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TITLE:

Using chicken embryo as a powerful tool in assessment of developmental cardiotoxicities

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Air cell injection; microinjection; lentivirus-mediated *in ovo* silencing; air cell inhalation; right ventricular wall thickness

SUMMARY:

Chicken embryos, as a classical developmental model, are used in our lab to assess developmental cardiotoxicities following exposure to various environmental contaminants. Exposure methods and morphological/functional assessment methods established are described in this manuscript.

ABSTRACT:

Chicken embryos are a classical model in developmental studies. During the development of chicken embryos, the time window of heart development is well-defined, and it is relatively easy to achieve precise and timely exposure via multiple methods. Moreover, the process of heart development in chicken embryos is similar to mammals, also resulting in a four-chambered heart, making it a valuable alternative model in the assessment of developmental cardiotoxicities. In our lab, the chicken embryo model is routinely used in the assessment of developmental cardiotoxicities following exposure to various environmental pollutants, including per- and polyfluoroalkyl substances (PFAS), particulate matter (PMs), diesel exhaust (DE) and nano materials. The exposure time can be freely selected based on the need, from the beginning of development (embryonic day 0, ED0) all the way to the day prior to hatch. The major exposure methods include air-cell injection, direct microinjection, and air-cell inhalation (originally developed in our lab), and the currently available endpoints include cardiac function (electrocardiography), morphology (histological assessments) and molecular biological assessments (immunohistochemistry, qRT-PCR, western blotting, etc.). Of course, the chicken

embryo model has its own limitations, such as limited availability of antibodies. Nevertheless, with more laboratories starting to utilize this model, it can be used to make significant contributions to the study of developmental cardiotoxicities.

INTRODUCTION:

The chicken embryo is a classic developmental model, which has been used for over two hundred years[1]. The chicken embryo model has various advantages compared to traditional models. First of all, as early as over 70 years ago, the normal development of the chicken embryo had been illustrated very clearly in the Hamburger-Hamilton staging guide[2], in which a total of 46 stages during chicken embryo development were defined with precise time and morphological characteristics, facilitating detections of abnormal development. Additionally, the chicken embryo model has other features such as being relatively low-cost and redundant in quantity, relatively accurate exposure-dose controls, an independent, closed system within the shell and easy manipulation of the developing embryo, all of which guarantees its potential to be used as a powerful toxicological assessment model.

In cardiotoxicity, the chicken embryo features a four chambered heart, similar to mammalian hearts but with thicker walls, allowing easier morphological assessments. Additionally, the chicken embryo allows for developmental inhalation exposure, which is not possible in mammalian models: during the later stage of development, the chicken embryo will transition from internal respiration to external respiration (getting oxygen via the lung); the latter requires that the embryo penetrates the air cell membrane with the beak, and starts to breathe air[3], making the air cell a mini-inhalation chamber. Utilizing this phenomenon, the toxicological effects of gas contaminants on the heart (and other organs) may be assessed without the need of dedicated inhalation chamber instruments.

In this manuscript, several exposure/endpoint assessment methods are described, all of which serve to make the chicken embryo a powerful tool in the assessment of development cardiotoxicity following exposure to environmental contaminants.

PROTOCOL:

All procedures described were approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University. In our lab, the eggs were incubated in two incubators. Eggs were held upright in the incubator and randomly placed on the shelves. The incubation conditions for the eggs were as follows: incubation temperature started at 37.9 °C, and gradually decreased to 37.1 °C as incubation proceeded; the humidity started at 50% and gradually increased to 70%.

1. Exposure methods

NOTE: Exposure of environmental contaminants to chicken embryos may be achieved in several ways. In this section, three routinely used methods are described in detail.

1.1. Air cell injection (**Figure 1**)

NOTE: This is the classical exposure method to chicken embryos[4-6], suitable for a wide range of materials, and may be performed at a very wide time window, from the beginning of development (embryonic day zero, ED0) all the way to the day prior to hatch (ED20). Sunflower oil is used as the vehicle. Previous studies have shown that no significant changes in mortality, hatchability, or body weight have been observed between untreated embryos and embryos injected with sunflower oil[7].

1.1.1. Prepare the following necessary reagents/tools: 75% ethanol, tissue paper, metal probe (can substitute with any sharp metal needle/stick/awl), melted paraffin, brush, povidone iodide solution, pipette, pipette tips, candling lamp, dosing mixture. Prepare the dosing mixture with sunflower oil (recommended)[4]. To use other diluents, perform vehicle control (versus untreated embryos).

1.1.2. Clean the surface of the eggs with povidone iodide solution (commercially available povidone iodide solution 1:5 diluted with deionized water), and dip-dry the egg shell with tissue paper without scrubbing. Scrubbing will break the protective layer coating the outside of the shell.

1.1.3. Candle the eggs in a dark room and mark the air cell with pencil. Exclude eggs with cracks on the shell. Exclude eggs with air cells on the side instead of blunt tip, as those are highly unlikely to hatch normally.

1.1.4. Sanitize the air cell area with 75% ethanol, and then drill a small hole at the center of air cell area with the metal probe. Do not stick the probe deep into the air cell or the inner membrane may be damaged, instead, only break the shell with the tip of the probe. If the hole is not large enough to fit in a fine pipette tip, break the shell again at the vicinity of existing hole, until the hole is large enough to allow insertion of 10 µL pipette tip.

1.1.5. Vortex the dosing mixture vigorously, and immediately draw solution to pipette tip. The recommended injection volume is 1 µL per 10 gram of egg (e.g., 5 µL injection volume for a 50-gram egg) as larger injection volumes may create hypoxic or anoxic conditions for the developing embryo. Calculate the concentration of the dosing solution for desired mg/egg kg dose.

1.1.6. Insert the pipette tip into the hole, with the tip touching the inner membrane. Slowly eject the dosing mixture, hold for at least ten seconds (allow the viscous oil to be fully dispensed), and then remove the tip.

1.1.7. Seal the hole with a brush and a drop of melted paraffin. Be careful not to drip melted paraffin onto the inner membrane.

1.1.8. Once sealed, place the eggs in the incubator until they reach the desired embryonic stage. On already developing embryos, perform the whole process as soon as possible to prevent potential embryo loss due to low environmental temperatures.

1.2. Microinjection (**Figure 2**)

NOTE: This is a more direct exposure method, resulting in definitive exposure to the substance-of-interest, and especially suitable for compounds with a short duration of action (e.g., lentivirus), since the classical air cell injection requires time for the compounds to penetrate the inner membrane. This method may also be tried if satisfying results could not be achieved by air cell injection. This method is best suitable for early embryos (up to ED2), but also can be performed on older embryos (with higher risk of embryo loss).

1.2.1. Prepare the following necessary reagents/tools: 75% ethanol, povidone iodide solution, micro-injector (5 µL), metal probe (can substitute with any sharp metal needle/stick/awl should work), fine forceps, tape. Prepare the dosing mixture with sterile saline, which also serves as an injection control without affecting hatchability significantly. Ensure the sterility of the saline, as a contaminated injection will dramatically increase mortality.

1.2.2. Clean the eggs as described in 1.1.2.

1.2.3. Candle the eggs as described in 1.1.3.

1.2.4. Sanitize the air cell area with 75% ethanol, and then drill a small hole at the center of air cell area with the metal probe. Do not stick the probe deep into the air cell or the inner membrane may be damaged, instead, only break the shell with the tip of the probe. Then use the fine forceps to carefully enlarge the hole until the diameter is approximately 2 mm, allowing visual confirmation of the inner membrane.

1.2.5. Load the solution into microinjector (maximum injection volume: 0.5 µL/10 g egg. (e.g., 2.5 µL for a 50 g egg) and carefully insert the needle through the hole into the inner membrane for approximately 2-3 mm. Gently dispense the solution and remove the needle. Keep the needle as perpendicular to the membrane as possible.

1.2.6. Seal the hole with a small piece of tape. Completely cover the hole to prevent embryo dehydration and death during subsequent incubation. Nevertheless, avoid pieces of tape that are too large to prevent hypoxia.

1.2.7. Once sealed, place the eggs in the incubator until they reach the desired embryonic stage. On already developing embryos, perform the whole process as soon as possible to prevent potential embryo loss due to low environmental temperature.

1.3. Air cell inhalation (**Figure 3**)

NOTE: This is a novel inhalation method taking advantage of the air cell, from which the late-stage chicken embryo will start to breathe air. It is suitable for gas or aerosol exposures and may achieve very early-in-life inhalation exposures, and fill the lungs with the target gas/aerosol when they open for the first time in life.

1.3.1. Prepare the following necessary reagents/tools: Sampling bag (PVF bag, for storage of gas/aerosol sample before exposure), catheter needle, syringe, metal probe (can substitute with any sharp metal needle/stick/awl should work), tape, fume hood.

1.3.2. Clean the eggs as described in 1.1.2 and candle them as described in 1.1.3 (it is not necessary to mark air cell prior to incubation), and then incubate eggs without treatment until ED17.

1.3.3. Candle the eggs at ED17 to mark the air cell area.

1.3.4. At ED18, take an egg out from the incubator, sanitize the air cell area with 75% ethanol, and then carefully drill two small holes on two sides of the air cell. One is for injection of gas/aerosol, the other is for expelling of air. Carefully control the size of the holes so that the size of the injection hole is just enough for the catheter needle to be inserted, while the diameter of the expelling hole is a bit larger (approximately 1 mm).

1.3.5. Gently inject 10 mL of target gas/aerosol from the injection hole with a syringe attached to the catheter needle. Inject air for an inhalation control group, which should have no significant differences to a negative control group[8]. Apply pressure against the catheter needle (with appropriate amount of pressure the elastic needle can be pushed against the shell) to minimize the leakage from injection hole. Seal both holes immediately with tape afterwards, and return the egg to incubator.

NOTE: This procedure should be performed in a fume hood to prevent inhalation of gas/aerosol by the operator.

1.3.6. Repeat the described procedure after one hour to further ensure the whole air cell is filled up with target gas/aerosol (optional).

1.3.7. Repeat the described procedure at ED19 again (optional). Repeating the exposure helps to ensure consistent exposure until hatch. Record the hatch time for an approximate estimation of exposure duration.

1.3.8. Once desired exposures have been performed and sealed, place the eggs in the incubator for hatching. Minimize the time the egg spends outside of the incubator to prevent death from low environmental temperature.

2. Endpoint assessment methods

NOTE: Following exposure of contaminant-of-interest to the developing embryo, several toxicity parameters can be evaluated, including cardiotoxicity. In this section, two frequently used specific methods are described in detail.

2.1. Electrocardiography (**Figure 4**)

NOTE: It is impossible to do non-invasive electrocardiography in hatchling chickens due to the presence of feathers. Thus, subcutaneous implantation of electrodes is essential, requiring anesthesia. The dose used in the lab is 33 mg/kg pentobarbital via intraperitoneal injection (some chickens may require up to 50% dose increase). This method will result in stable electrocardiography in over 90% of animals, allowing for analyzation of heart rate.

2.1.1. Prepare the following necessary reagents/tools: 1% (10 mg/mL) pentobarbital solution in saline, syringe, electrical balance, heater (if necessary), an electrocardiography instrument attached (e.g., BL-420E+) with two-channel metal needle electrodes.

2.1.2. Weigh the chickens with a balance and calculate the necessary volume of pentobarbital solution and inject the chickens. For a chicken that weighs 30 g, 0.1 mL of pentobarbital solution is needed. Make sure that the injection is done on the lateral side of the abdomen, as the yolk is located at the middle and injection there may not be effective.

2.1.3. Wait until the injected chickens are anesthetized (hold the chicken in hand, if the neck has no tension and the head can be swung freely, the anesthetization is sufficient). Place chickens on the operation table (heaters are necessary if room temperature is below 20 °C).

2.1.4. Insert two needle electrodes from two sides of the abdomen, subcutaneously. Make sure that the needles do not enter the abdominal cavity by lifting the skin a little bit and inserting needle from there. Once inserted, carefully push the needle forward until it reaches the side of chest cavity. Make sure that the needle does not go deep into the body or stick out from the skin.

2.1.5. Make the measurements with the electrocardiography instrument. Other similar instruments capable of electrocardiography can be used.

2.1.6. If the chickens are to be sacrificed, perform euthanasia after the electrocardiography since they are already under anesthesia. If chickens are to survive, place them back into their cages and warm until waking up. Returning them to the incubator is another option.

2.2. Histomorphometry (**Figure 5**)

NOTE: A specific method is developed to assess the right ventricular wall thickness in transverse sections of the heart. Morphological assessment of right ventricle dimension via echocardiography is not 100% accurate due to the irregular shape of right ventricle, and this method may serve as a good supplement in the morphological assessments for the right ventricle.

2.2.1. Prepare the following necessary reagents/tools: 4% phosphate buffered formaldehyde, sharp blade, phosphate buffered saline, paper towel, electrical balance, small scissors, general histological processing agents (graded ethanol, xylene, paraffin).

2.2.2. Once animals are sacrificed, use water to wet the feathers. This is to minimize the potential contamination due to flying feathers while opening up the chest.

2.2.3. Open the chest cavity carefully without damaging the heart. Use small scissors to cut the vasculature and gently remove the heart from the chest cavity. Leave a small piece (approximately 1-2 mm) of vasculature attached to the heart as this may be convenient for subsequent handling of the heart without damaging the heart.

2.2.4. Once removed, rinse the heart in cold phosphate buffered saline to remove blood and relax the muscle. Then dip dry the heart on paper towel before weighing for accurate weight reading. Put the heart into 10x volume fixative (4% phosphate buffered formaldehyde) for 24 hours at room temperature. Fixed tissues may be subsequently processed to paraffin blocks, or be stored at 4 °C for years (not recommended if immunohistochemistry is planned).

2.2.5. Before embedding, cut the tissues at approximately 60% length of the heart counting from the apex (**Figure 5A**), for easier subsequent processing. A microtome blade is recommended for a quick and vertical clean cut. For chickens older than one day, make another cut at approximately 25-30% length from the apex for easier paraffin penetration and to allow the tissue to fit in tissue cassettes.

2.2.6. Process the tissues with the following conditions (adjust as needed): 70% ethanol for 1 h, 80% ethanol for 1 h, 95% ethanol for 1 h x2, 100% ethanol for 30 min x2, xylene for 5 min x2, paraffin (melting point 52-54 °C) for 1.5 h, paraffin (melting point 62-64 °C) for 1 h, and then embed the tissues in a 3:1 mixture of paraffin (melting point 62-64 °C) and paraffin (melting point 52-54 °C).

2.2.7. Section the tissue at 6 µm thickness. Carefully maintain identical relative positions of the cross-sections by confirming the presence and size of an anatomical landmark (septomarginal trabecula) in the right ventricle. Confirm a landmark with moderate length on each section (**Figure 5B**, arrow).

2.2.8. Make two electronic rulers with Logo programmer: Ruler 1 is a straight line with 7 radius measure lines attached to the middle point, with 22.5° in between two adjacent measure lines. Ruler 2 is just two perpendicular lines in a T shape (**Figure 5B**).

2.2.9. Measure with two software programs: Adobe Photoshop and ImageJ.

2.2.9.1. In Photoshop, resize ruler 1 (NO reshaping) to place the two ends of the ruler on the two ends of free right ventricular wall, so that the seven measure lines on ruler 1 will each meet the inner right ventricular wall. Then use ruler 2 to make perpendicular measurements from the inner to external ventricular wall (**Figure 5B**).

2.2.9.2. Use ImageJ to make the seven measurements for each heart.

2.2.10. Depending on specific needs, analyze the seven measurements or average for one representative right ventricular wall thickness. Normalize to whole heart weight for specific ventricular wall thickness changes.

REPRESENTATIVE RESULTS:

Exposure results

Air cell injection

Air cell injection can effectively expose developing chicken embryos to various agents, which may be subsequently detected in the collected samples (serum, tissue, etc.) of embryos/hatchling chickens. Here is an example, in which perfluorooctanoic acid (PFOA) was air-cell injected, and serum PFOA concentrations were then determined with Ultra-performance liquid chromatography-mass spectrometry. The serum concentrations corresponded with the injected doses, indicating the effectiveness of this procedure (**Figure 6**).

Microinjection

Microinjection may expose the developing embryos to agents that may not effectively penetrate the inner membrane, or with a short duration of action, such as lentivirus. Here is an example, in which lentivirus was injected at embryonic day two with this method and then significant green fluorescence was observed in the heart of embryonic day 15 embryos, indicating the effectiveness of lentivirus transfection (**Figure 7**).

Air cell infusion

Air cell infusion is a novel method, which may work very well for small amount of gas/aerosol inhalation exposure during the initiation stage of external respiration. Here is an example, in which diesel exhaust was infused into air cell at embryonic day 18 and 19, resulting in significant fibrotic changes in the cardiac as well as pulmonary tissues (**Figure 8**).

Endpoint assessment results

Electrocardiography results

Due to the limitation of two electrodes, only 3 channels of electrocardiography may be shown. But they are sufficient to distinguish r waves, thus they may be used for functional assessments. In a real-life example, electrocardiography of chickens exposed to diesel exhaust indicated significantly shortened R-R interval, indicating functional changes (**Figure 9**).

Histopathology results

Our method of right ventricular wall thickness assessment was successfully used in several studies[5, 7-12]. In one of our previous studies, diesel exhaust exposure resulted in thickened right ventricular wall (**Figure 10**).

FIGURE AND TABLE LEGENDS:

Figure 1: Demonstration of air cell injection. An undeveloped fertile egg is shown in the picture, but embryos at all different stages may be exposed with this method.

Figure 2: Demonstration of microinjection. An early embryo is shown in the picture, which is the

preferred exposure time point for this method, but other time points may also be tried.

Figure 3: Demonstration of air cell infusion. A late-stage embryo undergoing internal pipping is shown in the picture, which is the preferred exposure time point for this method. Four stages of the operation were shown. 1: Intact embryo. 2: Two holes have been made. 3: Infusion is being performed. The PVF sampling bag is also shown at the bottom left. 4: Infusion is finished, holes sealed with tape.

Figure 4: Demonstration of electrocardiography. Top left panel showed how a hatchling chicken was anesthetized and undergoing electrocardiography measurement. Top right panel shows the electrocardiography instrument with the electrodes attached. Bottom panel shows a representative electrocardiography acquired from the chickens.

Figure 5: Demonstration of histopathological assessment of right ventricular wall thickness (Hematoxylin and eosin staining). (**A**) Demonstration of the cutting position of chicken hearts prior to embedding. (**B**) Demonstration of the right ventricular wall thickness measurement. Scale bars represent 1000 µm. Blue circles demonstrate the seven measurement points on the inner right ventricular wall. Red circle demonstrates a measurement point on the outer right ventricular wall. Arrow demonstrates the anatomic landmark for the appropriate cross-section position. This figure has been modified from Jiang et al. *Toxicology*. **293** (1-3), 97-106 (2012)[7].

Figure 6: Serum concentration of perfluorooctanoic acid from hatchling chickens following air cell injection with 0, 0.5, 1 or 2 mg/egg kg perfluorooctanoic acid prior to incubation. The resulting serum concentrations corresponded with the injected doses, indicating the effectiveness of air cell injection. This figure has been modified from Jiang et al. *Toxicology*. **293** (1-3), 97-106 (2012)[7].

Figure 7: Demonstration of lentivirus transfection efficacy following microinjection exposure (Direct observation following cryo-sectioning). Left panels showed light field images, while right panels showed green fluorescence for the same tissue sections. Embryonic day two chicken embryos were injected with lentivirus or control, and then incubated until embryonic day 15. The hearts were frozen-sectioned and directly visualized under fluorescent microscope. (**A**) Control group, little green fluorescence was present. (**B**) Lentivirus exposed group, significant green fluorescence was observed, indicating the effectiveness of lentivirus transfection following microinjection. Scale bars represent 125 µm. This figure has been modified from Zhao et al. *Environmental Toxicology and Pharmacology*. **56**, 136-144 (2017)[11].

Figure 8: Demonstration of the effectiveness of air cell infusion. Chicken embryos were infused with diesel exhaust at embryonic day 18 and 19, and then the hatched chickens were kept for 0, 1 or 2 weeks and then sacrificed. The heart tissues were assessed with Masson Trichrome staining for fibrotic lesions. Arrows showed the fibrotic lesions (blue staining). *: statistically different from control ($P<0.05$ from analysis of variance and least significant difference tests). This figure has been modified from Jiang et al. *Environmental Pollution*. 264, 114718 (2020)[8].

Figure 9: Demonstration of the effectiveness of electrocardiography. Chicken embryos were infused with diesel exhaust at embryonic day 18 and 19, and then the hatched chickens were kept for 0, 1 or 2 weeks and then electrocardiography was performed. Significantly shortened R-R intervals were observed in the chickens exposed to diesel exhaust via air cell infusion, indicating the effectiveness of the method. *: statistically different from control (P<0.05 from analysis of variance and least significant difference tests). This figure has been modified from Jiang et al. *Environmental Pollution*. 264, 114718 (2020 [8].

Figure 10: Demonstration of the effectiveness of right ventricular wall thickness measurement (Hematoxylin and eosin staining). Chicken embryos were infused with diesel exhaust at embryonic day 18 and 19, and then the hatched chickens were kept for 1 week, and then histological assessment of the right ventricular wall thickness was performed. A: Representative pictures of the heart cross-sections. Note the presence of anatomical marker in all the right ventricles (In older chickens, the marker tends to be a bit longer at desired position, which does not affect the accuracy of measurements). B: Quantification of the right ventricular wall thickness, which were firstly converted to actual length with standard slides, and then normalized with whole heart weight thus were represented in the form of um/ug. Blue arrows: two ends of the free right ventricular wall. Red arrows: the middle points of the right ventricular wall. Black arrows: anatomical marker. *: statistically different from control (P<0.05 from analysis of variance and least significant difference tests). Scale bars represent 1000 μm. This figure has been modified from Jiang et al. *Environmental Pollution*. 264, 114718 (2020)[8].

DISCUSSION:

The chicken embryo has been a classical model in developmental studies for 200 years[1]. Our methods presented in this manuscript have been used in the assessment of several environmental contaminants, including perfluorooctanoic acid, particulate matter, and diesel exhaust with success[5, 7-12]. With these methods, developmental cardiotoxicity was indicated cost-effectively and clearly. Furthermore, it is not difficult to expose chicken embryos with other compounds-of-interest and assess potential developmental cardiotoxicity.

The air-cell injection method is a classical method used previously in many studies[13-15], which is convenient and effective. Compared to other developmental exposure methods, such as rodent models[16-18], it features direct exposure into a closed system, which greatly reduces the variabilities due to maternal effects and varied excretion. Microinjection is an enhancement of the air-cell injection method, ensuring definitive exposure on or in the vicinity of developing early embryo, which may achieve similar effects as in utero injections in rodent models[19,20]. Comparing to in utero injections, our method allows visual confirmation of the injection with relatively easy manipulation steps, and accurate injection is easily achieved by controlling for the egg weight, which is not possible in the in utero injection, where the actual quantity and weight of embryos are not easily acquired. The infusion method is mainly for assessment of inhaled agents on the pulmonary system, but cardiotoxicity and pulmonary toxicity often co-occur. This method takes advantage of the air cell, into which a small amount of gas or aerosol are infused, allowing continuous inhalation of gas/aerosol without the need of specific inhalation chambers. Counterpart rodent models need to use relatively large amounts of gas/aerosol and large,

expensive inhalation instruments[21,22].

The two routinely tested endpoints in our lab, electrocardiography and histomorphometrical assessment of right ventricular wall thickness, represent functional and morphological changes following toxicant exposure, respectively. The assessment of right ventricular wall thickness has specific advantages in getting a comprehensive understanding of the right ventricular wall, as the traditional echocardiography-based assessment on right ventricle is usually challenging and not very accurate, due to the asymmetrical and complex crescent shape of right ventricle[23]. Our method may help to overcome this inaccuracy by supplementing with additional information about the right ventricular wall thickness at a representative position. Currently it is all manual, in the future, the measurements may be made automatically and the number of measurement points may be increased considerably, further improving the accuracy of this method.

Chicken embryo-based developmental models have several advantages in toxicological studies, such as the ability to deliver a relatively accurate exposure dose, an independent exposure system within the shell, and easy manipulation of the developing embryo. With respect to cardiotoxicity, chickens have relatively large hearts and thick ventricular walls, allowing easy histomorphometrical assessments. There are some shortcomings, such as availability of antibodies/primers and extra cage space requirements comparing to rodents if rearing chickens after hatch. Nevertheless, the chicken embryo is still a good alternative toxicological model to be used for potential developmental cardiotoxicity assessments.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

Click here to access/download;Figure;Figure 1.tif

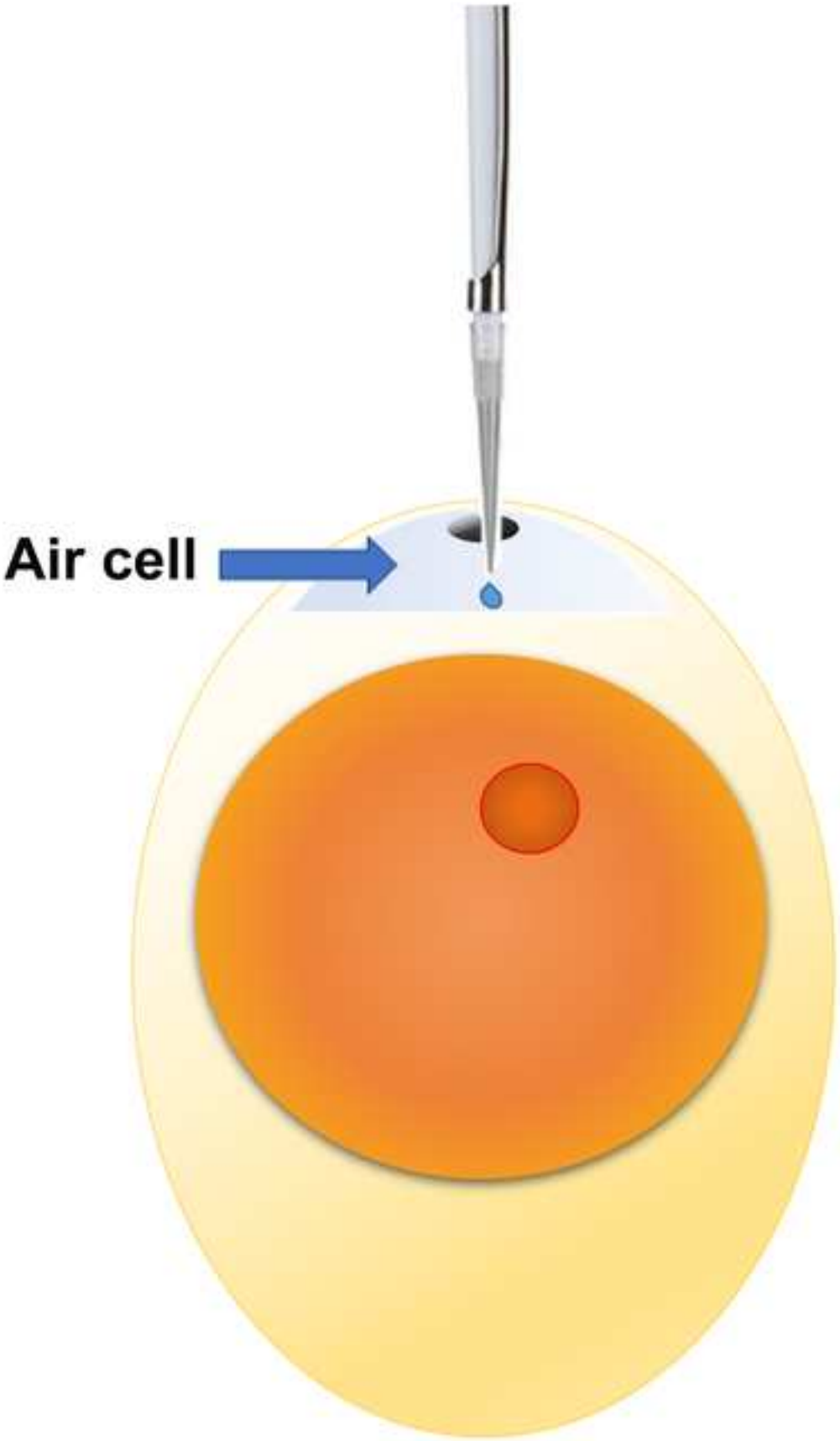


Figure 2

Figure 3

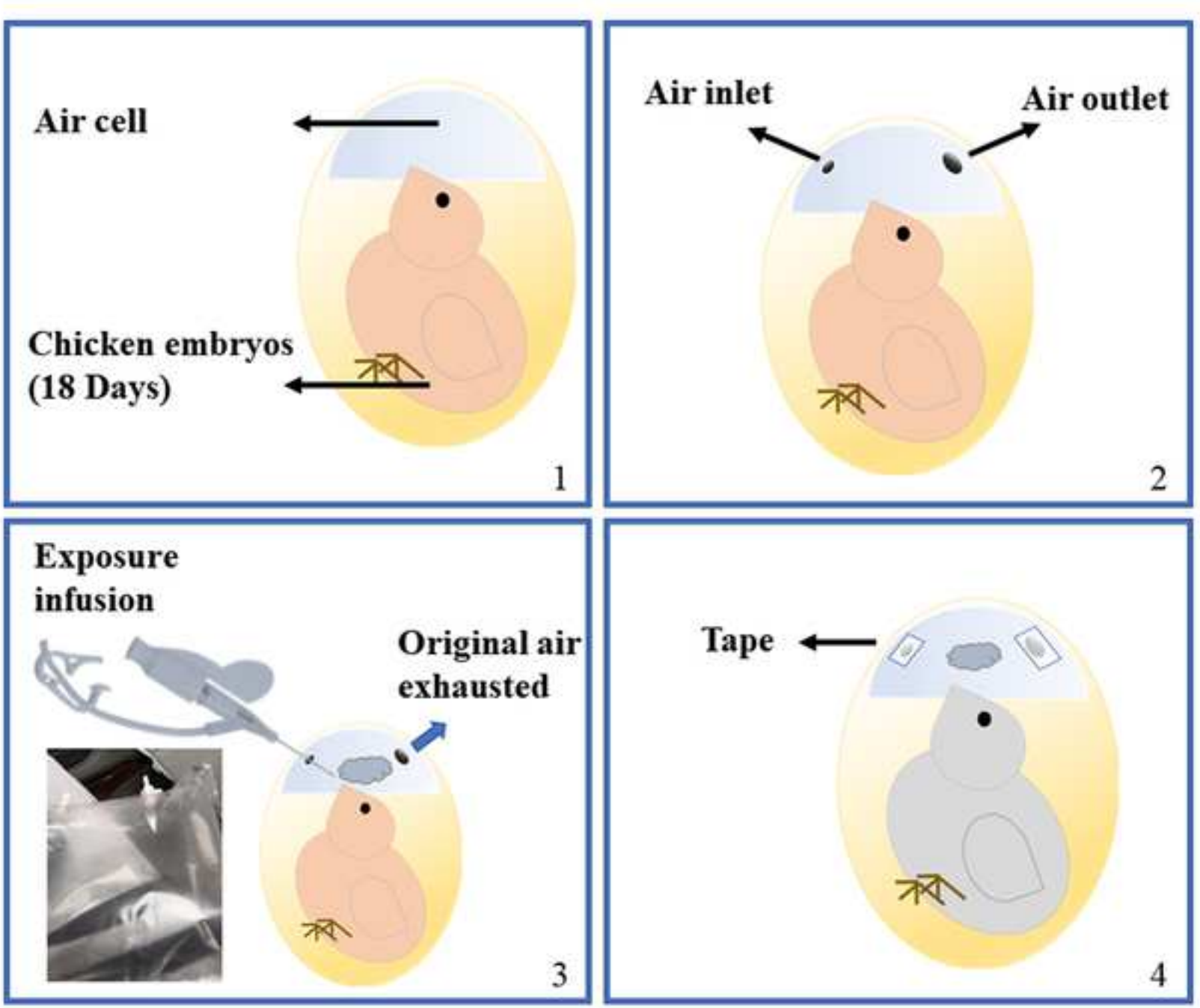


Figure 4

Click here to access/download;Figure;Figure 4.tif

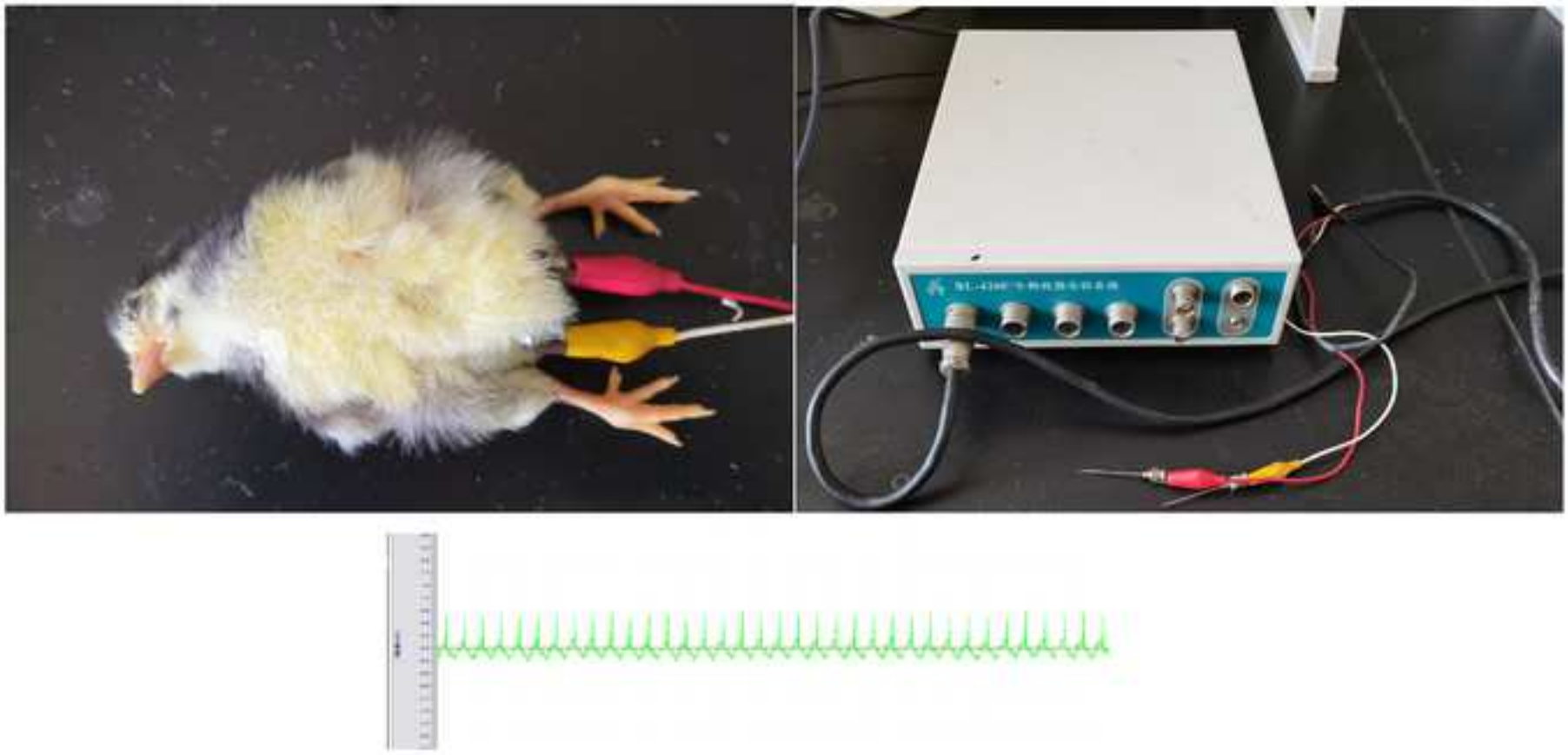


Figure 5

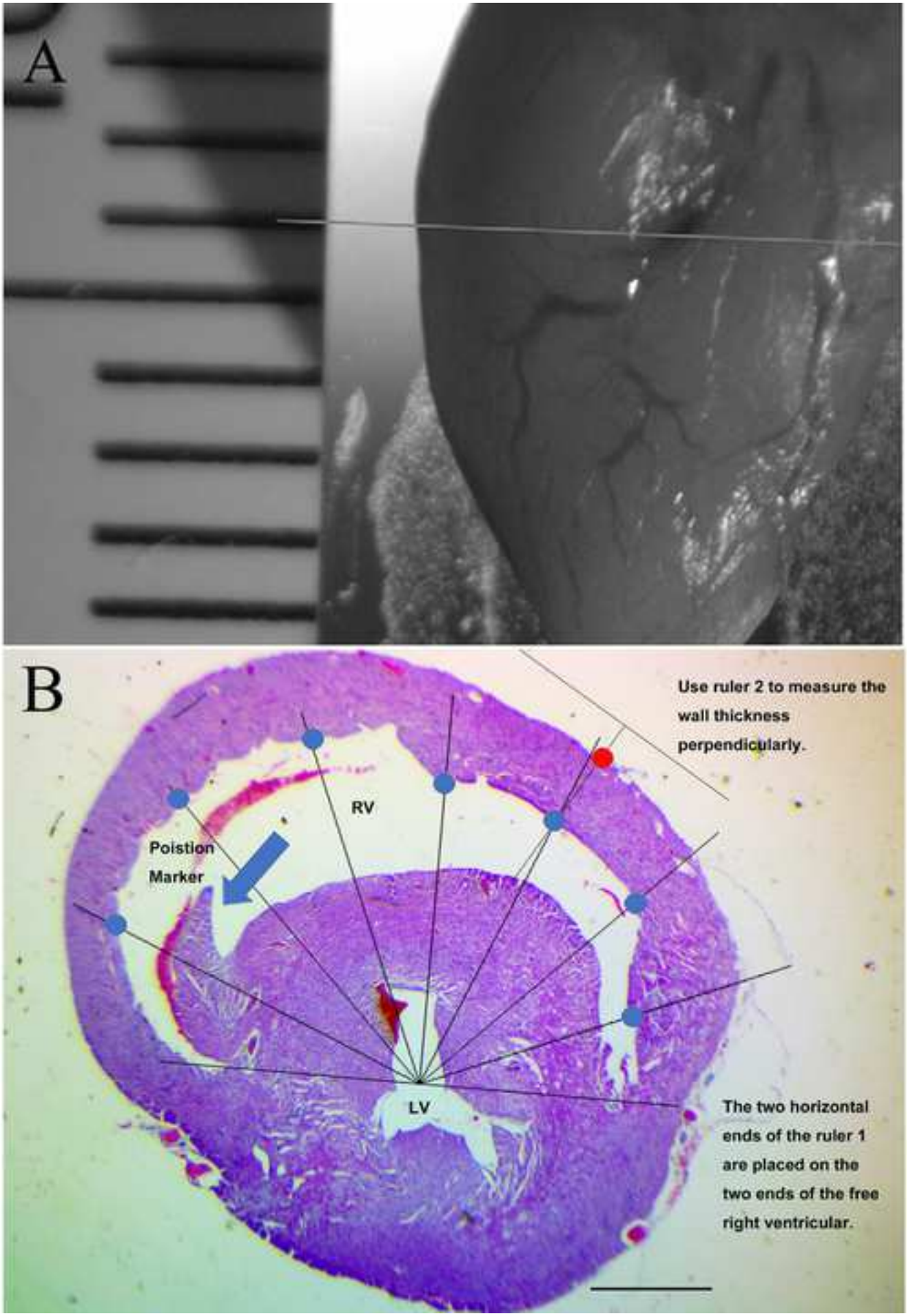


Figure 6

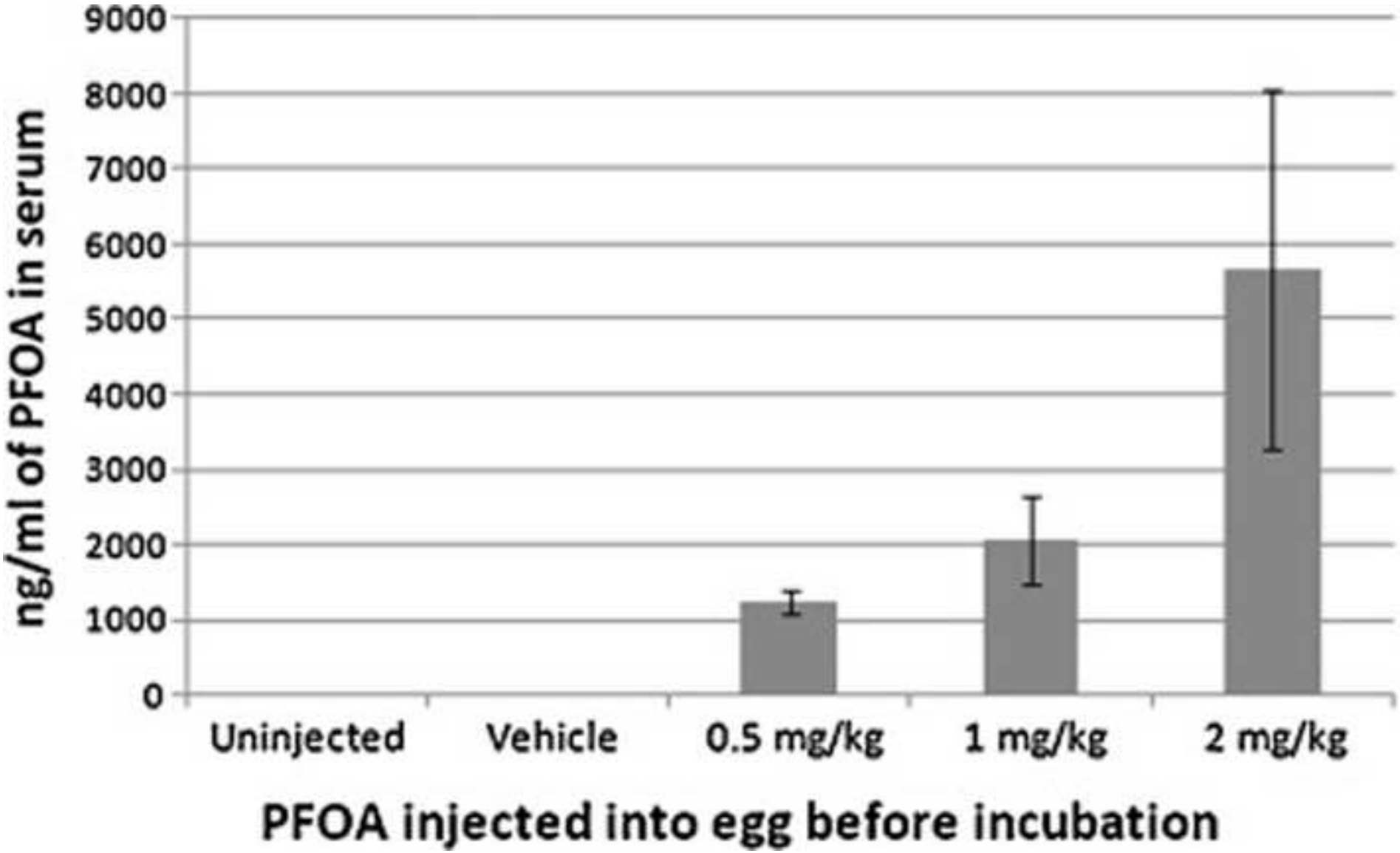


Figure 7

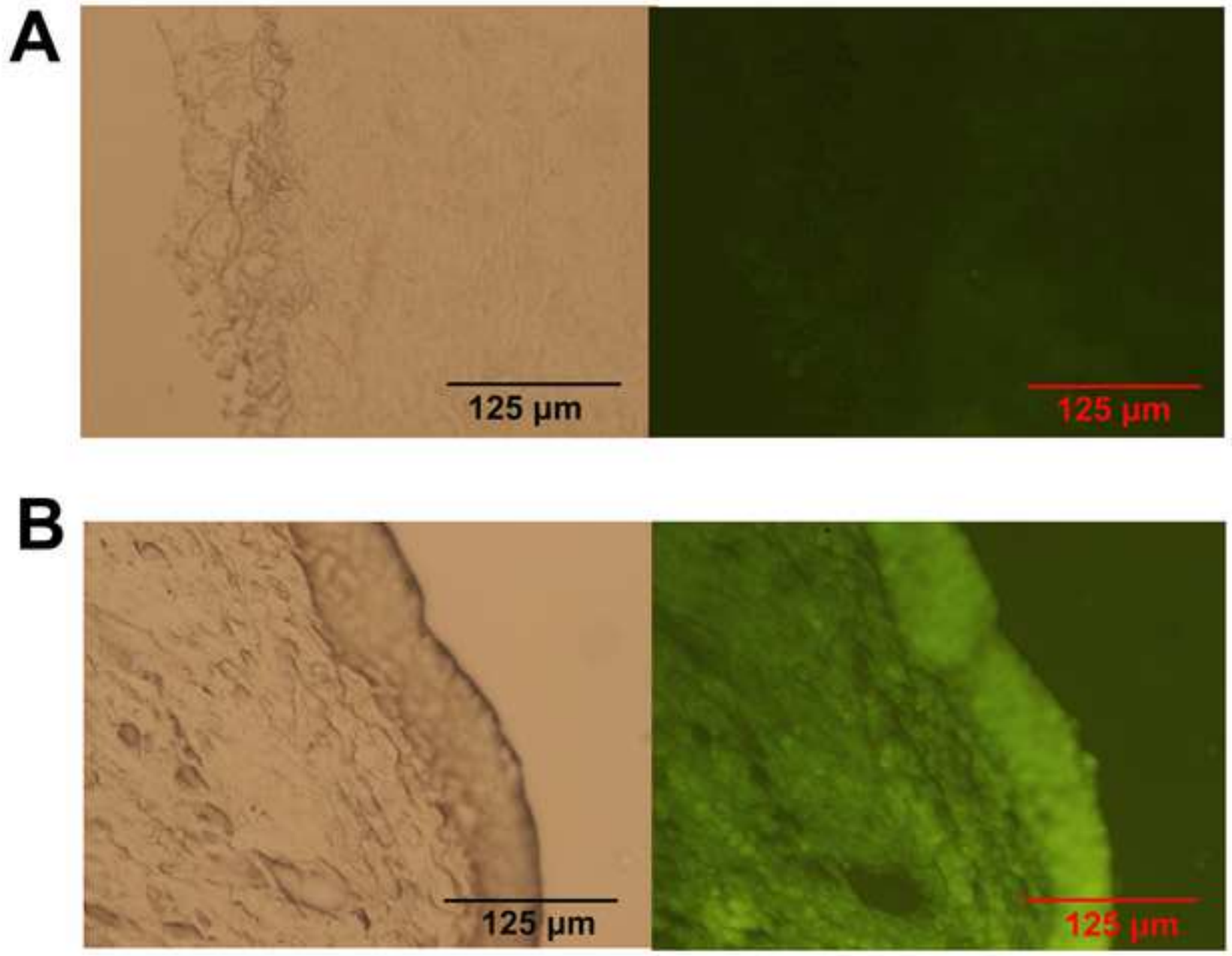


Figure 8

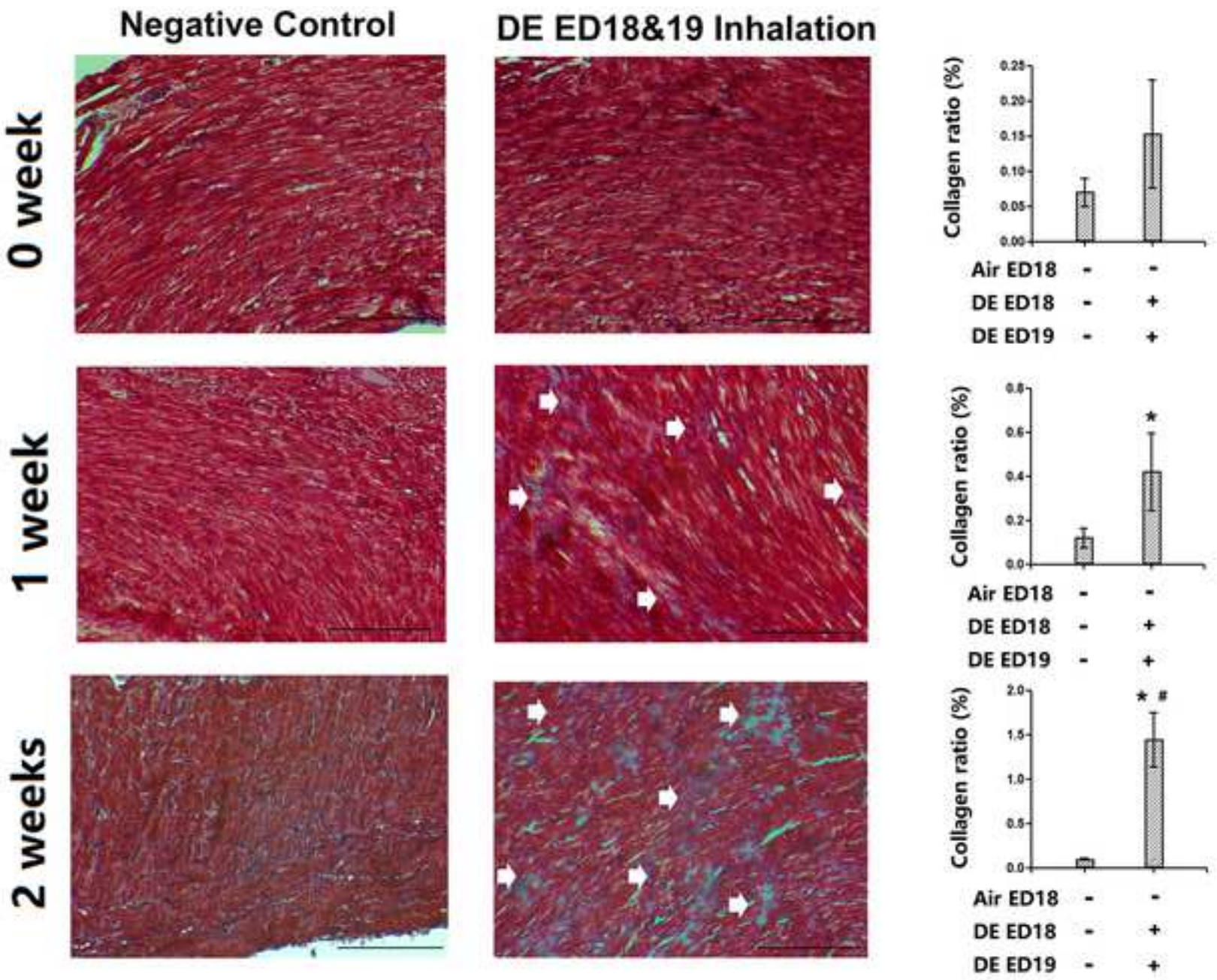


Figure 9

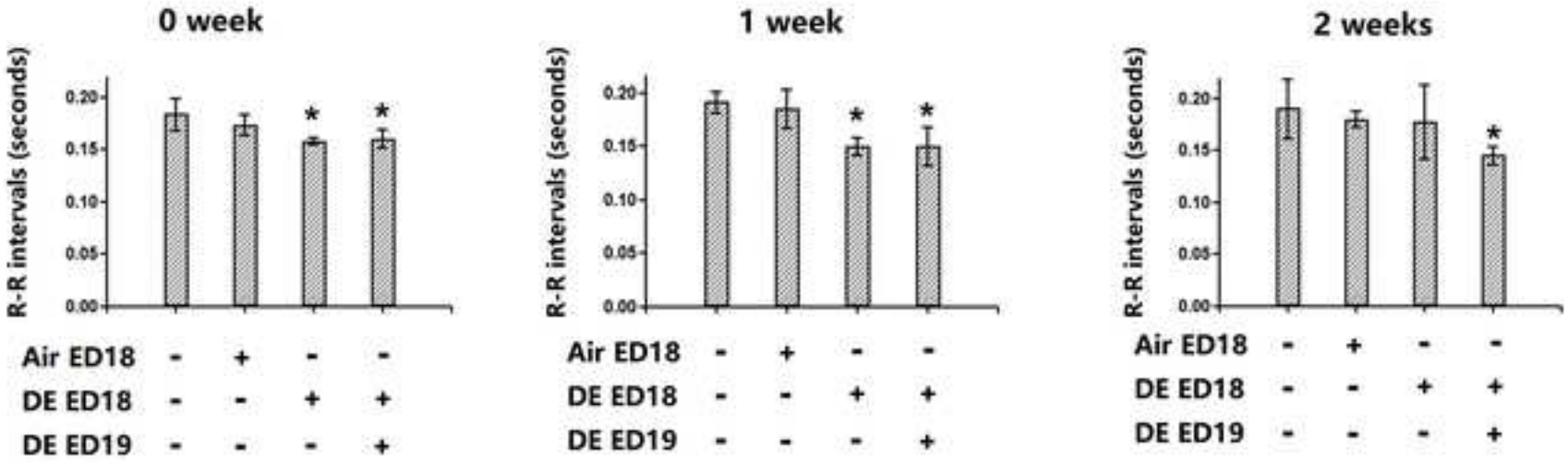
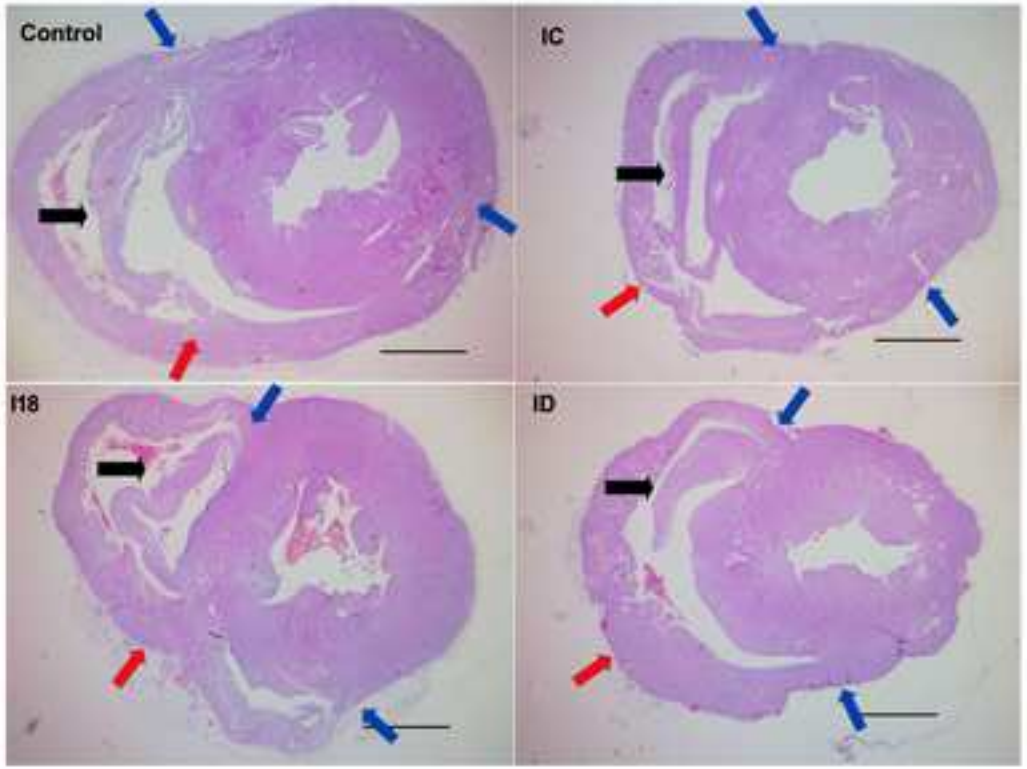
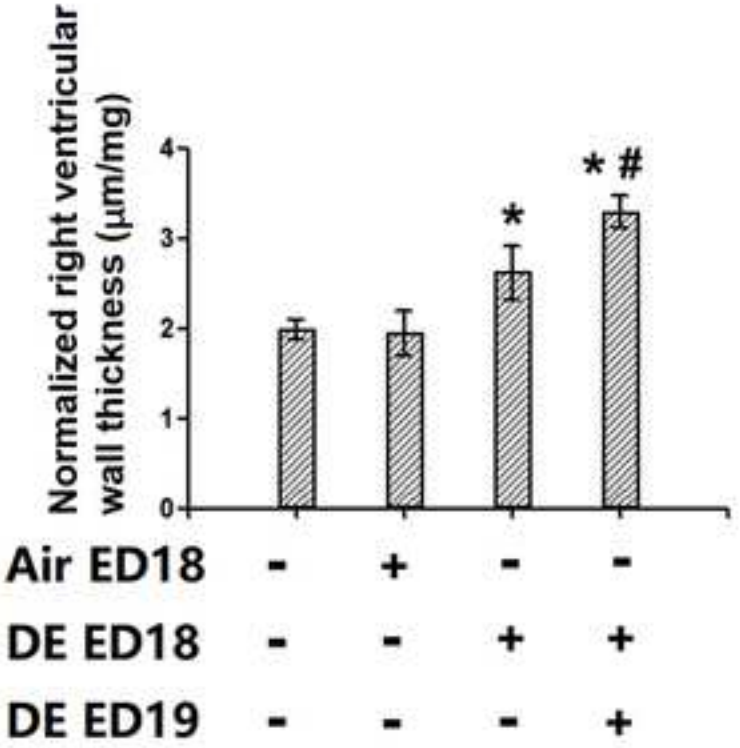


Figure 10





Name of Material/ Equipment	Company	Catalog Number	Comments/Description
4% phosphate buffered formaldehydefixative	Biosharp, Hefei, China	REF: BL539A	
75% ethanol	Guoyao,Shanghai,China	CAS:64-17-5	
Biosignaling monitor BL-420E+	Taimeng, Chengdu, China	BL-420E^{+}	
Candling lamp	Zhenwei, Dezhou, China	WZ-001	
Disposable syringe	Zhiyu, Jiangsu, China		
Egg incubator	Keyu,Dezhou, China	KFX	
Electrical balance	OHAUS, Shanghai, China	AR 224CN	
Electro-thermal incubator	Shenxian, Shanghai, China	DHP-9022	
Ethanol absolute	Guoyao,Shanghai,China	CAS:64-17-5	
Fertile chicken egg	Jianuo, Jining, China		
Hematoxylin and Eosin Staining Kit	Beyotime, Bejing, China	C0105	
Histology paraffin	Aladdin, Shanghai, China	P100928-500g	Melt point 52～54℃
Histology paraffin	Aladdin, Shanghai, China	P100936-500g	Melt point 62～64℃
IV catheter	KDL, Zhejiang, China		The catheters have to be soft, plastic
Lentivirus	Genechem, Shanghai, China		The lentivirus were individually desig
Masson's trichrome staining kit	Solarbio, Beijing, China	G1340	
Metal probe	Jinuotai, Beijing, China		
Microinjector (5 uL)	Anting,Shanghai, China		
Microscope	CAIKON, Shanghai, China	XSP-500	
Microtome	Leica, Germany	HistoCore BIOCUT	
Microtome blade	Leica,Germany	Leica 819	
Pentobarbitual sodium	Yitai Technology Co. Ltd., Wuhan, China	CAS: 57-33-0	
Pipetter(10ul)	Sartorius, Germany		
Povidone iodide	Longyuquan, Taian, China		
Scissor	Anqisheng,Suzhou, China		
Sterile saline	Kelun,Chengdu, China		
Sunflower oil	Mighty Jiage, Jiangsu, China		Any commerical sunflower oil for hun
Tape	M&G, Shanghai, China		
Tedlar PVF Bag (5L)	Delin, Dalian, China		

Vortex mixer	SCILOGEX, Rocky Hill, CT, US	MX-F
Xylene	Guoyao,Shanghai,China	CAS:1330-20-7

ones.
ned/synthesized by Genechem.

nan consumption should work

Dear Dr. Nguyen,

Thanks for the excellent review work from you and the reviewers. Our revised manuscript entitled “Using chicken embryo as a powerful tool in assessment of developmental cardiotoxicities” has been submitted. The editorial and each of the reviewers’ concerns are addressed in the submitted responses. Please find point-to-point responses to the comments below. The changes made in the manuscript were highlighted with tracked changes. We appreciate the opportunity to revise our manuscript and have it considered for publication in the Journal of Visualized Experiments.

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Detailed responses to editor and reviewer comments

Editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

A native speaker has proofread the manuscript to fix any spelling or grammar issues.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the

Protocol.

The protocols have been revised following your instructions.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Ethics statement has been added to the manuscript.

4. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

More details have been added to the manuscript and the figures (Figure 3), but some of them (e.g., the infusion process in the air cell inhalation method) are difficult to be described just in text. The video to be filmed will demonstrate the steps much better.

5. What are the egg incubation conditions?

Incubator model and incubation conditions have been added.

6. Please mention how proper anesthetization is confirmed.

Anesthetization is confirmed by holding the chickens and check their neck tensions. The detail has been added.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

The protocols have been revised to decrease the long sentences and now each step does not have too much information.

8. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or

a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Copyright permission have been obtained through the Rightslink service. Both PDF version and docx version of licenses (link is attached at the beginning of each license) are now included in the submission. Permission from Toxicology is for Figure 5 and 6. Permission from Environmental Toxicology and Applied Pharmacology is for Figure 7. Permission from Environmental Pollution is for Figure 8, 9 and 10.

9. Figure 5/7: Please include scale bars.

Scale bars have been added to Figure 7, but the original Figure 5 was prepared over 10 years ago and it is not possible to add scale bars now. To fix this, those pictures had been substituted with a new picture (with scale bar). This substitution is also recommended by reviewer 1.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript titled as "Using chicken embryo as a powerful tool in assessment of developmental cardiotoxicities" was submitted to JoVE. In the present manuscript, they evaluated the application of different exposure methods and morphological / functional evaluation methods for the study of cardiotoxicity in chicken embryos.

Major Concerns:

Throughout the manuscript, the wording used makes it difficult to understand the points being made by the authors. This manuscript would be greatly improved by having a native English speaking person edit the document. I noticed some typographical and grammatical errors but I will leave the correction to be made by the journal's internal team.

Overall, this study was well planned and carried out because the data have already been published but the way they are presented here is not clear. I advise the authors to make the text more didactic, for an easier understanding and reproduction of the data presented in the manuscript. My comments are as follow:

Thanks for the kind comments and very through review work. We have revised the manuscript following your instructions.

Specific comments:

KEYWORDS:

line 13: I suggest deleting the word "chicken embryo" from the keywords because it already appears in the title.

“Chicken embryo” has been removed from the keywords.

ABSTRACT:

line 23: "Chicken embryo is a classical model in developmental toxicological studies." The chicken embryo is a classic model for developmental biology and embryology, but not for toxicology.

“toxicological” has been removed from the sentence.

lines 25-26: "…… such as air-cell injection, direct microinjection, etc, without maternal effects." I suggest removing this part of the sentence because it is written again later.

This part has been removed.

INTRODUCTION:

The introduction is weak and disconnected. The authors should make a concise but complete introduction that makes sense to understand the importance of publishing this manuscript. 157 words were used and, according to the journal's rules, the introduction must have at least 2 paragraphs and between 150 and 1500 words.

Introduction has been mostly rewritten to strengthen it.

lines 43-44: "Chicken embryo is a classical developmental toxicity model, which had been put to use for over two hundred years1." As written above, the bird embryo is not a classic model for toxicity. In addition, the article cited (Kain, K. H. et al.) discusses the chick embryo for the investigation of cardiac and cancer biology, and about the utility of the chick as an experimental model system, but does not comment on toxicology.

“toxicity” had been removed from the sentence.

lines 45-46: Only rodent models?

“rodent” had been removed from the sentence.

lines 47-49: "During the development, chicken embryo needs to undergo a transition from internal respiration to external respiration, in which the beak of the embryo will enter the air cell and started to breath air from there2." This sentence was lost here, I suggest removing or reformulating it, calling it later, and providing more information and references. This part could be better explained, perhaps talking about internal pipping, which can also

be used as a parameter to assess the toxicity of compounds.

The sentence has been rewritten and explained with more details.

lines 50-51: "the normal development of chicken embryo had been demonstrated very clearly in the Hamburg-Hamilton staging3 …." Comment here about the stages of development because they will be shown in the procedures below. Some of the readers will probably not be familiar with embryonic development.

More description about Hamburg-Hamilton staging has been added.

line 51: Please change "Hamburg-Hamilton" to "Hamburger-Hamilton".

The typo has been corrected.

PROTOCOL:

In general, this part should be more detailed because this way it is not reproducible.

In the exposure methods, the controls were never mentioned.

It would be interesting to comment if there is no reduction in embryonic viability with the different forms of exposure. If this test has not been carried out, articles that demonstrate whether or not there are changes due to the type of processing could be mentioned.

More details had been added following your comments. Information about the controls (sunflower oil for air cell injection, sterile saline for microinjection and air for air cell inhalation) had been added to each exposure method. Viability (mortality, hatchability, body weight, organ index, etc.) had been demonstrated that no statistical significant changes were observed following these methods, if only vehicle controls were used. References had been added.

line 60: "This is the classical exposure method to chicken embryo…". It is a classic method of exposure, so at least one reference must be added. Suggestions:

1. https:// doi.org/10.1016/j.etap.2012.06.010

2. https://doi.org/10.1016/j.tox.2019.152286

3. https://doi.org/10.1016/j.taap.2018.05.028

References added. Thanks for the suggestion.

line 65: Add "povidone iodide solution", and "pipette tip". It is necessary to make clear whether the authors want to put ALL the necessary reagents and tools or just the main ones. Standardize for all methods.

Missed reagents and tools have been added for all the methods.

line 66: Remove "brush" because it does not appear anywhere.

The brush is used to seal opened injection holes (with melted paraffin). It has been added to the protocol.

line 67: "The dosing mixture is usually prepared with sunflower oil,…". It is better not to write "usually" because it will depend on the type of contaminant. As it is an article of methods I think it is important to say that! Or simply say that it is diluted in an appropriate vehicle and controls must be carried out.

This part has been modified to be more precise.

line 70: "(1:5 of commercially available povidone iodide solution)". What is this diluted on?

Deionized water. This information has been added.

line 78: "Sanitize the air cell area with 75% ethanol,… " . This area is usually cleaned with 70% ethanol. Why was 75% ethanol used?

It is a commonly used, commercially available sanitizer in the lab.

lines 83-84: "The dose is 1 ul per 10 gram of egg…" Dose of what? Won't the injected dose depend on the contaminant used? Make this clear to the reader.

Sorry for the confusion. This sentence has been rewritten. The injection volume is 1 ul per 10 grams of egg, the dose depends on the contaminant concentration used in the dosing solution.

line 86: "…, hold for at least ten seconds, …." Why? Some of the readers will probably not be familiar with the technique and so you should complement the sentence explaining why you should hold it.

Thanks for the suggestion. It is because of the viscosity of the sunflower oil. It takes a little while for the oil (and the contaminant in it) to fully be dispensed. This information had been added.

line 89: "Return the eggs to incubator." Return? But nowhere during the text said that the eggs were ever in the incubator. How long do they remain in there? Describe a little about the incubation (egg position, temperature, and humidity of the incubator, how long the eggs remained incubated, ...). Were the eggs randomly placed on the incubator shelves to avoid the influence of the location of the egg?

Please, change the sentence to: Once sealed, the eggs are placed in the incubator until they reach the desired embryonic stage.

Thanks for the suggestion. One additional paragraph was added to describe the egg

incubation. And the sentence had been revised as you instructed.

line 100: Here the same comment made on line 65.

Povidone iodide solution has been added, but no pipette or pipette tips were used in this procedure.

lines 100-101: Please change "micro-injector" to micro injector and "find forceps" to fine forceps.

Sorry for the typos. They have been fixed.

lines 101-102: "The dosing mixture is usually prepared with sterile saline." Here the same comment made on line 67.

It has been modified to exclude “usually”. Sterile saline is the best-known option here.

lines 111-115: The microinjection should be better described, with more details. It is difficult to reproduce this technique as described.

I agree that this technique is hard to reproduce as-is. It probably is best demonstrated with the video (to be filmed soon). Details are added as much as possible for now.

line 111: What kind of micro injector it was used? a manual or automatic one?

It is a manual 5 ul microinjector from Anting, Shanghai. Please refer to the attached picture.

line 112: What kind of needle? Remember that in a technical paper every detail is necessary!!!

Needle is part of the microinjector we used (permanently attached), that is why it was not mentioned separately.

lines 112-113: With an insertion of 2-3 mm in the inner membrane, could it not cross the thin body of the embryo and reach the yolk? Where will the needle be inserted?

This is a very good question. To develop this method, many different methods were tried (and failed). Eventually, the currently presented injection method has the best survivability (no statistical difference to control) and effective enough when injecting lentivirus for *in ovo* gene silencing. This depth is approximately where the embryos are located and yes, sometimes it may reach the yolk. With practice, most will not, and those do can be easily identified and excluded from the study, as a yellow trail can be seen on the tip of the needle. The insert location is the center of the air cell membrane, since most embryo will be located there (or slightly off position but not far away). Nevertheless, by ED2, the blood ring is large enough so that the injected solution will effectively get in touch with the blood circulation

and be transported to embryo even if not injected directly on the embryo body.

line 115: "Seal the hole with a small piece of tape…." Why isn't paraffin used now?

Because the holes are larger, approximately 2 mm in diameter. Melted paraffin would easily drip in and kill the embryo.

line 118: Here the same comment made on line 89.

The statement about putting eggs back to incubators has been revised.

lines 118-120: How long can the embryo stay out of the incubator without impairing development? And without significant reduction in viability? It is important to be more specific sometimes.

I agree this topic is very interesting and we may perform a study on this topic in future. It depends on many factors. Egg size, embryo staging, environmental temperature, etc. Generally, early-stage embryos (ED2 etc.) have a low tolerability to low temperature, while later stage embryos (ED18 etc.) can hold for over 10 minutes without problems.

line 122: "Air cell inhalation". This topic is very confusing, difficult to replicate. It is necessary to describe the steps better. I suggest making some schemes to facilitate the understanding of the method, even more, because it is a novel method.

Thanks for the suggestion. Yes, this is a novel method and is the one we would like to describe in the most details. We have revised the corresponding figure (Figure 3) to demonstrate it clearer.

line 127: Add "fume hood".

Fume hood has been added to the tools list.

line 127: "Sampling bag (PVF bag,…)" To facilitate understanding, it would be interesting to have a figure or scheme of PVF bag because some of the readers will probably be like me and not be familiar with this specific methodology.

Thanks for the suggestion, a PVF sampling bag sample picture is included in Figure 3.

lines 130-133: "Eggs are cleaned as described in 1.1.2, candled as described in 1.1.3 (it is not necessary to mark air cell prior to incubation) and incubated without treatment until ED17. 1.3.3 Eggs are candled at ED17 to mark the air cell area. At this stage, the air cell area should be quite large, ready for the internal pipping." This part should be better described as it is confusing. It is necessary to describe how the incubation is carried out - temperature and humidity of the incubator, turning of the eggs are important factors for the correct development of the embryo, especially in the later stages (ED17-ED18). How are eggs incubated? vertically? horizontally?

We apologize for the confusion. The incubation conditions for all the methods described are identical and they are now described in a dedicated paragraph.

line 131: Why was ED17 chosen? Couldn't it have been done in earlier stages? For this methodology, is this the best development window?

During incubation, the eggs slows dehydrate and the air cells get larger over time. By the time around internal pipping (the exposure time window of this method), the air cell area is

significantly larger comparing to those in early incubation stages. The two holes are to be made at two sides of the air cell area (to be as far away as possible from each other), so it is necessary to mark the air cell area close to the exposure time point (ED18), thus ED17 is chosen.

lines 139-141: Is this process done manually? A photo or scheme would be interesting to elucidate the method.

Yes, it is done manually. We are trying to develop some automatic instruments in the lab as well, but for now it is all manual. We have expanded Figure 3 to elucidate the method better. And when the actual video is filmed, it should be very clear (and it is quite easy to perform).

line 141: "Seal both holes…." Seal with what? adhesive tape, paraffin?

We used adhesive tape here, since the holes needs to be re-opened for a few times, tape can be easily removed so we chose it.

line 142: "The volume of air cell is no more than 10 ml at this stage…" How to ensure the volume / dose injected into each egg?

This is a disadvantage of this method, since egg sizes vary. By repeating the infusion twice in one hour, we just filled the whole air cell up with whatever gas we chose, but it is not possible to obtain accurate volume. It is possible, though, to get the gas concentration before hand and quantify the dose in concentration rather than mass weight, just like any inhalation chamber exposure method.

lines 146-147: How is the gas concentration calculated? Divided into different exposure days? Does the inserted gas not escape from the pores of the shell? Does this total filling of the air chamber not affect the chick's breathing after internal pipping?

Answer to this question is like the previous one. For now, we can only fill up the air cell with the gas-of-interest. By infusing on different exposure days, we did induce more prominent effects in the published results (Jiang et al., Environ Pollut. 264 114718, (2020)., which indicated that the gas (diesel exhaust in that study) will be depleted over time due to inhalation and/or escaping from the shell pores. Luckily, egg incubators will ventilate frequently so the escaped gas should have minimal effects. Nevertheless, we still recommend to use separated identical incubator for control animals. Regarding to the affection on breathing, from the hatchability data, the diesel exhaust we collected did not seem to have remarkable effects on the viability of the animals.

Endpoint assessment methods

lines 156-157: Other analyzes can be done in addition to cardiotoxicity. It may be better to write that several toxicity parameters can be taken, including cardiotoxicity.

Thanks for the suggestion. It has been revised following your instruction.

lines 158-159: "Common methods such as western blotting and immunohistochemistry are also used in the toxicity assessments, but will not be described here." Several other methodologies can be applied in the toxicity assessments, in addition to those mentioned (western blotting and immunohistochemistry). As they will not be used, it may not be

necessary to specify them. The text is confusing because sometimes it talks about cardiotoxicity and sometimes about toxicity in general. Be careful.

This sentence has been removed. Thanks.

lines 161-162: In rodents, trichotomy is performed. For chicken is it not possible to remove the feathers and then use the electrodes?

In the phase of method development, we tried to use ultrasound gel or saline for better transduction. Removal of feathers had also been tried. But the only stable method to acquire ECG, in our case, is to use invasive electrodes. We are fully aware that this requires anesthesia and is potentially introducing extra variables to the experiments, but all other methods will not work with our instrument (BL-420E+ is not a very sensitive instrument). In future, should we have access to more advanced instruments, non-invasive methods will be pursued again.

lines 163-165: Phenobarbital can cause irregular heartbeat, was that considered? In addition, it is reported that the dosage was 33mg/kg. In adults, the reference dosage is 4-5mg/kg. Has an allometric extrapolation calculation been performed to calculate this dosage?

It is pentobarbital 33 mg/kg, via i.p. injection. This dose was tested out in our lab with hatchling chickens previously, which can typically induce anesthesia in a few minutes, and the chickens can wake up after a few hours safely. In some sensitive individuals, we indeed

observed av block, premature beats or even ventricular tachycardia following anesthesia. But these can be easily identified and excluded from analysis.

lines 166-168: Add "electrical balance", and "heater, if necessary".

Manuscript has been revised following your instruction.

line 182: "If the chickens are to be sacrificed.." What is the euthanasia method used?

The chickens are already anesthetized, so a quick decapitation was performed. This is probably the one with least pain, and facilitates the collection of blood samples.

line 187: Can it be said that histopathology was performed? It seems to be more of a histomorphometry than histopathology.

We agree. The term has been changed to histomorphometry.

line 192-194: Add "phosphate buffered saline", "paper towel", "weight balance", "small scissor"… Review the materials used in this item (2.2).

Materials have been revised to include full lists of supplies.

line 202: Put which was the fixative used.

It is 4% phosphate buffered formaldehyde, and has been added.

line 213: "Process the tissue to paraffin blocks with standard procedures,..". Standard procedures? What's the pattern? Can any organ/tissue be processed in the same way? Of course not. It is necessary to describe the detailed histological procedure for the specific embedding of the heart. Or at least a reference article must be inserted.

OK. These procedures vary from lab to lab, that was why we did not include the specified procedure here. Now we include the detailed procedures. This method (with a little adjustment on 95% and 100% ethanol dehydration duration) works very well for us on most of the small chicken/mouse tissues (hearts, livers, spleens, pancreas, muscles, intestines, ears, testis, lungs, etc.).

lines 219-233: "Right ventricular wall thickness measurement." The description of the measurement should be better described. Although the photos have already been published previously (then they have already been evaluated by the reviewers and editor of the journal) I suggest making some schemes to facilitate the understanding of the technique and if possible that other photos replace the current ones.

We agree that this figure is not sufficient by itself. Hereby we substituted most of it with a new figure (this picture has not been published before). In this new figure, more annotations have been made for better understanding of the measurements. In the planned video, we will use screen-recording to show how it is done.

line 232:"(Figure 5B, C)." Standardize the figures citation. On line 218 is (Figure 5D-F).

Sorry for the mistake. The whole figure has been remade so it is no longer applicable.

REPRESENTATIVE RESULTS:

line 241: This is the first time that the term LC/MS-MS appears. LC/MS-MS is Liquid chromatography-mass spectrometry? Abbreviations need to be written in full when they

are first used.

Yes, it has been spelled out now.

line 245: Are there papers with microinjection of contaminant instead of lentivirus? It would be interesting to mention at least one.

Not yet, but two of them are in preparation. We will work on them as soon as possible.

lines 248-250: "RT-PCR, western blotting as well as immunohistochemistry …" These techniques have not been described, I see no sense in putting them in the results. In addition, in my view, they are not adding to the objective of the work. I suggest its deletion.

This sentence has been deleted.

line 255: (Figure 9). It is not necessary to place all images. It will be better to put a few, with better quality and size.

line 255: (Figure 9). It is not necessary to place all the graphics and any information about the lung.

OK. Now only six pictures of myocardium and corresponding quantifications are kept.

line 265: (Figure 11). It is not necessary to place all images.

OK. Now only one time point (1 week) is kept.

FIGURE AND TABLE LEGENDS: add the staining used in the histological images. Only in photo 9 is cited.

Staining methods have been added to the legends.

line 268: Figure 1. Make an indication of the air chamber.

Indication has been added.

line 274: Figure 3. It would be more didactic if 2 openings of different sizes were made, as described in the methodology.

Size difference is made more apparent now, and the figure has been expanded for better demonstration of the process.

line 277: Figure 4. Could show in the image the instrument used and reduce the electrocardiography a little.

The instrument is shown now with an improved ecg picture.

line 281: Figure 5. You might tell readers what are the arrows, LV, RV, and thick arrow. It took me a while to figure that out.

Figure 5 has been revised, and now these annotations are all explained in the legend.

line 283: C= With the employed staining it is not possible to observe the myosin layer. Improve the sentence.

This part has been removed (mostly irrelevant anyway).

line 284: D-F= Add the name of the anatomical marker.

It has been added to the new figure.

lines 304-312: Figure 8 is not necessary as the methodology was not described in the work.

Figure 8 has been removed.

line 314: In figure 9 remove images and information about the lung because it is not the focus of the paper. Enlarge photos for better viewing. It's hard to see the letters.

Lung pictures has been removed. Photos have been enlarged.

line 336: Figure 11= A: indicate the anatomical marker in the image.

The markers have been indicated.

line 336: "(In older chickens, the marker tends to be a bit longer at desired position, which does not affect the accuracy of measurements)." Is it possible to observe these markers in the images? if yes, indicate.

Same as previous question. Markers have been indicated.

line 340: Arrows: differentiate the colors of the arrows as it is in the original work (two ends and middle points).

Now the middle point arrow is in red.

DISCUSSION:

line 345: The chicken embryo is a classic model for developmental biology and embryology, but not for toxicology as commented on line 23.

The word “toxicological” has been changed to “developmental”.

line 348-350: As the paper is with cardiotoxicity, I suggest the lung part deletion. Perhaps it could be complemented with a separate sentence that the methods presented could also be observed for pulmonary toxicity.

Pulmonary toxicity has been deleted.

line 357: In the vicinity of developing early embryo?? In the methodology and scheme shown in figure 2, this is not clear because it appears that the microinjection is performed directly on the embryo. Furthermore, with the egg in an upright position, due to the chalaza the embryo is positioned in contact with the inner membrane, so how is it possible to guarantee that it is not reaching the embryo? And if that was the goal, how to ensure the correct injection site?

We apologize for the inaccurate description. This method is meant to reach the embryo as close as possible, however, during the actual operations, we observed that some of the ED2 embryos are not located perfectly at the center of the air cell membrane, they can be slightly “off-center”, but, for most if not all the cases, the blood ring covers the center of the air cell membrane, thus the injection will either be on the embryo, or at least fall within the blood ring. From our previous work, it seems like this method works well for both lentivirus and nanomaterials (data not published yet). The description has been revised to “on or in the vicinity”.

line 368: "..right ventricular wall…" Is this an endpoint? Wouldn't it be histopathology? or better histomorphometry, according to the images?

“right ventricular wall” has been revised to “histomorphometrical assessment of right ventricular wall”.

line 370-375: This part is confusing. Please make it clearer.

We apologize for the confusion. It has been rewritten.

lines 381-391: When reading this last paragraph, the reader will give up using the bird as a model. It must be rewritten, valuing the model in relation to others, considering cardiotoxicity. Despite presenting some negative points, I believe that the bird embryo is a great model to be used in toxicology and cardiotoxicity.

Thanks for the suggestion. We were meant to mention the shortcomings of the model so that people have a more comprehensive idea of this model. It probably contained too much

negative idea in the current form. This paragraph has been rewritten.

Name of Material/ Equipment:

Arrange in alphabetical order. Makes viewing easier.

Add "BL-420E+", "Masson's trichrome", "metal probe", "tape", "4%phosphate buffered saline"

The material/equipment lists have been arranged in alphabetical order, and the requested information had been added.

Reviewer #2:

Manuscript Summary:

In this manuscript the Jiang et al has explained a detailed protocol to utilized chick embryo in ovo to study developmental cardiotoxicity. The Zheng group and previous studies from other labs has shown chick embryo an excellent to model to study development and developmental toxicities, however being a non-mammalian system, it has some limitation. As chick heart development is well defined and resembles mammalian heart development that make it a good candidate to investigate cardiac developmental toxicities. The author has described precise and timely exposure of chemicals using air-cell injection, direct microinjection, and air-cell inhalation methods. The methods are explained in detail with echocardiography and histology end points. The other end points like western blot and PCR has also been used previously with these exposure methods.

Thanks for your through review work. We have revised the manuscript following your instructions. We sincerely hope it now meets your standards now.

Minor Concerns:

The corrections mainly involve figures and graph quality.

Figure 4C: It's difficult to see echocardiograph waves.

We apologize for the waves being a bit too narrow. We have substituted the waves with a different one, which should be much clearer.

Figure 8B and C: It's difficult to read text.

Figure 8D: Scale bar is difficult to read

Figure 8 has been removed as recommended by another reviewer.

Figure 9: Picture not very clear and text is difficult to read

We apologize for the inconvenience. This is a large figure, maybe it got compressed when you download it. On our end, this figure is 30+ mb in size, not easy to deal with, but should not be blurry. We rearranged the design of the figure, hopefully it can look better on your screen. Texts are also enlarged. Note that this is now Figure 8.

Figure 10 and 11: Text is difficult to read

Texts have been enlarged for easier reading. Note that these two figures are now Figure 9 and 10.

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