Retinal developmental neurotoxicity of trimethyltin chloride: in terms of excitotoxicity and excitatory amino acid transporters

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Abstract

Trimethyltin chloride (TMT), which is an organotin compound widely used in the agricultural and industrial fields, is a well-known neurotoxicant. In particular, excitotoxicity is suspected to be an important mechanism underlying TMT toxicity; however, the effect of TMT exposure on the retina during development and the mechanisms have not been fully elucidated to date. Therefore, in this study, we exposed postnatal mice to TMT and performed a comprehensive analysis of the retina in terms of developmental abnormalities, histopathology, apoptosis, electrophysiological function, glutamate concentration, gene expression, and fluorescence immunostaining. Exposure to 0.75 and 1.5 mg/kg of TMT up to postnatal day 14 caused a decrease in body weight and length, delayed eye opening, and induced thinning of the inner nuclear layer of the retina. In addition, apoptosis was observed in the retinal layer along with b-wave changes and a decrease in retinal ganglion cell spiking activity in the micro-electroretinogram. This change was accompanied by an increase in the concentration of glutamate in the retina, upregulation of astrocyte-related genes, and increased expression of excitatory amino acid transporter (EAAT) 1 and 2. Conversely, EAAT 3, 4, and 5, located in the retinal neurons, were decreased, and this was consistent with the immunostaining results. Our results are the first to prove that TMT induces excitotoxicity and changes in EAAT expression in the retina, and this mechanism causes functional as well as morphological retinal developmental toxicity.

1. Introduction

Trimethyltin chloride (TMT) is one of the most commonly used organotin compounds in industry and agriculture as a fungicide, antifouling agent, and plastic stabilizer (Tang et al. 2013). It is known as a classic neurotoxicant that leads to a major effect on the limbic system, especially hippocampus in adults (Liu et al. 2021). In particular, several studies have reported the effects of TMT exposure during development. Specifically, exposure to [14C]-labeled TMT during pregnancy lead to transmission and detection of TMT in the fetus (Lipscomb et al. 1989). In addition, TMT has been reported to inhibit neuronal differentiation or eye malformation in the zebrafish embryo (Kim et al. 2019; Kim et al. 2016). However, how TMT exposure affects ocular development, especially retinal layer, has not been clarified. Therefore, more detailed investigations related to the various toxic mechanisms of TMT are needed.

Although calcium overload, neuroinflammation, oxidative stress, and mitochondrial dysfunction have been considered as causes of TMT toxicity, excitotoxicity is one of the highly associated mechanisms. Overexcitation of glutamate receptors due to excessive glutamate in the synaptic cleft leads to activating various enzymes, damages cell structure, and causes neuronal pathological abnormalities (Christensen et al. 2019; Hulsebosch et al. 2009). Excitatory amino acid transporters (EAATs) are the major transporter systems involved in removing extracellular glutamate from the synaptic cleft of the CNS neurons and thereby function as a buffer to reduce excitotoxicity (Liang et al. 2008). It has been reported changes in EAAT expression in relation to diseases, drugs or environmental toxicants may induce glutamate spillover, leading to various neuropathological features (Castro-Coronel et al. 2011; O'Donovan et al. 2017).

However, in the case of TMT, although it is known that it enhances the release of glutamate, the relationship of retinal development with excitotoxicity, and with EAAT, has not yet been established.

Therefore, we designed the present study to confirm the retinal developmental toxicity in mice exposed to TMT. In the case of mice, the shape of the eye can be recognized from embryonic day 13, but complete development as a functional organ occurs by PND 14. Most retinal neurons differentiate between PND 0 to 14, all the synapses and circuits are formed, and thus, it has been reported that this period is extremely important in retinal development (Fan et al. 2016). In this study, chronic TMT exposure was performed during this period at every 4-day interval, which is the half-life of TMT (Friberg et al. 1986), and mice were administered with 0.75, 1.5, or 3.0 mg/kg of TMT considering 3.0 mg/kg as the maximum dose that can be administered in adult mice (Chang et al. 1982). We analyzed the retina of mice at PND 14 and tried to identify the mechanism by which exposure to TMT is toxic to retinal development.

We hypothesized that TMT induce retinal developmental toxicity due to changes in EAAT expression and in the extracellular release of glutamate. To reveal this, (1) overall growth abnormalities and changes in the size of eye were analyzed; (2) changes in retinal thickness and apoptosis were analyzed through histopathology and TUNEL assay; (3) electrophysiological abnormalities were analyzed with a multi-electrode array (MEA); (4) glutamate concentration changes were measured, and (5) Genetic analysis and immunofluorescent (IF) staining were performed.

2. Materials And Methods

2.1. Mice husbandry

Mice at day 17 of pregnancy were purchased through OrientBio (Seongnam, Gyeonggi-do, South Korea). All experiments using mice were approved by Seoul National University Institutional Animal Care and Use Committee (SNU-190413-3-2). All animals were maintained in a 12 h light/dark cycle at 23 ± 1°C and 50 ± 10% relative humidity, and food and water were provided *ad libitum*. Mouse pups were weighed daily from PND 4 for stabilization and returned to the mother after weight and administration.

2.2. TMT exposure and developmental defect analysis

TMT (CAS 1066-45-1) was purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA). 6 mg/mL TMT stock solution was prepared by dissolving PBS and used for dilution by concentration. Since the concentration range of acute TMT toxicity in adult rodent has been reported through previous studies, the present study performed a preliminary study and set the target concentration. Mouse pups were intraperitoneally injected at concentrations of 0.75, 1.5 and 3.0 mg/kg according to body weight from PND 4. Repeat administration was performed every 4 days, which is the half-life of TMT (Friberg et al. 1986). On PND 14, which is the period when eye opening occurs, euthanasia and enucleation through CO_2 were performed. At the sampling time, body length from the tip of the nose to the end of the tail were measured and the ratio of eye opening was calculated for group comparison.

2.3. Histological analysis and TUNEL assay

The extracted eyes of mice were fixed with formalin by puncturing cornea with a needle, dehydrated with ethanol for each concentration gradient (70, 80, 90, and 100%), transferred to xylene for 2 h incubation, and infiltrated with paraffin. Paraffin blocks were made using the Tissue-TeK TEC5 tissue embedding console system (Sakura Finetek, Tokyo, Japan), and the blocks were sectioned into 4 μm using a Microm HM 340E (Thermo Scientific, Walldorf, Germany). Tissue sections were placed on a slide glass and then stained with hematoxylin and eosin (H&E). H&E slides were photographed using an Olympus IX70 fluorescence microscope (Olympus, Japan) and a Nikon D2X (Nikon, Japan). The total area of the eyeball and the thickness of each retinal layer were measured through ImageJ software (National Institutes of Health, Bethesda, MD, USA) and compared by group.

To detect apoptotic cells, we performed TUNEL assay using Click-iT™ Plus TUNEL Assay for *In Situ* Apoptosis Detection kit (C10617, Life Technologies, OR, USA). According to the manufacturer's instructions, paraffin sections of 4 μm were used, and the nuclei were stained using a VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken using a Nikon eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA) and NIS element software (Nikon).

2.4. Isolation of retina and recordings with MEA system

All procedures were carried out by modifying previous studies (Alarautalahti et al. 2019; Fujii et al. 2016; Reinhard et al. 2014). Animals were dark-adapted 24 h before use in the experiment and transferred to a light-sealed box for use in the experiment. After the excised eye was transferred to 30°C oxygenated Ames' medium, the cornea was punctured using a fine eye scissor, and the lens was removed with fine forceps. The separated retina was incised in four directions so that it could be spread flat, and after 30 min of recovery, it was placed upside down on an MEA 6 well plate and measured with the Maestro Edge MEA system (Axion Biosystems, Atlanta, GA, USA).

Retinal electrical signals were measured and amplified between 0.1 Hz and 5 kHz. Signals were filtered between 1 Hz and 5 kHz to measure (micro-ERG) and between 100 Hz and 5 kHz to measure retinal ganglion cells (RGCs) spike responses. Spikes were detected using a threshold set to four times the standard deviation of the filtered data. RGC spikes were classified into spontaneous and light-evoked activity. We first measured spontaneous activity for 60 sec, followed by light-evoked acitivity for 60 sec. As light stimulation, full-field light pulses of 12.84 log photons/cm^2/s were applied at 2 sec using a White LED stimulation system, and the interval between light stimulation was 10 sec. Through the measured data, the waveform of micro-ERG, mean firing rate (MFR), number of bursts and synchrony index were analyzed.

2.5. Measurement of glutamate in retina

After sampling, the retina was weighed, and the level of glutamate was measured according to the manufacturer's instructions using a glutamate assay kit (ab83389, abcam, Cambridge, MA, USA). In summary, after washing in cold PBS, the tissues were resuspended in 100 μl of assay buffer and homogenized on ice. Following incubation on ice for about 15 to 30 min, centrifuge was performed at 4°C

for 5 min to remove insoluble materials, and 50 µl of supernatant was transferred to a clean tube. A reaction mixture prepared by mixing 90 µl of glutamate assay buffer, 8 µl of glutamate developer, and 2 µl of glutamate enzyme was applied to the sample well and incubated at 37°C for 30 min by blocking light. The absorbance was measured at OD = 450 nm using Epoch Microplate Spectrometer (BioTek Instruments Inc., VT, USA). The measured glutamate concentration was expressed in nmol/mg.

2.6. IF analysis

For preparing frozen section of the retina, the anterior segment of the formalin-fixed eye was dissected away, and then the lens was removed. After incubation in sucrose solution (15 and 30%), it was transferred to OCT embedding compound (Sakura Finetek, CA, USA) and frozen at -70°C. Thereafter, the samples were sectioned into 10 µm thick using a Microm HM525 cryostat (Thermofisher Scientific, MA, USA) and used for IF staining. Cryosection samples were washed with PBS and pre-incubated for 30 min using a blocking reagent. Thereafter, primary antibodies Glial fibrillary acidic protein (GFAP) (AB5804, Chemicon International, Temecula, CA, USA), EAAT1 (ab41751, abcam), EAAT2 (3838S, Cell Signaling Technology, Danvers, MA, USA), and EAAT3 (14501S, Cell Signaling Technology) were used for 4°C overnight incubation, followed by incubation for 2 h using secondary antibodies. Finally, after washing with PBS, nuclei were stained using VECTASHIELD mounting medium with DAPI (Vector Laboratories), and then photographed and merged using a Nikon eclipse Ti fluorescence microscope (Nikon Instruments Inc.) and NIS element software (Nikon).

2.7. Realtime PCR

For RNA isolation, four mice from each group were used, and after the eyes of the mice on PND 14 were enucleated, retinas were isolated from the sclera using #5 dumont forceps. The isolated retina was stored frozen, and then used for RNA extraction. After homogenization with 1 ml of Hybrid-R™ (GeneAll, Seoul, Korea) solution, RNA was extracted and complementary DNA was synthesized from 0.5 µg of the extracted RNA using the M-MLV cDNA synthesis kit (Enzynomics, Daejeon, Korea). 1 µl of synthesized cDNA was treated with 10 µl of TOPreal^{TM}qPCR 2X PreMIX (Enzynomics), mixed with forward and reverse primers of the target gene (Table 1), and amplified using CFXConnect™ Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Realtime PCR was performed as follows: denaturation at 95°C for 10 sec and 40 cycles of annealing at specific temperature for 15 sec and elongation at 72°C for 30 sec. The $2^{-\Delta\Delta Ct}$ method was used for gene expression analysis. For gene-specific expression analysis, Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh)* was used as a housekeeping gene for normalization. Relative mRNA expression for each gene was calculated as fold change compared to the control group.

Table 1
Sequences of primers used for real-time PCR analysis.

Gene name	Sequence of the primer (5’ – 3’)	GenBank accession No.
Caspase3 [*Casp3*]	Forward: TCTGGTACGGATGTGGACGC Reverse: CGGCAGTAGTCGCCTCTGAA	NM_009810.3
Caspase8 [*Casp8*]	Forward: TCTGCTGGGAATGGCTACGG Reverse: GCTGTGGCATCTGCTTTCCC	NM_001277926.1
Caspase9 [*Casp9*]	Forward: ATGGAGATGGCACACCGGAA Reverse: TGTCCCATAGACAGCACCCG	NM_015733.5
Glial fibrillary acidic protein [*Gfap*]	Forward: AAGGACGTGGTGATGTGAGG Reverse: AGCACTGATTGAGCCTTGGG	NM_010277.3
Aquaporin4 [*Aqp4*]	Forward: GCTGTGACTGTAGCCATGGT Reverse: GAACCGTGGTGACTCCCAAT	NM_001308647.2
Excitatory amino acid transporter1 [*EAAT1*]	Forward: CCCGTGCCTGGATCTGTGAA Reverse: TCCCAAGGGCTGTGATGAGC	NM_148938.3
Excitatory amino acid transporter2 [*EAAT2*]	Forward: GCCATGGCATCAACAGAGGGTG Reverse: TGACCACATCAGGGTGGATGG	NM_001077514.4
Excitatory amino acid transporter3 [*EAAT3*]	Forward: TGGTGATCGTGCTGAGTGCT Reverse: CTCTACGATGCCCGTCCCAA	NM_009199.3
Excitatory amino acid transporter4 [*EAAT4*]	Forward: GCGCTGTGATCATTGGTGTC Reverse: CCCTGTCGCCTTGTTGTCCA	NM_009200.3

Gene name	Sequence of the primer (5’ – 3’)	GenBank accession No.
Excitatory amino acid transporter5 [*EAAT5*]	Forward: GCTCATCCTGTCTGTGCTCTC Reverse: CACCAGTGGTAGGATCAGCAT	NM_146255.3
Glyceraldehyde-3-phosphate dehydrogenase [*Gapdh*]	Forward: GTGTTCCTACCCCCAATGTG Reverse: AGGAGACAACCTGGTCCTCA	NM_001289726.1

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) followed by the Tukey HSD test was performed in SPSS Statistics (version 26). Experimental data were checked to determine if there was a significant difference. The level for statistical significance was set at the 0.05, 0.01, and 0.001. All data are shown as mean ± SD.

3. Results

3.1. Effect of TMT exposure on the normal development of mice pups

Mice pups were administered TMT at concentrations of 0.75, 1.5, and 3.0 mg/kg from PND 4 to PND 14, and the induction of abnormalities in the developmental stage was investigated. Subjects exposed to 3.0 mg/kg of TMT did not survive until PND 14 and all these subjects died. At 0.75 and 1.5 mg/kg, all subjects survived, but inhibition of development was apparent with increase in TMT concentration (Fig. 1A). For the body weight measurement, the group exposed to 0.75 mg/kg showed an overall decrease in body weight compared to the control, but the difference was not significant. On the contrary, at 1.5 mg/kg, a significant decrease in body weight was observed from PND 10, and the difference gradually increased until PND 14. On PND 14, the body weight was 6.8 ± 0.7 mg/kg, which was about 26% lower than that of the control (Fig. 1B). Along with body weight, the long axis of the body was measured. Control group had a length of 10.32 ± 0.52 cm, but the pups exposed to 1.5 mg/kg were observed to have a significantly shorter body length of 8.89 ± 0.32 cm (Fig. 1C). In addition, when comparing the proportion of subjects with eye opening at PND 14, all eyes were opened in control group, whereas 0.19 and 0.4 of subjects treated with 0.75 and 1.5 mg/kg of TMT had closed eyes (Fig. 1D). When total area of the eyeball were analyzed, control was 2.22 ± 0.21 mm^2, but it was significantly decreased to 1.63 ± 0.40 and 1.55 ± 0.15 mm^2 at 0.75 and 1.5 mg/kg of TMT, respectively.

3.2. Retinal layer thickness analysis and TUNEL assay after TMT exposure

To analyze the effect of TMT on retinal development, a layer thickness analysis of retina was performed. The retina can be histologically divided into five layers: the ganglion cell layer (GCL), inner plexiform layer, inner nuclear layer (INL), outer plexiform layer, and outer nuclear layer (ONL). Thickness analysis was performed on the overall thickness of retina as well as individual three layers: GCL, INL, and ONL. The total thickness was measured to be 161.2 ± 17.4 μm in the central region, but it was confirmed that the thickness gradually decreased to 155.9 ± 18.2 and 143.3 ± 11.5 μm as the TMT exposure concentration increased. INL showed significant thinning consistent with the change of total thickness, but changes of ONL and GCL were not significant (Fig. 2A, B). In peripheral region, the total thickness showed a significant decrease from 123.7 ± 6.4 μm to 127.8 ± 18.5 and 111.0 ± 8.5 μm. In addition, thinning of INL and ONL was also confirmed to be statistically significant (Fig. 2C, D).

TUNEL assay was performed to confirm that change in thickness of retinal layer is also related to cell death, and TUNEL-positive cells in the entire layer were counted (Fig. 2E, F). In the control group, almost no cell death was observed. However, as the treatment concentration increased to 0.75 mg/kg, cell death significantly increased to 6 ± 2.5 and 7 ± 3.4 cells, and as the treatment concentration increased to 1.5 mg/kg, it was confirmed that the number of apoptotic cells increased to 13.4 ± 5.6 and 14.2 ± 5.5 cells (central and peripheral regions, respectively) (Fig. 2G). Specifically, apoptotic cells were mainly observed in the INL, which was found to show significant thinning in the thickness analysis of retinal layer.

3.3. Retinal electrophysiological function analysis: micro-ERG and spontaneous spike

We measured retinal electrical signals using MEA for 1 min with or without light stimulation and performed micro-ERG and spike analysis. Following TMT exposure, no significant change was observed in the a-wave, but it was confirmed that the amplitude decreased in the b-wave (Fig. 3A). Spike measurements were performed by applying a filter of 100 to 5000 Hz to the corresponding waveform, and plot analysis and quantification were performed. In the dark condition, it was confirmed that the MFR significantly decreased from 0.60 ± 0.08 to 0.20 ± 0.08 and 0.02 ± 0.03 Hz at 0.75 and 1.5 mg/kg TMT exposure, respectively (Fig. 3B, D). The synchrony index, which is based on the synchrony of spikes between electrodes, also decreased from 0.044 ± 0.005 to 0.031 ± 0.008 and 0.027 ± 0.002 (Fig. 3F). In the presence of light stimulation, it was confirmed that the MFR significantly decreased from 0.62 ± 0.04 to 0.18 ± 0.07 and 0.14 ± 0.07 Hz (Fig. 3C, D). Bursts were measured in response to light stimulation in the control group, and the number of bursts came out to be 29.7 ± 16.2, and the network burst frequency was noted as 0.14 ± 0.05 (Fig. 3E). However, 0.75 and 1.5 mg/kg group did not respond to the light stimulus. The synchrony index was 0.081 ± 0.004, but significantly decreased to 0.031 ± 0.008 and 0.027 ± 0.003 at 0.75 and 1.5 mg/kg, respectively (Fig. 3F). Through functional analysis, it can be inferred that toxic effects were mainly induced in the cells constituting the INL rather than ONL.

3.4. Retinal glutamate concentration and mRNA expression changes

It is known that excessive glutamate in certain pathological conditions can damage retinal cells. Therefore, we measured the concentration of glutamate to confirm whether the retinal toxicity of TMT is related to glutamate. When the retina was isolated and the concentration of glutamate was measured in the homogenate, the control was 2.14 ± 0.20 nmol/mg. As TMT exposure increased to 0.75 mg/kg and 1.5 mg/kg, the concentrations increased significantly to 2.55 ± 0.32 and 2.51 ± 0.44 nmol/mg, respectively (Fig. 4A).

In addition, real-time PCR was performed to check whether changes in gene expression occurred in response to TMT exposure, and a total of 10 genes were analyzed. First, with respect to apoptosis, Caspase3 (*Casp3*), Caspase8 (*Casp8*), and Caspase9 (*Casp9*) showed a tendency towards increased expression. In *Casp8* and *Casp9*, the expression was analyzed to increase significantly when exposed to TMT. *Casp8* significantly increased to 1.38 ± 0.17 and 1.37 ± 0.25 fold, and *Casp9* significantly increased to 1.31 ± 0.11 and 1.29 ± 0.07 fold at 0.75 and 1.5 mg/kg, respectively (Fig. 4B).

When an excitotoxic situation occurs, the EAAT reactively removes excess glutamate from the synapse. *EAAT1* and *EAAT2*, which are mainly located in astrocytes, have a major role in relieving toxicity, and thus, when the expression changes of *GFAP* and Aquaporin4 (*AQP4*) (which indicate astrocyte activity) were examined, the expression of both were found to be increased. *GFAP* significantly increased to 1.92 ± 0.75 and 2.27 ± 0.76 fold at 0.75 and 1.5 mg/kg, and *AQP4* was significantly increased to 1.31 ± 0.18 fold at 1.5 mg/kg (Fig. 4C). Furthermore, the expression changes of five types of *EAAT* were investigated, and *EAAT1* and *2* showed increasing changes in accordance with the changes in *GFAP* and *AQP4*. *EAAT1* significantly increased to 1.37 ± 0.18 fold at 1.5 mg/kg, and *EAAT2* significantly increased to 1.49 ± 0.15 and 1.55 ± 0.27 fold at 0.75 and 1.5 mg/kg, respectively. On the contrary, *EAAT3*, *4*, and *5*, which are located in the neurons, showed a decreasing tendency. The expression of *EAAT3*, *4* and *5* was significantly reduced to 0.64 ± 0.03, 0.72 ± 0.09, and 0.64 ± 0.20 fold at 1.5 mg/kg (Fig. 4D).

3.5. EAAT expression changes in retina following TMT treatment

IF staining was performed for GFAP, EAAT1, EAAT2, and EAAT3 to confirm whether changes similar to gene expression changes were observed at the protein level. GFAP is mainly stained in the GCL, and like the gene expression changes, the fluorescent signal was seen to increase at 0.75 and 1.5 mg/kg TMT compared to the control. In addition, a cell shape extending toward the INL was observed (Fig. 5A). Along with the above change in GFAP expression, EAAT1 and EAAT2, also showed increased signals in the GCL and INL (Fig. 5B, C). It was confirmed that the change was more prominent in EAAT1 than in EAAT2. EAAT3 is the most representative carrier in glutamatergic synapses among EAAT3, 4, and 5, and was used to analyze changes in EAAT expression in neurons. As shown in Fig. 5D, the signal strongly expressed from GCL to INL in the control, decreased as the treatment concentration increased to 0.75 and 1.5 mg/kg, showing a similar tendency as seen in the genetic analysis (Fig. 5D). Thus, it can be interpreted that TMT exposure during development increases EAAT expression of astrocytes, while decreasing the expression in retinal neurons.

4. Discussion

In this study, postnatal mice in the ocular developmental stage were treated with TMT to determine the mechanisms underlying retinal developmental toxicity. Although TMT is known to cause various neurological abnormalities, changes of the retinal development have been rarely reported. Kim et al. reported that TMT affects zebrafish ocular development, and stated ROS generation as a possible mechanism (Kim et al. 2019). To reveal underlying mechanism, this study examined the effect of TMT toxicity on retinal development by treating postnatal mice with TMT. We hypothesized that excitotoxicity and EAAT, among several potential neurotoxic mechanisms of TMT, would cause retinal developmental toxicity.

We administered TMT from PND 4 to PND 14 when eye opening and functional eye development were completed, and thereafter developmental abnormalities were observed. First, body weight was measured to evaluate general toxicity, and as the administration progressed, it was confirmed that the weight decreased significantly compared to the control group. In addition to body weight, it was also observed that the length of the body was shortened; in particular, it was confirmed that the proportion of mice with open eyes and the size of the eye decreased. Such delayed eye opening or microphthalmia, may be due to one or more genetic or chromosomal abnormalities, insufficient nutritional intake during pregnancy, radiation exposure, or infection. In addition, it has been reported that exposure to certain substances, including alcohol, may cause ocular developmental abnormalities during the development process (Kashyap et al. 2011; Stromland and Pinazo-Duran 2002). Environmentally toxic substances, such as perfluorooctane sulfonate or perfluorooctanoic acid, which are perfluorinated compounds remaining in the environment after industrial use, may cause eye dysgenesis when passed through mother to the child. However, the mechanisms by which these substances affect eye development have not yet been clearly elucidated (Abbott et al. 2009; Abbott et al. 2007).

To further analyze eye malformation, we performed retinal layer thickness measurements and TUNEL assay. It was confirmed that the overall retinal thickness decreased with TMT exposure, and among the various layers constituting the retina, INL was significantly thinned in both the central and peripheral parts. Previous studies have reported that sodium L-glutamate has a toxic effect on the inner layer of the developing retina (Lucas and Newhouse 1957). After oral administration of glutamate to mice pups at PND 2–4, pyknotic nuclei were observed in the cells of the INL, and after PND 6 to 8, INL was found to be thinner than control. On the other hand, changes in ONL were insignificant and there was little effect on retinal differentiation. Although these results are different from ours in that external glutamate was administered, it has been reported that TMT also induces glutamate release in the nervous system (Patel et al. 1990). Since the concentration of glutamate in the retina also increased in our experiment (Fig. 4A), it can be presumed that this change in retinal thickness, especially the decrease in INL thickness, is due to excessive glutamate exposure of the retina. In addition to the thinning, TUNEL assay confirmed that cells constituting the retina exhibited apoptosis and apoptotic marker genes were upregulated at the mRNA level. In a previous study, chromatin clumping of nuclear membrane and apoptotic cells with dense cytoplasm occurred by the activation of endonuclease in the GCL and INL of the central and peripheral

retina when N-methyl-D-aspartate (NMDA), which induces excitotoxicity, is intravitreally introduced in rat. However, no obvious changes were observed in the ONL (Lam et al. 1999). These changes are consistent with our histopathological changes and apoptosis assay, and therefore, it can be inferred that these toxic effects are due to excitotoxicity caused by exposure to TMT.

With respect to developmental toxicity of the retina, it is important to determine whether not only histopathological lesions but also impairment of retinal function occur. To confirm this, we conducted a functional evaluation of the retina using MEA. Micro-ERG is the sum of the changes in the electrical potential of various retinal cells (Fujii et al. 2016), and the spontaneous spike is the reaction of ganglion cells that convert light into electrical signals, which are transmitted to the brain (Marrese et al. 2019). Micro-ERG consists of two waves, and the first negative a-wave that occurs following light stimulation is known to originate from the photoreceptor cells. The positive b-wave that follows comes from bipolar cells and Müller glial cells. It can be inferred that nerve cells in the retina were damaged through ERGs, and a decrease in the b-wave was observed in our results. In a previous study, when kainic acid (KA) and NMDA, which are glutamate agonists, were applied, it was observed that the thickness of the inner plexiform layer and INL decreased with the decrease in b-wave (Calvo et al. 2020), which is consistent with our micro-ERG and histopathological changes. As a follow-up study, researchers measured spike signals in ON, OFF, and ON/OFF RGCs, and it was confirmed that a significant decrease in spike signals occurred (Milla-Navarro et al. 2021). This is consistent with the tendency of decreasing MFR in dark conditions and decreasing MFR, burst, and synchrony index under light stimulation in our results. In addition, others also reported that excitotoxins, NMDA and glycine, when introduced intravitreally in concentrations of 15 nmol and 10 nmol, respectively, caused retinal cell apoptosis, decrease in spontaneous spike activity, and abnormalities in light-evoked response (Seki et al. 2010). The toxicity of TMT also causes functional changes in the retina layer, possibly inducing excitotoxicity and affecting the cells present in the INL, which appears in the form of b-wave and spike reduction.

Glutamate concentration of retina as well as the expression of Müller cells, astrocytes of the retina, and EAAT changes in various pathological conditions (Ishikawa 2013). It has been reported that when high pressure is applied to the *ex vivo* retinal model, ischemia is induced, and the level of extracellular glutamate increases (Louzada-Junior et al. 1992), thereby increasing the expression of GFAP, a pathological marker of Müller cell stress (Ishikawa et al. 2010). In addition, it is also known that when astrocytes are activated in a glutamate-excess condition, AQP4, a water channel protein present in the astrocyte endfeet is activated to maintain water and ion homeostasis (Shi et al. 2017). These changes were consistent with our mRNA changes and changes seen in IF staining. In particular, AQP4 regulates synaptic plasticity and neural function by regulating EAAT expression as well as ion and water balance (Szu and Binder 2016). EAATs work to remove synaptic glutamate in response to increased glutamate concentration. EAAT1 and 2 located in astrocytes, exhibit a neuroprotective effect by increasing their expression (Rothstein et al. 2005; Woldemussie et al. 2004), and neurotoxicity occurs when abnormal down regulation occurs (Rothstein et al. 1996). In particular, EAAT2 normally limited on the photoreceptors in humans and rats, but when the concentration of glutamate is increased, it has been reported that additional expression appears in the RGC layer as a protective mechanism (Sullivan et al.

2006). This was consistent with the increased expression of these two transporters in our mRNA and IF results.

EAAT3-5 are mainly located in retinal neurons, and among them, EAAT3, also called excitatory amino acid carrier 1, is mainly expressed in neurons of the inner retina and in the inner and outer plexiform layers (Sarthy et al. 2005). It is known that in EAAT3 deficient mice, abnormal postnatal development retinal development occurs such as thinner inner retinal layer and decreased visual response (Semba et al. 2014). These changes are similar to our results in that the INL was thinned, and the MEA signal was reduced. It is presumed that the gene and protein expression of EAAT3-5 proportionally decreases with a decrease in retinal layer thickness and neuronal death (Arriza et al. 1997; Vorwerk et al. 2000).

In conclusion, our results show that TMT induces retinal developmental toxicity. When the developing mammalian retina is exposed to TMT, excitotoxicity is induced by changes in the expression of EAATs and overexposure of glutamate. These events lead to functional impairment in retina and related histological abnormalities. In response to TMT-induced high concentration of glutamate in the retina, the expressions of EAAT1 and EAAT2 located in Müller cells are increased, and the expression of GFAP and AQP4 increases due to the activation of Müller cells. However, it seems that cytotoxicity induced in the neurons constituting the retina, leads to a decrease in EAAT3-5 expression, reduced apoptosis, and a decrease in the thickness of the INL. These changes induced abnormalities in the electrophysiological function of the retina, reducing the b-wave and RGC spike reactivity (Fig. 6). To the best of our knowledge, this study is the first to reveal the retinal developmental toxicity of TMT at the mammalian model and analyze the molecular, functional as well as morphological aspects of elucidate possible underlying mechanisms. These mechanisms may suggest not only a method to treat TMT toxicity, but also a clue to prevent the damaging effects of other environmental toxic substances with similar toxicity profiles.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Induction of developmental abnormalities in mice pups exposed to Trimethyltin chloride (TMT) at postnatal day (PND) 14. (A) Morphological alterations, Scale bar = 3 cm, (B) Body weight, (C) Body length, and (D) Proportion of subjects with eye opening. (E) Sagittal section and (F) Size of eyeball. n = 5. Scale bar = 500 μm. Data represent the mean ± SD. *P < 0.05; ***P< 0.001

Figure 2

Histopathological analysis and TUNEL assay of mice pups exposed to TMT for PND 14. Histological image of the (A) central and (C) peripheral part of retina exposed to TMT (control, 0.75, and 1.5 mg/kg). Scale bar = 50 μm. The thickness analysis of total layer, the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) in the (B) central and (D) peripheral part of retina. TUNEL assay of (E) central and (F) peripheral part of retina exposed to TMT (control, 0.75, and 1.5 mg/kg). Scale bar = 100 μm. (G) Quantification of apoptotic cells in retinal layer. n = 5. Data represent the mean ± SD. *P < 0.05; **P < 0.01; ***P< 0.001

Figure 3

Electrophysiological recordings using multi-electrode array system. (A) The micro-electroretinogram (micro-ERG) responses of retina from mice pups exposed to TMT (control, 0.75, and 1.5 mg/kg) for PND 14. Raster plots of (B) spontaneous retinal ganglion cell (RGC) spike activity and (C) light-evoked RGC activity from retina. (D) Mean firing rate, (E) number of bursts, and (F) Synchrony index with or without light stimulation. n = 3. Data represent the mean ± SD. *P < 0.05; **P < 0.01; ***P< 0.001

Figure 4

Glutamate level and gene expression changes following TMT exposure. (A) Measurement of glutamate concentration in retina of mice pups exposed to TMT (control, 0.75, and 1.5 mg/kg) for PND 14. n = 9. (B)

The expression of three genes (Caspase3, 8, and 9 (*Casp3, 8*, and *9*)) related to apoptosis, (C) two genes (Glial fibrillary acidic protein (*GFAP*) and Aquaporin4 (*AQP4*)) which indicate astrocyte activity, and (D) five genes (Excitatory amino acid transporter 1, 2, 3, 4, and 5 (*EAAT1*, *2*, *3*, *4*, and *5*)) of major transport systems involved in relieving excitotoxic situation. Data are quantified with standard curves using Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a reference gene and normalized to control. n = 4. Data represent the mean ± SD. *P < 0.05; **P < 0.01

Figure 5

Immunofluorescence staining of retina from mice pups exposed to TMT (control, 0.75, and 1.5 mg/kg) for PND 14. Fluorescent images (A) GFAP, (B) EAAT1, (C) EAAT2, and (D) EAAT3 with DAPI double staining. Scale bar = 100 μm

Figure 6

Possible mechanism of the TMT retinal developmental neurotoxicity. (A) In normal condition, an adequate amount of glutamate releases from the pre-synaptic neuron. EAAT1, 2 located at astrocyte and EAAT3 located at post-synaptic neuron eliminate glutamate from the synaptic cleft. (B) In the presence of TMT, presynaptic neuron releases excessive amount of glutamate and astrocytes are activated with upregulated expression of EAAT1, 2, GFAP, and AQP4. On the contrary, EAAT3 expression downregulates in post-synaptic retinal neuron. As a result, retinal function impairs with micro-ERG b-wave and spike activity decrease, and further, morphological changes such as retinal cell death, thinner retinal layer, and microphthalmia occur