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The effects of triclosan on pluripotency factors and development of mouse embryonic stem cells and zebrafish

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Abstract Triclosan (TCS) poses potential risks to reproduction and development due to its endocrine-disrupting properties. However, the mechanism of TCS's effects on early embryonic development is little known. Embryonic stem cells (ESC) and zebrafish embryos provide valuable models for testing the toxic effects of environmental chemicals on early embryogenesis. In this study, mouse embryonic stem cells (mESC) were acutely exposed to TCS for 24 h, and general cytotoxicity and the effect of TCS on pluripotency were then evaluated. In addition, zebrafish embryos were exposed to TCS from 2- to 24-h post-fertilization (hpf), and their morphology was evaluated. In mESC, alkaline phosphatase staining was significantly decreased after treatment with the highest concentration of TCS (50 μM). Although the expression levels of *Sox2* mRNA were not changed, the mRNA levels of *Oct4* and *Nanog* in TCS-treated groups were significantly decreased compared to controls. In addition, the protein levels of *Oct4, Sox2* and *Nanog* were significantly reduced in response to TCS treatment. MicroRNA (*miR)-134,* an expression inhibitor of pluripotency markers, was significantly increased in TCS-treated mESC. In zebrafish experiments, after 24 hpf of treatment, the controls had developed to the late stage of somitogenesis, while embryos exposed to 300 μg/L of TCS were still at the early stage of somitogenesis, and three genes (*Oct4*, *Sox2* and *Nanog*) were upregulated in treated groups when compared with the controls. The two models demonstrated that TCS may affect early embryonic development by disturbing the expression of the pluripotency markers (*Oct4*, *Sox2* and *Nanog*).

Keywords Triclosan · Mouse embryonic stem cells · Pluripotency · MicroRNA · Zebrafish embryos · Developmental retardation

Xiaojiao Chen, Bo Xu and Xiumei Han have contributed equally.

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Abbreviations

TCS	Triclosan
mESC	Mouse embryonic stem cells
hpf	Hour post-fertilization
MTT	3-(4,5)-Dimethylthiahiazo(-z-y1)-3,5-di-pheny-tetrazoliumromide
AP	Alkaline phosphatase

Introduction

Triclosan (TCS) is an efficient, broad-spectrum antibacterial and anti-fungal agent used in a broad variety of

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products, including cosmetics, detergents, toothpaste, mouthwash, toys, plastics and textiles (Stoker et al. 2010). TCS was produced and used in large quantities. In the USA, the usage amount of TCS was more than 3 million tons per year, and it was more than 3.5 million tons per year in Europe (Halden and Paull 2005). Human exposure to TCS is also widespread. In Sweden, the exposure level of TCS in human milk was 20–300 ng/g, and in blood it was 0.01–38 ng/mL (Adolfsson-Erici et al. 2002; Allmyr et al. 2006). TCS can be bioaccumulated and produce endocrine-disrupting effects in some exposed fish and mammals (Veldhoen et al. 2006; Crofton et al. 2007). Many studies have shown that TCS posed potential risks to reproduction and development (Russell and Montgomery 1980; Orvos et al. 2002; Ishibashi et al. 2004; Oliveira et al. 2009; Rodriguez and Sanchez 2010). However, the exact mechanism of TCS's effects on early embryonic development is not well understood.

Mouse embryonic stem cells (mESC), derived from the inner cell mass of a blastocyst stage embryos, have the distinct capacity to both self-renewal and differentiation into specialized cell types of the adult organism (Marson et al. 2008). *Oct4*, *Sox2* and *Nanog* are transcription factors that regulate stem cell pluripotency and self-renewal (Niwa 2007; Jaenisch and Young 2008). ESC have been widely used to screen embryo toxicants in many in vitro studies (Chen et al. 2010; Stummann et al. 2008; Balmer et al. 2012; Vallier and Pedersen 2005).

The zebrafish (*Danio rerio)* is a small cyprinid species, which is widely used as a model organism in studies of developmental physiology and developmental toxicology. Its short time to sexual maturity, transparent embryos, ease of manipulation and in vitro fertilization, as well as its genetic similarity with human, have made it a very robust and popular model system (Berry et al. 2007). Since the late 1970s, zebrafish have been used in acute toxicity studies of chemicals. In the early 1990s, zebrafish were used for acute and chronic toxicity testing of mixed compounds. Currently, zebrafish embryos are widely used as an important model in high-throughput detection of the developmental toxicity and mechanism of action studies of a variety of environmental chemicals such as phenols, polybrominated diphenyl ethers and pesticides (Chan and Chan 2012; Chandrasekar et al. 2011; Shi et al. 2011; Usenko et al. 2012). In short, the above mentioned features make the zebrafish an excellent model for high-throughput toxicity screening, and zebrafish embryos have played an increasingly important role in the field of developmental toxicology studies with broader application prospects for human risk assessment.

Previous literature has demonstrated that ESC and zebrafish are valuable models for assessing the effects of environmental chemicals on early embryogenesis. Kleinstreuer et al. (2011) found that TCS at the high concentration (100 μM) had significant cytotoxicity on human ESC. Oliveira et al. (2009) showed that TCS had deleterious effects on zebrafish embryos and adults at 300 μg/L and above. However, the mechanistic information they provided about TCS's effects on early embryonic development was limited.

The purpose of the present study was to elucidate the mechanism of toxicity of TCS on early embryonic development. We specifically tested the effects of TCS on general cytotoxicity and pluripotency of mESC. In addition, zebrafish embryos were used to examine the developmental effects of TCS exposure and to explore its mechanism of action.

Materials and methods

Chemicals and reagents

TCS (CAS NO. 3380-34-5, 99.5 % purity) was purchased from Dr. Ehrenstorfer GmbH (Bürgermeister-Schlosser, Augsburg, Germany). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), diethylpyrocarbonate (DEPC), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and TCS treatment

D3 mouse ES cells were a gift from Cell Institute of Shanghai (Shanghai, China). Undifferentiated mESC were grown on mitomycin C-treated mouse embryonic fibroblast feeder cells (MEF) in knock-out Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 20 % ES qualified fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), 0.1 mM β-mercaptoethanol (Sigma Chemical, St Louis, MO), 0.1 mM L-glutamine (Gibco BRL), 0.1 mM pyruvate sodium, 100 unit/mL penicillin/streptomycin (Gibco BRL) and 1,000 U/mL of leukemia inhibitory factor (LIF) (Millipore, Billerica, MA). Cells were maintained at 37 °C and 5 % CO_2 in humidified air, received fresh medium every day and were passaged every 3 days. Cells were dislodged using trypsin/EDTA (Gibco BRL). Before the start of experiments, the ES cell cultures were depleted of feeder cells by incubating trypsinized cells in complete ES cells medium on cell culture dishes for 30 min, during which time feeder cells attached to the dish. Feeder cells were again depleted before counting and plating at the appropriate density. The ES cells were then plated on gelatin-coated dishes and cultured overnight at 37 °C in a 5 % CO_2 incubator with a relative humidity of 95 %. TCS was firstly

dissolved in DMSO and then adjusted to a concentration of 100 mM and with mESC medium as stock solution which was further diluted to lower concentrations for testing. In formal experiments, the final DMSO concentration was 0.1 % (v/v). All chemicals were of analytical grade.

Cell viability assay and morphological study

Feeder-depleted ES cells were seeded on gelatin-coated plates at a density of about 1.5×10^4 per well in 96-well plates and 1×10^6 per well in 6-well plates and incubated overnight. ES cellular viability was evaluated using the MTT proliferation assay in a dose range of 0.001–100 μM MTT (5 mg/mL) dissolved in PBS, sterilized by filtration through a 0.22-μm Millipore® filter and stored at 4 °C. After exposing to TCS at different concentrations, the cells were washed twice with PBS. Then, 25 μl of MTT were added to each well, and the cells were incubated for another 4 h at 37 °C to allow MTT metabolism. The medium was replaced with 150 μl DMSO, plates were shaken for 10 min, and the absorbance was determined at 490 nm. Results were presented as percentage of the control. The mESC were examined and recorded morphologically using a phase contrast microscope (Olympus, CK41, Japan) after exposure to TCS (0.01, 0.1, 1, 10 and 50 μM) or control medium (0.1 % DMSO).

Cell cycle analysis and apoptosis assay

To determine whether TCS could affect the cell cycle and induce apoptosis of mESC, flow cytometry was used to determine the state of cell cycle and the DNA fragmentation. ES cells were feeder depleted twice and seeded on gelatin-coated 6-well plates at a density of about 1×10^6 cells per well. Cells were incubated overnight and subsequently exposed to TCS (0.01, 0.1, 1, 10 and 50 μM) and control medium containing 0.1 % DMSO. After 24 h, cells were washed with PBS and harvested with trypsin/EDTA. Cells were fixed in 75 % ethanol for 2 h or washed in cold PBS, then stained with propidium iodide (PI) and annexin V for 30 min protected from light. The fixed/stained cells were analyzed by FACS Calibur Flow Cytometry (BD Biosciences, NJ, USA) to quantify cell cycle or cell apoptosis.

Alkaline phosphatase staining and activity assay

Alkaline phosphatase (AP) staining was performed with the Alkaline Phosphatase Complete Kit (Sidansai Corporation of Biotechnology, Shanghai, China) according to the manufacturer's protocol. The cells were washed twice with PBS and fixed for 2 min with fixing solution at room temperature. The cells were washed with PBS and incubated with AP staining solution for 30 min, protected from light. After washing with PBS, the cells were photographed using a microscope (Olympus, CK41, Japan).

AP activity was determined from whole cell lysates by analyzing the rate of *p*-nitrophenyl phosphate disodium hexahydrate (pNPP) hydrolysis. AP activity was normalized to cellular protein levels determined using the Bradford Kit (Beyotime Institute of Biotechnology, Shanghai, China). The pNPP product was generated using a *p*-nitrophenol phosphate stock (Cell Biolabs, Inc., San Diego, CA, USA) and analyzed according to the manufacturer's instructions to produce a soluble yellow end product that was determined spectrophotometrically. The pNPP reaction was then terminated using the stop solution and immediately analyzed at 405 nm on a microplate reader and compared against a standard curve.

RNA isolation and quantitative real-time PCR assay

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Dried RNA pellets were dissolved in ddH_2O containing 0.1 % DEPC and quantified by measuring the absorbency at 260 nm. All real-time PCR reactions were carried out on ABI7900 Fast Real-Time System (Applied Bio systems, Foster City, CA, USA) according to the manufacturer's instructions for quantification of gene expression.

cDNA synthesis for coding genes and miRNAs was performed with 1 μg of total RNA according to the manufacturer's instructions (Takara, Tokyo, Japan). The expression levels of pluripotency markers and miRNAs were measured with SYBR PCR Master Mix reagent kits (Takara) according to the manufacturer's instructions. All primers sequences are given in Supplemental Table S1. All oligonucleotide primers were synthesized by Invitrogen (Shanghai).

Western blot analysis

The total cellular proteins (60 μg) were solubilized in the sample buffer (25 mM Tris, pH 6.8, 1 % SDS (w/v), 5 % β-mercaptoethanol(v/v), 1 mM EDTA, 4 % glycerol and 0.01 % bromophenol blue) and then fractionated by electrophoresis on a 12.3 % polyacrylamide-SDS gel at 90 V for 3 h. The proteins were then transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA). The membrane with transferred proteins was incubated in buffer containing specific rabbit polyclonal antibodies for Sox2/Nanog or goat polyclonal antibodies for Oct4 (Abcam, Kendall square, MA, USA, 1:1,000 dilution), followed by incubation with goat anti-rabbit or donkey

anti-goat secondary antibody conjugated with horseradish peroxidase at 1:1,000. The specific signals were detected by the enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Life Science Limited). The amount of GAPDH (34 kDa) in each lane was used as a control to correct the expression of Oct4 protein (45 kDa), Sox2 (43 kDa) and Nanog protein (35 kDa).

Zebrafish embryos assay

Stock solutions of TCS were prepared by dissolving neat standards in DMSO. All exposure solutions were prepared by serial dilution of stock solutions with embryo medium. In formal experiments, the final DMSO concentration was 0.01 % (v/v).

Adult wild-type zebrafish (AB strain) were obtained from stocks at Biology Institute, Shandong Academy of Sciences, Jinan. Adult fish were housed at 28 °C on a 14:10 light–dark photoperiod under semi-static conditions with charcoal filtered water.

Zebrafish were spawned in the morning when the light was turned on. Zebrafish embryos were obtained from spawning adults in groups of about 18 males and 18 females. Embryos were collected and examined under the microscope. Those that had developed normally were selected for TCS exposure. Embryos were randomly distributed into plastic petri dishes containing 5 mL of TCS test solutions (0.3, 3, 30, 300 μg/L) and solvent control (0.01 % DMSO). One hundred embryos were used per replicate per experiment. Exposure was from 2- to 24 hpf. The embryos' mortality and morphological changes were recorded at 10 and 24 hpf. Morphological evaluation of the embryos was performed according to the general morphology score (GMS) system as described by Hermsen et al. (2011). They were collected at 10 and 24 hpf and quickly stored at −70 °C until RNA extraction. Three biological replicates were performed for each assay. Whole embryos were extracted using Trizol reagent. RNA extraction, cDNA synthesis and real-time PCR were performed as mentioned before. The housekeeping gene *β-actin* was used as an internal control. Primers sequences specific for *Oct4, Sox2, Nanog* and *β-actin* are given in Supplemental Table S2.

Data analysis

Values are expressed as mean ± standard error of the mean (SE) for all experiments. Statistically significant differences between the treatments and the control were determined by a one-way ANOVA, followed by Dunnett's multiple comparison test. All tests of statistical significance were two-sided, and the statistical significance was set at $p < 0.05$.

Results

Effects of TCS on colony morphology and cell viability in mESC

To examine the effects of TCS on colony morphology and cell viability, D3 mESC were exposed to increasing concentrations of TCS for 24 and 48 h. After 24 h of treatment with TCS, the colony morphology of mESC in 50 μM group was smaller than that in the control (Fig. 1a). Cell viability was assessed quantitatively using the MTT assay. After 24 h of exposure, TCS significantly decreased cell viability of mESC at 50 and 100 μM compared with the control group (Fig. 1b). However, after 48 h of exposure, the decrease in viability was observed at doses of 10, 50 and 100 μM (Fig. 1c). Thus, we used the 24 h as treatment time and various TCS concentrations (0.01, 0.1, 1, 10 and 50 μM) as treatment doses for following experiments.

Effects of TCS on cell cycle and apoptosis in mESC

To assess cytotoxicity of TCS on mESC, we examined the cell cycle and apoptosis at various concentrations of TCS for 24 h using flow cytometry. No significant differences in cell cycle were found between treatment groups and the control group (Fig. 2a, b), and there were no significant differences in apoptosis between treatment groups and the control group (Fig. 2c, d).

Effects of TCS on alkaline phosphatase staining and activity in mESC

To detect the effect of TCS on the pluripotency of mESC, we stained mESC with alkaline phosphatase (AP) and measured AP activity of them. We also described the density of colony staining by image J software. As shown in Fig. 3a, similar to the control group, mESC colonies treated with TCS were round and appeared similar to the control. However, AP staining at the highest TCS concentration (50 μM) was significantly reduced compared to the control, which was consistent with the results in Fig. 3b. This was also confirmed using an in vitro assay for AP activity (Fig. 3c). These results demonstrated that TCS affected AP staining and activity at the highest concentration (50 μM).

Effects of TCS on pluripotency in mESC

The mRNA and protein levels of key marker genes associated with pluripotency were evaluated using PCR and Western blotting. Compared with the control group, mRNA levels of *Oct4* and *Nanog* in TCS-treated groups were significantly decreased, while mRNA levels of *Sox2* were not changed (Fig. 4a). However, the protein levels of Oct4,

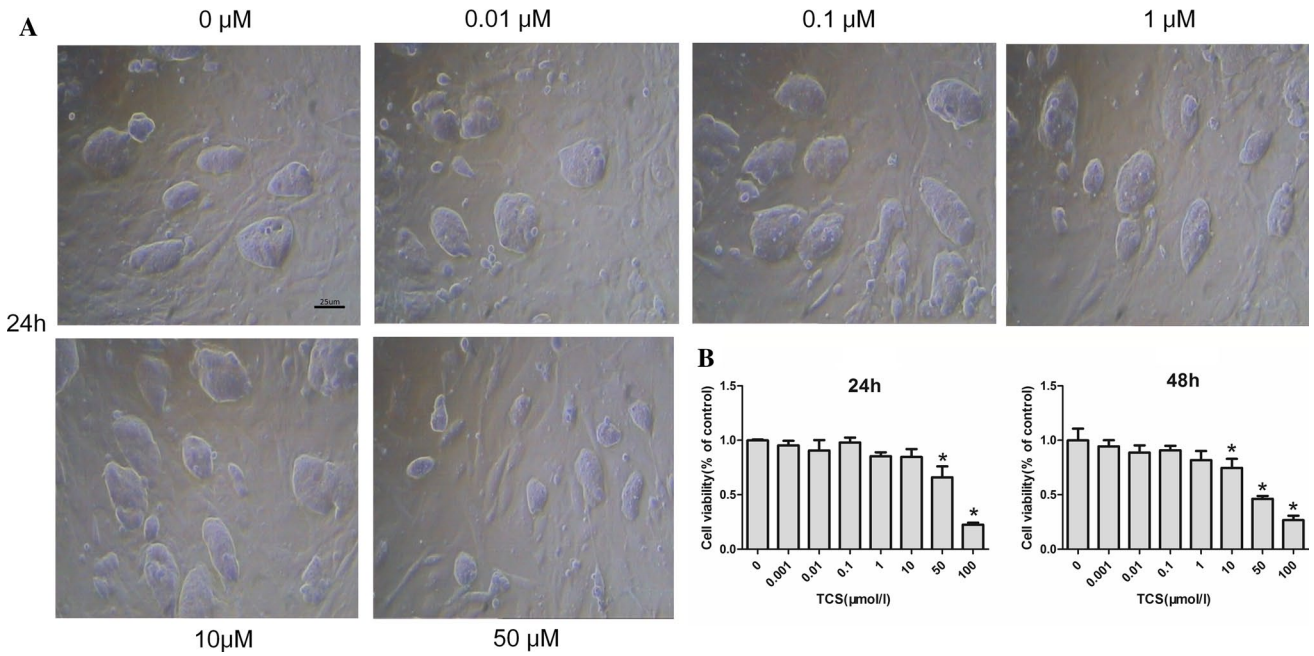

Fig. 1 Effects of TCS on colony morphology and cell viability in mESC. **a** Expose D3 mESC to TCS, colony morphology was observed. *Scale bar* 25 μm. Cells were cultured with various concentrations of TCS (0.01, 0.1, 1, 10 and 50 μM) or DMSO as control for 24 h. **b** Cell viability was determined by MTT assay after exposure to various concentrations of TCS for 24 h and 48 h. Values of the experiment were represented as the percentages of cell viability compared with that of the control and expressed as mean ± SE from three separate experiments in which treatments were performed in quadruplicate. *Asterisk* indicates significant difference when the values were compared to that of the control at $p < 0.05$

Sox2 and Nanog were reduced in a dose-dependent manner in response to TCS treatment, as shown in Fig. 4b.

Effect of TCS on the expression of *miR-134* in mESC

miR-134 has emerged as a key regulator of gene silencing that acts by targeting specific mRNAs of pluripotency (*Oct4*, *Sox2* and *Nanog*) (Tay et al. 2008a, b). To investigate the potential mechanism by which TCS disturbed pluripotency in mESC, the expression of *miR-134* was tested by real-time PCR. We found that the expression of *miR-134* was increased in a dose-dependent manner after TCS exposure, as shown in Fig. 4c.

Effects of TCS on development of zebrafish embryos

Zebrafish embryos were exposed to various TCS concentrations (0, 0.3, 3, 30, 300 μg/L) from 2 h post-fertilization (hpf) to 24 hpf. The exposure mainly covered the period from gastrula to somitogenesis (Kimmel et al. 1995).We recorded mortality and morphological changes in zebrafish embryos at 10 and 24 hpf. The proportions of the dead embryos for the control groups at 10 and 24 hpf were all below 10 % as is required for test validity (Fig. 5a). During the whole exposure time, no significantly increased mortality was observed in any of the TCS-treated groups.

Figure 5b showed that only 300 μg/L TCS resulted in decrease in GMS at 24 hpf as compared to the controls. At 10 hpf, embryo development in the control and TCS treatment groups was normal. However, 300 μg/L TCS induced developmental retardation at 24 hpf (Fig. 5c). At 24 hpf, while embryos in control group had developed to the late stage of somitogenesis, embryos exposed to 300 μg/L of TCS all were at the early stage of somitogenesis. No other concentrations of TCS induced a change in developmental progression over the course of the study.

Effects of TCS on gene expression in zebrafish embryos

Three genes commonly associated with pluripotency (*Oct4, Sox2* and *Nanog*) were quantified by real-time PCR (Fig. 5d). At 10 hpf, TCS exposure decreased the expression levels of *Oct4* and *Nanog,* but not *Sox2*. Embryos exposed to 3, 30 and 300 μg/L of TCS presented a significant decrease in the expression of *Oct4,* while the differences of *Nanog* expression were significant at the concentration of 30 and 300 μg/L of TCS. After 24 hpf of TCS exposure, the expression of *Sox2* and *Nanog* were significantly upregulated at the concentrations of 30 and 300 μg/L, while only TCS at 300 μg/L significantly increased the expression levels of *Oct4.*

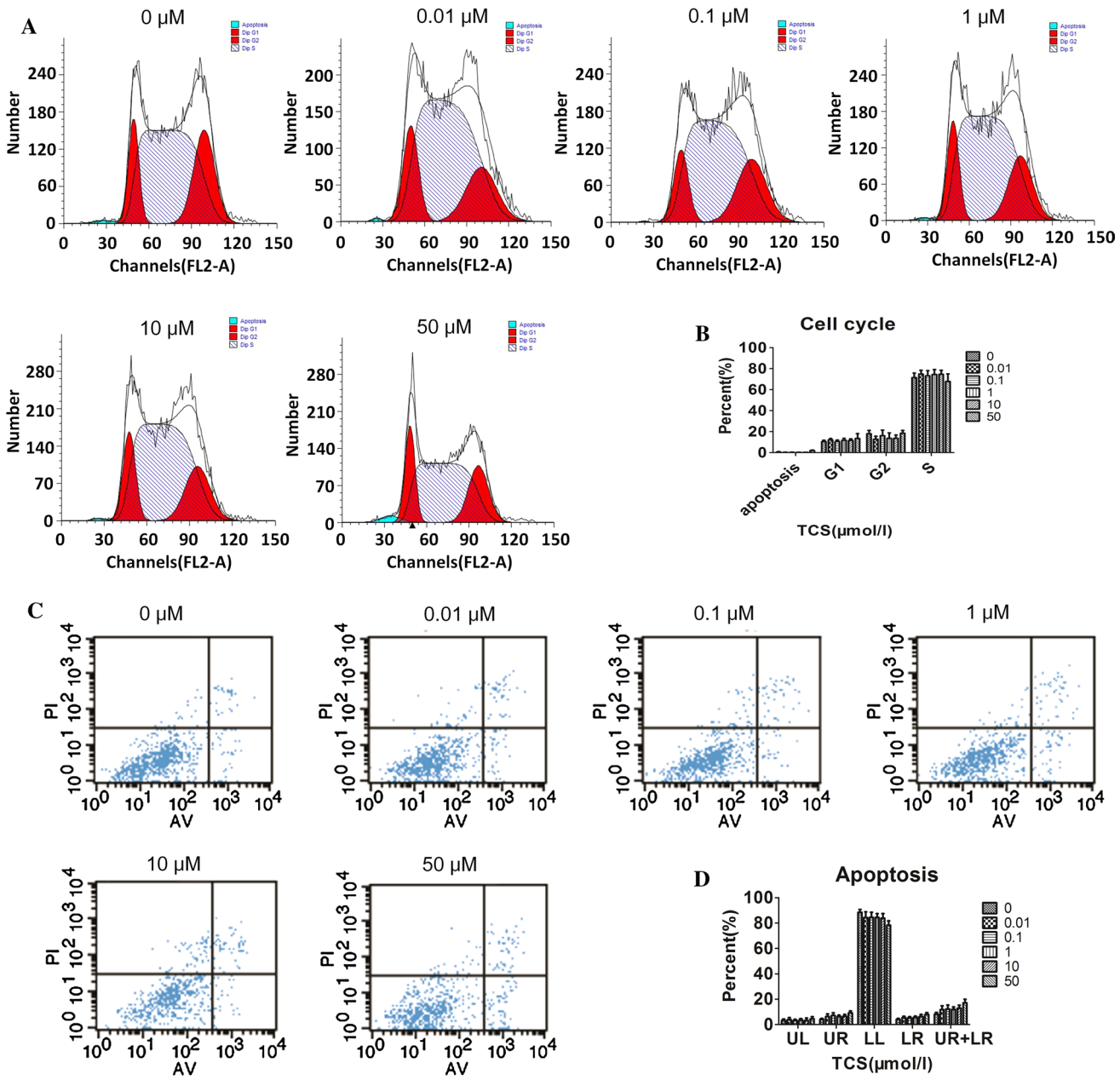

Fig. 2 Effects of TCS on cell cycle and apoptosis in mESC. Cells were cultured with various concentrations of TCS (0.01, 0.1, 1, 10 and 50 μM) or DMSO as control for 24 h. The cell cycle and apoptosis were analyzed by flow cytometry. 10,000 cells were analyzed for each sample. **a**, **b** Data of the experiment were expressed as a percentage of total cells. Results quantitated in cell cycle were shown in (**b**), respectively. **c**, **d** Cells in the LL quadrant indicated that they were live cells. Cells in the LR quadrant were in the early stages of apoptosis. Cells in the UR quadrant were late apoptotic (**c**). The percentage of apoptotic cells was also presented in histogram (**d**). Each data point represented the mean ± SE from three separate experiments in which treatments were performed in triplicate

Discussion

Experimental and animal studies have demonstrated that TCS posed potential risks to reproduction and development (Orvos et al. 2002; Ishibashi et al. 2004; Oliveira et al. 2009). However, the effect and possible mechanism of TCS on early embryonic development are not well understood. mESC were isolated from pre-implantation embryos and can be cultured in an undifferentiated status. Thus, it is an appropriate model for pre-implantation toxicity screening. Zebrafish embryos are a valuable model for assessing the effects of environmental chemicals on early embryogenesis. In this study, we firstly examined the effects of TCS on cytotoxicity and pluripotency using mESC and evaluated

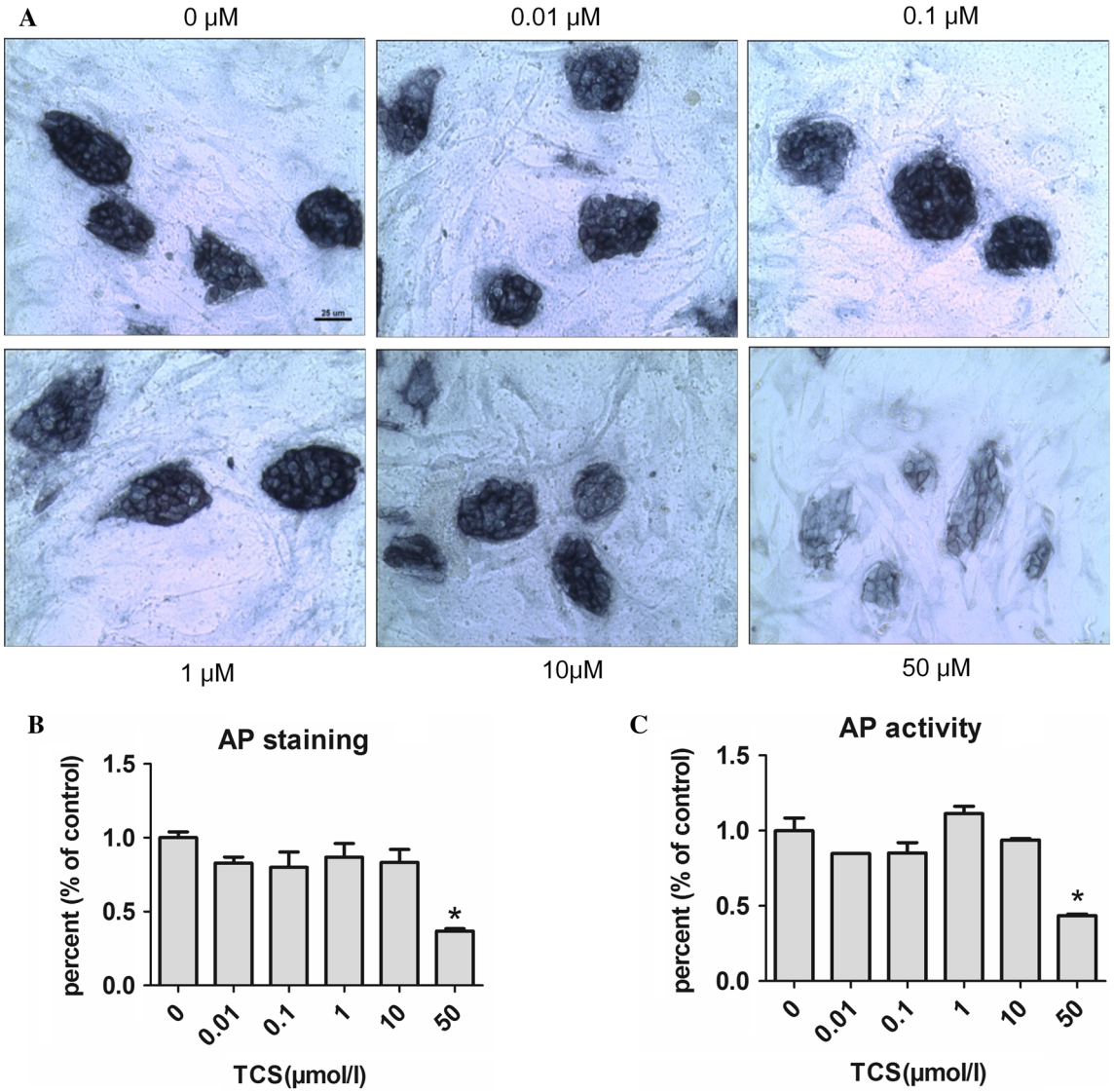

Fig. 3 Effects of TCS on alkaline phosphatase staining and activity in mESC. Cells were cultured with various concentrations of TCS (0.01, 0.1, 1, 10 and 50 μM) or DMSO as control for 24 h. **a**, **b** AP staining. **c** AP activity assay. *Scale bar* 25 μm. Each data point represented the mean ± SE from three separate experiments in which treatments were performed in triplicate. *Asterisk* indicates significant difference when the values were compared to that of the control at $p < 0.05$

the effects of TCS on development of zebrafish embryos before 24 h. This study provides new information on the possible mechanism of TCS's effects on early embryonic development in two models.

In this study, we detected the general toxic effects of TCS on mESC, including cell morphology, proliferation capability, cell cycle distribution and apoptosis. After 24 h of treatment with TCS, the colony of mESC at 50 μM was obviously smaller than that of the control, while cell viability at the 50 μM group was significantly decreased compared with the control group. The result of cell morphology was consistent with that of cell viability. Compared with somatic cells, mESC have a distinct cell cycle structure characterized by a short G1 phase and a rather large S phase which was important for unlimited proliferate of mESC (Savatier et al. 1994; Kapinas et al. 2013). Thus, we used cell cycle as a target of toxicity testing. It was found that TCS did not affect cell cycle of mESC. A previous study has demonstrated that environmental chemicals could induce the apoptosis of ESC (Kim et al. 2006). So, we analyzed the effect of TCS on apoptosis of mESC. Our results showed that TCS exposure could not induce apoptosis of mESC. In short, the general cytotoxicity tests of TCS on mESC indicate that TCS affected proliferation and morphology of mESC.

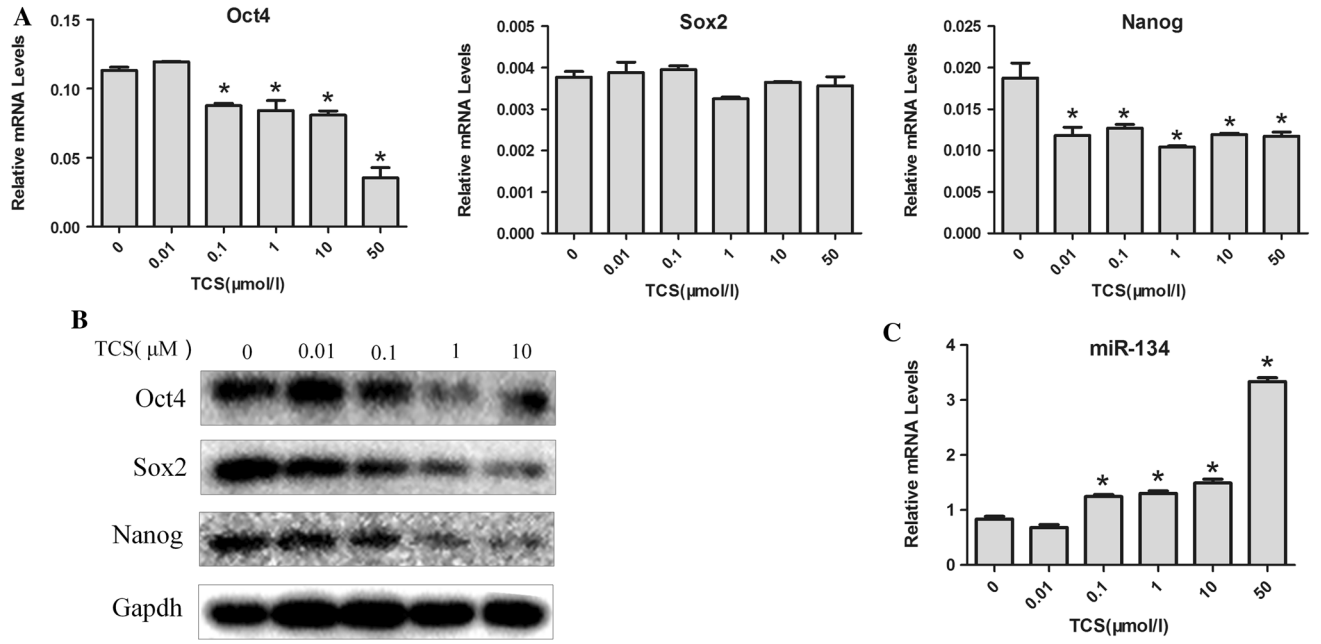

Fig. 4 Effects of TCS on pluripotency state and expression of *miR-134* in mESC. Cells were cultured with various concentrations of TCS (0.01, 0.1, 1, 10 and 50 μM) or DMSO as control for 24 h. **a** *Oct4,Sox2* and *Nanog* mRNA levels were determined by quantitative real-time PCR using a housekeeping gene *GAPDH* as an internal control. **b** The protein levels of Oct4,Sox2 and Nanog were determined by Western blot analysis using GAPDH as an internal control. **c** miRNA assay. *miR-134* was analyzed using total RNA from mESC and were normalized to U6 as an internal control. Each data point was normalized to the control (DMSO) and represented the mean ± SE from three independent experiments. *Asterisk* indicates significant difference when the values were compared to that of the control ($p < 0.05$)

Pluripotency, one of the most significant features in ESC, is the capacity of a single cell to generate all cell lineages of tissues and organisms. Many studies indicate that environmental chemicals can disturb the pluripotency of embryonic stem cells (Aoki and Takada 2012; Jung et al. 2010). Markers, including alkaline phosphatase (AP) and the pluripotency transcription factors (*Oct4*, *Sox2*, and *Nanog*), are involved in maintaining pluripotency of embryonic stem cells (Wang et al. 2006; Chambers and Smith 2004). As previously reported (Ivanova et al. 2006), a mark of differentiation is outgrowth and expansion of colonies, as well as by decreased AP staining. Our results demonstrated that TCS decreased AP staining/activity at the highest concentration (50 μM), which suggested that TCS disturbed the pluripotency of mESC. *Oct4* and *Nanog* were identified as key regulators in maintaining a robust pluripotent state of mESC (Mitsui et al. 2003; Chambers and Smith 2004). *Sox2* can act synergistically with *Oct4* to activate Oct–Sox enhancers, which regulate the expression of stem cell-specific genes, including *Nanog, Oct4* and *Sox2* itself (Masui et al. 2007). The expression levels and functional status of the three factors will directly affect the pluripotency and self-renewal of ESC. Niwa et al. demonstrated that up- or down-regulation of Oct4 could disturb the balance between self-renewal and differentiation of ES cells (Niwa et al. 2000). In agreement with the AP data, the mRNA levels of *Oct4* and *Nanog* in TCS-treated groups were significantly decreased compared to the control, while *Sox2* mRNA levels were not decreased. Moreover, the protein levels of Oct4, Sox2 and Nanog were significantly reduced in response to TCS treatment. We conclude that TCS disturbed the pluripotency of mESC by decreasing the expression of the pluripotency markers (*Oct4, Sox2* and *Nanog*), which in turn lead to decreased protein for these markers and loss of a pluripotent state.

miRNAs are important parts of the core ES cell transcriptional regulatory network and participate in controlling self-renewal and pluripotency of ES cells (Kanellopoulou et al. 2005; Murchison et al. 2005; Wang et al. 2007). *miR-134* can target and down-regulate *Oct4*, *Sox2* and *Nanog* in mESC (Tay et al. 2008a, b). Our results showed increase in *miR-134* levels and decreased expression of Oct4, Sox2 and Nanog in TCS-treated mESC. Therefore, TCS decreased Oct4, Sox2 and Nanog expression which may be associated with the increases of *miR-134* in the upstream induced by TCS.

Zebrafish embryos are an important model organism in toxicological and the related mechanistic studies (Kimmel et al. 1995; Peterson et al. 2008). Zebrafish are largely homologous to other vertebrates including humans

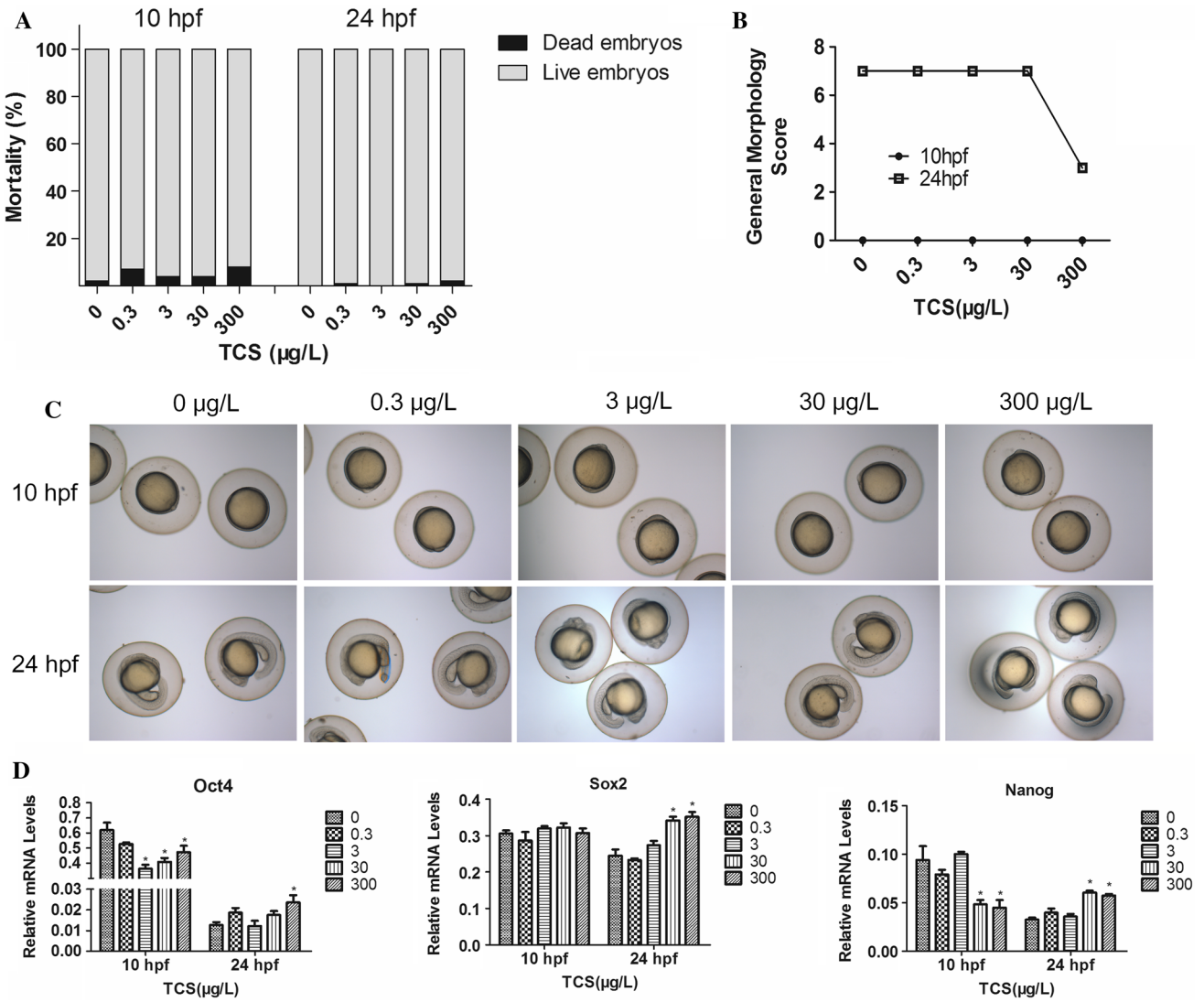

Fig. 5 The effects of TCS on zebrafish embryonic development. **a** General overview of TCS effects on zebrafish embryo during the 24 h post-fertilization (hpf) of exposure. **b** Curves of general morphology score at 10 and 24 hpf. **c** Representative photos of the observations regarding developmental retardation at 10 and 24 hpf. **d** The effects of TCS on genes (*Oct4, Sox2* and *Nanog*) expression in zebrafish embryos. Zebrafish embryos at 2 hpf were exposed to TCS (0.3, 3, 30, 300 μg/L) and 0.01 % DMSO (control). The expression levels of genes were determined by quantitative real-time PCR using a housekeeping gene *β-actin* as an internal control. Quantification was performed on three independent experiments. Data are expressed as fold of DMSO-treated embryos (control). Significant differences between treatment group and the corresponding control group are indicated by *asterisk*—$p < 0.05$

at the anatomical, physiological and molecular level (Wilson et al. 2002; Moens and Prince 2002). The zebrafish' genome is fully sequenced and shows high homology (60–80 %) with the human. More importantly, the amino acid sequences of functionally relevant protein domains are highly conserved evolutionary (de Esch et al. 2012). Therefore, the zebrafish model can help understand the effect of environmental chemicals on reproduction and development in humans. A previous study suggested that hydroxylated PBDEs induced developmental arrest of zebrafish in a concentration-dependent manner (Usenko et al. 2012). Oliveira et al. (2009) found that TCS seemed to be teratogenic at concentrations higher than 0.7 mg/L. Embryos exposed to 0.5 mg/L TCS suffered a significant hatching delay, spine and edematous deformations. At 0.3 mg/L, TCS firstly changed developmental biomarkers levels rather than the other parameters (Oliveira et al. 2009). In this study, we selected 300 μg/L as the highest exposure concentration. We found no significant increase in mortality after TCS treatment; however, 300 μg/L TCS caused developmental retardation of zebrafish embryos at 24 hpf. From 10 to 24 hpf, the expression levels of Oct4 and Nanog were significantly decreased and Sox2 levels were slightly decreased (Robles et al. 2011; Schuff et al.

2012). Zebrafish *Oct4* deficient maternal and zygotic spiel ohne grenzen (MZspg) mutant embryos have defective development in endoderm formation and develop severe gastrulation defects which are a severe delay in epiboly progression. Absence of endoderm in MZspg embryos is caused by a requirement for Pou5f1 during maintenance of sox32 and activation of sox17 expression, downstream of Nodal signaling. Overexpression of *Oct4* in zygotic spg mutant zebrafish may rescue aspects of the mutant phenotype (Lachnit et al. 2008). "Stem cell gene" *Sox2* in SOXB1 family is thought to play an essential role in these embryonic processes from the blastoderm stage to the neural stage. *Oct4* cooperates with SOXB1 class transcription factors, to play a key for early development of zebrafish embryo. Knockdown of *Nanog* impaired endoderm formation of zebrafish through the Mxtx2-Nodal pathway (Xu et al. 2012). So, pluripotency factors seem to be very important in embryonic development process of zebrafish, and their expression level will decrease in the late stage of development. Thus, TCS induced later development retardation of zebrafish embryos at 24 hpf may be caused by the preceding decreases of the pluripotency factors as observed at 10 hpf. Meanwhile, while the controls had already developed to the late stage of somitogenesis, embryos exposed to 300 μg/L of TCS were still at the early stage of somitogenesis. Therefore, the expression levels of pluripotency factors in exposure groups at 24 hpf were relatively higher than that of the control.

Considering that TCS and its metabolites are excreted primarily in urine (Queckenberg et al. 2010), TCS measured in human urine can reflect individual internal exposure level. The National Health and Nutrition Examination Survey of a US population showed that TCS was detected in 74.6 % of the samples at concentrations of 2.4–3,790 μg/L (Calafat et al. 2008). In the cell culture experiments of our study, we chose the concentrations (0.01, 0.1, 1, 10 μM) as the exposure doses which represented human exposure. The TCS concentrations in the zebrafish embryo experiment (3, 30, 300 μg/L) were equivalent to the concentrations (0.01, 0.1, 1 μM) in mESC experiment. According to our results, these exposure concentrations could cause some adverse effect. TCS disturbed pluripotency of mESC and TCS of the highest dose (300 μg/L) caused developmental retardation of zebrafish embryos at 24 hpf which may be associated with the change of pluripotency markers levels (Oct4, Sox2 and Nanog). Previous work indicted that TCS exposure is ubiquitous and likely occurs daily and may exist in a relatively pseudosteady state over the course of months or years (Queckenberg et al. 2010). Given the long-term exposure to TCS for human body, the effect of TCS on human may be still underestimated in vitro. Therefore, the biological significance of our findings regarding the effects of TCS on pluripotency of mESC and development of zebrafish embryos with different levels should be taken into consideration. There is sufficient reason to be concerned about the impact of TCS on human health. The research will help to clarify the possible mechanism of TCS exposure on early embryonic development. This provides scientific basis for risk assessment and reducing the potential risk of TCS on reproduction and development.

In conclusion, these results indicate that TCS disturbed pluripotency of mESC and that *miR-134* may play a key role by inhibiting the expression of downstream transcription factors (*Oct4, Sox2* and *Nanog)*. In addition, TCS at the highest dose (300 μg/L) caused developmental retardation of zebrafish embryos at 24 hpf which may be associated with the change of pluripotency markers levels (*Oct4, Sox2* and *Nanog).*The two models demonstrated that TCS may affect early embryonic development by disturbing the expression of the pluripotency markers.

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