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A Pleural Effusion Model in Rats by Intratracheal Instillation of Polyacrylate/Nanosilica

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Dear editor- in-chief,

We would like to submit the enclosed manuscript entitled “Making a pleural effusion model in rats with polyacrylate/nanosilica and isolating nanoparticles in the pleural effusion ”, which we wish to be considered for publication in “JOVE”. No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication. We certify that the submission is original work and is not under review at any other publication. All the authors listed have seen and approved the manuscript that is enclosed.

In the study, we aim to make a model of pleural effusion in rats by intratracheal instillation of polyacrylate/nanosilica, and to introduce the method of nanoparticles isolation in pleural effusion. Ultrasound and CT examinations were performed to confirm the presence of pleural effusion. This model may be useful for the further study of nanotoxicology and pleural effusion diseases.

We deeply appreciate your consideration of our manuscript, and we look forward to receiving comments from the reviewers.

Sincerely yours,

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TITLE:

A Pleural Effusion Model in Rats by Intratracheal Instillation of Polyacrylate/Nanosilica

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KEYWORDS:

Model, Pleural effusion, Polyacrylate/nanosilica, Ultrasound examination, Nanoparticle, Detection, Isolation

SUMMARY

Here, we present a protocol to construct a pleural effusion model in rats by intratracheal instillation of polyacrylate/nanosilica.

ABSTRACT:

Pleural effusion is a prevalent clinical finding of many pulmonary diseases. Having a useful animal pleural effusion model is very important to study these pulmonary diseases. Previous pleural effusion models paid more attention to biological factors rather than nanoparticles in the environment. Here, we introduce a model to make pleural effusion in rats by intratracheal instillation of polyacrylate/nanosilica, and a method of nanoparticle isolation in the pleural effusion. By intratracheal instillation of polyacrylate/nanosilica with concentrations of 3.125, 6.25 and 12.5 mg/kg·mL, the pleural effusion in rats presented on day 3, peaked at days 7-10 in 6.25 and 12.5 mg/kg·mL groups, then slowly decreased and disappeared on day 14. When the concentration of polyacrylate/nanosilica increased, the pleural effusion is produced more and faster. This pleural fluid was detected by ultrasound examination or CT chest scanning and

confirmed by dissection of rats. Silica nanoparticles were observed in the rats' pleural effusion by transmission electron microscope. These results showed that the exposure to polyacrylate/nanosilica leads to the induction of pleural effusion, which was consistent with our previous report in humans. Additionally, this model is beneficial for the further study of nanotoxicology and the pleural effusion diseases.

INTRODUCTION:

Pleural effusion is a very common clinical manifestation of pulmonary diseases with a variety of causes. Having a useful animal pleural effusion model is very important to study these pulmonary diseases, the roles of the two pleural membrane layers, the mechanisms of pleural effusion, and its treatment. However, some reported pleural effusion models mainly focus on the malignant pleural effusion or biological factors rather than the nanoparticles in the environment[1,2]. Here, we introduce a new model of pleural effusion that is simple, safe and effective.

With the development of nanotechnology and the extensive use of nanoproducts, there is a concern about the potential hazards of nanomaterials to the environment and the human health[3,4]. Nanomaterials introduce risk factors and potentially lead to novel hazards within the workplace or through environmental contamination. In vitro and in vivo studies show that nanomaterials can result in multi-organ damage to the lungs, the heart, the liver, the kidney, and the nervous system, as well as the reproductive and immune systems[5,6]. Additionally, some studies reported that the specific toxicity of nanomaterials was due to their unique physicochemical properties[3,4,7].

We have reported that a group of workers with occupational exposure to nanomaterials clinically presented with pleural and pericardial effusion, pulmonary fibrosis and granuloma[8,9]. Silica nanoparticles were isolated in these patients' pleural effusion[9]. In order to reproduce and verify the pleural effusion induced by the inhaled nanoparticles in human, we conducted the experiment by instilling the polyacrylate/nanosilica (PA/NPSi) via the respiratory tract in rats, which mimicked human respiration in a real environment, and found that intratracheal instillation of PA/NPSi could result in pleural effusion in rats. Here, we introduce how to make pleural effusion in rats by intratracheal instillation of PA/NPSi, and how to isolate nanoparticles in the pleural effusion. This model may be useful for the further study of nanotoxicology and pleural effusion diseases.

PROTOCOL:

The study followed guidelines of Capital Medical University (Beijing, P.R China) for the care and use of experimental animals. All procedures were approved by the Animal Ethical Committee of Capital Medical University in China.

1. Experimental preparations

NOTE: Acclimate the male specific pathogen-free Wistar rats (weight: 200 ± 10 g) to the experimental environments for a week before administration (Environmental conditions: light/dark:12h/12h, temperature 22 ± 2 °C, humidity 50 ± 10 %).

1.1. Use a fresh 10 mL of PA/NPSi suspensions (nanosilica Ø:20 ± 5 nm by in situ emulsion polymerization) diluted in normal saline at concentrations of 3.125, 6.25, and 12.5 mg/mL, respectively[10]. Before administration, sonicate the suspensions for 20-30 min and vortex for 10 min in order to prevent nanoparticles aggregation.

1.2. Equally divide a total of 20 rats into four groups: one group for each concentration of PA/NPSi (0, 3.125, 6.25, and 12.5 mg/mL).

1.3. To anesthetize them, place the rats in a closed container with 1.5 mL of ether (99.5%) or any other IACUC approved protocols. After 60-90 s of anesthesia, check for the lack of response to pedal reflex. Ensure that the rats are breathing.

1.4. Put the anesthetized rat on the board and fix its front teeth with a line of nylon on the board too.

1.5. Open its mouth and expose its fissure of glottis with the help of a surgical forceps and frontal lens.

1.6. Instill the rats with 0.5 mL of PA/NPSi suspension to each rat's lung for a total of 1 mL using a using fine tube into the bilateral bronchus.

1.7. Place the rats on a plastic board in a supine position and let the rats recover slowly in 5-10 min.

2. Ultrasound examination for pleural effusion

2.1. Use an ultrasound system with a linear array transducer (frequency: 8 MHz) to examine rats on days 1, 3, 7 and 14[10].

2.2. Give anesthesia (10% chloral hydrate, 0.35 mL/100 g, i.p.) to the rats and check for the lack of pedal reflexes.

2.3. Remove the hair from rats' chest and upper abdomen using an electric shaver. Then place the rat on a mounting plate in a supine position.

2.4. Cover the skin with the coated gel, and then place the transducer on the intercostal space and subcostal area to detect the pleural fluid.

NOTE: In order to detect the effusion accurately, the left and the right lateral positions were selected to perform an ultrasound examination.

2.5. Put the rat on a plastic board in a supine position after ultrasound examination and let the rats recover slowly in 10 min.

3. Chest CT scanning for pleural effusion

3.1. On days 7 and 14 post-administration, anesthetize the rats with 10% chloral hydrate (i.p). Consider it sufficient depth of anesthesia when the rat does not react to pedal reflexes.

NOTE: Day 7 post-administration is the most appropriate time to observe pleural effusion by CT scanning.

3.2. Place the rat on a plastic sheet in a prone position and then scan its chest to investigate pleural effusion using a 64-channel CT. Use the following settings: 64 mm x 0.625 mm detector configuration, 120 kV (peak), and 350 mAs.

4. Collection of pleural effusion and isolation of nanoparticles in the pleural effusion

4.1. After chest CT scanning of rats and under anesthesia of chloral hydrate, check the pedal reflex of the rats, shave the hair from abdomen to chest, and then disinfect the skin by iodine.

4.2. Bring the rats to the surgical area.

4.3. Under anesthesia, quickly cut 1-1.5 cm of the skin and abdominal muscles to the xiphoid along the midline with the intact diaphragm.

4.4. Carefully open the chest and inspect bilateral pleural cavities with the help of tweezers, especially the bilateral costal phrenic angles. Collect 1-2 mL of the light-yellow pleural effusion with a 2 mL sterile syringe.

4.5. Once done, sacrifice the rats with IACUC approved protocol.

4.6. Centrifuge the pleural effusion in a 2 mL tube for 15 min at 300 x *g* in order to isolate the nanoparticles.

4.7. Use a drop of the upper layer which is the bright liquid and observe under a transmission electron microscope (TEM,) at an accelerating voltage of 60-80 kV.

REPRESENTATIVE RESULTS:

Using a thoracic ultrasound, we found no pleural effusions on day 1 in all groups. However, on day 3, the pleural effusion appeared in the 6.25 and 12.5 mg/kg·mL groups. The effusion was mainly in the right costal phrenic angle, while the pericardial effusion only presented in 12.5 mg/kg·mL group. Furthermore, on day 7, both pleural effusion (**Video 1**) and pericardial effusion (**Video 2**) were detected in 6.25 mg/kg·mL group (**Figure 1**). Pleural effusion increased slowly to the greatest extent on days 7-10 and then decreased gradually. On day 14, no pleural effusion was found anymore but with the sign of adhesion of pleura in all groups.[10]

At days 7 and 14, there were no signs of the pleural effusion in 3.125 and 6.25 mg/kg·mL groups[10]. However, in the 12.5 mg/kg·mL group, the chest CT scanning was abnormal with the blunt posterior costophrenic angle, which hinted at a small amount of pleural effusion (**Figure 2a**,**b**). No signs of the fluid level were observed, which was explained due to an insufficient amount of water.

Upon dissection of rats, we observed amber or colorless effusions in the 6.25 mg/kg·mL and 12.5 mg/kg·mL groups on days 3 and 7. The volumes of pleural effusion vary from 1-1.8 mL in each pleural cavity in the 6.25 mg/kg·mL and 12.5 mg/kg·mL groups. In the group of 3.125 mg/kg·mL, no fluid in the pleural cavities appeared in the full experimental process.

With the TEM, the NPSi nanoparticles presented individually and clusters formed in the drained pleural fluid. The average diameter (Ø: 20 ± 5 nm) and the morphology in the pleural fluid were consistent with the NPSi in the prepared suspension. The nanoparticles were mostly spherical and well dispersed, and the average size of an individual nanoparticle was ~20 ± 5 nm (**Figure 3a**, **b**).

FIGURE LEGENDS:

Figure 1: Representative images of pleural effusion by sonographic findings on day 7. (**a, b**) Sonographic images from a rat in the 3.125 mg/kg·mL group with no fluid in pleural and pericardial cavities. (**c, d**) Sonographic images from a rat in the 6.25 mg/kg·mL group with apparent pleural effusion and pericardial effusion. (**e, f**) Sonographic images from a rat in the 12.5 mg/kg·mL group with much more fluid in pleural and pericardial cavities.

Figure 2 Representative images of thoracic CT images in rats. CT image from a rat in the 3.125 mg/kg·mL group with no pleural effusion (**a**) and CT image from a rat in the 12.5 mg/kg·mL group with a negative finding of free fluid but the blunt posterior costophrenic angle in the pleural cavity (**b**).

Figure 3. Silica nanoparticles in polyacrylate/nanosilica suspension and the pleural effusion of a rat. (**a**) Silica nanoparticles in polyacrylate/silica nanocomposite. (**b**) Silica nanoparticles in a rat's pleural effusion with clusters or individual form. Scale bar: 200 nm.

Video 1. The pleural effusion in a rat in the 6.25 mg/kg·mL group.

Video 2. The pericardial effusion in the rat in the 6.25 mg/kg·mL group.

DISCUSSION:

Sonography is the most convenient tool for determining pulmonary diseases, due to its excellent sensitivity to the free fluid in the pleural cavity[11]. That is because sonography can immediately detect the contrast in acoustic impedance of air and fluids in the lung[12]. Besides, sonography is more flexible in a small animal's model than CT. Nevertheless, the air in the lung reflected the sound wave and impeded from observing the intrapulmonary changes after nanoparticles instillation. Therefore, we combined chest CT scan and lung sonography to investigate the intrapulmonary changes and the pleural fluid.

After exploring the imaging data, we found the imaging results remarkable. Firstly, our model demonstrated that the PA/NPSi, indeed induced the unusual toxicity, which was manifested as the pleural and the pericardial effusion at the early stage in the rat model. Secondly, this model successfully reproduced the occurrence and the development of human polyserous effusions; meanwhile, these processes were observed in our patients, who presented with pleural and pericardial effusion, pulmonary fibrosis and granuloma[8,9]. Thus, these facts implied that the serous membrane such as the pleural membrane or the pericardial membrane was one of the injury targets of PA/NPSi, which was similar in nature to the one caused by asbestos. Also, the timeline of polyserous effusions was meaningful as concluded by our findings.

As for the design of our model, the intratracheal instillation was the critical step. This method ensured that the toxicity of nanoparticle entered the body through the tracheal, which was different from the previous study[13]. However, the cons of this method were as follows: the PA/NPSi was instilled into bilateral bronchus by the fine tube, which required highly experimental skills to prevent the mechanical damage to the trachea and the cough caused due to its irritation.

Thus, the critical point was the proper depth of intratracheal instillation. Meanwhile, maintaining proper anesthesia was critical to completing the aforementioned step.

The use of nanoparticles, the fine particles for the research purposes are picking up more and more attention. The smaller the diameter of the fine particles, the more challenging it is to protect them. On the other hand, the nano-silica with a diameter of 20 ± 5 nm indeed required a high-tech preparation method to prepare for this study, caused an increase in difficulty with the decrease in diameter. Thus, one of the pros of our technique was the diameter of nano-silica, which was smaller than the previous study[13,14]. Another advantage of this study was that we induced the nanoparticle via tracheal rather than skin or circulation[13,15,16]. For example, the intravenous exposure hampered us in investigating the target organ, which was difficult to distinguish the injury of the target organ triggered by the primary or secondary damage. Hence, in our opinion, the intratracheal instillation shall be the best way to investigate the nanoparticle toxicity of the lungs in the coming future. Besides, the dosage of nanoparticle was lower than the previous study[13], which presented a higher cost-effectiveness ratio.

As for the pleural and the pericardial effusion induced by PA/NPSi, the inflammation reaction and production of reactive oxygen system (ROS) would be the cause of that. We explained it as follows: firstly, the nanosilica increased ROS concentrations, induced inflammatory production, caused mitochondrial depolarization and reduced glutathione levels both in vivo and in vitro[5,6]. Secondly, inflammation and production of ROS increased the interstitial fluid in the lung or permeability of the pleural capillaries, which promoted the formation of pleural effusion at the end. Besides, the potential impairment of pleural lymphatic drainage might also be involved in the accumulation of the pleural fluid. With more accumulation of pleural fluid, the oncotic pressure had increased, which finally induced the deposition of PA/NPSi in pleural cavities. This result was consistent with our previous animal experiments and reported patients[8,17].

For the pleural effusion itself, it was prevalent in the clinic. Nevertheless, many respiratory or systemic diseases could cause pleural effusion. Therefore, constructing an animal model would benefit the etiological study of the pleural effusion. The previous study reported the lung toxicity of nanosilica[13]. However, the previous reported pleural effusion models mainly focused on the biological factors rather than nanoparticles[18,19]. Therefore, the dosage of nanoparticle remained an open issue. Our model demonstrated that pleural effusion occurred on day 3 after a PA/NPSi concentration of 6.25 mg/kg·mL was administered by intratracheal instillation and peaked on days 7-10. Furthermore, with increasing concentrations of PA/NPSi, the pleural effusion produced more and rapidly. Moreover, compared with biological models[18,19], our model of pleural effusion was well-controllable and effective. To sum up, our model would be beneficial for the future studies of pleural effusion diseases, as well as for the further study of nanotoxicity in particular.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure1

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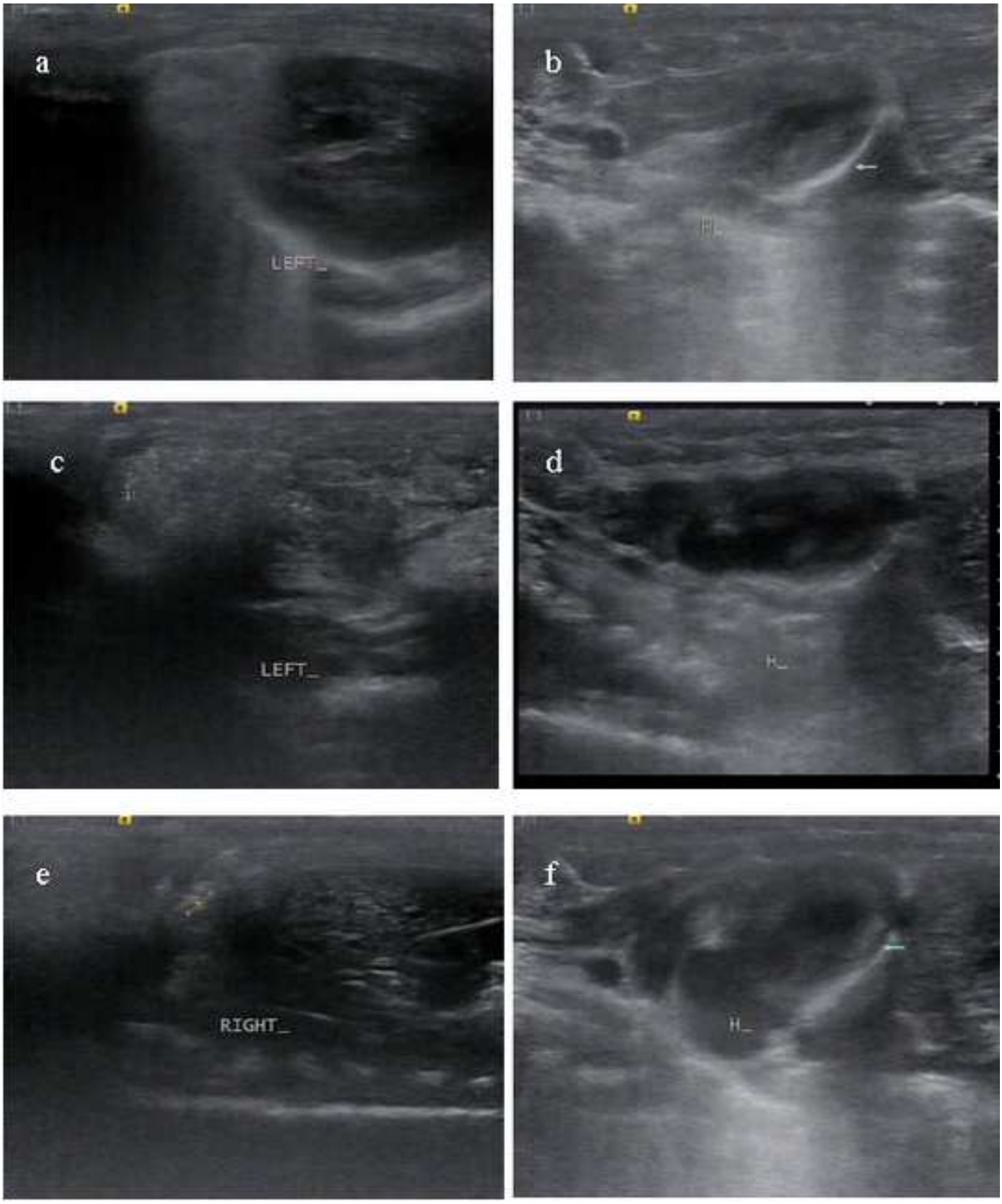

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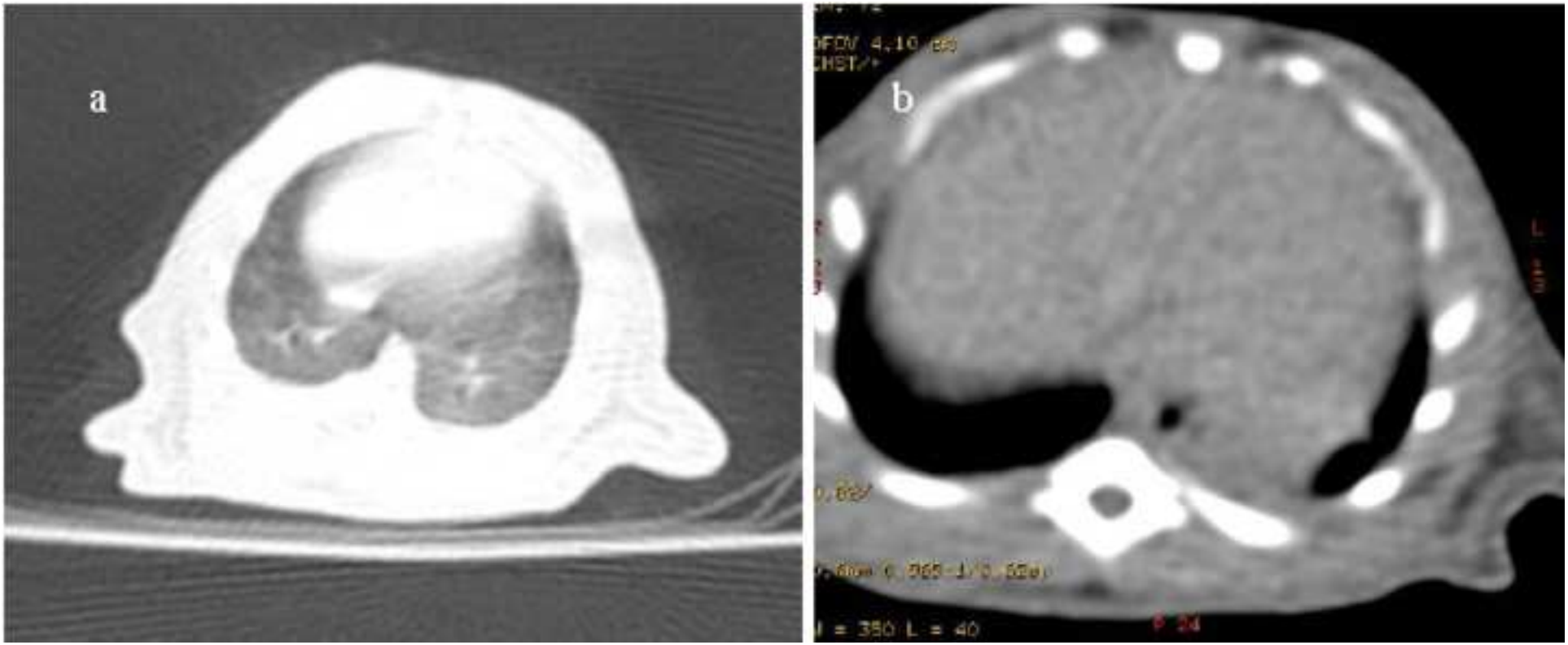

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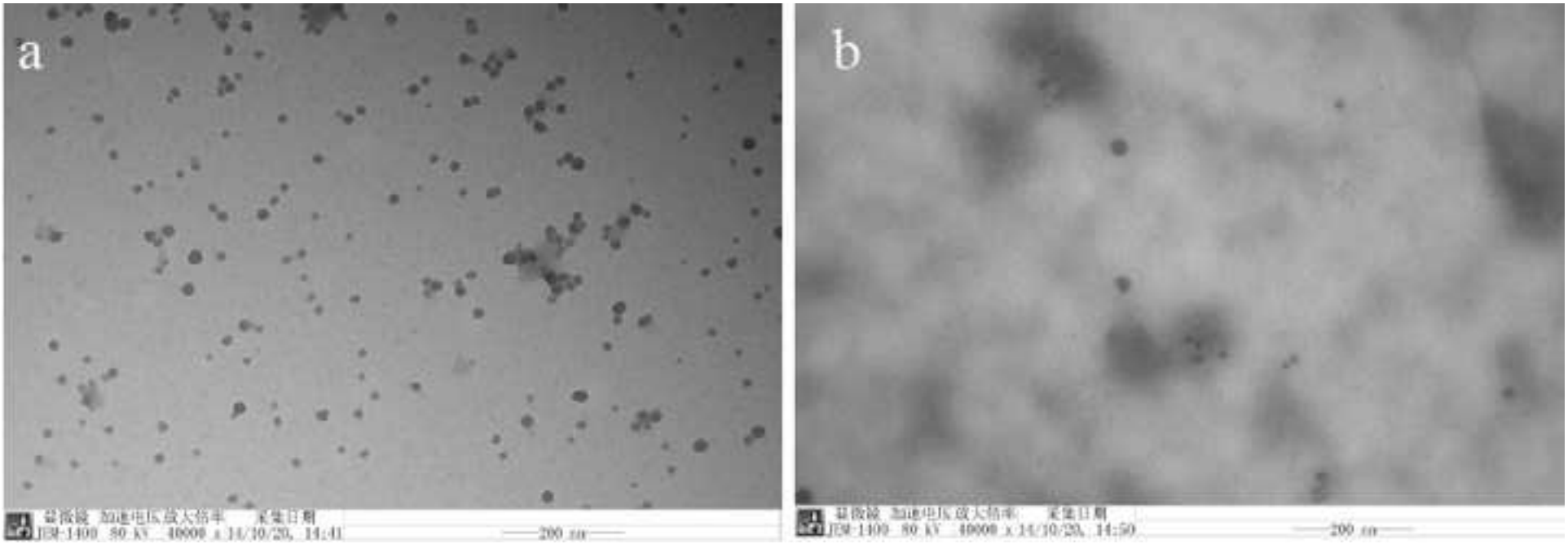
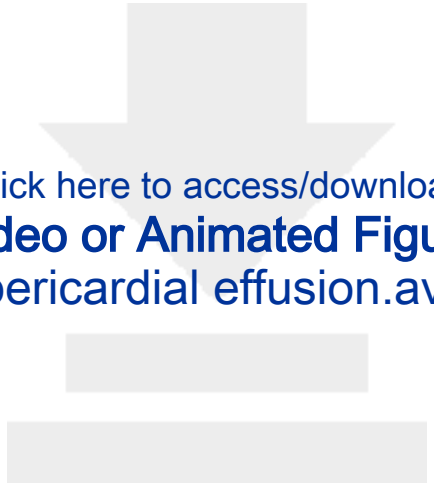


Video of pleural effusion

Video of pericardial effusion



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Acuson S2000 Color Doppler ultrasound system	Siemens Medical Solutions, Mountain View ,CA		
Polyacrylate/nanosilica	Fudan University,Shanghai, China		made by order with nanosilica(20±5)nm
10% chloral hydrate	Beijing Chemical Works	302-17-0	
Transmission electron microscope	JEM-1400Plus，JEOL Ltd., Japan.		
Light speed 16 spiral computed tomography	GE Healthcare, US		
Specific pathogen-free Wistar	Animal Center of Lianhelihua (Beijing, China)		Wistar rats

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Department: Department of Emergency

Institution: Beijing Chao-Yang Hospital

Article Title: making a pleural effusion model in rats with polyacrylate [illegible] and isolating [illegible]

Signature: Ziren Tang Date: May 31. 2018

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Dear editor,

Thank you very much for your reviewing our manuscript and giving us so much good suggestions. We have carefully revised our manuscript and corrected them point by point.

Thank you again!

Editorial comments:

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

1. Please edit the manuscript to be clear and free from grammatic mistakes.

Thank you. Done as suggested.

2. Figure 1: Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible.

Done as suggested.

3. Please delete F1/F2/F3 etc. in the panel labels, i.e., use only a, b, c, etc. to label the panel.

Done as suggested.

4. Please revise the title to be more concise if possible.

Thanks. Done as suggested.

5. Please provide an email address for each author.

Thanks. Done as suggested.

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

Thanks. Done as suggested.

7. Please revise the Long Abstract to focus on the method being presented rather than the results of a specific experiment. A detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.

Thanks. Done as suggested.

8. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to

previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Done as suggested.

9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Thanks. Done as suggested.

10. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Thanks. Done as suggested.

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Done as suggested.

12. Please revise the protocol to contain only action items that direct the reader to do something (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. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Done as suggested.

13. Please add more details 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. 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.

Thank you. **Done as suggested.**

1.3: Please describe how ether anesthesia is done and how proper anesthetization is confirmed. How many rats are included in the

experiment? What concentration of the PA/NPSi suspension is used in this step? How to ensure that the suspension is instilled to the lung of the rats?

Have given more information.

2.2: Please describe how to perform and maintain anesthesia. Is shaving gel used before removing the rat's hair? Which area of rat's hair is removed?

Have given more information.

4.1: Please describe how to open and inspect the rat's bilateral pleural cavities. Please specify all surgical instruments used.

Have given more information.

14. Please reference Figure 1, Video 1, and Video 2 in the Representative Results section.

Done as suggested.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

These are very good idea, we have revised the manuscript.

16. References: Please do not abbreviate journal titles.

Done as suggested.

17. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Done as suggested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The underestimation of nanotoxicity may induce that some diseases are considered as "idiopathic". In fact some times nanoexposure may induce granulomatosis or inflammatory diseases . It's important to have animal models to test nanosafety and study and look for nanoparticles in the biopises of the tissue or on effusions such as pleural or pericardial fluids.

So the model in rats with polyacrylate/nanosilica and the technics for

isolating nanoparticles in the pleural effusion is very intersting for other searchers needing reproducing it.

Major Concerns:
Ther is no major concern

Minor Concerns:
It would be good to have repeated explainments about the synergy between polyacrylate and nanosilica. Why not test only nanosilica toxicity? Is there a synergistic effect between nanosilica and polyacrylate..???We need more détails about that.
Thanks. We will try and do further tests just by giving rats nanosilica

Reviewer #2:

Manuscript Summary:
The authors describe generation of pleural effusion in a rat model with subsequent detection of polyacrylate/nanosilica nanoparticles in the effusion.

Major Concerns:
There are some open questions regarding the application of the model. Is this method intended as a toxicity test model for nanoparticles and chemicals? Relevance of the model for the human situation is questionable as only very high doses, >2g polyacrylate/nanosilica particles in the lung of the standard reference human, cause the effect. How reproducible/variable is the system.

Our test shows that the model is very effect and it can be used for nanotoxic study or pulmonary diseases with pleural effusion

Minor Concerns:
Description of the protocol (phrasing) is unusual.
We have revised the protocol.

Reviewer #3:

Manuscript Summary:
This study by Wen Cao and colleagues exposed the method to make pleural effusion by intratracheal instillation of polyacrylate/nanosilica (PA/NPSi) and how to isolate these nanoparticles in pleural effusion. This study is helpful to understand the potential toxicity of nanoparticles. The method used is clearly exposed and describe. However, some results have to be precise.

Major Concerns:

1) According to the title, this study is about pleural effusion induced by PA/NPSi exposure. However, the authors conclude also in pericardial effusion which is not described as well as the pleural effusion in this study. In particular, there are no descriptions of pericardial effusion on CT scanner and no confirmation by dissection of rats. This data should not appear in the conclusion of the abstract.

Thanks. we reviewed and modified the abstract

2) I understand that the group with 3.125mg/kg of PA/NPSi is used as a control group. It should be clearly describe in the methods.

Thanks, We have revised the protocol.

3) At day 14 the authors described signs of adhesion of the pleura in all groups with ultrasound examination which is not confirmed in the section "dissection/inspection" of pleural effusion. Please clarify.

Ultrasound examination is more sensitive to detect pleural effusion or pericardial effusion than just inspection

4) Figure 1, it could be helpful if there was some annotations on the figure to understand the different anatomical structures on the images exposed.

Thanks. The videos with arrow should in the manuscript give a hand to understand

5) Figure 2a,b is unclear. A pleural effusion is normally described as a declivitous, elliptic or crescent-shaped which is not found on this figure. In addition there is no annotation or arrows on the figure which may help readers who are not used to this technique.

Figure 2a,b are representative images of thoracic CT images in rats. Signs of a pleural effusion normally described as a declivitous, elliptic or crescent-shaped were not observed is that becuase CT scanning is not sensitive as Ultrasound examination. The amount of pleural effusion in rats is very small.

6) Concerning the overall presentation of the results it might be useful to add synthetic(s) additional(s) figure(s) to summarize your results obtain with the different groups of rats and the different techniques.

Thanks.

7) In the discussion, do you have any hypothesis concerning the spontaneous resolution of pleural effusion after 14 days? What do we know about the elimination of PA/NPSi in this model?

After 14 days, pleural effusion was observed and we can’t find anymore.

8) Line 145, in this protocol there was no histological findings of granuloma or fibrosis, this result is different from your clinical study on patients. This is probably related that the duration, concentration and the type of exposure were different between the two models. It is therefore difficult to compare the two models without giving the limits.
Thanks. It is very good suggestion.

9）as for others, we have carefully revised and corrected them point by point.