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Using immunofluorescence to detect PM2.5-induced DNA damage in zebrafish embryo hearts

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Corresponding Author:	Tao Chen Soochow University Suzhou, Jiangsu CHINA
Corresponding Author's Institution:	Soochow University
Corresponding Author E-Mail:	tchen@suda.edu.cn
Order of Authors:	Yujie Huang
	Yizhou Tao
	Chang Cai
	Jin Chen
	Cheng Ji
	Stanley Aniagu
	Yan Jiang
	Tao Chen
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TITLE

Using immunofluorescence to detect $PM_{2.5}$-induced DNA damage in zebrafish embryo hearts

AUTHORS AND AFFILIATIONS

Yujie Huang[1,*], Yizhou Tao[1,*], Chang Cai[1], Jin Chen[1], Cheng Ji[1], Stanley Aniagu[2], Yan Jiang[1], Tao Chen[1]

[1]School of Public Health, Soochow University, Suzhou, China
[2]Toxicology, Risk Assessment and Research Division, Texas Commission on Environmental Quality, Austin, TX, USA

[*]These authors contributed equally

Corresponding author:
Tao Chen (tchen@suda.edu.cn)

Email addresses of co-authors:
Yujie Huang (2535997172@qq.com)
Yizhou Tao (616379417@qq.com)
Chang Cai (1287045427@qq.com)
Jin Chen (chenjin2015@163.com)
Cheng Ji (jichenggreen@163.com)
Stanley Aniagu (stanley.aniagu@tceq.texas.gov)
Yan Jiang (yjiang@suda.edu.cn)

KEY WORDS

Immunofluorescence' $PM_{2.5}$, DNA damage, heart, embryo, zebrafish

SUMMARY

This protocol uses an immunofluorescence assay to detect $PM_{2.5}$-induced DNA damage in the dissected hearts of zebrafish embryos.

ABSTRACT

Ambient fine particulate matter ($PM_{2.5}$) exposure can lead to cardiac developmental toxicity but the underlying molecular mechanisms are still unclear. 8-hydroxy-2'deoxygenase (8-OHdG) is a marker of oxidative DNA damage and γH2AX is a sensitive marker for DNA double strand breaks. In this study, we aimed to detect $PM_{2.5}$-induced 8-OHdG and γH2AX changes in the heart of zebrafish embryos using an immunofluorescence assay. Zebrafish embryos were treated with extractable organic matters (EOM) from $PM_{2.5}$ at 5 μg/mL in the presence or absence of antioxidant N-acetyl-L-cysteine (NAC, 0.25 μM) at 2 h post fertilization (hpf).

DMSO was used as a vehicle control. At 72 hpf, hearts were dissected from embryos using a syringe needle and fixed and permeabilized. After being blocked, samples were probed with primary antibodies against 8-OHdG and γH2AX. Samples were then washed and incubated with secondary antibodies. The resulting images were observed under by fluorescence microscopy and quantified using ImageJ. The results show that EOM from $PM_{2.5}$ significantly enhanced 8-OHdG and γH2AX signals in the heart of zebrafish embryos. However, NAC, acting as a reactive oxygen species (ROS) scavenger, partially counteracted the EOM-induced DNA damage. Here, we present an immunofluorescence protocol for investigating the role of DNA damage in $PM_{2.5}$-induced heart defects that can be applied to the detection of environmental chemical-induced protein expression changes in the hearts of zebrafish embryos.

INTRODUCTION

Air pollution is now a serious environmental problem facing the world. Ambient fine particulate matter ($PM_{2.5}$), which is one of the most important indicators of air quality, can carry a large number of harmful substances and enter the blood circulatory system, causing serious harm to human health[1]. Epidemiology studies have demonstrated that $PM_{2.5}$ exposure can lead to an increased risk of congenital heart defects (CHDs)[2,3]. Evidence from animal experiments also showed that $PM_{2.5}$ can cause abnormal cardiac development in zebrafish embryos and the offspring of mice but the molecular mechanisms of the cardiac developmental toxicity of $PM_{2.5}$ is still largely unknown[4-6].

DNA damage can cause cell cycle arrest and induce apoptosis, which may extensively destroy the potential of progenitor cells and, consequently, impair heart development[7)]. It has been well documented that environmental pollutants, including $PM_{2.5}$, have the potential to attack DNA through oxidative stress mechanisms[8,9]. Both human and zebrafish cardiac development are sensitive to oxidative stress[10-12]. 8-OHdG is an oxidative DNA damage marker, and γH2AX signal is a marker of DNA double strand breaks. N-acetyl-L-cysteine (NAC), a synthetic precursor of intracellular cysteine and glutathione, is widely used as an anti-oxidative compound. In this study, we use NAC to investigate the role of oxidative stress in $PM_{2.5}$-induced DNA damage[13].

Zebrafish as a model vertebrate has been widely used to study cardiac development and human cardiovascular diseases because mechanisms of cardiac development are highly conserved among vertebrates[14,15]. The advantages of using zebrafish as a model include their small size, strong reproductive ability, and low feeding cost. Of particular interest to these studies, zebrafish embryos do not depend on the circulatory system during early development and can survive severe heart malformation[14]. Moreover, their transparency allows the entire body to be directly observed under a microscope. Thus, zebrafish embryos provide an outstanding opportunity for assessing the molecular mechanisms involved in the induction of cardiac developmental toxicity as a result of exposure to various environmental chemicals[5,16,17]. We have previously reported that $PM_{2.5}$-induced oxidative stress leads to DNA

damage and apoptosis, resulting in heart malformations in zebrafish[18]. In this study, we provide a detailed protocol for investigating $PM_{2.5}$-induced DNA damage in the heart of zebrafish embryos.

PROTOCOL

Wild type zebrafish (AB) used in this study were obtained from the National Zebrafish Resource Center in Wuhan, China. All animal procedures outlined here have been reviewed and approved by the Animal Care Institution of The Ethics Committee of Soochow University.

1. $PM_{2.5}$ sampling and organic compound extraction

NOTE: $PM_{2.5}$ was collected in an urban area in Suzhou, China, August 1-7, 2015, as described previously[5].

1.1. Bake 47 mm quartz membrane filters in a 500 °C muffle furnace for 2 h to remove the organic components.

1.2. Place a filter in a $PM_{2.5}$ sampler for 24 h of uninterrupted sampling.

1.3. Remove the filter and dry for 24 h at room temperature.

1.4. Quantify the filter with an analytical balance.

1.5. Extract organic components from the filter by Soxhlet extraction using dichloromethane as a solvent[19].

1.6. Dry the EOM by a rotary evaporation in a 60 °C water bath and nitrogen flow. Dissolve EOM in DMSO and store at -20 °C.

2. Zebrafish embryo collection and treatment

2.1. Maintain the zebrafish at 28.5 ± 0.5 °C in a re-circulating aquaculture system with a 14 h light and 10 h dark photoperiod cycle.

2.2. Place healthy adult zebrafish into a tank at a 2:1 male to female ratio.

2.3. The next day, collect the embryos and wash them with system water (i.e., zebrafish breeding water).

2.4. Select and randomly divide zebrafish embryos demonstrating a normal development

(uniform size, full grains, and no egg coagulation) into 4 groups in individual glass Petri dishes with a diameter of 7 cm (about 50 embryos per dish).

2.5. Treat the embryos with $PM_{2.5}$ (5 mg/L) in the presence or absence of NAC at 0.25 μM from 2 hpf until 72 hpf. Use DMSO as a vehicle control to a final concentration at 0.1% (v/v).

3. Morphological observation of zebrafish embryos and cardiac dissection

3.1. At 72 hpf, transfer embryos to glass slides and observe under a stereo microscope. Record heart malformations, such as pericardial edema, altered looping, and decreased size.

3.2. Calculate malformation rates (the percentage of embryos with heart defects out of the total living embryos) and analyze differences between groups using one-way ANOVA followed by Turkey's Multiple Comparison Test ($p < 0.05$ = statistically significant).

3.3. Anesthetize the embryos with 0.6 mg/mL MS-222 to immobilize them on glass slides.

3.4. Record heart beats for 30 s and quantify heart rates using ImageJ software[5,20].

3.5. Dissect hearts from zebrafish embryos with a disposable syringe needle under a stereo microscope. Caution: Avoid destroying the heart shape.

4. Immunofluorescence Assay

4.1. To use an immunofluorescence assay to detect $PM_{2.5}$ induced DNA damage in the heart of zebrafish embryos, use a hydrophobic barrier pen to draw a circle on a clean glass side.

4.2. Add 50 μL of 4% paraformaldehyde to 1.25 mL of Phosphate Buffered Saline (PBS) to make a fixative solution.

4.3. Place 3 dissected hearts into one hydrophobic barrier pen circle and incubate for 20 min at room temperature.

4.4. Decant the solution under the microscope and dry the samples at room temperature for at least 5 min, so that the hearts completely attach to the glass slides.

4.5. Wash the slides three times in PBS with 0.1% Tween 20 (PBST) for 5 min per wash.

4.6. Add 50 μL of bovine serum albumin (BSA) to 1000 μL of PBST to obtain a 5% BSA solution and incubate the slides in a humid chamber for 1 h to block non-specific antibody binding.

4.7. Decant the solution and wash the samples three times with PBS for 5 min per wash.

4.8. Dilute 2 μL of mouse monoclonal antibody against 8-OHdG and 2 μL of rabbit polyclonal antibody against γH2AX in 296 μL of PBST to obtain a working primary antibody cocktail solution.

4.9. Incubate the heart samples with 50 μL of the primary antibody cocktail solution against 8-OHdG and γH2AX in a humidified chamber for at least one hour at room temperature or overnight at 4 °C (Overnight incubation can increase signal intensity).

4.10. Decant the solution and wash the samples three times with PBST for 5 min per wash.

4.11. Dilute 1 μL of FITC-labeled goat anti-mouse secondary antibody and 1 μL of cy3 goat anti-rabbit secondary antibody in 498 μL of PBST to obtain a working secondary antibody cocktail solution and incubate the samples with the secondary antibodies (1:500 in PBST) for 1 h at room temperature in the dark.

4.12. Decant the solution and wash the samples three times with PBS for 5 min per wash protected from light.

4.13. Add 20 μL of DAPI (4',6-diamidino-2-phenylindole) to the samples for nuclear staining for 30 min at room temperature.

4.14. Apply a coverslip to slide and seal with nail polish to prevent drying and movement. Then image the samples under a fluorescence microscope and quantify the fluorescence signal of heart area using ImageJ software. Calculate the relative changes with the average of DMSO control samples. Determine the statistical significance of the data as in step 3.2.

REPRESENTATIVE RESULTS

This immunofluorescence assay is a sensitive and specific method for measuring protein expression changes in the hearts of zebrafish embryos exposed to environmental chemicals.

In this representative analysis, embryos exposed to $PM_{2.5}$ in the absence or presence of the antioxidant NAC were evaluated for the presence the presence of heart malformations (**Figure 1**). As observed, EOM from $PM_{2.5}$ caused a significant increase in cardiac teratogenesis, such as pericardial edema, altered looping, and decreased size, compared to DMSO control-treated hearts. The heartbeat rate was also significantly decreased in embryos exposed to EOM (**Figure 1B**). The addition of NAC significantly attenuated EOM-induced heart defects (**Figure 1**).

Here the immunofluorescence assay was used to measure 8-OHdG and γH2AX expression in

zebrafish embryo to evaluate the extent of DNA damage in EOM-treated tissues. As shown in **Figure 2**, the levels of 8-OHdG and γH2AX expression were significantly increased in the hearts of zebrafish embryos treated with EOM group compared to the control, DMSO-treated hearts, indicating an increase in oxidative DNA damage and DNA double strand breaks, respectively. Furthermore, the EOM-induced DNA damage was partially counteracted by NAC supplementation (**Figure 2**).

FIGURE LEGENDS

Figure 1. Cardiac defects of zebrafish embryos at 72 hpf. (A) Images of zebrafish embryos at 72 hpf. Dotted lines indicate atria (red) or ventricles (blue). Scale bar, 200 μm. (B) Heart malformation and heartbeat rates. Results are presented as mean ± SEM. At least 50 embryos in each group were examined. EOM: EOM at 5 mg/L; NAC: NAC at 0.25 μM.. **, $P < 0.01$; ***, $p<0.001$

Figure 2. DNA damage in the heart of zebrafish embryos at 72 hpf. A) Immunofluorescence staining. Scale bar, 100 μm. B) Quantitative results. Results were presented as mean ± SEM. At least 15 hearts from each group were examined. EOM: EOM at 5 mg/L; NAC: NAC at 0.25 μM. **; $P < 0.01$; ***; $p<0.001$.

DISCUSSION

Although zebrafish is an excellent vertebrate model for studying the cardiac developmental toxicity of environmental chemicals, due to the small size of the embryo heart, it is difficult to obtain enough protein for western blot analysis. Therefore, we present a sensitive immunofluorescence method for quantifying the protein expression levels of DNA damage biomarkers in the hearts of zebrafish embryos exposed to $PM_{2.5}$.

During dissection, it is important to keep the integrity of the heart intact. In our experience, it is relatively easy to perform the isolation at 3 hpf. In addition, the heart needs to be put into fixation solution as soon as possible after collection. Another critical step is drying the samples to make sure that the dissected hearts are completely attached to the glass slide. Otherwise, the samples may be washed off from the slide during labeling.

Dual immunofluorescence staining is performed to detect both 8-OHdG and γH2AX signals in the isolated hearts. This method not only saves labor and allows the use of a reduced sample size, but also facilitates co-localization of the two signals. Although this antibody-based method can not detect fluorescence signals in living embryos, this rapid protocol can be used to detect protein expression in isolated zebrafish embryo hearts.

It has been frequently reported that oxidative stress mediates $PM_{2.5}$-induced DNA damag[8,9]. Excessive ROS production can lead to DNA damage and apoptosis during zebrafish embryonic

development[21-23]. As we have previously reported [18], an increased 8-OHdG and γH2AX signal expression is observed in the hearts of zebrafish embryos exposed to EOM, the expressions of which are significantly counteracted by treatment with the ROS scavenger NAC. It is noteworthy that NAC does not completely reverse $PM_{2.5}$-induced DNA damage signal expression, indicating that oxidative stress may only contribute partially to the DNA damage observed in the hearts of zebrafish embryos exposed to $PM_{2.5}$.

In conclusion, this method uses a sensitive technique for detecting $PM_{2.5}$ induced DNA damage in the intact hearts of zebrafish embryos. In addition, the method can be applied to detect protein expression changes in the hearts of zebrafish embryos exposed to environmental chemicals.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. World Health Organization (WHO), Ambient (outdoor) air quality and health http://www.who.int/mediacentre/factsheets/fs313/en/ (Acessed Oct 10, 2020) (2018).
2. Zhang, B. et al. Maternal Exposure to Air Pollution and Risk of Congenital Heart Defects. *European Journal of Pediatrics*, **175**, 1520-1520 (2016).
3. Huang, C.C., Chen, B.Y., Pan, S.C., Ho, Y.L. and Guo, Y.L. Prenatal exposure to PM2.5 and Congenital Heart Diseases in Taiwan. *The Science of the Total Environment*, **655**, 880-886 (2019).
4. Mesquita, S.R. et al. Toxic assessment of urban atmospheric particle-bound PAHs: relevance of composition and particle size in Barcelona (Spain). *Environmental Pollution*, **184**, 555-562 (2014).
5. Zhang, H. et al. Crosstalk between AhR and wnt/beta-catenin signal pathways in the cardiac developmental toxicity of PM2.5 in zebrafish embryos. *Toxicology*, **355-356**, 31-38 (2016).
6. Duan, J. et al. Multi-organ toxicity induced by fine particulate matter PM2.5 in zebrafish (Danio rerio) model. *Chemosphere*, **180**, 24-32 (2017).
7. Lorda-Diez, C.I. et al. Cell senescence, apoptosis and DNA damage cooperate in the remodeling processes accounting for heart morphogenesis. *Journal of Anatomy*, **234**, 815-829 (2019).
8. Kouassi, K.S. et al. Oxidative damage induced in A549 cells by physically and chemically characterized air particulate matter (PM2.5) collected in Abidjan, Cote d'Ivoire. *Journal of*

287 *Applied Toxicology*, **30**, 310-320 (2010).
288 9. Gualtieri, M. et al. Gene expression profiling of A549 cells exposed to Milan PM2.5.
289 *Toxicology Letters*, **209**, 136-145 (2012).
290 10. Li, S.Y., Sigmon, V.K., Babcock, S.A. and Ren, J. Advanced glycation endproduct induces
291 ROS accumulation, apoptosis, MAP kinase activation and nuclear O-GlcNAcylation in human
292 cardiac myocytes. *Life Sciences*, **80**, 1051-1056 (2007).
293 11. Yamashita, M. Apoptosis in zebrafish development. *Comparative biochemistry and*
294 *physiology. Part B, Biochemistry & Molecular Biology*, **136**, 731-742 (2003).
295 12. Moazzen, H. et al. N-Acetylcysteine prevents congenital heart defects induced by
296 pregestational diabetes. *Cardiovascular Diabetology*, **13**, 46 (2014).
297 13. Sun, S.Y. N-acetylcysteine, reactive oxygen species and beyond. *Cancer Biology & Therapy*,
298 **9**, 109-110 (2010).
299 14. Tu, S. and Chi, N.C. Zebrafish models in cardiac development and congenital heart birth
300 defects. *Differentiation*, **84**, 4-16 (2012).
301 15. Asnani, A. and Peterson, R.T. The zebrafish as a tool to identify novel therapies for human
302 cardiovascular disease. *Disease Models & Mechanisms*, **7**, 763-767 (2014).
303 16. Li, M. et al. Toxic effects of polychlorinated biphenyls on cardiac development in zebrafish.
304 *Molecular Biology Reports*, **41**, 7973-7983 (2014).
305 17. Massarsky, A., Prasad, G.L. and Di Giulio, R.T. Total particulate matter from cigarette
306 smoke disrupts vascular development in zebrafish brain (Danio rerio). *Toxicology and Applied*
307 *Pharmacology*, **339**, 85-96 (2018).
308 18. Ren, F., et al. AHR-mediated ROS production contributes to the cardiac developmental
309 toxicity of PM2.5 in zebrafish embryos. *The Science of the Total Environment*, **719**, 135097
310 (2020).
311 19. van Berlo, J.H. and Molkentin, J.D. An emerging consensus on cardiac regeneration.
312 *Nature Medicine*, **20**, 1386-1393 (2014).
313 20. Yue, C. et al. Protective effects of folic acid on PM2.5-induced cardiac developmental
314 toxicity in zebrafish embryos by targeting AhR and Wnt/beta-catenin signal pathways.
315 *Environmental Toxicology*, **32**, 2316-2322 (2017).
316 21. Zhao, X., Ren, X., Zhu, R., Luo, Z. and Ren, B. Zinc oxide nanoparticles induce oxidative
317 DNA damage and ROS-triggered mitochondria-mediated apoptosis in zebrafish embryos.
318 *Aquatic Toxicology*, **180**, 56-70 (2016).
319 22. Zhao, X., Wang, S., Wu, Y., You, H. and Lv, L. Acute ZnO nanoparticles exposure induces
320 developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. *Aquatic*
321 *Toxicology*, **136-137**, 49-59 (2013).
322 23. Zhu, L. et al. DNA damage and effects on glutathione-S-transferase activity induced by
323 atrazine exposure in zebrafish (Danio rerio). *Environmental Toxicology*, **26**, 480-488 (2011).

Figure 1

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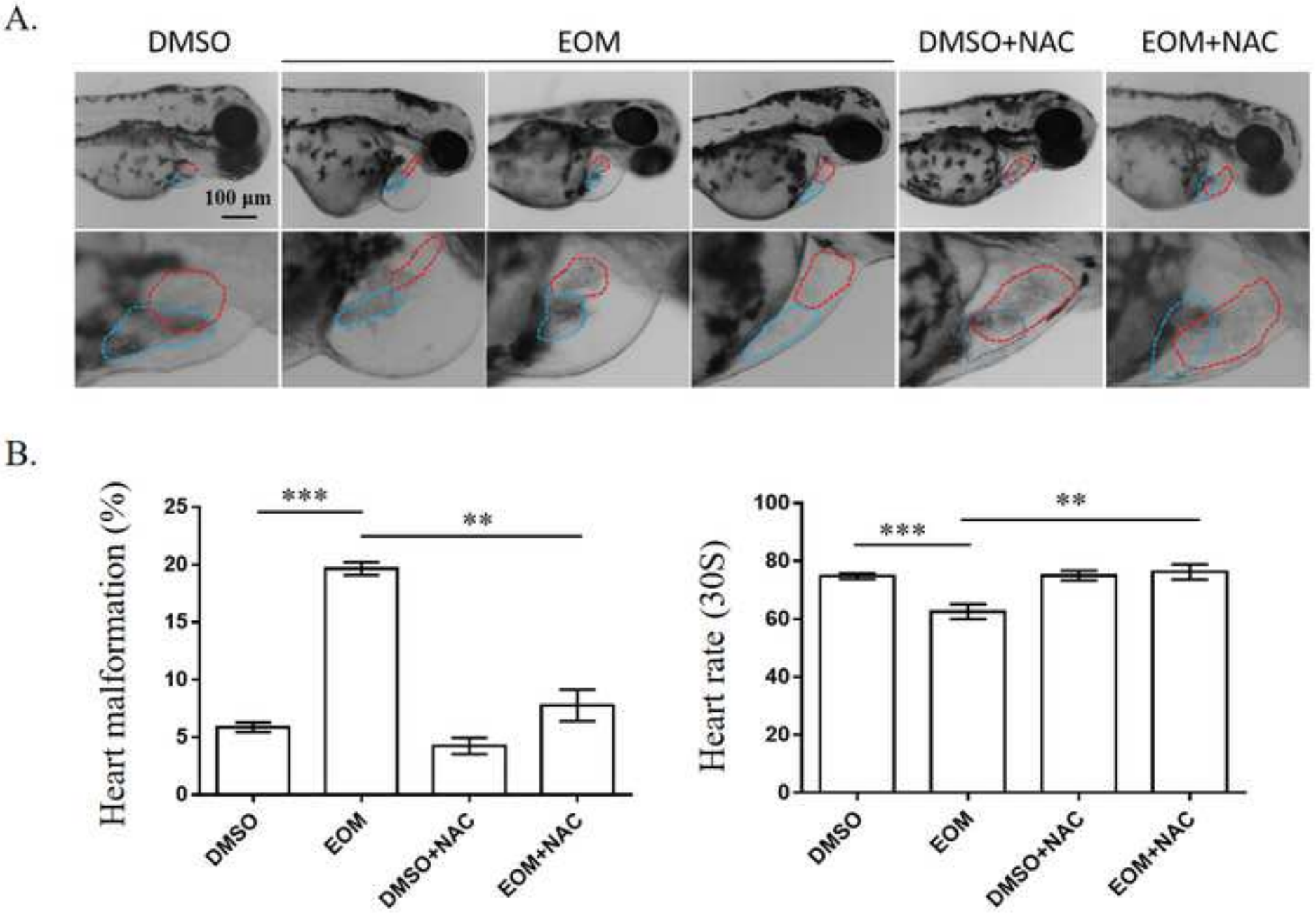

Figure 2

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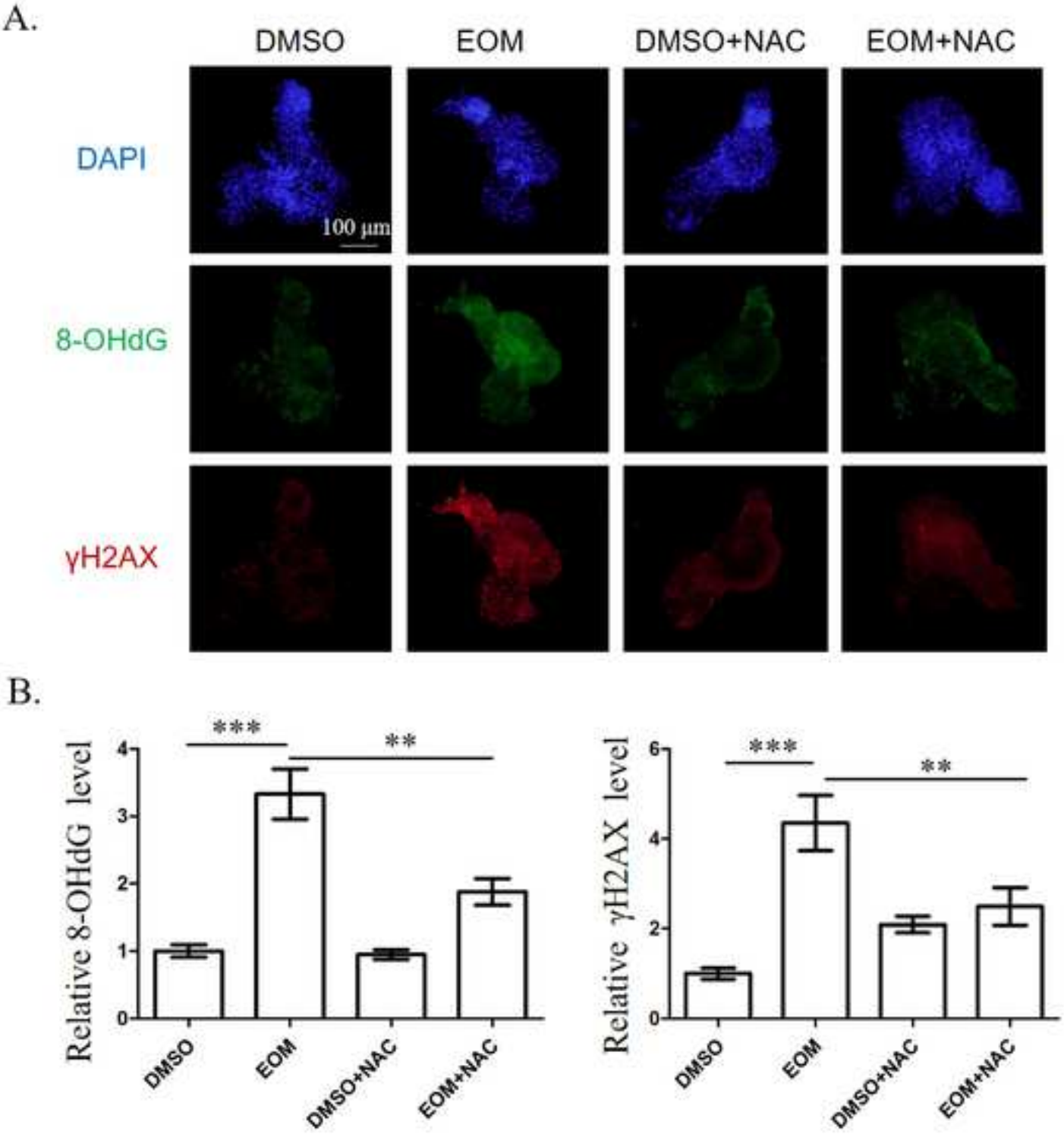

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
8-OHdG Antibody	Santa Cruz Biotechnology, USA	sc-66036	Primary antibody
Analytical balance	Sartorius,China	BSA124S	
BSA	Solarbio,Beijing,China	SW3015	For blocking
DAPI	Abcam, USA	ab104139	For nuclear counterstain.
DMSO	Solarbio,Beijing,China	D8371	
Fluorescence microscope	Olympus, Japan	IX73	For imaging fluorescence signals/
Goat Anti-Rabbit IgG Cy3	Carlsbad,USA	CW0159	Secondary antibody
Goat Anti-Rabbit IgG FITC	Carlsbad,USA	RS0003	Secondary antibody
N-Acetyl-L-cysteine(NAC)	Adamas-Beta, Shanghai, China	616-91-1	
Orbital shaker	QILINBEIER,China	TS-1	
Paraformaldehyde	Sigma,China	P6148	Make 4% paraformaldehyde for fixation.
Phosphate Buffered Saline	HyClone,USA	SH30256.01	Prepare 0.1% Tween in PBS for washing.
PM2.5 sampler	TianHong,Wuhan, China	TH-150C	For 24-hr uninterrupted PM2.5 sampling.
Re-circulating aquaculture system	HaiSheng,Shanghai,China		The zebrafish was maintained in it.
Soxhlet extractor	ZhengQiao,Shanghai, China	BSXT-02	For organic components extraction.
Stereomicroscope	Nikon,Canada	SMZ645	For heart dissection from zebrafish embryos.
Tricaine methanesulfonate（MS22	Sigma,China	E10521	To anesthetize zebrafish embryos
Tween 20	Sigma,China	P1379	
γH2AX Antibody	Abcam, USA	ab26350	Primary antibody

Rebuttal Letter
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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have made changes as suggested.

2. Please provide an email address for each author.

We have added email addresses.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We have rephrased the Summary "Here, we present an immunofluorescence protocol to investigate the role of DNA damages in PM2.5-induced heart defects, which can be used to detect environmental chemical-induced protein expression changes in the heart of zebrafish embryos."

4. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 56 (sensitive to…)-60; 157-160; 183 (Excessive…)-186;

Thanks for the reminder. We have made changes as suggested.

5. Please remove the headings (Objective, Methods etc) from the abstract. As we are a methods journal, please rephrase the conclusion to emphasize that this method (immunofluorescence assay) can be used to obtain the results of your study.

Thanks for the advice. We have rephrased our conclusion as suggested.

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

We have made changes as suggested.

7. Please move the ethics statement (lines 90-93) to the beginning of the protocol, before you start the numbered steps.

We have made changes as suggested.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Tianhong TH-150C PM2.5 sampler (Wuhan, China); National

Zebrafish Resource Center, Wuhan, China; CAS 616-91-1, Adamas-Beta, Shanghai, China; TRNzol A+ reagent (Tiangen, Beijing, China); Santa Cruz Biotechnology, USA; Abcam, USA

We have made changes as suggested.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have made changes as suggested.

10. 1.5: If this step is not part of the video, please cite a reference so that readers can replicate the protocol.

We have added a reference for Soxhlet extraction in "1.5".

11. 1.6: As you store the EOM at -20 °C, please specify if there is a temperature that should not be exceeded during the concentration (rotary evaporator water bath).

During the concentration step (rotary evaporator water bath), the temperature was set at 60 °C. We have made corresponding changes.

12. 2.3: What do you mean by "system water"?

'System water' means zebrafish breeding water. We have added a note in the manuscript.

13. 2.4: How do you determine "normal" zebrafish embryos? How many zebrafish embryos per group per Petri dish?

We selected embryos with uniform size, full grains, and no egg coagulation, and put about 50 embryos into each petri dish. We have put the relative information in the manuscript.

14. 3.1: What do you look for to infer heart malformation?

We modified the 3.1: "Heart malformations such as balloon shaped heart chambers and pericardial edema were recorded, and malformation rates were then calculated."

15. 3.2: How do you anesthetize the embryos? Do you add MS-222 to the Petri dishes or glass slides> How much MS-222 is to be added?

To make it clear, we modified the 3.2: “Anesthetize the embryos with 0.6 mg/ml MS-222 to immobilize them on glass slides.”

16. 3.3: Do you use a specific module in ImageJ to count heart rates?

No, we don’t need a specific module in ImageJ to count heart rates.

17. After including a one line space between each protocol step, if your protocol exceeds 3 pages, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

The protocol does not exceed 3 pages. All the steps except part 1 (PM2.5 sampling and organic compounds extraction) will be filmed.

18. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend. Please indicate whether error bars are derived from standard deviation or standard error of the mean.

Sorry for the confusion. All the images in one figure share the same scale bar. We have added explanations for scale bars and error bars in the two Figure legends.

19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Any modifications and troubleshooting of the technique

b) Any limitations of the technique

c) The significance with respect to existing methods

d) Any future applications of the technique

We have added more sentences to address these issues.

20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal names and capitalize the first letters of all words of the journal names, e.g., Journal of Applied Toxicology.

We have made changes as suggested.

21. Please sort the Materials Table alphabetically by the name of the material.

We have made changes as suggested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Overall this is an interesting manuscript and useful method to be available. A few overall comments and section specific comments are detailed below. Throughout the manuscript fine particulate matter (PM2.5) the "2.5" should be subscripted which is the common presentation of this abbreviation. More information on how the statically significant differences were identified is needed both in the methods and results sections.

Thanks for the advice. We have changed all the "PM2.5" to "$PM_{2.5}$", and added statistical analysis steps in the protocol.

Major Concerns:

Representative Results

144-149: clearly stating what these "Significant" differences are being compared to would be helpful (i.e. vehicle control, NAC, etc.)

Sorry for the confusion. We have made relative changes.

Further explanations in the captions about what each letter of significance indicates is needed. How many animals were used to determine these findings?

Thanks for the suggestion. We have made changes as suggested.

Protocol

In the abstract it says that embryos were treated at 2 hpf but in the methods it says 3 hpf.

Sorry for the mistake. For this study, the embryos were treated within 2 hours after fertilization. We have made corresponding changes.

Was a blank extracted filter used as a methods control?

Thanks for the suggestion. We agree that it would be ideal to use a blank extracted filter as a control. However, since the extractions were dried before being dissolved in DMSO, we chose DMSO as a vehicle control.

Minor Concerns:

Introduction

The use of NAC was not mentioned in the introduction which is an important element of this study.

Thanks for the suggestion. We have added an introduction for NAC.

Protocol

1.2 - What are the details for when and where this sample was collected?

PM2.5 was collected in an urban area in SuZhou, China in August 1-7, 2015. We have put more information in the protocol.

1.4 - Quantify the filter is unclear? Was gravimetric analysis pre- post-sampling being conducted to determine the PM mass collected? Was a temperature/humidity-controlled chamber used for weighing the filter?

We routinely use the 47 mm quartz membrane filters for PM2.5 collection. Yes, the members were weighed before and after sample collection. The temperature and humidity were under control during the weighing process.

2.5 - Details on the zebrafish exposure are lacking. How many animals were in each group to get these representative images?

Sorry for the confusion. We have put more details into this section. For each exposure group, there were about 50 embryos, and the experiments were repeated at least 3 times.

Reviewer #2:

This manuscript described an immunefluorescence assay to detect DNA damage in the heart of zebrafish embryos. Some concerns are outlined below.

1. Line 22. Consider removing "maternal" since the exposure condition in this study is a waterborne exposure with the zebrafish embryo. Similarly, it is not necessary to emphasize "maternal" or "offspring" in Introduction section.

Thanks for your advice. We have made changes as suggested.

2. Line 99. Consider removing "with transparent color".

We have deleted "with transparent color".

3. Line 103. The exposure began at 3 hpf. However, in the abstract, it stated that " embryos at 2 h post fertilization (hpf) were treated with …"

Sorry for the mistake. For this study, the embryos were treated within 2 hours after fertilization. We have made corresponding changes.

4. Line 113-114. Consider remove this sentence.

Thank you for your suggestion. We have removed this sentence.

5. Line 119. Please define PAP in the first place.

We have used hydrophobic barrier pen to replace "PAP" pen.

Reviewer #3:

The study looks interesting at first sight, but some important questions should be addressed. There are also important shortcomings.

In protocol section;

1. (line 77) The content analysis of the sampled PM2.5 must be done and the content must be given.

Thank you for your suggestion. We did conduct content analysis of the sampled PM2.5, and the results have already been published in Toxicology in 2016. We have cited the article in the PM2.5 sampling section.

1.6 What is the concentration of DMSO in which the EOM is dissolved?

Final DMSO concentration was 0.1% (v/v) in all the groups.

2.4. (line 99) How did they decide that the embryos were normal? Criteria should be given for the selection of healthy embryos.

We selected embryos with uniform size, full grains, and no egg coagulation. We have put this information in the manuscript.

Detailed information should be given about the application equipment (petri dishes or cell culture plates?)

The petri dish used in this study was a glass dish with a diameter of 7 cm. We have put this information in the manuscript.

How many groups were embryos divided into? Clearer information about groups should be given. It was necessary to have a control group with no treatment.

Embryos were divided into 4 groups: DMSO (vehicle control), EOM, DMSO+NAC, EOM+NAC. We did have a control group with no treatment at the beginning, but find no difference between no treatment and DMSO groups.

How many embryos did they select for each group? How many repetitions have been

done?

About 50 embryos were exposed in each group, and the experiments were repeated at least three times. We have added the relative information in the manuscript.

Why was a single dose used?

In our previous study, we have tested the effects of PM2.5 in different dose levels (Zhang *et al*, Toxicology 2016). We have cited the article in the PM2.5 sampling section.

3.1. The criteria(s) for heart malformation should be clearly stated. how many embryos were used? Was the pericardiac edema given in Figure 1 evaluated as a heart malformation? If so, I must say that this is not true. Because, percardiac edema is considered as body malformation in the literature. Malformations observed in the heart should be given and identified.

Thanks for your advice. We identify heart malformation based on not only pericardial edema but also altered looping and decreased size. The relative information has been added in section 3.1.

3.3. Why did they record the heart rate for 30 seconds? reference should be given.

We have put a reference in section 3.3 (Zhang *et al*, Toxicology 2016)

3.4. Dissecting the heart from a 72-hour embryo is a very successful and interesting practice. More detailed information should be given about this application.

Thank you for your comment. The technique is a little bit tricky, but all we need are practise and to be careful. Thus, I added this sentence "Need to be careful to avoid destroy the heart shape."

4.1. how many embryos were used?

About 50 embryos were exposed in each group. We have added this information in the manuscript.

4.2. If only 3 hearts were used for each group, this would not be sufficient.

About 50 embryos were exposed in each group.

4.3. I doubt that waiting for the samples for 5 minutes at room temperature would be sufficient for the hearts to completely attached to the glass slides. Are they sure about this time?

In our experience, the zebrafish heart can adhere to the glass slide in about 5 min at room temperature if the fixation solution was discarded extensively. Otherwise, the time

need to be extended. We have changed “5 min” to “at least 5 min” in section 4.3.

4.7. Primary antibodies dripped onto the same sample? this part needs to be given much more detail.

For the two primary antibodies used in this study, they can be added at the same time. We have added more information in section 4.7.

4.9. What was used as a secondary antibody? The images (Figure 2) look like the picture of the same marker (ec. DAPI) in different filters.

The two secondary antibodies used in this study were FITC-labeled goat anti-mouse and cy3 goat anti-rabbit as mentioned in section 4.9. The images in Figure 2 did look similar but we have double checked the results.

4.11. How was the quantification of fluorescent images done?

We used ImageJ software to quantify the fluorescent signals from at least 5 hearts in each group.

In Discussion section

The discussion is very weak. Must be strengthened.

There are some sentences that should not be in the discussion section (lines 173-180). these should be moved to the method part.

Thanks for your advice. We have added more sentences in the Discussion section to strengthen it. We put these sentences in the discussion because we are asked to explain the advantages and limitations of the technique in this section.