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Teratogenic effects of sodium thiosulfate on developing zebrafish embryos

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1. ABSTRACT

Sulfuric derivatives are potentially hazardous to human health, especially during embryogenesis. Zebrafish were used to study the toxic effect of sodium thiosulfate (STS) ($1\sim1\times10^{-6}$ mol/L) on embryo development with real-time *in vivo* imaging. Motor neuron differentiation and proliferation were analyzed by detecting the dynamics of acetylated tubulin (alpha-tubulin) and of proliferating cell nuclear antigen (PCNA). The expression pattern of brain- and muscle-specific microRNAs was detected by whole-mount *in situ* hybridization. The development of embryos exposed to 0.1~1 mol/L STS was severely retarded and was accompanied by malformation of multiple organs; embryos exposed to 10 μmol/L~10 mmol/L STS had circulatory, nervous and maxillofacial malformations. Embryos were more sensitive to STS at 48 hours post fertilization (hpf) compared with 24 and 96 hpf. STS can destroy the normal development of motor neurons and can affect cell proliferation. We also found differential expression of miR-124a and miR-133a in STS-treated embryos. STS interferes with the normal cytoskeleton structure, inhibits cell proliferation and leads to nervous, cardiac and maxillofacial malformations. MiR-124a and miR-133a were involved in STS malformation induction.

2. INTRODUCTION

Sulfuric derivatives are potentially hazardous to human health, especially during embryogenesis. Sulfuric derivatives consist mainly of sulfur dioxide (SO_2), hydrogen sulfide (H_2S), sulfur hexafluoride (SF_6), sulfite and thiosulfate. SO_2 is one of the most pervasive air pollutants. In mice and rabbits, inhalation of SO_2 resulted in an increased incidence of minor skeletal variants and in lower body mass of newborn animals (1, 2). Large doses of maternally administered sodium sulfite (up to 3.3 g/kg) resulted in rat embryos having dilation of the renal pelvis and of the lateral ventricles in the brain, and in decreased feed consumption and decreased body weight of the maternal rats (3, 4). These results show that some sulfuric derivatives are toxic during embryogenesis.

STS is a sulfuric derivative that has been widely used in food processing, papermaking, the photographic industry, dye manufacture and in the pharmaceutical industry and resides in industrial wastewater at low levels. Previous research has indicated that STS can injure neural stem cells, and can be used to generate a hypoxia model in neural stem cells (5). STS can induce the development of regeneration hypertrophy and marked epimorphosis in

experimental pancreatitis but can also prevent the pancreatitis from progressing to its chronic form (6). Some reports indicate that using STS to treat eczema by intravenous injection may induce anaphylaxis (7-11). These reports also suggest that STS has toxic properties.

Currently, it is not clear how STS exposure effects embryo development. In this study, zebrafish were used to study the effect of STS on embryo development. Our aim was to explore the developmental toxicity of STS to gain understanding of the risks of sulfur compound exposure to pregnant women.

3. MATERIALS AND METHODS

3.1. Zebrafish maintenance and collection of embryos

The zebrafish (strain AB) feeding program used was in accordance with the method of Westerfield (12). The day-night cycle was controlled with an automatic timer (14 hr light/10 hr dark) and males and females were separated. Adults were fed with brine shrimp (Artemien, Salina) twice a day. Embryos were generated by natural pair-wise mating in our aqua-culture facility (13) and then collected with a pipette. Embryos were maintained in fish water (5 g instant ocean salt in 25 L of distilled water, pH 7.4) at 28.5°C. Developmental stages were distinguished based on morphological characteristics.

3.2. Stock solutions and exposure protocols

Embryos were incubated for 4 hpf and then blastula stage embryos were selected according to developmental characteristics. Embryos were then exposed to 1, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5} and 1×10^{-6} mol/L of STS in a volume of 50 mL water. For each group, 200 eggs were collected and incubated to 24, 48 and 96 hpf. Subsequently, half the embryos of each group, the experimental group, were transferred to the STS solution while the remaining embryos, the control group, were transferred to embryo water. After exposure to STS, the eggs were rinsed three times with embryo water, and then incubated until observation. 0.003% phenylthiourea (Sigma-Aldrich Corp., St Louis MO, USA) was administered at 24 hpf to prevent melanin formation.

3.3. Morphological Observation and Statistics

The entire processes of cardiovascular system and nervous system development were observed in zebrafish embryos, incubated from 12 to 96 hpf, using *in vivo* live imaging. We used a modified scoring system based on C. Zhang *et al.* (14). Compound-exposed and control zebrafish were scored at 48 and 96 hpf (5 zebrafish were scored at 1 mmol/L), and multiple parameters were used to determine the developmental malformation index (DMI) for each STS concentration. DMI at a given concentration was defined as the ratio of the overall positive count to the overall parameter.

The following developmental endpoints were scored at 48 and 96 hpf:

Heart rate: Because the heart rate is sensitive to temperature, MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4) and methylcellulose, the heart rate was the first parameter to be measured. The ventricular beat rate of each zebrafish was measured for 15 sec using a stopwatch and counter, and multiplied by 4 to obtain beats per min.

Circulation: Movement of blood cells through the heart and major vessels and the rate and pattern of blood flow was visually examined. Abnormal circulation includes slow or fast circulation and defects in the circulatory pattern (e.g., circulation only in the head or trunk).

Number of red blood cells (48 hpf only): By 48 hpf, zebrafish red blood cells (RBCs) can be seen circulating in transparent blood vessels. A significant decrease in the number of RBCs in circulation was scored as abnormal.

Edema: Edema is visualized as an accumulation of fluid in the interstitial space surrounding an organ or tissue. We assessed the presence of heart and trunk edema, which can reflect circulatory or excretory problems, respectively.

Hemorrhage: Hemorrhage may be caused by blood vessel malformation or rupture of tissues or organs after STS exposure. In zebrafish, hemorrhage is visualized as a pool of blood clustered in an area outside of the normal circulation. The presence of a hemorrhage anywhere in the body was scored as abnormal.

Ventricle swelling (48 hpf only): The third brain ventricle is visible at 48 hpf. Accumulation of fluid in this ventricle was scored as abnormal.

Brain necrosis: Normal brain tissue is transparent, while necrosis results in cloudy, white, or brown areas. The appearance of 1 or more necrotic areas in the brain was scored abnormal.

Jaw formation (96 hpf only): The jaw develops from the 7 pharyngeal arches and by day 5 has a characteristic shape. Malformations of the jaw are commonly associated with problems in bone formation or of neural crest migration. An abnormal jaw was scored abnormal.

Caudal embryo morphology: Abnormalities scored as positive include a bent or misshapen caudal region of the embryo. Such defects reflect possible problems in development of muscle or of the nervous system.

Motility: To assess potential neuronal and muscular disorders, motility was examined. A dissecting needle was used to touch the caudal region of the embryo. In wild-type zebrafish, this touch normally elicits a rapid and brief swim response; a lack of response indicates a defect in neuromuscular transmission and was scored as abnormal.

3.4. Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was

Table 1. Developmental index parameters and results following exposure to 1 mmol/l sodium thiosulfate

Concentration (mmol/L)		1 (48 hpf)	1 (96 hpf)
Parameter Heart rate (bpm)	Measure	168	156
>2 SD from control	Yes(+)	(+)	(+)
Circulation	Absent (+)	(-)	(-)
Number of RBCs (48 hpf only)	<50% wt (+)	(-)	NA
Heart edema	Present(+)	(+)	(+)
Trunk edema	Present(+)	(-)	(-)
Hemorrhage	Present (+)	(-)	(-)
Ventricle swelling (48 hpf only)	Present (+)	(+)	NA
Brain necrosis	Present (+)	(+)	(+)
Jaw malformation (96 hpf only)	Present (+)	NA	(+)
Abnormal caudal region	Present (+)	(-)	(-)
Motility	Absent (+)	(-)	(-)
Length (mm)	Measure	2.54	3.12
>2 SD from control	Yes (+)	(+)	(-)
Total positive measures		5/11	3/10

Note: Wt: wild-type; bpm: beat per min; NA: not applicable

performed according to the method described by Rivera-Pérez *et al.* (15). Embryos, incubated to 24, 48 and 96 hpf, were selected, rinsed with PBST (0.1 mol/L PBS + 0.1% Tween20), and then dehydrated sequentially using 25%~100% methanol. Embryos were fixed with 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffered saline (PBS) and stored at -20°C until use. Primary antibodies used were mouse monoclonal anti-PCNA (Sigma-Aldrich Corp., St Louis MO, USA) and mouse monoclonal anti-alpha-tubulin (Sigma-Aldrich Corp., St Louis MO, USA). After rinsing in PBS, the embryos were exposed to fluorescein isothiocyanate conjugated goat anti-mouse IgG secondary antibodies (Bridge Biotechnology Corp., Beijing, China).

3.5 Whole-mount *in situ* hybridization

Unstained 24 and 48 hpf embryos, after being stripped, were fixed immediately in 4% PFA in PBS at 4°C overnight, then dehydrated with methanol and stored at -20°C until use. 5'-DIG was used to label locked nucleic acid (LNA)-miRNA probes: miR-124a, miR-1 and miR-133a (Table 1), synthesized by Takara. On the first day 10 embryos were put into a 1.5 mL microcentrifuge tube and successively rehydrated for 5 min in each of 75%, 50% and 25% methanol solutions. Embryos were then rinsed for 5 min in PBST four times and then digested with 10 ng/μl Proteinase K, (1 min for 24 hpf embryos and 2 min for 48 hpf embryos). Embryos were then fixed immediately in 4% PFA. Prehybridization was then performed in 800μl of hybridization solution [Formamide 50-65%, 5X SSC, Tween-20 0.1%, citric acid to pH 6.0 (460 μl from 1 M stock of 50 ml), heparin 50 μg/ml, tRNA 500 μg/ml] at a temperature, 20-22°C lower than the Tm value of the probe, for 4 hours. Hybridization was performed in 200 μl of hybridization solution containing 100 pmol 3'-DIG labeled LNA-miRNAs probes in a water bath at 60°C overnight. On the second day, embryos were sequentially rinsed with 2X SSC, 0.2X SSC, and PBST, blocked with goat serum/PBST, and incubated with anti-DIG/PBST (1:5000) at 4°C overnight. On the third day, embryos were rinsed with PBST three times, rinsed with staining buffer (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$) three times and the staining was then developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and observed and photographed under a Zeiss stereo microscope.

4. RESULTS

4.1. Effects of STS Exposure on Early embryo Development

Embryos exposed to 0.1~1 mol/L STS were severely retarded in development with malformation of multiple organs, slow blood flow and localized hemorrhage. Embryos exposed to 10 μmol/L~10 mmol/L STS presented with circulatory and nervous system malformations, mainly as maxillofacial malformation, pericardial expansion and slow blood flow and encephalocoele. Embryos exposed at 24 hpf didn't show any significant differences compared with controls and showed essentially normal axon and organ partition (Figure 1A and B), while embryos exposed at 48 hpf (Figure 1C and D) and at 96 hpf (Figure 1E and F) presented with marked pericardial expansion, optic tectum and maxillofacial malformation. Both the mortality and malformation rates at 48 hpf, for all STS concentrations, were higher compared with those at 24 hpf. STS at 1 mmol/L had an extensive effect on the cytoplasmic skeleton (Figure 2) and on the nervous and circulatory systems. The DMI was 25/ (11×5) =45.45% at 48 hpf and 20/ (10×5) = 40% at 96 hpf and mainly reflected nervous system and maxillofacial development (Table 1). The sensitivity to STS was, therefore, highest at 48 hpf.

4.2. Effects of STS on the development of the nervous system

Alpha-tubulin plays in a key role in maintaining the functions of motor neurons and can be used as a marker for axon localization (16). Axon tracts in the brain and in the caudal regions of embryos at 24 hpf (Figure 2A and B), 48 hpf (Figure 2C and D) and 96 hpf (Figure 2E and F) were visually assessed by immunohistochemistry with an alpha-tubulin antibody. In the brain, the pattern and size of axon tracts can be used as an indicator of malformation. The control group showed that alpha-tubulin was highly expressed in the brain and spinal cord at each developmental stage (Figure 2A, C and E). Exposure to 1 mmol/L STS caused alpha-tubulin in motor neurons to be

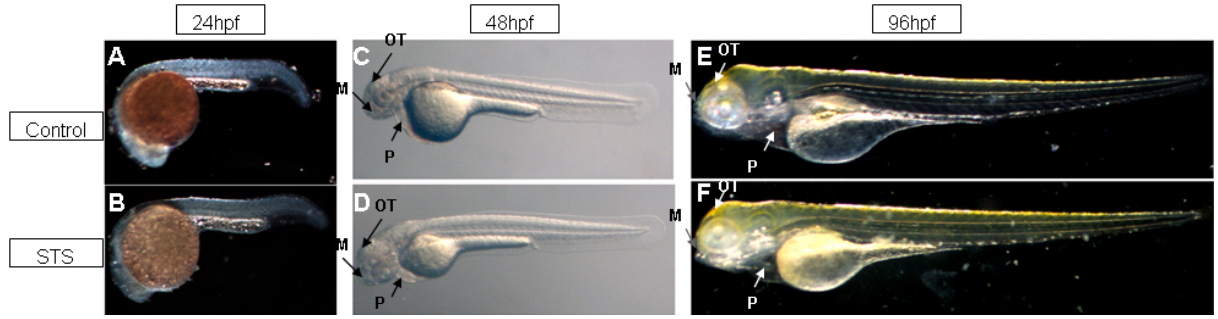

Figure 1. In untreated controls, zebrafish embryos underwent normal development at 24 hpf (A), 48 hpf (C) and 96 hpf (E); abnormal development in 1 mmol/L STS-exposed zebrafish embryos at 24 hpf (B), 48 hpf (D) and 9 6hpf (F).OT: optic tectum, P: pericardial expansion, M: maxillofacial.

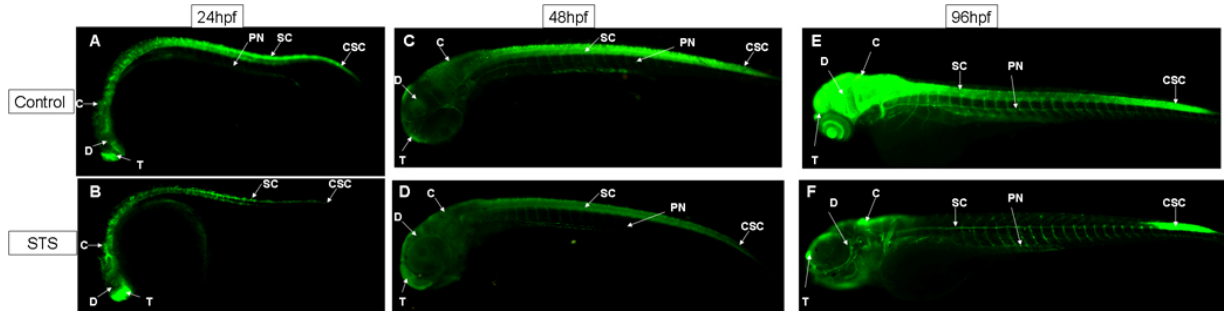

Figure 2. Examination of motor neurons (A-F): Motor neuron at 24, 48 and 96 hpf were examined by immunohistochemistry with an anti-alpha-tubulin antibody (green signal). In untreated controls, the motor neurons were highly arranged along the brain, the spine cord and the peripheral nerves (A, C, E). However, after treatment with 1 mmol/L STS, loss of motor neurons was observed at 48 and 96 hpf (D, F). T: telencephalon, D: diencephalon, C: cerebellum, PN: peripheral nerves, SC: spinal cord, CSC: coccygeal spinal cord.

up-regulated in the brain at 24 hpf (Figure 2B), but down-regulated in the brain, the spinal cord in the trunk region and in the peripheral nerves at 48 hpf (Figure 2D) and at 96 hpf (Figure 2F). Alpha-tubulin was mainly expressed in the telencephalon, diencephalon, cerebellum, coccygeal spinal cord and peripheral nerves at each developmental stage. Motor neuron axon tracts in the trunk region of zebrafish exposed to STS are thin and truncated (Figure 2B and F).

4.3. Effects of STS on cell proliferation

PCNA is a cofactor for DNA polymerase and is expressed in all cells during early development and is then down-regulated during morphogenesis and differentiation, being only expressed in late developing organs and tissues, such as the pectoral fin and the pharyngopalatine arch (17). The control group showed that PCNA was mainly expressed in the brain, sarcomere and spinal cord at 24 hpf (Figure 3A). At 48 hpf (Figure 3C and E), PCNA was expressed in a punctiform pattern in the hindbrain region and abdomen but it was expressed at lower levels in the telencephalon, cerebellum, sarcomere and coccygeal spinal cord. At 96 hpf (Figure 3G) PCNA was highly expressed in the brain and palatal arch. Exposure to 1 mmol/L STS caused PCNA expression in neuronal progenitors to be highly increased compared with control levels, with PCNA expression increased in the telencephalon, cerebellum, sarcomere and coccygeal spinal cord at 24 hpf (Figure 3B) and at 48 hpf (Figure 3D and F), while PCNA was highly expressed in optic tectum, cerebellum, posterior commissure abdomen and branchial arches at 96 hpf (Figure 3H). These changes indicated that STS could disturb cellular proliferation and differentiation in anterior rather than posterior regions during early development and in brain, muscle and tail during late development.

4.4 Effects of STS on the expression pattern of central nervous system- and muscle-specific microRNAs

Among the microRNAs that are enriched in the brain are miR-9, miR-9*, miR-29, miR-124a, miR-125a and miR-125b (18). The decrease or absence of miR-124a expression can possibly influence the development of motor neurons and dorsal root ganglia (DRG) (19). Muscle specifically expresses the miRNAs, mir-1, mir-133 and mir-206 (20). We choose one of the brain-specific microRNAs (miR-124a) and the muscle-specific microRNAs (miR-1 and miR133a) to study the effects of 1 mmol/L STS on the expression pattern of brain- and muscle-specific microRNAs. MiR-124 is most abundantly expressed in mature neurons and may have a prominent function of maintaining neuron-specific gene expression profiles (21). For miR-1-targeted isoforms, the strongest repression of expression is observed in cardiovascular and musculoskeletal tissues (22, 23). MiR-1 and miR-133, which are clustered at the same chromosomal locus, are transcribed together in a tissue-specific manner during development (24). Zebrafish embryos exposed to STS

Table 2. LNA Modified miRNAs probes

Probes	Sequence
P-LNA -mir-124a	5'- DIG*-gGcaTtcACCgCgtGccTtaa-3'
P-LNA-mir-1	5'- DIG*-tAcaTacTtCtTtaCatTcCa-3'
P-LNA-mir-133a	5'- DIG*-acAgcTggTtGaAggGgaCcAa-3'

Note: The capitalized letters represented LNA modified bases.

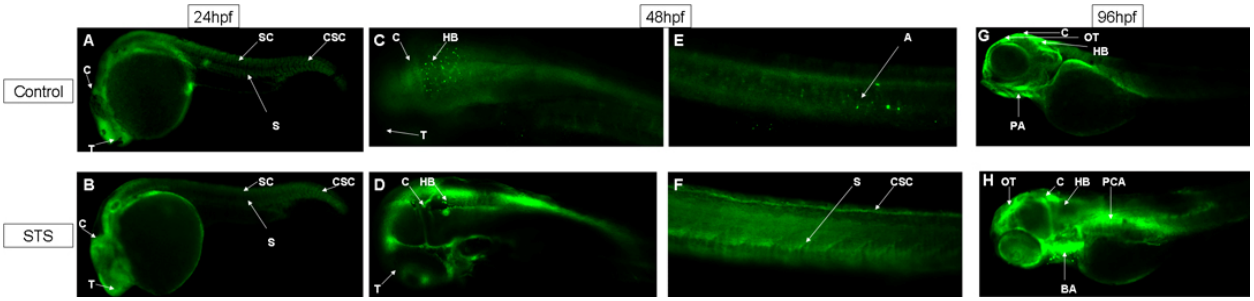

Figure 3. Assessment of neuronal proliferation (A-H): Neuronal proliferation was examined by immunohistochemistry with an anti-PCNA antibody (green signal). In untreated controls, the neuronal proliferation was highly arranged along the somites (A, C, E and G). In 1 mmol/L STS treated embryos the pattern of proliferating neuronal cells in the brain was different from controls with the proliferation zone being larger at 48 hpf (D, F) and at 96 hpf (H), but smaller at 24 hpf (B). T: telencephalon, C: cerebellum, S: sarcomere, SC: spinal cord, CSC: coccygeal spinal cord, HB: hindbrain, BA: branchial arches, PA: palatal arch, A: abdomen, OT: optic tectum, PCA: posterior commissure abdomen.

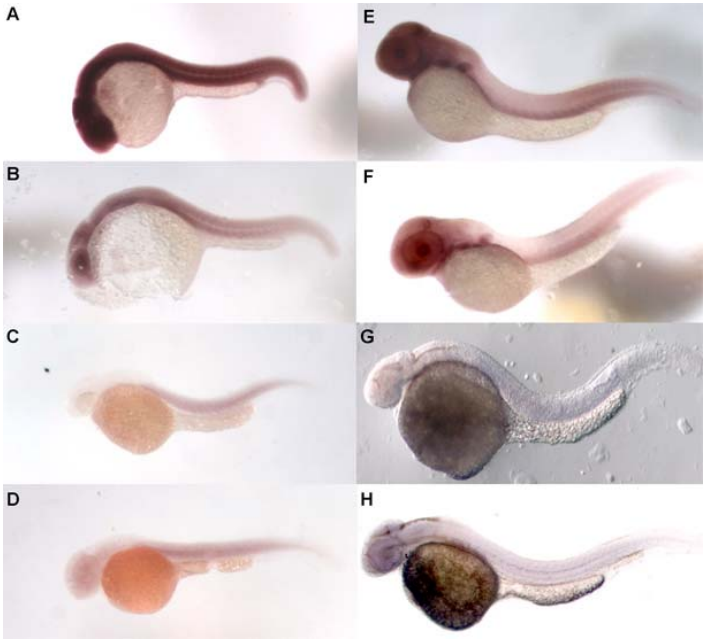

Figure 4. Expression of miRNA124a, miRNA 133a and miRNA 1 in embryos treated with 1mmol/L STS. Expression of miR124a at 24 hpf (A, B) and at 48 hpf (E, F); expression of miR133a at 48hpf (C, D); expression of miRNA1 at 48hpf (G, H); A, C, E and G: untreated embryos; B, D, F and H: embryos treated with 1mmol/L STS.

displayed differences in the expression of miRNAs (Figure 4) compared with controls. MiR-124a was highly expressed in normal mature neural cells (Figure 4A and E), however, their localization was significantly altered after exposure to STS (Figure 4B and F), mainly in the diencephalon, cerebellum and lateral fins. Exposure to 1 mmol/L STS caused the down-regulation of miR-124a in neurons in the maxillofacial region and caudal spinal cord (Figure 4B and F), while miR-133a was down-regulated in skeletal muscleand myocardium (Figure 4D) at 48 hpf. MiR-1 expression was not enriched or constitutively expressed throughout development (Figure 4G and H), as was shown previously (25), and it was not significantly altered following STS exposure. See Table 2 for the miRNA sequences.

5. DISCUSSION

We examined the effects of STS on the early development of zebrafish embryos. Zebrafish embryos were treated with STS at various concentrations and observed at different developmental phases. Our study found that STS treatment resulted in different effects on zebrafish embryogenesis depending on the dose of STS and on the development phase of the zebrafish embryos. High dose (0.01~1mol/L) STS exposure mainly caused developmental retardation in multiple organs and localized hemorrhage; 1 mmol/L STS exposure resulted in nervous system, circulatory and maxillofacial malformations. Embryos were more sensitive to STS at 48 hpf than at 24 hpf or 96 hpf. These results indicate that STS can induce developmental abnormalities, particularly in a sensitive phase 48 hpf, and that target organs were mainly in the nervous system, circulatory system and maxillofacial region.

STS exposure mainly interfered with neural development, indicating that further study on zebrafish neural development in response to STS exposure is warranted. Embryogenesis was examined using *in vivo* micro-imaging and whole-mount immunohistochemistry with the key molecular markers of neural development, alpha-tubulin and PCNA. This enabled the development of zebrafish embryonic motor neurons and of neuronal proliferation to be tested after exposure to STS (26).

PCNA is a cofactor for DNA polymerase and is expressed in all cells during early development and is then down-regulated during morphogenesis and differentiation, being only expressed in late developing organs and tissues, such as the pectoral fin and the pharyngopalatine arch (17). In this study, at 24, 48 and 96 hpf, whole-mount immunohistochemistry showed that PCNA was down-regulated in maxillofacial regions of controls, mainly in late developing organs, such as pectoral fin and pharyngopalatine arch, while it was highly expressed in head, branchia, trunk spinal cord and somites. This indicates that STS might interfere with cellular proliferation, in anterior rather than posterior regions during early development. In brain, muscle and tail, however, during late development, STS may interfere with development of the nervous system and motility.

Alpha-tubulin is a cytoskeleton protein that plays a key role in maintaining the functions of motor neurons and can be used as a marker for axon localization and is specifically regulated by thyroid hormones. At all periods of cerebral development, reduction of thyroid hormone levels can delay the expression of alpha-tubulin, leading to changes in microtubule assembly and stability (27). Our results show that the expression patterns and levels of alpha-tubulin changed significantly during embryogenesis in both normal controls and in STS treated embryos. In controls alpha-tubulin was mainly expressed in the telencephalon, diencephalon, cerebellum, coccygeal spinal cord and peripheral nerves. After exposure to STS, motor neuron axons displayed thin and truncated in the head and in the caudal two thirds of the tail, at 48 hpf. The caudal two thirds of the embryo is the main region for motility and it includes the notochord, muscles and nerve fibers. Each somite contains a simple pattern of axonal distribution, with only motor neurons distributed ventrally. In STS treated embryos, α-tubulin was up-regulated at 24 hpf, while down-regulated at 48 hpf and at 96 hpf. This suggested that teratogenesis of STS mainly interfered with the microtubules of the cytoskeleton, the expression patterns of axons and the functions of motor neurons, thereby altering motor neuron differentiation.

MiRNA is not only able to regulate gene expression but is also associated with the metabolism of pharmaceuticals. MiRNA expression is likely to be altered in response to any exogenous substance that can alter gene regulation and determination of cell fate. As a novel regulatory mechanism of gene expression, small RNAs play critical roles in embryogenesis, cellular differentiation, proliferation, programmed cell death and metabolic death though interfering with signal transduction pathways (28, 29). Thus small RNAs, especially the target genes of miRNAs, are being extensively investigated (30). In this study, the results of the whole-mount ISH indicated that, as an exogenous teratogen, STS interfered with embryogenesis of nerves and muscles. Down-regulation of miR-124a in the nervous system and of miR-133a in muscle tissue was likely to be a critical molecular event in the teratogenic effect of STS. The down-regulation of miR-133a in skeletal muscle and myocardium and the low level of expression of miR-1 indicated that the skeletal muscles were hypertrophic at 48 hpf, in agreement with McCarthy et al. (31). This result was in agreement with the report of Chen et al. (24). Furthermore, our results showed that miR-124a was highly expressed in normal mature neural cells, and that this localization was significantly altered after exposure to STS, mainly in the diencephalon, cerebellum and lateral fins, indicating the complexity of miR-124a effects in zebrafish teratogenesis. The endogenous targets of miR-124, laminin-1 and integrin-1, have been determined by Cao *et al.* (32). Both are highly expressed in neural progenitors but their expression is inhibited during neural differentiation. Thus miR-124a appeared to ensure that progenitor genes were post-transcriptionally inhibited in neurons, the mechanism of which has yet to be investigated.

In summary, we found that STS is toxic to development and leads to neuronal, cardiac and maxillofacial malformations. STS could interfere with the normal structure of cytoskeletons and inhibit cellular proliferation. MiR-124a and miR-133a were involved in the process of STS-induced embryo malformations. This study will contribute the evaluation of sulfur compound exposure risks of pregnant women to help to decrease the sulfur compound exposure-related birth risk.

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Abbreviations: STS: sodium thiosulfate; PCNA: proliferating cell nuclear antigen; SO_2: sulfur dioxide; H_2S: hydrogen sulfide; SF_6: sulfur hexafluoride; DMI: developmental malformation index; RBCs: red blood cells; PFA: paraformaldehyde; PBS: phosphate buffered saline; LNA: locked nucleic acid; NBT/BCIP: nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; DRG: dorsal root ganglia; OT: optic tectum; P: pericardial expansion; M: maxillofacial; T: telencephalon; D: diencephalon; C: cerebellum; PN: peripheral nerves; SC: spinal cord; CSC: coccygeal spinal cord; S: sarcomere; HB: hindbrain; BA: branchial arches; PA: palatal arch; A: abdomen; PCA: posterior commissure abdomen.

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