent staining or *in situ* hybridization staining, the stained embryos and the regions of interest were pictured using a stereo-fluorescence microscope and processed with Image-Pro Plus 7.0 (IPP 7.0). The sliced embryos were pictured by using an Olympus IX51epi-fluorescent microscope, and the obtained pictures were analyzed with a CW4000 FISH Olympus software.

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from the HH10 chick embryos using a total RNA kit (R6834-01, Omega, USA) based on the manufacturer's instructions. Some of the RNA isolated was subjected to high-throughput sequencing, while others were used to reverse transcription. Agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, USA) were employed to evaluate integrity, concentration, and purity of RNA for reverse transcription, respectively. First-strand cDNA was generated from 1 μg of total RNA by using a HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). The integrity, concentration, and purity of RNA for high-throughput sequencing were determined by an Agilent 2100 Bioanalyzer and RNA Nano 6000 assay kit (Agilent Technologies, CA, USA).

Specific primers employed in this study were designed by Primer 5.0. Reference genes were screened using geVanNorm, NormFinder and BestKepper. Among the six candidate genes, including glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ubiquitin A-52 (*UBA52*), cyclophilin-A, succinate dehydrogenase complex subunit A (*SDHA*), ribosomal protein S15e (*RPS15*) and ribosomal protein L30 (*RPL30*). *RPS15* and *RPL30* exhibited the most stable expression. Therefore, the two genes were employed as reference genes to normalize expression of target genes. The primer characters used in qRT-PCR are summarized in Table 2. The PCR reaction system and procedure were performed as described in our previous paper [30].

The comparative Cq method was employed to analyze the relative expression of target genes as described by [31], in which multiple reference genes and inter-run calibration algorithms were considered. Standard curves were generated to check the efficiency of PCR amplification [32]. Amplification efficiency for each reaction should vary from 0.900 to 1.110, while correlation coefficients range between 0.990 and 0.999.

RNA-seq assay

The sequencing was conducted in BGI-Shenzhen (Shenzhen, China). Total RNA was qualified and quantified using a NanoDrop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, USA). Oligo (dt)-attached magnetic beads were used to purified mRNA. Purified mRNA was randomly fragmented into

small pieces, and sequencing libraries were established using a MGIEasy RNA-seq library prep kit (BGI-Shenzhen, China) based on the manufacturer's instructions. The library quality was assessed on the Agilent Bioanalyzer 2100 system. The final library was then sequenced on BGISEQ-500 platform (BGI-Shenzhen, China) at paired-end mode (PE150).

Trimmomatic (Version 0.36) was employed to trim adapters and low-quality bases, and Q20 was chosen for quality trimming [33]. Bowtie2 (Version 2.2.5) was applied to align the clean reads to the reference coding gene set, then expression level of genes was calculated by RSEM (v1.2.12) [34]. Differential expressed genes (DEGs) analyses were performed using the DESeq2 (v1.4.5) [35] with |Fold Change | ≥2 and Q-values ≤ 0.001. Gene Ontology (GO, http://www.geneontology.org/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) enrichment analyses of annotated different expressed genes were performed with Phyper based on hypergeometric test. The significant levels of terms and pathways were corrected by Q-values with a rigorous threshold (Q value ≤ 0.05) by [36].

Western blot

Total protein concentration was measured by using a BCA Protein Assay Kit (Beyotime, China) according to the manufacturer's instructions. The samples containing equal amounts of proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, USA). The membranes were blocked with 5% Difco™ skim milk (BD, USA), and then incubated with primary and secondary antibodies. All primary and secondary anti-bodies used were diluted to 1:1000 and 1:3000 in 5% skim milk, respectively. The protein bands were visualized with an ECL substrate kit (BIO-RAD, USA). The antibody-stripped membrane was then blocked again and re-incubated with other antibodies.

Detection of MDA content and SOD activity

Thirty HH10 chick embryos were harvested from each experimental group. The ten embryos within the same treatment were pooled together as one sample, and each group contains three pooled samples. The malondialdehyde (MDA) levels and superoxide dismutase (SOD) activities were detected in tree homogenized samples isolated from control or OA-treated groups according to the manufacturer's instructions. MDA content was measured by using a lipid peroxidation MDA assay kit (Beyotime, China). SOD activity was determined by using a total superoxide dismutase assay kit with WST-8 (Beyotime, China). A microplate reader (Tecan Sunrise, Switzerland) was available for absorbance detection in the experiments.

Flow cytometry analysis

The HH10 chick embryos were harvested in a cell culture dish on a clean bench. After being rinsed with sterilized PBS (phosphate buffer saline), the tissue was transferred to a sterile centrifuge tube. To the centrifuge tube, trypsin (0.25%) was added, and the tissue was blown to homogenate with a pipette. Cell culture medium (Gibco, USA) was introduced to terminate trypsin digestion. Finally, the mixture was

filtered with a 200-mesh sterile cell filter sieve (Jing An, China), and the filtered cell suspension was centrifuged at 1600 × g for 2 min, and liquid was discarded.

For the analysis of apoptosis, we re-suspended cells in 100 μl of binding buffer (BD, USA), then added 2.5 μl of Annexin V-FITC and propidium iodide (PI) to the cell suspension. Thereafter, another 200 μl of the binding buffer were added to the mixture. After being incubated for 15 min at room temperature in the dark, the cell suspension was transferred to the upper sample tube. For the detection of reactive oxygen species (ROS) content, we re-suspended cells in 200 μl of dichlorofluorescein diacetate (DCFH-DA) reagent and incubated at room temperature for 15 min in the dark. After being washed with sterile PBS, the cell suspension was centrifuged at 1600 × g for 5 min. Finally, we re-suspended cells in 300 μl of PBS and transferred it to the upper sample tube for ROS detection. Flow cytometry analysis was performed on the FACSCanto (BD, USA) system.

Statistical analyses

Statistical analyses were carried out by using GraphPad Prism 7 software (CA, USA). All data are presented as mean ± SD. After testing for homogeneity of variance, Student's t-test was employed to check the differences between the control and OA-treated groups.

Results

OA exposure induced craniofacial abnormality in early-stage chick embryos

To explore the possible toxicological effects of OA on chick embryo development, EC culture was performed as shown in Figure 1A. The HH10 chick embryos were exposed to the culture media containing different concentrations of OA (20, 50, 100, 200 and 500 nM). Three types of NTDs were observed, including cranial abnormality, trunk abnormality and both (Figure 1B), and some embryos died during the incubation. The mortality rate was the highest when embryos were exposed to OA at 200 nM or 500 nM, about 85% (Figure 1C), while the mortality rate was approximately 30% in 20 nM or 50 nM OA treatment group (Figure 1C). When the embryo was exposed to OA at 100 nM, both cranial and trunk abnormality was about 40% (the highest) (Figure 1D). The embryos that the neural tubes were not closed on the cranial and trunk were selected as the research object.

OA exposure caused craniofacial abnormality in late-stage chick embryos

After pre-incubated for 18 h, the chick embryo was injected with 100 μl of OA at 100 nM, 200 nM and 500 nM, respectively (Figure 2 E). After exposed to OA, we found some neural tube defects (NTDs) in 4.5-day chick embryos (Figure 2 A-D1). Compared with the control counterparts, embryo mortality and malformation rate of embryos were increased (Figure 2 G). In addition, the weight of the embryos showed a trend of decline after exposed to OA. The weight was distinctly lower than that of control when exposed to OA at 500 nM ($p<0.05$) (Figure 2 F and G). These results suggest that OA could cause neural tube defects in late-stage chick embryos.

OA exposure led to abnormal neurogenesis during chick embryo development

To investigate the effects of OA on early embryonic neurogenesis, we performed immunofluorescent staining with NF (neurofilament) and Tuj1 (class III β-tubulin) in OA-treated embryos (HH 10). As shown in Figure 3, the expressions of NF (Fig. 3 A-D2 and I) and Tuj1 (Fig. 3 E-H2 and J) were significantly reduced after exposed to OA. These indicate that OA exposure inhibit the neuronal differentiation, which might partially contribute to the OA-induced NTDs.

OA exposure inhibited cell proliferation but promoted cell apoptosis in the developing neural tubes

To explore whether OA exposure affect proliferation of neural progenitor cells during neural tube development, we evaluated cell proliferation of neural progenitors in the developing chick embryos treated with OA using pHIS3 as a cell proliferation marker. As demonstrated in Figure 4 A-D2 and E, the number of $pHIS3^{+}$ cells were significantly increased, suggesting that OA exposure promoted cell proliferation.

In the same way, we detected the changes in expression of c-Caspase3 and c-Caspase9 in the developing neural tubes after exposed to OA. As shown in Figure 5 A-D1, the expression of Caspase3 significantly decreased both in mRNA (Fig. 5 E) and protein levels after OA treatment (Fig. 5 G). However, C-caspase9 showed no significant changes in protein and transcriptional levels (Figure 5 E and G). Flow cytometry analyses showed that the apoptosis rate was obviously reduced compared with control counterpart (Figure 5 I-J). These results suggest that OA exposure could promote cell proliferation and inhibit cell apoptosis in the neural tubes of early chick embryos.

OA exposure could induce oxidative stress in early chick embryo

To understand whether oxidative stress is promoted in early chick embryo after OA exposure, flow cytometry was employed to detect ROS content in chick embryos. As in Figure 6, ROS content (Fig. 6A and H) and SOD activity (Fig. 6 B) were evidently increased after OA treatment. However, there were no significant changes in MDA level (Fig. 6 C). qPCR data demonstrated that *Nrf2* and *CBP* transcripts were markedly down-regulated, while *CREB* and *KEAP1* mRNA levels did not experience any changes (Fig. 6 D-G). Western blot analyses revealed that Nrf2 expression was distinctly reduced in protein level after OA exposure (Fig. 6 I). These results indicate that OA exposure induced oxidative stress in early chick embryo and inhibited the Nrf2 signaling pathway.

Effects of OA on BMP/Shh signaling molecules

As presented in Figure 7A, BMP4 and Shh signaling pathways play important roles in regulating the formation of dorsolateral hinge points (DLHP). The results of *in situ* hybridization demonstrated that the expression of BMP4 was significantly inhibited both at cranial and trunk after OA exposure compared with control group (Figure 7 B-C3). However, qPCR data showed no significant difference in *BMP4* transcription (Fig. 7 D). Compared with control group, the Shh expression in the dorsal neural tubes was

obviously reduced after OA treatment (Fig. 7 E-H4). Accordingly, the expression of Pax7 on the dorsal part of cranial and trunk neural tubes was decreased in the OA-treated embryos (Fig. 7 E'-H' and I-J). These results suggest that the formation of NTDs might be related to the inhibition of Pax7 and *BMP4* expression induced by OA exposure.

Transcriptome analysis and qRCR validation

Samples were sequenced on the BGISEq-500 platform, and an average of 6.94Gb data was produced for each sample. The raw data have been deposited in the NCBI SRA database (https://dataview.ncbi.nlm.nih.gov/object/PRJNA673393?reviewer=hcgkqljjc0sfffpsu7tdla9gem).The ratio of clean reads to raw reads was greater than 93.73%, and the ratio of clean reads Q2 was greater than 97.5%, indicating that the sequencing quality was reliable (Supplementary Table S1). All the correlation coefficients of biological replications were higher than 0.979, suggesting that the expression patterns of the three replications under the same treatments were highly similar (Supplementary Figure S1). The average ratio of samples to genome was 89.72% and the ratio of gene set was 78.82% (Supplementary Table S2).

Among the 782 DEGs detected, 485 genes were up-regulated (red) and 297 down-regulated (green) (Fig. 8 A). Based on the GO (Gene Ontology) enrichment analyses, these DEGs are mainly distributed in "Biological regulation", "Cellular process", "Metabolic process", "Response to stimulus", "Cell", "Catalytic activity", "Membrane" and "Binding" (Supplementary Figure S2). According to the KEGG pathway category, 45 DEGs were annotated to "Cell growth and death", 146 DEGs were annotated to "Signal transduction", 26 DEGs were annotated to "Folding classification and degradation", 102 DEGs were annotated to the "Immune system", 29 DEGs were annotated to the"Nervous system", and 38 DEGs were annotated to"Development" (Supplementary Figure S3). In the KEGG pathway enrichment analyses, "Drug metabolism-cytochrome P450 (CYP450)", "Legionellosis" and "Malaria" had the highest proportion of DEGs (about 0.14) (Figure 8 B). In addition, the enrichment ratios of Toll-like receptor signaling pathway, IL-17 signaling pathway and TNF signaling pathway were also close to 0.14, with high number of DEGs and low Q-values (Fig. 8 B).

The representative differentially expressed genes induced by OA exposure are summarized in Table 2, which indicated that the multiple key genes involved in Toll-like receptor signaling pathway such as *LBP* (lipopolysaccharide-binding protein), *JUN* (transcription factor AP-1), *FOS* (proto-oncogene protein c-fos) and *CCL4* (C-C motif chemokine 4), were significantly increased after exposed to OA. In addition, most assayed genes displayed similar expression levels as detected in transcriptome analysis, as demonstrated in Table 3 and Figure 8, which corroborated the transcriptome data.

Discussion

Several studies have revealed that OA exposure could delay zebrafish *Oryzias latipes* [18, 37], frog *Xenopus* [17], longfin yellowtail *Seriola rivoliana* [38] and *Gallus gallus* [19] embryonic development and increase embryo mortality. More importantly, it has been shown that OA can cross the placental barrier,

suggesting that OA may cause more harmful to fetuses compared to adults since fetus is more vulnerable [20]. Because the symptoms of DSP are very similar to gastroenteritis, OA's neurodevelopmental toxicity is often overlooked by investigators. Hence, it is absolutely necessary to evaluate the neurodevelopmental toxicity of OA exposure during pregnancy.

We found that OA exposure could increase the incidences of NTDs and fetal mortality. When the developing chick embryos were exposed to OA at 200 nM and 500 nM, the embryonic mortality rate was about 85% and 65%, respectively. When the embryos were exposed to OA at 100 nM, the embryo malformation rate was at its highest, about 50%. Therefore, we finally chose 100 nM as the experimental concentration and analyzed the abnormal phenotype in both cranial and trunk levels.

To explore the underlying mechanisms of neural tube malformation induced by OA, we first analyzed the neuronal differentiation in the neural tube of chicken embryos. Neurofilament (NF), an intermediate filament protein in the cytoplasm of neurons, is the most abundant component in the cytoskeleton and myelinated axons of mature neurons [39]. Normal expression of NF is closely related to the growth and regeneration of axon and plays an important role in maintenance of neuronal homeostasis [40]. Abnormal development of neurofilament may lead to a variety of diseases, including ALS (amyotrophic lateral sclerosis), AD (Alzheimer's disease) and CMT (Charcot-Marie-Tooth) [39]. Tuj1 is a class III member of the β-tubulin protein family. Its expression correlates with the earliest phases of neuronal differentiation. As a marker for the recognition of positive neurons, it has been widely used in many studies since the human brain was found to produce new neurons from neural stem cell pools [41]. The decrease in NF and Tuj1 expressions suggest that OA exposure disrupted the neuronal differentiation and might eventually facilitate the NTDs.

To further understand the causes for the inhibition of neuronal differentiation induced by OA exposure, we used pHIS3, a proliferation marker, to detect cell proliferation in the neural tube. The results showed that the number of neural tube cells in the proliferative state increased, suggesting that OA exposure could promote cell proliferation. Similarly, we also found that OA exposure could inhibit the cell apoptosis in the neural tube revealed by the immunofluorescent staining of c-Caspase3. As the specific inhibitors of protein phosphatases PP1 and PP2A, it has been reported that OA could induce cell apoptosis in many cell types [42]. However, several studies have also shown that OA could also block apoptosis through inhibiting PP2A activity [43–45]. OA could protect SH-SY5Y cells from 1-methyl-4-phenylpyridinium ion-induced apoptosis [45]. These conflicting outcomes obviously imply the complexity of OA effect on cell apoptosis.

There has been reported that oxidative stress could cause NTDs through suppressing the expressions of related genes [46]. Therefore, we speculate that OA-induced oxidative stress may play an important role in this process. Transcription factor NFE2-related factor (Nrf2) is an important transcription factor, conferring protection against oxidative damage by orchestrating antioxidant and detoxification responses to oxidative stress [47]. After exposed to OA, the increase in ROS content and SOD activity indicate that OA exposure indeed could cause oxidative stress in early chick embryos. Down-regulation of

Nrf2 corroborate with OA exposure-inhibited Nrf2 signaling pathway, and this in turn aggravated oxidative stress in chick embryos [47]. These results suggest that oxidative stress induced by OA also account for the formation of NTDs.

The formation of dorsolateral hinge points (DHLP) plays an important role in neural tube closure during neurogenesis [48]. Bone morphogenetic protein (BMP) signaling and Sonic hedgehog (Shh) signaling jointly regulate the formation of DHLP, subsequently affecting the closure neural tube [49]. Neural tube development is highly dependent on the precisely spatiotemporal regulation of bone morphogenetic protein 4 (BMP4), paired box 7 (Pax7) and sonic hedgehog (Shh) genes in the dorsal side of the tube [50]. After exposed to OA, the inhibition of BMP4 and Shh expression in the dorsal neural tube suggests that OA exposure could also affect the formation of DHLP, and then disturb the subsequent folding process, and ultimately lead to incomplete closure of the neural tube.

To understand the underlying molecular mechanisms by which OA exposure induces neural tube defects, we performed transcriptomic sequencing on the early chicken embryos. A total of 782 differentially expressed genes were obtained with 485 of up-regulated genes and 297 of down-regulated ones. These DEGs were mainly enriched in cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, IL-17 signaling pathway and TNF signaling pathway. To further assess if OA exposure activated Toll-like receptor signaling pathway in the early chicken embryos, we observed the expression of some genes related to Toll-like receptor signaling pathway by using qPCR. As our expected, the expressions of *LBP*, *JUN*, *FOS* and *CCL4* in Toll-like receptor signaling pathway were significantly increased after exposed to OA, which is consistent with the results of transcriptome sequencing. The slight discrepancy between transcriptome analysis and qPCR might be due to the diversity in the statistical processing of data [51].

Toll-like receptors (TLRs) play a crucial role in the innate immune system by recognizing pathogen-associated molecular patterns derived from various microbes [52]. FOS, the immediate early transcription factor of neurons, is closely connected with neuronal programmed cell death [53]. c-Fos is a member of FOS family proteins (Fra-1, Fra-2, FosB), which can heterodimerize with members of JUN family (c-Jun, JunB, JunD) to form transcription factor activator protein 1 (AP-1) [54]. AP-1 transcription factor complexes can affect cell proliferation and differentiation via regulating gene expression in response to positive and negative stimuli [55]. Shaulian and Karin (2001) [56] found that cell proliferation and cell cycle were inhibited in mouse fibroblasts and erythroleukemia cell lines when the expressions of *FOS* and *JUN* were suppressed by antisense RNA. Kovary and Bravo (1991) [57] reported that microinjection of anti-Fos and anti-Jun antibodies efficiently prevented serum-stimulated or asynchronously growing cells from entering the S phase. In our study, *FOS* and *JUN* expressions were significantly up-regulated after OA exposure, which could explain why the numbers of apoptotic cells in the neural tube decreased while proliferating cells increased in the OA-treated embryos.

Chemokine CC (motif) ligand 4 (*CCL4*), also named macrophage inflammatory protein-1β (MIP-1β), is essential for chemotaxis of macrophages, natural killer cells, and lymphocytes [58]. *CCL4* is secreted from glial and astrocytes, and involved in the progression of various brain diseases, including Alzheimer's

disease, multiple sclerosis, and ischemic brain disease, though its function in the brain remains unclear [59]. Several reports have shown that the recombinant *CCL4* can attenuate the toxicity of methylmercury (MeHg) to primary neurons in mice, while *CCL4* knockdown in C17.2 cells results in higher MeHg sensitivity compared with control cells [59]. The up-regulation of *CCL4* in the OA-exposed embryo might be a protective response of embryos to OA exposure-induced toxicity.

As a key participant in the inflammatory response to infection, LBP is a type I acute phase response protein produced by a variety of cell types, which can enhance the recognition of endotoxins and pathogens by immune system [60]. Studies have manifested that LBP plays an important protective role in alcoholic-induced liver injury [61]. Recently, Pretorius et al. (2018) [62] found that LBP could reverse the presence or induction of fibrin amyloid in Parkinson's disease. Based on the significant reduction of egg-laying in *Biomphalaria glabrata* after silencing of LBP/BPI1 expression, Baron et al. (2013) [63] consider that LBP may be involved in prenatal immune protection of offspring. The increased expression of LBP after OA exposure might also be a protective response to OA exposure-induced toxicity.

Conclusion

OA exposure can cause neural tube defects in early chick embryos and increase the incidences of embryo mortality and malformation. OA exposure can alter the expressions of *BMP4* and *Shh*, affect the formation of DLHP, and ultimately hinder the closure of neural tube. OA exposure can cause oxidative stress in early chick embryos, which may be subsequently responsible for the formation of NTDs. OA exposure can affect cell proliferation and apoptosis through toll-like receptor signaling pathway. Our findings provide new basis for comprehensive evaluation of the neural developmental toxicity of OA during pregnancy. However, we should keep in mind that neural tube closure is a complex and precise process concerning about the regulation of multiple signaling pathways. There is no doubt that much more precise works are required to explore the molecular mechanisms of neural tube defects induced by OA exposure in the future.

Abbreviations

AD: Alzheimer’s disease; ALS:Amyotrophic lateral sclerosis; BMP:Bone morphogenetic protein; BMP4:Bone morphogenetic protein 4; CCL4:C-C motif chemokine 4; CMT:Harcot-Marie-Tooth; DCFH-DA:Dichlorofluorescein diacetate; DEGs:Differentially expressed genes; DLHP:Dorsolateral hinge points; DMSO:Dimethyl sulfoxide; DSP:Diarrheic shellfish poisoning; EC:Early chick; FOS:Proto-oncogene protein c-fos; GAPDH:Glyceraldehyde 3-phosphate dehydrogenase; JUN:Transcription factor AP-1; LBP:Lipopolysaccharide-binding protein; MDA:Malondialdehyde; MeHg:Methylmercury; NF:Neurofilament; Nrf2:NFE2-related factor; NTDs:Neural tube defects; OA:Okadaic acid; Pax7:Paired box 7; PBS:Phosphate buffer saline; PFA:Paraformaldehyde; PI:Propidium iodide; PP1:Serine/threonine protein phosphatase 1; PP2A:Serine/threonine protein phosphatase 2A; ROS:Reactive oxygen species; RPL30:Ribosomal protein L30; RPS15:Ribosomal protein S15e; SDHA:Succinate dehydrogenase complex subunit A; Shh:Sonic

hedgehog; SOD:Superoxide dismutase; TLRs:Toll-like receptors; Tuj1:Class III β-tubulin; UBA52:Ubiquitin A-52

Declarations

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets presented in the current study are available from the corresponding authors on reasonable request. And the transcriptome datasets analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra/) under PRJNA673393.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yu-hu Jiao performed the experiment and wrote the manuscript. Guang Wang was a contributor in the direction of the experiment. Da-wei Li contributed to transcriptome data analysis. Hong-ye Li and Jie-sheng Liu provided instructional advice in data analysis. Wei-dong Yang and Xuesong Yang helped perform the analysis with constructive discussions and modified the manuscript.

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Tables

Table 1 Primer sequences used in this study

Genes	Primer sequence 5’-3’	Products size (bp)
RPL30	F: CTGGTGATGAAAAGCGGTAA	108
	R: CAAAGCAGGACAGTTGTTGG	
RPS15	F: TTCCGCAAGTTCACCTACAG	165
	R: CTCCTTCTTGGCCTTACGAA	
CASP8	F: TGGGAAAGTGGACAAGAGCC	73
	R: CATCTCTCCTTCACCAAGTAAGT	
MAP2K4	F: GCATGCAGGGTAAACGCAAA	70
	R: AACCTTGCCGTGGACTTGAA	
SPP1	F: GAGCGTAGAGAACGACAGCC	139
	R: CTCTAGCGTCTGGTTGCTGG	
CCL4	F: AGCCTCCTCTGCCCCAG	153
	R: TCGCGCTCCTTCTTTGTGAT	
FOS	F: GCCGACATGATGTACCAGGG	101
	R: GACGGGTAGTAGGTGAGGCT	
IL12B	F: CACCAGCCGACTGAGATGTT	103
	R: GAGGTGGGTCTGGCTTTATGAT	
LBP	F: AAGGTTTGTGACAGCGTGGT	77
	R: ACGTTTGCTTCTGGCAAGGT	
HPGDS	F: GCCATTCCAACTGCATTCCC	84
	R: TTTTCTCCCTCTGCGAACCC	
CREB	F: AATGGATCTCTTGGGGCAGC	186
	R: ACCTGCCATTCCCATTTTTGT	
CBP	F: CCTCAACCACATGACGCACT	111
	R: GGACAGTCGTGCCGAGTACA	
Nrf2	F: GGCCGTCTTGAAGCTCATCTC	175
	R: TGCCTCTCCTGCGTATATCTCG	
Caspase3	F: CCACCGAGATACCGGACTGT	173
	R: TGCTTCGCTTGCTGTGATCT	

Caspase9	F: GGAATGAGGACGAGCCAGAC R: GTACCACGAGCCACTCACCTT	119
KEAP1	F: ACTTCGCTGAGGTCTCCAAG R: CAGTCGTACTGCACCCAGTT	142
IL1B	F: GGAGAGCAGCAGCCTCAG R: AGCCCTCCCATCCTTACCTT	78
JUN	F: CCTCCCCTGTCCCCTATTGA R: CCTTTTCCGGCATTTGGACG	99

Abbreviations: RPL30, ribosomal protein L30; RPS15, ribosomal protein S15e; CASP8, caspase 8; MAP2K4, mitogen-activated protein kinase kinase 4; SPP1, secreted phosphoprotein 1; CCL4, chemokine CC motif ligand 4; FOS, proto-oncogene protein c-fos; IL12B, interleukin 12B; LBP, lipopolysaccharide-binding protein; HPGDS, hematopoietic prostaglandin D synthase; CREB, cAMP-response element binding protein; KEAP1, Kelch-like ECH-associated protein 1; IL1B, interleukin 1 beta; JUN, transcription factor AP-1. CBP, CREB-binding protein; Nrf2, nuclear factor erythroid 2-related factor 2.

Table 2 Representative differentially expressed genes in the early chicken embryos after exposed to OA

ID	KO	Name	Product	Log2(FC)	Q-value
107056355	K10030	IL8	interleukin 8	-1.74	5.08E-85
107056614	K04398	CASP8	caspase 8	1.47	1.13E-62
107057160	K04430	MAP2K4	mitogen-activated protein kinase kinase 4	10.38	1.4E-101
395196	K04519	IL1B	interleukin 1 beta	3.86	1.76E-108
395210	K06250	SPP1	secreted phosphoprotein 1	1.03	0
395468	K12964	CCL4	chemokine CC motif ligand 4	3.54	0
395872	K10030	IL8L1	interleukin 8-like 1	7.46	2.33E-20
396330	K09447	IRF7	interferon regulatory factor 7	1.49	1.71E-128
396512	K04379	FOS	proto-oncogene protein c-fos	2.19	0
404671	K05425	IL12B	interleukin 12B	1.77	7.7E-20
416548	K17783	ERV1	mitochondrial FAD-linked sulfhydryl oxidase	-1.01	2.29E-19
424673	K04448	JUN	transcription factor AP-1	1.27	0
771461	K05399	LBP	lipopolysaccharide-binding protein	1.37	2.1E-10
395863	K04097	HPGDS	hematopoietic prostaglandin D synthase	1.75	3.71E-25

Table 3 Changes of partial differences in gene expression

Genes	Transcriptome results Log2(FC)	qPCR results
CASP8	1.47	1.16
MAP2K4	10.38	-
IL1B	3.86	-
SPP1	1.03	-
CCL4	3.54	-
FOS	2.19	2.73
IL12B	1.77	-
JUN	1.27	1.94
LBP	1.37	1.51
HPGDS	1.75	-0.62

Note: “-” stands for no significant changes detected