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Sodium thiosulfate exposure disrupts *in vitro* and *in vivo* heart development

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1. ABSTRACT

It is well-known that the majority of malformations found in the human population is based on complex gene-environment interactions. As an industrial chemical sodium thiosulfate (STS) is used heavily in many industries. Nevertheless, there is little known about the effects of STS on embryo development. In the present study, we have investigated the effects of STS on cardiac development in rat cardiomyocyte H9C2 cell line and chick embryos. As determined by MTT assays, the proliferation of H9C2 cells was inhibited by STS in a dose-dependent manner. Fertilized eggs injected via the yolk sac with STS at Hamburger-Hamilton (HH) stages 6, 9 and 12 showed significantly increased cardiotoxicity at HH stage 18, including cardiomyocyte apoptosis and animal mortality. Western blot analysis showed that STS significantly affected the expression of the apoptosis-related genes bcl-2, bax, and caspase-3 in a dose-dependent manner in the H9C2 cell line and in chick embryos. Dysregulation of apoptosis was correlated with embryonic heart malformations. Thus, STS may be a potent cardiac teratogen during embryo development.

2. INTRODUCTION

As an chemical reagent, sodium thiosulfate (STS) is used extensively as a component in various fields of industries such as photographic fixing, paper manufacture, medicine, food industry etc. It may release during the chemical's manufacture, use or disposal, therefore, STS is one of the environmental contaminants. While STS is widely used in many area related to our human closely, little attention has been paid to the effects of STS on embryo development. Therefore, in the present study, we used the rat heart H9C2 cell line and chick embryo models to assess the effects of STS on embryonic cardiac development. The H9C2 cells and chick embryos were treated with a range of STS concentrations and evaluated for cytotoxic and embryotoxic symptoms with respect to cell viability, embryo mortality, embryo morphology, and induction of apoptosis. H9C2 cardiomyocytes were used as they are morphologically similar to embryonic cardiocytes and are recognized as a well suited model for the study of cardiomyocyte biology(1). Understanding how these cells respond to STS may furnish novel insights into how cardiac and skeletal tissues respond to STS.

A number of recent studies have demonstrated that gene-environment interactions directly influence the development of embryo(2). Although the mechanisms by which these agents disrupt normal development are often not well understood, it is established that apoptosis is a common effect of many kinds of environmental stresses on the developing embryo(3). Many teratogens are well known to induce cell death in areas normally undergoing programmed cell death, resulting in abnormal development and structural malformations(4,5). Boya *et al.* (2000) found aberrant levels of apoptosis associated with abnormal development, often culminating in structural birth defects, in mouse mutants in which known apoptosis-related genes had been deleted (6). We therefore also used our *in vitro* and *in vivo* models to identify possible relationships between apoptosis and cardiac defects following exposure to STS.

3. MATERIALS AND METHODS

3.1. Cell culture and treatment

The H9c2 cell line was purchased from America Tissue Type Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in 75 cm2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO2. Cells were fed every 2–3 days, and sub-cultured once they reached 70–80% confluence in order to prevent the loss of differentiation potential. All cell culture products, including FBS, Dulbecco's phosphate buffered saline (PBS), and DMEM, were supplied by Invitrogen (Carlsbad, CA). Stock solutions of STS (Sigma Chemical Co., St. Louis, MO) were prepared in PBS and used immediately. STS was stored at -80°C in small aliquots, and thawed only once. Treatment dilutions were prepared in PBS immediately prior to use. Cells were grown to 60–70% confluence before treatment with STS. Cells were treated for 3 days with different concentrations of STS (0.1, 1, and 10 μM); PBS (solvent) was added to the control cells.

3.2. Cytotoxicity Assay

Cytotoxicities of STS in cultures of H9C2 cells were evaluated using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, a 5mg/ml solution of MTT (Sigma Chemical Co., St. Louis, MO) was prepared, filtered through a 0.2 μm filter, and used in the assays. Cell viability was determined on cells grown in 96-well plates. After STS treatment under appropriate conditions, MTT solution (40 μl) was added to each well, followed by incubation at 37°C for 4 h. The cells were washed twice in phosphate-buffered saline and acidic propanol (0.1 N HCl in absolute propanol) was added to each well. The absorbance was determined at 570 nm in a plate reader using acidic propanol as the blank.

3.3. Experimental animals and sodium thiosulfate treatment

White Leghorn chick eggs (Bovan strain) were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd (China). Approximately 250 eggs were randomly divided into five groups. To resume embryonic development, eggs were placed in automatic tilting racks in an incubator (Grumpatch, Savannah, GA, USA), with circulated air at 38ºC and 55% humidity. All embryos were staged according to the criteria of Hamburger and Hamilton(7). The compounds were directly injected into the center of the egg yolk via a small hole at the blunt end of the egg, using an established protocol(8). A hole was made at the blunt end of the egg with a sharp, thick sterile probe, and 50 μl of PBS and STS solution was slowly delivered into the center of each egg yolk using a 1-ml glass Hamilton syringe. Total dosages of 1, 2, 5 and 10μM were administered via three injections at stages 6 (24 h), 9 (33 h) and 12 (46 h), and were compared with vehicle-only controls (1×PBS). The eggs were sealed with electrical tape and returned to the incubator. Embryos were harvested at HH stage 18 (72 h) for examination of embryonic survival, cardiac function and molecular biology target parameters.

3.4. Detection of heart malformations

Experimental and control embryos were examined for the presence of congenital malformations at HH stage 18. Eggs were opened and the embryos were removed and transferred to a Petri dish filled with Locke's solution for morphological analysis. Embryos were dissected free from the extra-embryonic membranes and the gross morphological phenotypes were recorded using a Zeiss Lumar V12 stereomicroscope (magnification×12) (Carl Zeiss MicroImaging Co., Ltd. Germany).

3.5. Assessment of apoptosis by TUNEL assay

Apoptosis in H9C2 cells after STS or vehicle exposure was determined by performing TUNEL assays (FITC-dUTP nick-end labeling) using an *In situ* Cell-Death Detection kit (Roche Diagnostics) as the manufacturer's protocol described. H9C2 cells were grown on the coverslip of a 10 mm chamber. After STS treatment for 72 h, cells were washed twice with ice-cold PBS and then fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were washed twice with ice-cold PBS, and then permeabilized using freshly prepared permeabilization solution and incubated on ice for 10 min. The cells were washed twice with PBS, and then resuspended in 50 μl/slide TUNEL reaction mixture and incubated for 60 min at 37°C in a humidified atmosphere in the dark. The cells were washed twice with PBS and incubated with 1μM Hoechst 33342 (Sigma-Aldrich, USA) at room temperature in the dark. Cells were then washed three times with PBS and visualized using a laser scanning confocal microscope (LSM 510 META, Zeiss, Jena, Germany). All apoptosis assays were performed at least three times with similar results.

To further assess the extent of cell death and the presence of apoptosis in chick embryos, tissues were examined by TUNEL using the Apoptosis Detection System, DeadEndTM Colorimetric TUNEL System (Promega, Madison, WI, USA). Treated embryos were harvested at HH stage 18 and rinsed with 1×PBS. Hearts were excised, fixed overnight in freshly prepared 4% paraformaldehyde, dehydrated, and embedded in paraffin,

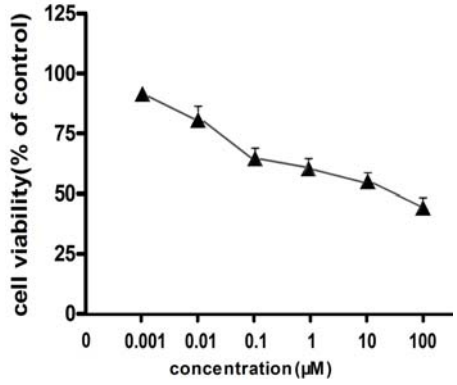

Figure 1. H9C2 cells were treated with the indicated concentrations of STS for 72 hours. Cell viability was measured by the MTT assay. Each bar represents the mean ± S.E.M. * Indicates statistically significant (P<0.05). Experiments were performed in triplicate.

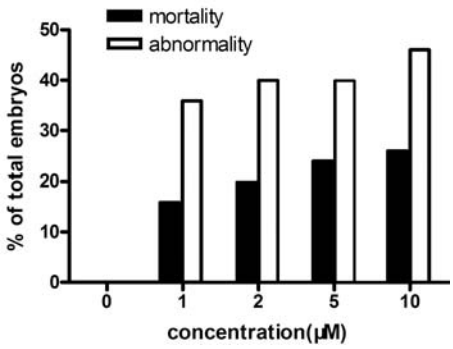

Figure 2. Effects of STS on mortality and abnormalities in chick embryo. Chick embryos were injected into the yolk sac with varying doses of STS dissolved in PBS. Embryos were were examined at HH stage 18 for mortality and abnormalities. Fifty bird embryos were used in each treatment group.

then sagitally sectioned at 5 μm. Sections were mounted on silanized slides, dewaxed in xylene and rehydrated with descending concentrations of ethanol. After washing in PBS (pH 7.4), treatment with proteinase-K, and refixation in methanol-free 4% formaldehyde, each section was covered with 50 μL of a solution consisting of equilibration buffer, nucleotide mix and TdT enzyme (all supplied with the kit). Negative control sections were treated as above, but minus the TdT enzyme. As external positive controls, sections were treated with 0.01 U/l deoxyribonuclease I (DNase I; AMPD1, Sigma Chemical Co., St. Louis, MO) for 10 min at 37ºC prior to reaction with the TdT mixture. After incubation for one h at 37ºC, the reaction was terminated by immersing the slides in 2×SSC for 15 min. The slides were then immersed in 0.3% hydrogen peroxide for 5 min and washed in PBS for 15 min. Streptavidin-horseradish peroxidase (100 μl) was added to each slide and incubated for 30 min at room temperature. Finally, sections were counterstained with diaminobenzidine (DAB) for 15 min and mounted in 50% glycerol under glass coverslips, which were sealed with nail varnish. Sections were examined and recorded using a 20x objective on a Nikon TE 2000-U microscope (Nikon Instruments, Inc., Melville, NY).

3.6. Western blot analysis

Western blot analysis was performed using standard procedures. Following treatment, H9C2 cell line and embryo hearts were lysed by ice-cold lysis buffer (CelLytic TM M Cell lysis reagent, Sigma Chemical Co.), then centrifuged at 12,000 g for 20 min at 4ºC. Aliquots were taken for protein quantification by Bradford assay (BCA assay, Bio-Rad Laboratories, Hercules, CA, USA) and equal amounts of protein from each sample in 2 × sodium dodecyl sulfate loading buffer were subjected to 15% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubation in blocking buffer (5% non-fat milk), the membranes were incubated overnight at 4ºC with the following antibodies: rabbit anti-actin (sc-1616-R, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-caspase-3 (sc-7148, Santa Cruz Biotechnology), rabbit anti-bcl-2 (sc-492, Santa Cruz Biotechnology), rabbit anti-bax (sc-526, Santa Cruz Biotechnology), at dilutions of 1:2000. After washing with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with IgG-alkaline phosphatase conjugate (diluted 1:1000 in TBS) for 1 h at room temperature. The membrane was stained with 1 mol/l Tris-HCl (pH 9.5) containing 5-bromo-4-chloro-3-indolyl phosphate p-toluidine and nitro blue tetrazolium (Promega) for 5–30 min. The nitrocellulose sheets were washed with deionized water and dried in air.

3.7. Statistical analysis

Data are presented as mean +/- S.E.M. Differences in mortality and abnormality rates between the groups were determined using Fisher's exact test. $p<0.05$ was taken to be significant. For the quantitative analysis of TUNEL-positive cells, one-way analysis of variance (ANOVA) was used for multiple comparisons between the STS-treated and control groups. All statistical analyses were done using the Instant and Prism software programs (GraphPad Software, San Diego, CA).

4. RESULTS

4.1. Viability of H9C2Cells after STS Treatment

H9C2 cells were treated with different concentrations (0.001, 0.01, 0.1, 1, 10, and 100μM) of STS for 72 h and then the rates of cell growth inhibition were evaluated based on the OD value of MTT assays. As shown in Figure 1, cell proliferation was inhibited by STS in a dose-dependent manner.

4.2. STS induces general structural effects in chick embryos

A total of 250 embryos were studied. As shown in Figure 2, treatment with STS significantly reduced embryo survival and increased the heart malformation rate. The incidence of embryonic death was significantly higher in treated embryos than in PBS controls. Embryos were examined carefully under a dissecting microscope. Heart malformations were detected in the treated embryos, but no

Figure 3. HH stage 18 chick embryo. The left picture demonstrates the normal appearance of the heart. In contrast, in the right picture the STS-treated embryo has a grossly abnormal heart. (the arrow indicates the difference shape of the heart between the normal and the STS-treated embryo)

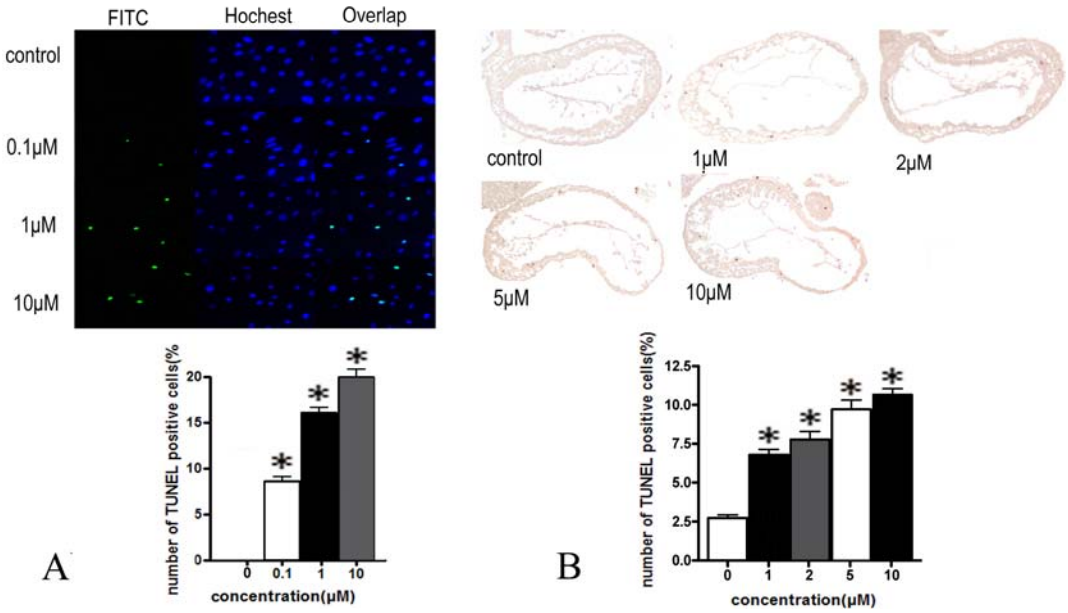

Figure 4. Cell death was measured by TUNEL assay, as detailed in methods. (A) Apoptosis was induced by treatment of H9C2 cells with varying concentrations of STS in medium for 72h. Cell death was assessed by TUNEL assay and photographed by the confocal microscope. The number of dead cells after STS exposure was increased significantly compared with the control ($p<0.05$). (B) Exposure to STS during the cardiac specification window alters the incidence of apoptosis in the HH stage 18 chick heart. (the arrow indicates the signal of the apopototic cells). Quantitative cell death analysis of TUNEL-positive cells. Number of TUNEL-positive cells in 1, 2, 5 and 10μM STS-treated and control embryos. The number of dead cells per field of view after STS exposure was increased significantly compared with the control ($p<0.05$).

congenital defects were found in the control group treated with vehicle only. We observed large, expansive ventricular heart abnormalities in STS-treated embryos (Figure 3), suggesting a teratogenic action of STS.

4.3. STS-induced *in vitro* and *in vivo* apoptosis

TUNEL assays in H9C2 cells exposed to STS (0.1, 1, or 10μM) for 72 h revealed dose-dependent increases in apoptotic cell numbers (Figure 4A). This observation was also confirmed *in vivo* in chick embryos by *in situ* TUNEL analysis. Six randomly chosen embryos from each group were harvested at HH stage 18 (72 h). As shown in Figure 4B, apoptotic myocardial cells were rarely found at this stage in control embryos. However, the number of TUNEL-positive myocardial cells in treated chick embryos was significantly higher than that found in controls. The ratio of apoptotic nuclei in the cardiomyocytes was significantly higher for STS-treated chick embryos than for the controls. The number of apoptotic cells was clearly increased in both *in vivo* and *in vitro* models in response to STS.

4.4. Effect of STS on expression of apoptosis-related proteins.

Western blot analysis showed that STS induced apoptosis by the mitochondrial pathway, as evidenced by the statistically significant increase in total bax and caspase-3 expression and the decrease in bcl-2 expression in both H9C2 cells and HH stage 18 embryos (Figure 5).

5. DISCUSSION

Cardiac malformations constitute the most common birth defects, about 1% of infants are born with one or more heart or circulatory problems. However it is often difficult to determine the cause of congenital heart

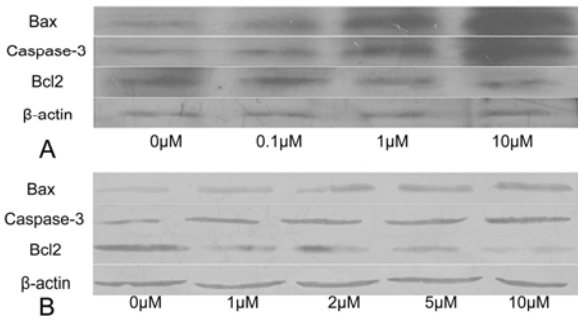

Figure 5. Quantitative western blot analysis of bax, caspase-3 and bcl-2 in H9C2 cells (A) and chick embryos (B) after treatment with different concentrations of STS. Equal amounts of protein were loaded into each lane. Protein loading was normalized based on beta-actin expression. Representative western blots illustrate significantly higher bax and caspase-3, and lower bcl-2 protein levels in STS-treated embryos, compared with control embryos.

disease. Specialists believe that about ten percent of heart defects are caused by specific genetic abnormalities, as in Down's syndrome, or from an abnormal gene that is passed down from one generation to the next, as in Marfan syndrome. As for the remaining 90 percent, a poorly understood combination of genetic predisposition and environmental factors is thought to be responsible (9). Environmental health scientists and developmental biologists put more emphasis on the development of those malformations causing by environmental agents, using mammalian and non-mammalian animal models, state-of-the-art molecular biology techniques to investigate the cellular and molecular mechanisms involved in the development of these malformations. There is increasing evidence to suggest that environmental agents such as trichloroethylene (10), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (11) etc are closely related to cardiac malformations. So much interest has been focused on the alterations causing by environmental agents in the development of the cardiovascular system and thereby lead to cardiovascular malformations.

The present study was designed to investigate whether STS treatment had any deleterious effects on embryonic cardiac development. Our previous study has suggested that zebrafish 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 (12). In this study we found that reatment with STS resulted in decreased cell viabilities in H9C2 cells and also increased the risk of congenital heart malformations in chick embryos. This is the first *in vitro* and *in vivo* experimental evidence that STS exposure can significantly induce dose-dependent cardiomyocyte apoptosis. Our findings suggest that STS exposure may cause serious congenital heart malformations, possibly brought about by this increased apoptosis. The dose-related increase in the expression of bax and caspase-3, as well as a decrease in the expression of bcl-2, indicated that STS activated the mitochondrial apoptotic pathway.

In this study, we have taken advantage of H9C2 cells and chick embryos as experimental model systems to assess the effects of STS on both *in vitro* and *in vivo* cardiomyocyte development. The H9C2 cells are commonly used as an *in vitro* model to study the cellular mechanisms and signaling pathways involved in drug-induced cardiotoxicity(13). These cells retain many of the molecular markers common to cardiomyocytes and show morphological characteristics of immature embryonic cardiomyocytes (14). The chick embryo was selected as the animal model in our study because it is known to be highly sensitive to teratogens, making it useful for detection of teratogenesis (15,16). In addition, cardiovascular development in avian and human embryos is almost identical, utilizing the same signaling molecules and progressing through very similar developmental steps at approximately equivalent embryonic stages(17). Chick embryos have specifically been used to examine the developmental effects of various agents on an embryonic system that mimics the human fetus in many important aspects, with regard to its toxicology.

When investigating the teratogenic effects of chemicals, the level of exposure and the stage of embryonic development are important factors. HH stages 6–12 have been established as the critical window of sensitivity for teratogenesis in chick embryos (18). Treatment of avian embryos with STS during this critical period subsequently resulted in the development of gross cardiac defects in the embryos. Significantly more ($p<0.05$) cardiac malformations occurred in experimental embryos than in controls. Our findings demonstrate that early developmental exposure of chick embryos to an environmentally relevant dose of STS significantly increased the risk of congenital heart malformations. We used an egg injection protocol in this study, because the determination of environmental contaminant concentrations in avian eggs is frequently used as part of environmental pollutant monitoring programs. Hence, STS appears to have teratogenic effects on cardiac development similar to those of a number of other well-known environmental pollutants. To our knowledge, this is the first report to demonstrate that STS can cause cardiac malformations in an avian model.

We also observed that STS exposure during HH stages 6, 9 and 12 induced apoptosis, and it seems possible that the cardiac abnormalities could have been related to increases in apoptosis. Although apoptosis has been more extensively investigated in other tissues (19,20), this process has only recently been implicated in development of the normal and diseased cardiovascular system(21,22). The finding of increased caspase-3 expression in response to STS exposure is particularly relevant in this respect, as this enzyme is known to target a variety of cellular proteins for proteolytic cleavage, as part of the process by which dying cells are eliminated (23). In contrast, the bcl-2 proteins are involved in the control of apoptosis upstream of caspase-3 activation and upstream of other irreversible stages of cellular damage; thus, their role may be to determine whether a cell will live or die(24) . This intrinsic pathway (also called the bcl-2-regulated or mitochondrial

pathway) is triggered by various developmental cues or cytotoxic insults, such as viral infection, DNA damage, and growth factor deprivation, and is strictly controlled by the bcl-2 family of proteins(25). Upon receiving a stress signal, the pro-apoptotic cytoplasmic proteins bax binds to the outer membrane of the mitochondria to initiate the release of caspase-3, the effector protein that initiates degradation (26). The transcriptional regulation of bax, caspase-3, and bcl-2 seems to be a critical factor in the regulation of cell death. Our results are consistent with previous work that indicate that this intrinsic pathway plays an important role in teratogen-induced apoptosis and cell cycle arrest.

In conclusion, the chicken may represent a suitable species to further investigate the effects of STS on cardiovascular development, it can be very helpful in determining the mechanism of teratogenesis. In view of the data obtained from this study, it would seem important that extreme care should be used for the safe disposal of waste STS so that it does not contaminate ground water and thus enter the ecological food chain.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. J. Hescheler, R. Meyer, S. Plant, D. Krautwurst, W. Rosenthal, G. Schultz: Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res* 69 (6), 1476-86 (1991)

2. S.S. El-Dahr, L.M. Harrison-Bernard, S. Dipp, I.V. Yosipiv, S.Meleg-Smith: Bradykinin B2 null mice are prone to renal dysplasia: gene-environment interactions in kidney development. *Physiol Genomics* 3,121-131 (2000)

3. J. Thompson, E. Hipwell, H.V. Loo, J. Bannigan: Effects of cadmium on cell death and cell proliferation in chick embryos. *Reprod Toxicol* 20, 539-548 (2005)

4. P.E. Mirkes: Cell death in normal and abnormal development. *Congenit Anom (Kyoto)* 48, 7-17 (2008)

5. I. Ivnitski, R. Elmaoued, M.K. Walker: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inhibition of coronary development is preceded by a decrease in myocyte proliferation and an increase in cardiac apoptosis. *Teratology* 64, 201-212 (2001)

6. P. Boya, E.J. de la Rosa: Cell death in early neural life. *Birth Defects Res C Embryo Today* 75, 281-293 (2005)

7. V.H.H. Hamburger: A series of normal stages in the development of the chick embryo. *J Morphol* 88, 49 –92 (1951)

8. V.J. Drake, S. L. Koprowski, J. W. Lough, S.M. Smith: Gastrulating chick embryo as a model for evaluating teratogenicity: A comparison of three approaches. Birth Defects Res. *A Clin Mol Teratol* 76,66-71 (2006b)

9. C.A. Loffredo: Epidemiology of cardiovascular malformations: prevalence and risk factors. *Am J Med Genet* 97, 319-325 (2000)

10. B.D. Hardin, B.J. Kelman, R.L. Brent: Trichloroethylene and dichloroethylene: a critical review of teratogenicity. *Birth Defects Res A Clin Mol Teratol* 73,931-955 (2005)

11. H.M. Goldstone, J.J. Stegeman: Molecular mechanisms of 2,3,7,8-tetrachlorodibenzo-p-dioxin cardiovascular embryotoxicity. *Drug Metab Rev* 38, 261-289 (2006)

12. W. Hu, L. Cheng, H. Xia, D. Sun, D. Li, P. Li, Y. Song, X.Ma: Teratogenic effects of sodium thiosulfate on developing zebrafish embryos. *Front Biosci* 1 (14), 3680-7 (2009)

13. B.Zordoky, A.El-Kadi: H9c2 cell line is a valuable *in vitro* model to study the drug metabolizing enzymes in the heart. *J Pharmacol Toxicol Methods* 56 (3),317-22 (2007)

14. M.Aboutabl, A.El-Kadi: Constitutive expression and inducibility of CYP1A1 in the H9c2 rat cardiomyoblast cells. *Toxicol In vitro* 21 (8),1686-91 (2007)

15. M.O. Cheung, E.F. Gilbert, R.E. Peterson: Cardiovascular teratogenicity of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in the chick embryo. *Toxicol Appl Pharmacol* 61 (2),197-204 (1981)

16. J. Manner, W. Seidl, F. Heinicke, H. Hesse: Teratogenic effects of suramin on the chick embryo. *Anat Embryol* 206 (3),229-237 (2003)

17. B.J.Martinsen: Reference guide to the stages of chick heart embryology. *Dev Dyn* 233 (4),1217-1237 (2005)

18. V.J. Drake, S.L. Koprowski, N.Hu, S.M. Smith, J. Lough: Cardiogenic effects of trichloroethylene and trichloroacetic acid following exposure during heart specification of avian development. *Toxicol Sci* 94 (1),153-162 (2006)

19. M.D. Jacobson, M. Weil, M.C. Raff: Programmed cell death in animal development. *Cell* 88 (3),347-354 (1997)

20. S. Nagata: Apoptosis by death factor. *Cell* 88 (3),355-365 (1997)

21. G. Olivetti, R. Abbi, F. Quaini, J. Kajstura, W. Cheng, J.A. Nitahara, E. Quaini, C. Di Loreto, C.A. Beltrami, S. Krajewski, J.C. Reed, P.Anversa: Apoptosis in the failing human heart. *N Engl J Med* 336 (16),1131-1141 (1997)

22. R.S. Williams: Apoptosis and heart failure. *N Engl J Med* 341 (10),759-760 (1999)

23. V. Anantharam, M. Kitazawa, J. Wagner, S. Kaul, A.G. Kanthasamy: Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. *J Neurosci* 22 (5),1738-1751 (2002)

24. D.T. Chao, S.J. Korsmeyer: BCL-2 family: regulators of cell death. *Annu Rev Immunol* 16,395-419 (1998)

25. Soleman, D., L. Cornel, S.A. Little, P.E. Mirkes: Teratogen-induced activation of the mitochondrial apoptotic pathway in the yolk sac of day 9 mouse embryos. *Birth Defects Res Part A Clin Mol Teratol* 67 (2),98-107 (2003)

26. L.Czerski, G.Nunez: Apoptosome formation and caspase activation: is it different in the heart? *J Mol Cell Cardiol* 37 (3),643-652 (2004)

Abbreviations: STS: sodium thiosulfate (STS); MTT:3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HH stages:Hamburger-Hamilton stages; DMEM:Dulbecco's modified Eagle's medium; PBS:phosphate buffered saline; FBS: fetal bovin serum; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

Key Words: Sodium thiosulfate, Chick Embryo, H9C2, Apoptosis

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