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A Silver Nanoparticle Method for Ameliorating Biliary Atresia Syndrome in Mouse

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Re: A Silver Nanoparticle Method for Ameliorating Biliary Atresia Syndrome in Mouse

Dear Professor myers

Enclosed please find the manuscript entitled "A Silver Nanoparticle Method for Ameliorating Biliary Atresia Syndrome in Mouse", by Ming Fu, Zefeng Lin, Huiting Lin...Yan Chen and Ruizhong Zhang. We would like to submit for consideration for publication in *The Journal of Visualized Experiments* as an Original article.

Biliary atresia (BA) is a severe type of cholangitis with high mortality in children, and the etiology is still not fully understood. Viral infection may be one possible cause. The typical animal model used for studying BA is established by inoculating a neonatal mouse with rhesus rotavirus. Silver nanoparticles have been shown to exert antibacterial and antiviral effects; their function in the BA mouse model is evaluated in this study. Currently, in BA animal experiments, the methods used to improve the symptoms of BA mice are generally symptomatic treatments given via food or other drugs. The aim of this study is to demonstrate a new method for ameliorating BA syndrome in mice by the intraperitoneal injection of silver nanoparticles and to provide a detailed demonstration of the intraperitoneal injection and detailed methods for preparing the silver nanoparticle gel formulation. This method is simple and widely applicable and can be used to research the mechanism of BA as well as in clinical treatments. Based on the BA mouse model, when the mice exhibit jaundice, the prepared silver nanoparticle gel is injected intraperitoneally to the surface of the lower liver. The survival status is observed, and biochemical indicators and liver histopathology are examined. This method allows a more intuitive understanding of both the establishment of the BA model and novel BA treatments.

This work has been approved by all contributing authors. The manuscript has not been published previously and has not been submitted elsewhere in any language, in whole or in part for consideration of publication. The authors have no competing interests that might be perceived to influence the results and discussion reported in this paper.

We very much appreciate this opportunity to submit and hope the reviewers and editorial staff find this study of interest and acceptable for publication after suitable review.

Thank you for your kind attention with regard to this submission and if you have any question please contact me at the address below.

Sincerely yours,

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TITLE:

A Silver Nanoparticle Method for Ameliorating Biliary Atresia Syndrome in Mice

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KEYWORDS:

Biliary atresia, silver nanoparticle, rhesus rotavirus, neonatal mouse, intraperitoneal injection, treatment

SUMMARY:

This article describes in detail a method based on silver nanoparticles for ameliorating biliary atresia syndrome in an experimental biliary atresia mouse model. A solid understanding of the reagent preparation process and the neonatal mouse injection technique will help familiarize researchers with the method used in neonatal mouse model studies.

ABSTRACT:

Biliary atresia (BA) is a severe type of cholangitis with high mortality in children of which the etiology is still not fully understood. Viral infections may be one possible cause. The typical animal model used for studying BA is established by inoculating a neonatal mouse with a rhesus rotavirus. Silver nanoparticles have been shown to exert antibacterial and antiviral effects; their function in the BA mouse model is evaluated in this study. Currently, in BA animal experiments, the methods

used to improve the symptoms of BA mice are generally symptomatic treatments given *via* food or other drugs. The aim of this study is to demonstrate a new method for ameliorating BA syndrome in mice by the intraperitoneal injection of silver nanoparticles and to provide detailed methods for preparing the silver nanoparticle gel formulation. This method is simple and widely applicable and can be used to research the mechanism of BA, as well as in clinical treatments. Based on the BA mouse model, when the mice exhibit jaundice, the prepared silver nanoparticle gel is injected intraperitoneally to the surface of the lower liver. The survival status is observed, and biochemical indicators and liver histopathology are examined. This method allows a more intuitive understanding of both the establishment of the BA model and novel BA treatments.

INTRODUCTION:

BA is a form of cholestasis characterized by persistent jaundice and has high mortality in the absence of liver transplantation. Viral infections are closely associated with the pathogenesis of BA. The cytomegalovirus, reovirus, and rotavirus have all been suggested as pathogens in BA[1-3]. During the neonatal period, the response of the immature immune system to a viral infection results in immune dysregulation against extra- and intrahepatic bile ducts, leading to biliary epithelial cell apoptosis, inflammatory cell infiltration in the portal area, intrahepatic and extrahepatic bile duct obstruction, and finally, liver fibrosis[4-6].

The commonly used animal model for BA studies involves the inoculation of a neonatal mouse with the rhesus rotavirus (RRV). The mouse typically develops jaundice after 5 - 6 days, showing a low body weight and acholic stools. The role of the immune response in the disease process is critical, especially for natural killer (NK) cells; the depletion of these cells with anti-NKG2D antibody greatly reduces BA-induced damage[7]. Furthermore, other cells, including $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells, and regulatory T cells, have all been shown to play roles in the disease[8-11]. All data suggest the indispensable nature of the immune system in the course of BA.

Silver nanoparticles (AgNPs) have been demonstrated to have beneficial effects against some infectious diseases, including bacterial infections[12] and viral infections[13-15]. However, other than dermatological usage, few studies have used AgNPs in a clinical treatment, mostly because of their potential toxicity. In animal experiments, researchers have generally studied the efficacy of AgNPs administered *via* oral[16] or intravenous methods[17]. However, no other researchers have studied the efficacy of AgNPs administered *via* an intraperitoneal (i.p.) injection in neonatal mouse experiments, which is a simple and rapid method leading to a more direct effect on the liver and bile ducts while reducing the toxicity to other systems, such as the immune system. AgNPs have been shown to affect NK cell activity[18]; therefore, we tested the therapeutic effects of AgNPs administered *via* i.p. injection in the BA mouse model.

PROTOCOL:

All animal experimental protocols have been approved by the Institutional Animal Care and Use Committee of the Sun Yat-Sen University Laboratory Animal Center (#IACUC-DB-16-0602).

1. Establishing the Biliary Atresia Mouse model

1.1. Maintain pregnant BALB/c mice in a specific pathogen-free environment under a 12-h dark/light cycle at 25 °C, with access to autoclaved chow *ad libitum*.

1.2. To prepare the RRV strain MMU 18006, amplify the virus in MA104 cells and measure the viral titers by a plaque assay[19].

Note: MA104 cells are cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in an incubator with a humidified atmosphere containing 5% CO_2. The amplification steps are briefly outlined below.

1.2.1. Infect MA104 cells (1.5 x 10^7) in a 150-cm^2 culture flask with trypsin-activated RRV [1.5 x 10^6 plaque-forming units (PFU)] in 30 mL of serum-free medium. Incubate the infected cells for 3 d in a humidified incubator at 37 °C with 5% CO_2.

1.2.2. Lyse the infected cells in the culture flask by three freeze-and-thaw cycles, with 20 min in a -80 °C freezer for each freeze phase and, then, thawing the cells back to room temperature; the cell-associated virus particle will release into the supernatant. Then, collect and transfer the cell lysate and culture supernatant into a 15-mL conical tube.

1.2.3. Remove large cellular debris from the lysate by low-speed centrifugation (300 x *g* at 4 °C for 3 min). Then, transfer the supernatant containing the virus (approximately 6 mL) into a new 15-mL conical tube for animal experiments.

Note: The RRV is then ready to be titered, aliquoted, and stored or used for additional rounds of amplification. Long-term exposure to room temperature will reduce the viral infection capacity; the virus should be placed on ice and stored at -80 °C or in liquid nitrogen.

1.3. Load the RRV into a small-volume (1-mL) insulin syringe with a 29-G needle for the neonatal mouse injection.

Note: The thick needles of volumetric syringes easily lead to drug leakage.

1.4. Within 24 h of birth, administer to each neonate 20 µL of 1.2 x 10^5 PFU/mL RRV *via* the i.p. route; use the same volume of saline as the control.

Note: The syringe used in this experiment is a 1-mL insulin syringe. Infected mice that were not fed by their mothers died within the first 2 d due to other reasons were not included in the analysis.

1.5. Observe all neonatal mice closely and weigh them daily. Typically, on the sixth day after the RRV inoculation, jaundice appears on the ears and bare skin, the stool becomes clay-colored, and the fur becomes oily, suggesting the establishment of the BA model; check for these symptoms.

Note: BA can be confirmed by a liver tissue section examination with H & E and immunohistochemical stating. The BA mice are then ready for the AgNP treatment.

CAUTION: The protocol presented is for use with contemporary animal and human RV strains, which must be handled under Biosafety Level 2 (BSL-2) conditions.

2. Silver Nanoparticle Synthesis

2.1. Prepare and characterize the AgNPs as previously described[12,20].

Note: The details of preparing and characterizing the AgNPs have been described in publications by C. M. Che's team at the Department of Chemistry, the University of Hong Kong[12,20]. The final concentration of the solution was 1 mM. The mean diameter of the AgNPs was 10 nm (ranging 5 to 15 nm) and confirmed by electron microscopy.

3. Preparation of the Silver Nanoparticle Collagen Mixture

Note: The AgNP collagen mixture is prepared and characterized as previously described[21] and stored at 4 °C. All procedures must be performed on ice.

3.1. First, for the collagen preparation, add 490 µL of type I collagen (4 mg/mL) to a 1.5-mL tube and place it on ice.

3.2. Add 100 µL of phosphate-buffered saline (PBS, 10x) to the collagen and mix it with a pipette.

3.2.1. To prepare 1 L of 10x PBS buffer, combine 80 g of NaCl, 2 g of KCl, and 35.8 g of $Na_2HPO_4 \cdot 12H_2O$ to 2.4 g of KH_2PO_4 and store the buffer at room temperature.

3.3. Then, add 10 µL of NaOH (0.2 M) to the above solution.

3.3.1. To prepare 0.2 M NaOH, add 8 g of NaOH powder to 1 L of distilled water.

3.4. Finally, add 400 µL of AgNPs (1 mM) to the collagen and mix them with a pipette.

Note: Add the AgNPs last for an even mixing. The AgNP collagen mixture should be stored at 4 °C; otherwise, it easily solidifies at room temperature.

4. Mouse Injection Method

4.1. Administer the infected neonatal mice in the treated RRV group with an i.p. injection of 50 µL of the AgNP collagen mixture after the appearance of jaundice; perform a second injection 3 d later.

Note: The mice in the control RRV (infected control) group are given the same volume of saline, and the mice in the normal control group are not given any treatment.

4.2. At the beginning of the injection, press the mouse's leg with the ring finger obliquely over the right thigh, and introduce the needle slowly at a 15° angle (**Figure 1**). Upon reaching the surface of the lower edge of the liver (**Figure 2**), about 0.5 cm in, inject the AgNP collagen mixture; then, withdraw the needle slowly.

Note: Be careful not to introduce any air into the syringe as, then, the neonatal mouse may be killed. In neonatal mice, the stomach and spleen are in the left abdomen, and the stomach is full of milk. If the injection is administered from this side, the needle could easily enter either the stomach, causing milk to flow into the abdominal cavity, or the spleen, causing bleeding.

CAUTION: Pay attention to the needle to prevent any finger injuries, and be sure to replace the needle cap, remove the needle, and place it in a sharps container.

4.3. After all the injections, keep the mice out of their cages for 10 min to allow the AgNP collagen mixture to gel and to prevent the mother from licking the injection site. Then, return the mice to their cages.

4.4. Observe and record the physical appearances of all mice daily, including jaundice and body weight, as well as the survival rate.

5. Blood Sample Collection

Note: Blood samples of approximately 120 µL are collected by inserting the needle into the heart. After centrifugation, the serum is collected (approximately 70 µL) for liver function testing. The blood collection method is as follows.

5.1. Anesthetize the mice on the ninth and 12th day after the RRV inoculation (which is 3 d after the AgNP treatment) using 0.5% sevoflurane.

5.2. Immobilize the limbs of the mouse and sterilize the upper and lower abdomen with 75% alcohol.

5.3. Expose the diaphragm by cutting the mouse skin, muscle, and peritoneum along the midline to the xiphoid with scissors; use a sterile cotton swab to remove the gastrointestinal tract to fully expose the diaphragm muscle.

5.4. Insert the needle (with a 1-mL unloaded insulin syringe) into the left ventricle of the heart and slowly pull back the syringe plunger to obtain the maximum blood volume. Then, transfer the blood to a 1.5-mL tube.

5.5. Allow the tube to stand for 30 min at room temperature and centrifuge it for 5 min at 400 x *g*. Then, using a transfer pipette, collect and save the serum for further use.

Note: Avoid damaging the diaphragm, as diaphragm defects easily lead to pneumothorax, death, and blood coagulation, thereby preventing the blood sample collection.

6. Biochemical Parameter Detection

6.1. Use the serum collected in step 5.5 for a biochemical parameter detection.

6.2. Use an automated biochemical analyzer to detect the following biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), globulin (GLO), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), and total bile acids (TBA).

7. Extrahepatic Cholangiography to Observe the Extrahepatic Bile Duct Patency

Note: Perform the entire process under a dissection microscope.

7.1. Fully expose the liver, gallbladder, and extrahepatic bile ducts with a cotton swab.

7.2. Observe and photograph the appearance of the liver and bile ducts under a dissection microscope.

7.3. Use ophthalmic forceps to gently hold the bottom of the gallbladder.

7.4. Load a 1-mL syringe with methylene blue solution (0.05 wt.% in H_2O). Slowly insert the syringe needle into the gallbladder cavity; then, grasp the needle with the ophthalmic forceps, and slowly infuse 10 - 20 µL of methylene blue.

7.5. Observe under a microscope whether the blue color passes through the extrahepatic bile ducts to the jejunum and take a photograph.

8. Collection of Fresh Liver Samples for Hematoxylin and Eosin Staining

8.1. Fix the fresh mouse liver tissues overnight in 10% formalin.

8.2. Then, embed the fixed liver tissues in paraffin and section them.

8.3. Dewax the sections, rehydrate them with an ethanol series (such as 100%, 95%, 80%, and 70% ethanol in distilled water, each for 5 min), stain the tissue sections with hematoxylin, subject them to a 1% hydrochloric acid alcohol differentiation, and finally, stain the sections with eosin.

8.4. Finally, observe the histopathology of the liver under a 40X microscope.

9. Immunohistochemical Staining of the Hematoxylin-and-Eosin-stained Tissue Sections

9.1. After dewaxing and rehydrating the sections, perform an antigen retrieval by submerging the sections in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution; pH 9.0) and heating them in a microwave for 10 min at 95 °C.

9.2. Remove endogenous peroxidase by exposing the tissue sections for 10 min to a 3% hydrogen peroxide solution.

9.3. Treat the sections with 5% goat serum, to block nonspecific binding.

9.4. Add primary antibodies rabbit-mouse NKG2D (1:100) to the sections, and incubate them overnight at 4 °C.

9.5. Incubate the sections with the appropriate secondary antibodies (HRP-labeled polymer anti-rabbit system) for 30 min at room temperature.

9.6. Visualize the immunohistochemical staining using 3,3'-diaminobenzidine (DAB) as chromogen.

9.7. Observe the sections under a 40X microscope, acquire images, and proceed to analyze them as desired.

10. Flow Cytometric Analysis

10.1. Gently mince the liver tissue, pass it through a 70-µm cell strainer, and centrifuge it 2x at 270 x *g* at 4 °C for 4 min.

10.2. Resuspend the cell pellet in RPMI 1640 medium and analyze it by two-color immunofluorescence using monoclonal antibodies.

10.3. Perform cellular phenotyping using specific cell-surface markers, including fluorescein isothiocyanate- and phycoerythrin-conjugated anti-NKp46 (NK lymphocytes; 1:1,000) and anti-CD4 (T-cell subtype; 1:1,000), with a flow cytometer and analyze the data with flow cytometry data analysis software.

10.4. Select cell populations according to forward/side scatter, gate according to the isotype controls to account for any background fluorescence, and subject the data to a secondary analysis based on the fluorescence signals from individual antibodies.

REPRESENTATIVE RESULTS:

Based on the established BA mouse model, the infected neonatal mice were administered an i.p. injection of the prepared AgNP collagen mixture 2x after exhibiting jaundice. Mouse survival was checked for daily, and liver function testing, liver pathology, and flow cytometry were performed. Compared to the untreated control BA mice, the AgNP-treated mice showed reduced jaundice and maintained their normal body weight (**Figure 3**). The levels of bilirubin metabolism and hepatic transaminase dropped to normal control values, suggesting that the AgNPs greatly improved the liver function (**Table 1**). Extrahepatic cholangiography (**Figure 4**) with methylene blue staining confirmed the bile duct patency after the AgNP treatment. H&E staining (**Figure 5**) showed a significantly decreased inflammatory cell infiltration in the hepatic portal area of mice treated with AgNPs, compared to the control mice. The flow cytometry results showed significantly less NK cells in the livers after the AgNP treatments (both on days 9 and 12) than in the RRV mice (**Figure 6**). Immunohistochemical staining revealed a substantially reduced expression of the NK cell marker NKG2D (**Figure 7**) in the portal triad of the AgNP-treated mice, compared to the RRV mice.

FIGURE & TABLE LEGENDS:

Figure 1: Initial syringe penetration position. The red dotted line indicates the line parallel to the abdomen of the P6 neonatal mouse; the yellow arrow indicates the needle point; the red arrow indicates the needle angle.

Figure 2: Needle reaching the surface of the lower edge of the liver. The yellow dotted line indicates the lower edge of a mouse liver; the red arrow indicates the needle position during the injection.

Figure 3: Effect of AgNPs on BA syndrome in an experimental BA mouse model. (**A**) This panel shows the appearance of neonatal mice on days 9 and 12 after an injection with RRV alone and 3 days and 6 days after an injection with AgNPs (RRV + AgNPs). (**B**) This panel shows the weight of the mice in each group at different time points; the *x*-axis indicates the number of days after the mouse was born and the *y*-axis indicates the fold-change in body weight. $^{**}P < 0.01$ with Student's *t*-test comparing the RRV + AgNp group to the RRV control group; $n = 16$ in the control group, $n = 18$ in the RRV group, and $n = 17$ in the RRV + AgNP group. (**C**) This panel shows the survival rate of the mice in each group. This figure has been modified from Zhang *et al.*[18].

Figure 4: Extrahepatic cholangiography. A contrast agent was used to detect the patency of the extrahepatic bile ducts and to capture images. The blue dotted line indicates the direction of the extrahepatic bile duct; the red arrow indicates a narrowing of the common bile duct; the black arrow indicates BA. The scale bar shown is 1 mm.

Figure 5: H&E staining. Liver tissues of the mice in each group on days 9 and 12 were collected, fixed, sectioned, and stained with H&E. Abbreviations: PV = portal vein, BD = bile duct. The scale bar shown is 50 µm. This figure has been modified from Zhang *et al.*[18].

Figure 6: Percentage of NK cells in the liver tissue. (**A**) The livers of mice were processed into cell suspensions on days 9 and 12, and the proportion of NK cells was detected by flow cytometry. (**B**) This panel shows the percentage of NK cells ($NKp46^{+}CD4^{+}$) in each group at different time points after the AgNP injection. The *y*-axis indicates the fold-change in the percentage of NK cells, which was calculated relative to the percentages of the control group on day 9 and day 12.**$P < 0.01$ and *$P < 0.05$, with Student's *t*-test comparing the RRV + AgNp group to the RRV control group, $n = 10$ in each group. This figure has been modified from Zhang *et al.*[18].

Figure 7: Immunohistochemical staining for the NK cell marker NKG2D in the portal area of the mice in each treatment group. The expression of the NK cell marker NKG2D in the portal area of the mice in each treatment group was detected by immunohistochemical staining. The long arrows indicate bile ducts; the short arrows indicate NK cells. Abbreviations: PV = portal vein, BD = bile duct. The scale bar shown is 50 µm. This figure has been modified from Zhang *et al.*[18].

Table 1: Clinical laboratory examination of liver function-related molecule serum levels. Peripheral blood was used to measure the liver function in the mice in each group. ALT = alanine aminotransferase, AST = aspartate aminotransferase, ALP = alkaline phosphatase, TP = total protein, ALB = albumin, GLO = globulin, TBIL = total bilirubin, DBIL = direct bilirubin, IBIL = indirect bilirubin, and TBA = total bile acids. *$P < 0.05$ and **$P < 0.01$, with Student's *t*-test for each cohort compared to the RRV alone group, $n = 10$ in each group. All biochemical indicator data are displayed as the mean ± SD. All mice in the three groups were 12 days old. This table has been modified from Zhang *et al.*[18].

DISCUSSION:

AgNPs exhibit potent broad-spectrum antibacterial properties and a strong permeability[22]; additionally, they are used to produce a range of antibacterial medical products[23]. However, AgNPs can take a long time to clear once they accumulate in organs, and this persistence may lead to toxic effects[24,25]. A previous study examined the acute toxicity and genotoxicity of AgNPs after a single i.v. injection in a rat experiment, and the results showed that AgNPs could cause acute liver and kidney damage. AgNPs accumulated in the main immune system organs, including the thymus and the spleen[17]. In this mouse BA model, the treatment with AgNPs ameliorated BA syndrome, which our data suggest is partially mediated by NK cell inhibition. However, the long-term effects of AgNPs require a further investigation, to assess the potential toxicity to the mouse development and immune regulation.

In terms of the method, some additional notes for a successful surgery are as follows: (i) The process of preparing the AgNP collagen mixture must be carried out on ice because, at room temperature, the AgNP collagen mixture will quickly become a semi-solid gel, which cannot be

used for injections. After the preparation, the AgNP collagen mixture should be stored at 4 °C. (ii) Only 1-mL insulin syringes should be used because of the small diameter, which reduces the leakage of the injected drug. (iii) Previous studies have generally examined the effect of AgNPs administered orally[16] or *via* intravenous injection[17]. In our animal experiments, the experimental subjects are neonatal mice; thus, intravenous injection is almost impossible, and we used an i.p. injection. The injection of AgNPs improved the symptoms of BA in the mice. (iv) At the beginning of the injection, the lower limbs of the mouse are fixed by hand to prevent the mouse from moving. This method ensures that the right amount of the drug is injected into the abdominal cavity without any leakage and further guarantees the efficacy of the experiment. (v) In neonatal mice, the stomach and spleen are in the left abdomen, and the stomach is full of milk. To inject the AgNP mixture to the surface of the lower edge of the liver, the needle is inserted from above the right thigh of the mouse. If the needle is inserted from the left side, it could easily puncture either the stomach, causing milk to flow into the abdominal cavity, or the spleen, causing bleeding. (vi) Because the abdominal wall in neonatal mice is thin, drug leakage can be prevented by diagonally advancing the needle at a 15° angle close to the abdominal wall to reach the lower edge of the liver.

We have observed the encouraging effect of the AgNPs in this RRV-induced mouse BA model. Together with previous studies that used AgNPs in the treatments of varying virus infections and diseases, these AgNP data suggest the possibility of an *in vivo* application in anti-virus infections. The limitation of these experiments is that the pharmacokinetics of AgNPs is not totally clear as due to a lack of measurement methods for the AgNPs, which makes the control of AgNP dosages difficult. Further study is also needed for the intracellular target of the AgNPs, which will help us to understand the mechanism and reduce the side-effects in future disease treatments.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose**.**

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Figure 1

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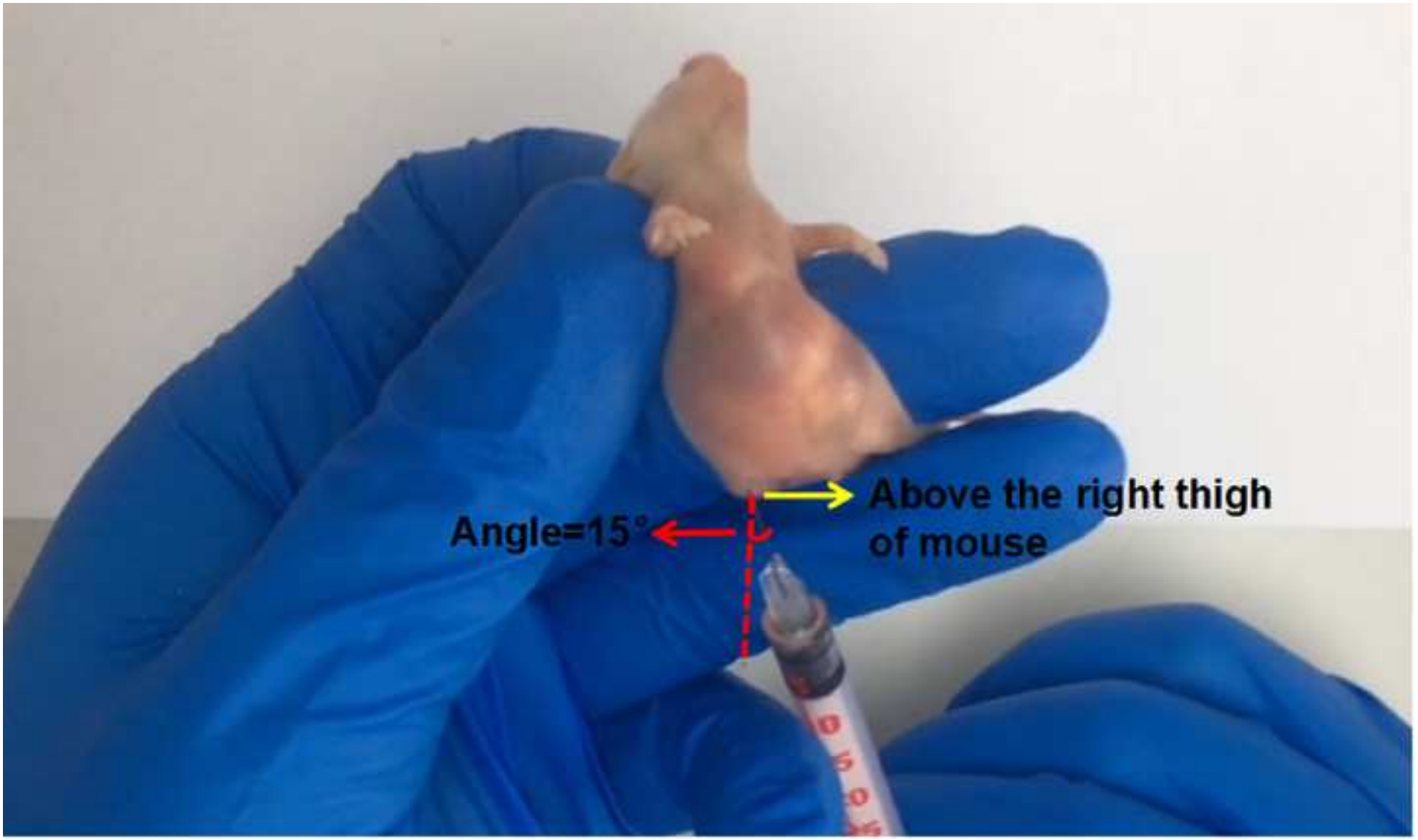

Figure 2

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Figure 3

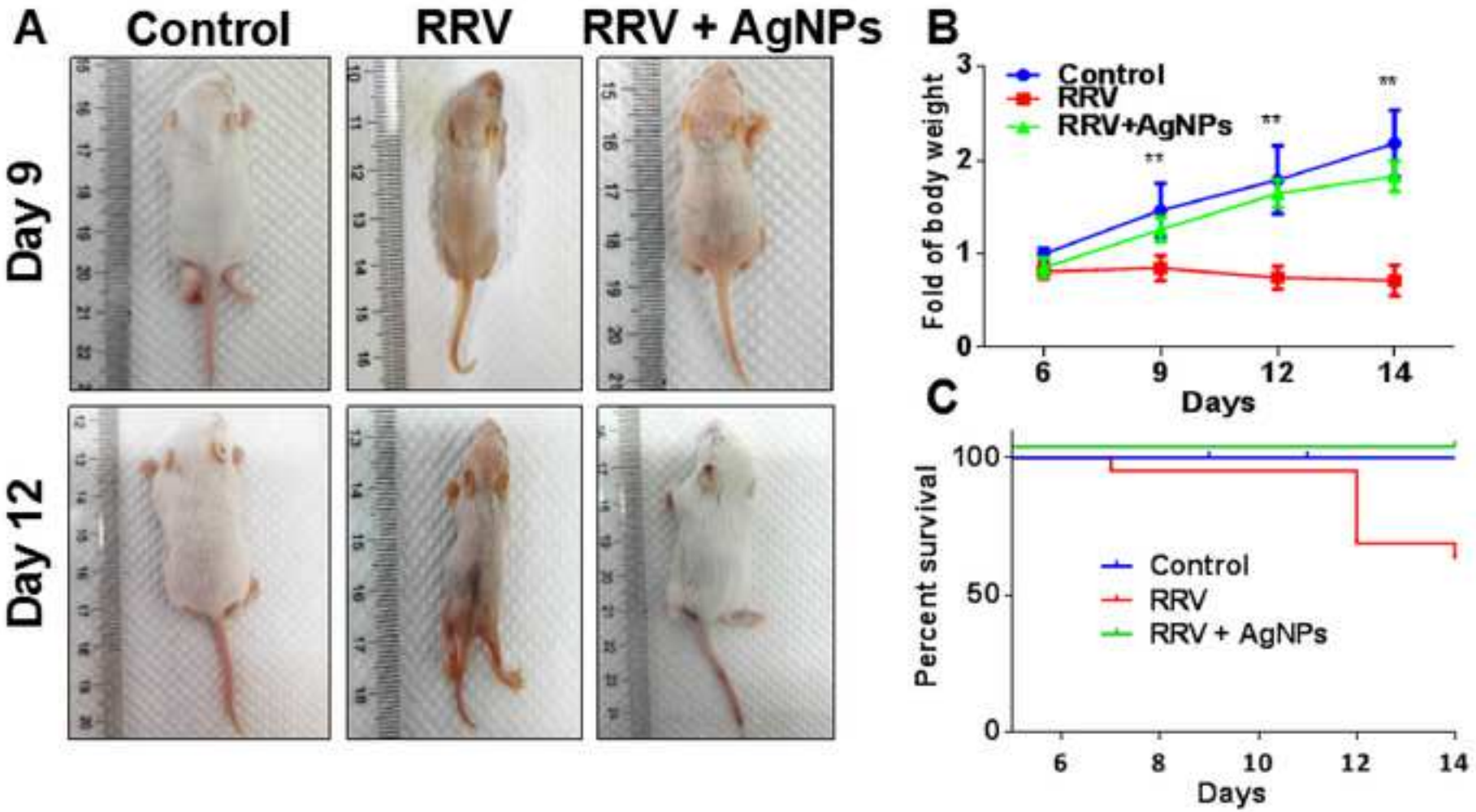

Figure 4

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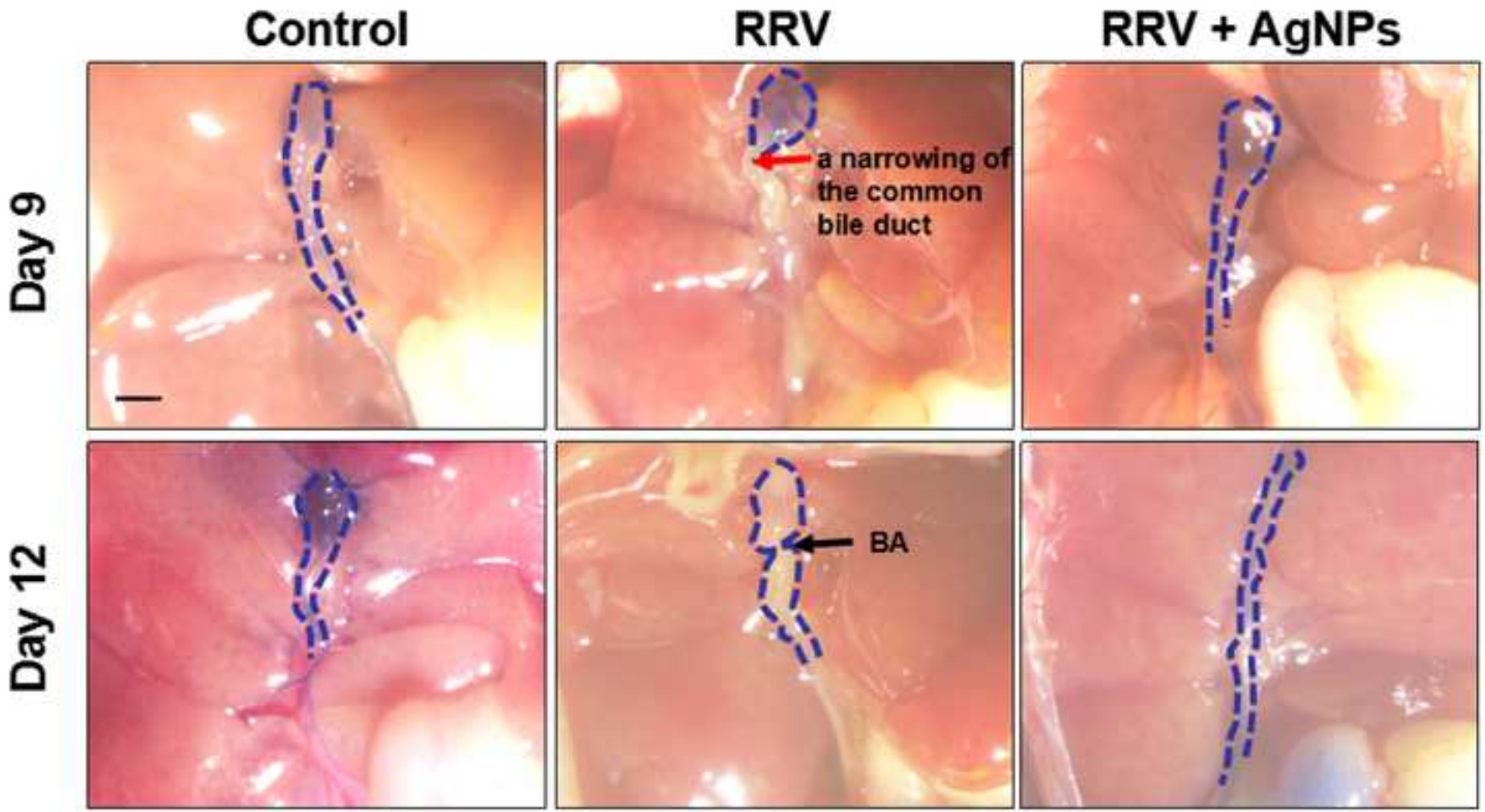

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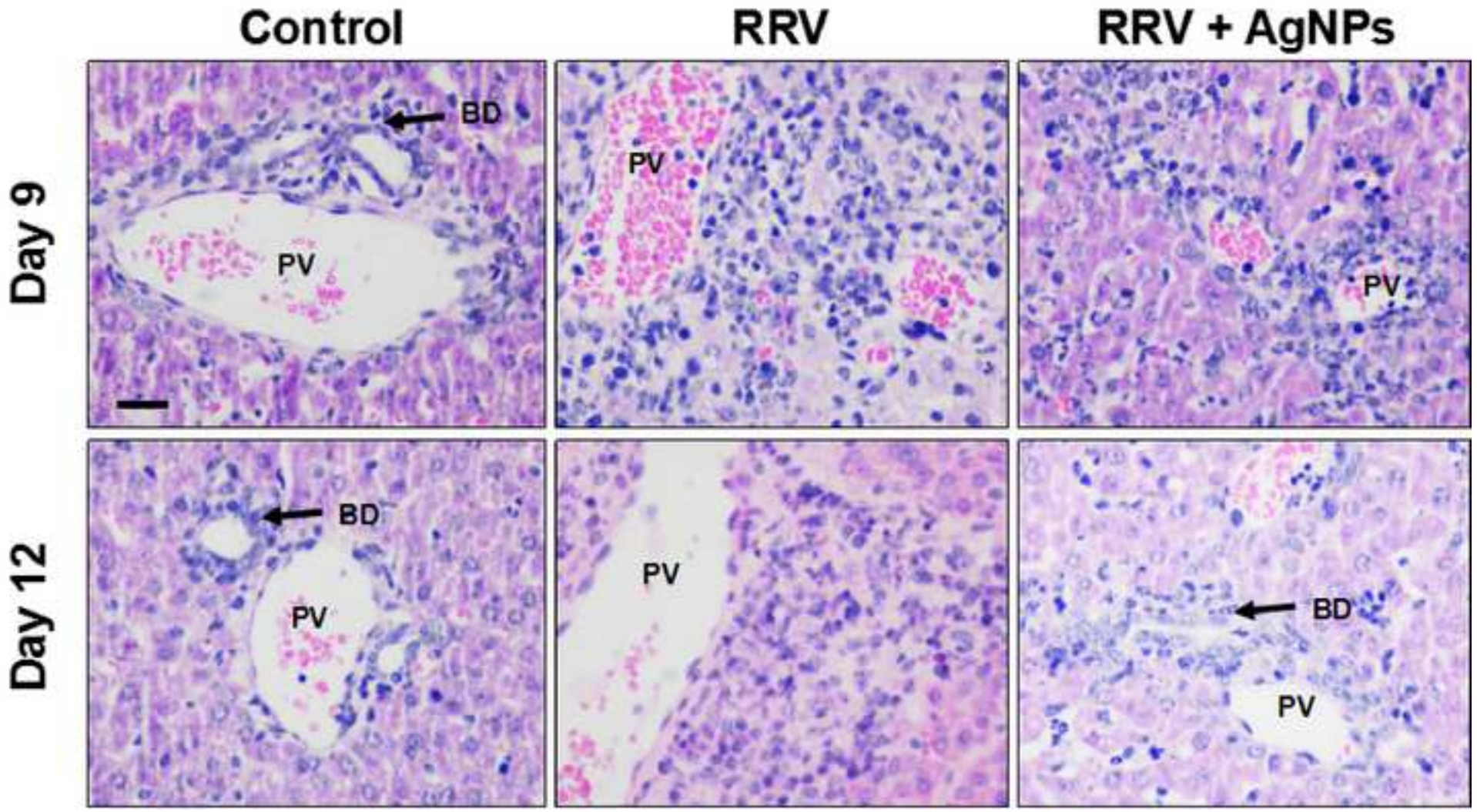

Figure 6

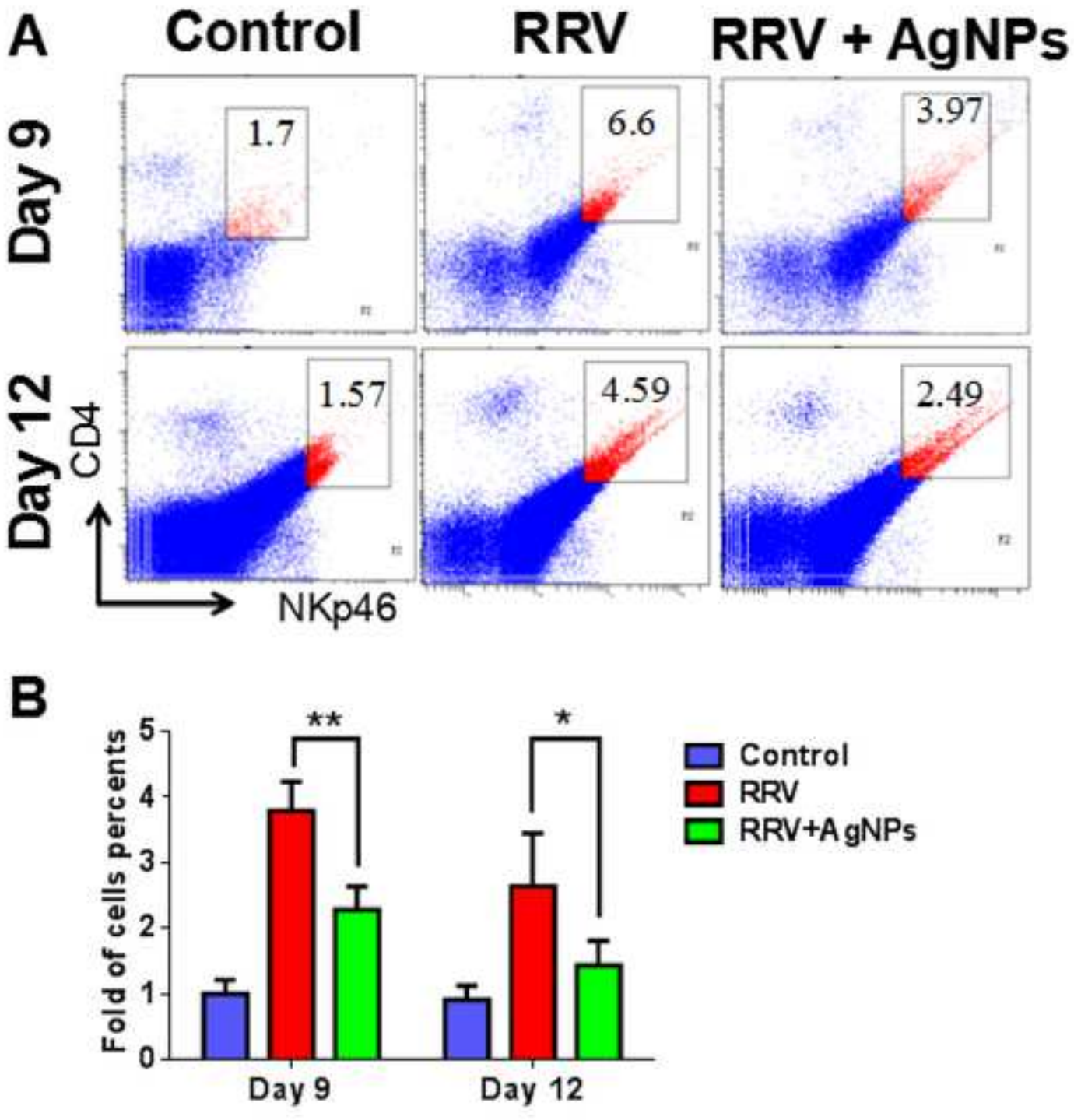

Figure 7

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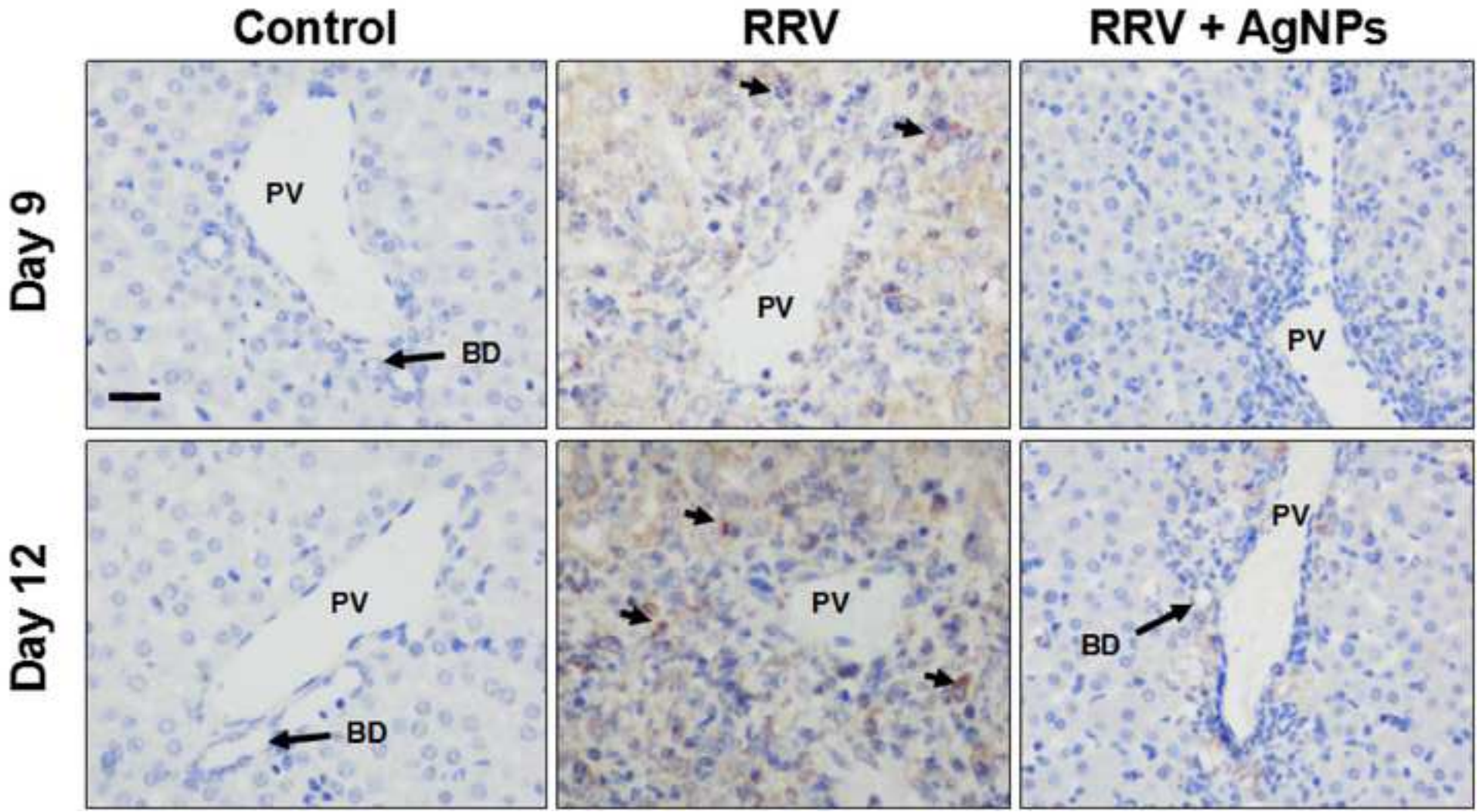

Table 1. Clinical laboratory examination of liver function related mol

	ALT(U/L)	AST(U/L)	ALP(U/L)
Control (n=10)	36.7±7.5	175.0±63.4	441.7±167.6
RRV (n=10)	87.2±53.5	784.4±423.9	1161.7±423.9
RRV+AgNps (n=10)	37.8±14.2*	175.0±116.8**	401.9±115.1**

lecules in the blood.

TP(g/L)	ALB(g/L)	GLO(g/L)
43.7±0.9	25.5±1.4	18.2±1.0
43.4±11.0	22.9±5.0	18.0±3.4
41.6±2.1**	24.4±1.4	17.2±1.2

A/G	TBIL(μM)	DBIL(μM)	IBIL(μM)
1.4±0.13	3.5±0.71	0.58±0.73	2.9±1.0
1.2±0.3	1545±58.2	147.3±51.7	7.2±7.2
1.4±0.1	1.2±0.6**	1.0±0.4**	0.4±0.4**

TBA(μM)
12.5±3.6
464.1±120.6
5.7±3.6**

Name of Material/ Equipment	Company
BALB/c mouse	Guangdong Medical Experimental Animal Center
Rhesus rotavirus (RRV)	ATCC
MA104 cells	ATCC
DMEM	Thermo Fisher
Fetal Bovine Serum	Thermo Fisher
collagen Type I	CORNING
PBS buffer	OXOID
NaOH	Sigma
AgNP	
Insulin syringe with integrated needl	BD
15-mL Centrifuge Tube	Corning
1.5-mL Microcentrifuge Tube	GEB
Microscope	Nikon
Dissecting/Intravital microscope	Nikon
anti-Mouse NKp46 FITC	eBioscience
anti-Mouse CD4 PE-Cyanine5	eBioscience
Monoclonal Mouse Anti-Human CD4	DAKO
anti-NKG2D	RD
BD FACSCanto Flow Cytometer	BD Biosciences

Catalog Number	Comments/Description	Note
SYXK2017-0174	Animal experiment	
ATCC VR-1739	Establish biliary atresia mouse model	
ATCC CRL-10569010	For laboratory use only Mammalian Cell Culture	
10099141	Mammalian Cell Culture	
354236	For research use only	
BR0014G	For washing	
1310-73-2	Adjust the PH value	
	Antibacterial	The AgNps was
9161635S	For medical use	
430791	For laboratory use only	
CT0200-B-N	For laboratory use only	
ECLIPSE-Ci	For laboratory use	
SMZ 1000	For laboratory use	
11-3351	For research use only	
15-0041	For research use only	
20001673	For research use only	
MAB1547	For research use only	
FACS Canto Plus	For laboratory use only	

ity of Hong Kong.

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Title of Article: The Method of Silver Nanoparticle Treatment Ameliorating Biliary Atresia Syndrome in Rhesus Rotavirus Inoculated Mice

Author(s): Ming Fu, Zefeng Lin, Huiting Lin, Yanlu Tong, Hezhen Wang, Hongjiao Chen, Yan Chen, Ruizhong Zhang

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8 June 2018

Editor

Alisha DSouza, Ph.D.

e-mail: em@editorialmanager.com

Re: A Silver Nanoparticle Method for Ameliorating Biliary Atresia Syndrome in Mouse

Dear Dr. DSouza

We would like to express our sincere thanks to the editor for the positive and constructive comments. In the revised version, we have revised our manuscript according to the editor's comments. We also want to thank you for your time and courtesy in the editorial management of our manuscript, hope the revised version met for the requirements and suitable for publishing.

Sincerely yours,

Dr. Ruizhong Zhang

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Replies to Editor

1. References?

Answer (60): Reference 1-3 have been added the revised version.

2. I don't understand you why call this an i.p injection since you inject into the liver.

Answer (78): We actually injected into the surface of the lower edge of the liver in mice and did not inject into the liver.

3. I don't understand you why call this an i.p injection since you inject into the liver.

Answer (82): Same as above, we injected into the surface of the lower edge of the liver in mice but not injected into the liver.

4. We have highlighted sections for filming.

Answer (84): We noticed.

5. Mention culture temperature, humidity, and CO2 levels.

Answer (96): All culture incubations should be performed in a humidified atmosphere containing 5% CO_2 incubator. The information was added in the revised version.

6. What is the MOI? How much virus per mL of cell culture? What is the cell density in the culture? Mention all environmental conditions; Are the cells in a flask? What is the total volume?

Answer (100):

MOI means Multiplicity Of Infection;

$1.5x10^6$ PFU virus /30mL culture medium;

A confluent $150cm^2$ flask contains approximately $1.5x10^7$ MA104 cells.

In a humidified 37℃, 5% CO2 incubator;

Yes, the cells are in a confluent 150 cm^2 flask;

Total volume is 30 ml.

The information was added in the revised version.

7. At what temperature? At what temperature? When were the cells lysed and how? What is the lysing agent? How much is added? For how long?

Answer (104): Freeze at -80℃ in a freezer and thaw at room temperature the flask contained infected cells for three times, the cell

membrane is breakdown and the cell-associated virus particles are released into the supernatant. The information was added in the revised version.

8. Duration?

Answer (109): Remove large cellular debris from the lysate by low-speed centrifugation for 3 min (300 × g, 4℃). The information was added in the revised version.

9. What kind of pathological examination?

Answer (132): Hematoxylin and eosin (H&E) staining and immunohistochemical staining. The sentence was rewritten in the revised version.

10. Please revise? In contact with mouse or human skin? Please also recommend wearing gloves throughout.

Answer (137): The sentence was rewritten as a general cultural condition under Biosafety level 2 (BSL-2).

11. Please mention additional characteristics eg, size of the NPs used.

Answer (142): AgNPs were synthesized based on the following

method (The AgNPs used here are a gift from Prof. CM Che in the Department of Chemistry, the University of Hong Kong. The details of preparing and characterizing the AgNPs have been described in their publication[9,17]). Final concentration of solution was 1 mM. The mean diameter of the nanosilver particles was 10 nm (range 5 to 15nm) and confirmed by electron microscopy. The information was added in the revised version.

12. I don't understand you why call this an i.p injection since you inject into the liver.

Answer (174): We actually injected into the surface of the lower edge of the liver in mice and did not inject into the liver.

13. Isn't the RRV group the infected group? Why do you give them saline? This is confusing! Do you have an RRV-only group?

Answer (176): The sentence was rewritten to clarify the three groups, treated RRV group, control RRV group and normal control group.

14. Mention needle gauge and syringe volume. Should the needle and syringe be prechilled to avoid collagen solidifying prematurely?

Answer (181): A small-volume (1.0 mL) insulin syringe with a 29G needle for the neonatal mouse injection was mentioned previously

(1.3). It is not necessary that the needle and syringe are prechilled.

15. Introduce it where in the body? How deep should the needle be pushed?

Answer (181): Introduce the needle into the surface of the lower edge of the liver in mice. The needle should be pushed 0.5cm. The information was added in the revised version.

16. At what age? How long after AgNP treatment?

Answer (205): Anesthetize the mice on the 9^{th} and 12^{th} day after RRV inoculation which is 3 days after AgNP treatment using 0.5% Sevoflurane. The information was added in the revised version.

17. Mention dosage? Also add oxygen flow rate?

Answer (206): Anesthetize the mice on the 9^{th} and 12^{th} day after RRV inoculation using 0.5% Sevoflurane. The time for collecting the specimen is very short, there is no need to give oxygen in our study.

18. Please describe the surgery steps in greater detail. How is anesthesia maintained? How and when is the heart exposed?

Answer (211):

Expose the diaphragm fully exposed with scissors: Cut mouse skin,

muscle, and peritoneum along the middle line to the xiphoid with scissors; use a sterile cotton swab to remove the gastrointestinal tract to fully expose the diaphragm muscle. The information was added in the revised version.

We don't need to cut the diaphragm to expose the heart. We can see the heart well and take blood through the diaphragm.

19. Mention needle gauge,

Answer (215): Use a small-volume (1.0 mL) insulin syringe with a 29G needle.

20. How? By centrifuging? Mention speed and duration. Are the animals euthanized after this? If not, how is the surgery completed? How do you keep the neonates alive for the rest of the procedures (cholangiography) after so much blood has been drawn? How is anesthesia maintained? Do you use a heating pad to keep the animals warm (and alive)?

Answer (218): Allow the tube to stand for 30 min at room temperature and centrifuge for 5 min at × 400 g. Collect and save the serum using transfer pipette. After blood taken, the animal often dead, but this is not affecting the procedure of extrahepatic cholangiography and later samples collection.

21. Whole blood? Not plasma from 5.5? If whole blood is kept overnight, do you use heparin? Plasma? You have already mentioned this in 5.5, correct?

Answer (226): Yes, use the collected and saved serum (step 5.5).

22. Immediately after 5.4?

Answer (235): Yes.

23. How? Using a camera?

Answer (237): Yes. Using a dissecting/ inverted microscope (Nikon, SMZ 1000) and imaging system (Nikon, DS-Ri1), image under a NIS software.

24. Mention volume and concentration.

Answer (243): Use 10-20uL of methylene blue solution (0.05 wt. % in H_2O).

25. How?

Answer (238): Use a dissecting/ inverted microscope (Nikon, SMZ 1000) and imaging system (Nikon, DS-Ri1).

26. How?

Answer (257): Rehydration with a series of ethanol such as 100%, 95%, 80%, 70% ethanol, distilled water each for 5 minutes.

27. Magnification?

Answer (261): Finally, observe and image the histopathology of the liver under a light microscope (Nikon, ECLIPSE-Ci), 40× was used in the study.

28. For how long?

Answer (265): After dewaxing and hydrating the sections, perform antigen retrieval using Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, pH 9.0) in a microwave for 10 min at 95℃.

29. Which antibodies? Mention concentrations.

Answer (272): Add rabbit-mouse NKG2D antibodies (1:100) to the sections, which are incubated overnight at 4°C.

30. Which antibodies? Mention concentrations.

Answer (275): Incubate the sections with appropriate anti-rabbit liquid (HRP Labelled Polymer Anti-Rabbit) for 30 min at room temperature.

31. Please replace the commercial name with a generic alternative.

Answer (278): Visualize immunostaining using DAB as chromogen.

32. Isn’t this a staining system and not a visualization system? If you have to visualize the stained slides under the microscope, it is unclear why you state this here. Please check!

Answer (278): The paragraph was rewritten in the revised version.

33. Poor phrasing. Please revise. Please replace the commercial name with a generic alternative. Mention magnification, fluorescence excitation and emission filter settings. What do you analyze?

Answer (280): The paragraph was rewritten in the revised version.

34. Duration?

Answer (285): Gently mince the liver tissue, pass through a 70-µm cell strainer, and centrifuge for 4 min at 270 ×g and 4°C twice.

35. This step comes out of the blue. What is the source of the MNCs? You cannot assume that all the liver cells are MNCs. Which antibodies? Why is this step in this section?

Answer (288): The phrase was rewritten: “Resuspend the cell pellet in RPMI 1640 medium and analyze by two-color immunofluorescence using monoclonal antibodies”.

36. Please properly describe the staining procedure. Mention concentrations of all antibodies.

Answer (291): Perform cellular phenotyping using specific cell-surface markers, including fluorescein isothiocyanate- and phycoerythrin-conjugated anti-NKp46 (NK lymphocytes, 1:1000) and anti-CD4 (T cell subtype, 1:1000), with a flow cytometer and analyze with a flow cytometry data analysis software.

37. Remove this from the manuscript and add to the table of materials.

Answer (295): Perform flow cytometry using a flow cytometer, and analyze the data using a flow cytometry data analysis software. The brand has been removed from the manuscript and added to the table of materials.

38. Remove this from the manuscript and add to the table of materials.

Answer (295): Perform flow cytometry using a flow cytometer, and

analyze the data using an analyzing software. This has been removed from maniscript and added to the table of materials.

39. I don't understand you why call this an i.p injection since you inject into the liver.

Answer (301): As indicated before, we actually injected into the surface of the lower edge of the liver in mice and did not inject into the liver.

40. Livers?

Answer (311): The flow cytometry results showed significantly less NK cells in the livers after AgNP treatments (both days 9 and 12) than that in the RRV control mice.

41. Please remove the figure legends from the figure files. Please provide each figure (if multiple panels are present per figure, keep them within 1 file) as an individual SVG, EPS, AI, TIFF, or PNG file.

Answer (316): Correction has been made in the revised version (Figure files), and the figures were separated individually with TIFF fomat.

42. Our in-house editors suggest that this does look like a rat, please double check. Our in-house editors suggest that this does look like a rat, please double check.

Answer (319, 323): We confirmed that this is a neonatal mouse at 6th day after birth.

43. Mention the statistical test performed.

Answer (330): **P<0.01 with student's t-test compared the RRV+AgNp group to the RRV control group, changes was made in the revised version

44. Please add this to the reference list and use superscripted citations throughout.

Answer (332): Correction has been made in the revised version.

45. Please provide scale bars for reference.

Answer (336): Correction has been made in the revised Figures.

46. Please provide scale bars for reference.

Answer (341): Correction has been made in the revised version.

47. Mention statistical test.

Answer (351): “with student’s t-test compared the RRV+AgNp group to the RRV control group” was added in the revised version

48. Please provide scale bars for reference.

Answer (357): Correction has been made in the revised version.

49. Mention statistical tests and sample sizes.

Answer (369): “with student’s t-test compared the RRV+AgNp group to the RRV control group” was added in the revised version, n=10 in each group.

50. Please add and discuss the following: Limitations of the method, future applications.

Answer (407): The sentences have been added in the Discussion in the revised version to address this issue.

51. Reference? Reference?

Answer (378): Reference 22 has been added the revised version. The sentence “they lead to little drug resistance” has been deleted in the revised version.

52. How would you test for this? It does not look like this was really

studied here, so please avoid making this claim.

Answer (384): The phrase was deleted in the revised version.

53. I don’t understand you why call this an i.p injection since you inject into the liver.

Answer (395): We actually injected into the surface of the lower edge of the liver in mice and did not inject into the liver.

54. In your steps you inject into the liver not the abdominal cavity.

Answer (398): We actually injected the abdominal cavity.

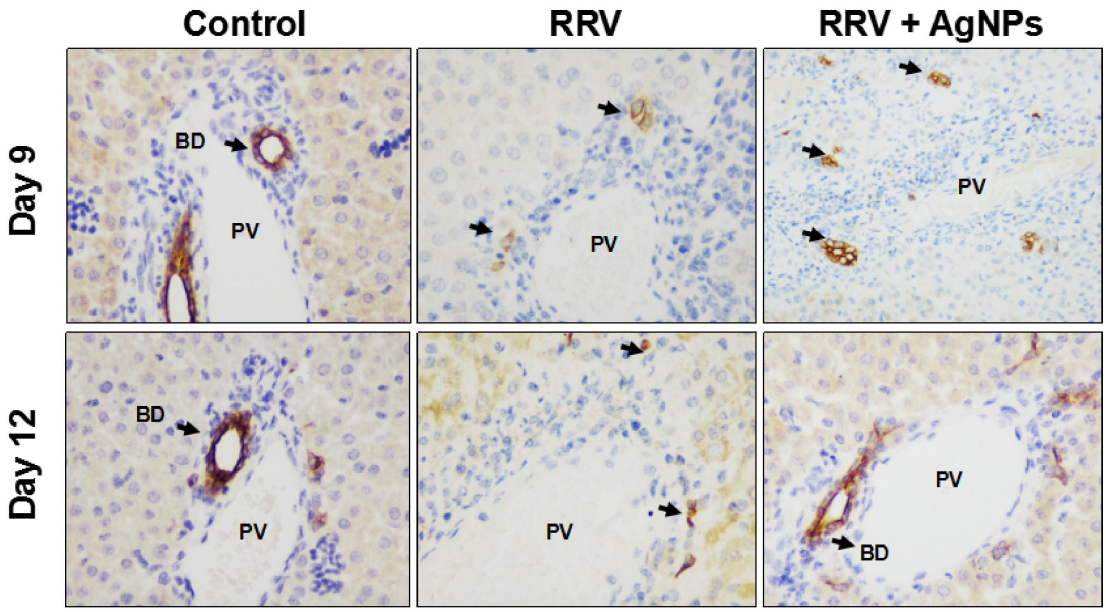

Figure A. biliary epithelial cell marker CK-19 immunohistochemistry staining of the portal area of each treatment group are shown. The black arrow indicates CK19 immunopositive cells. Abbreviations: PV, portal vein and BD, bile duct.

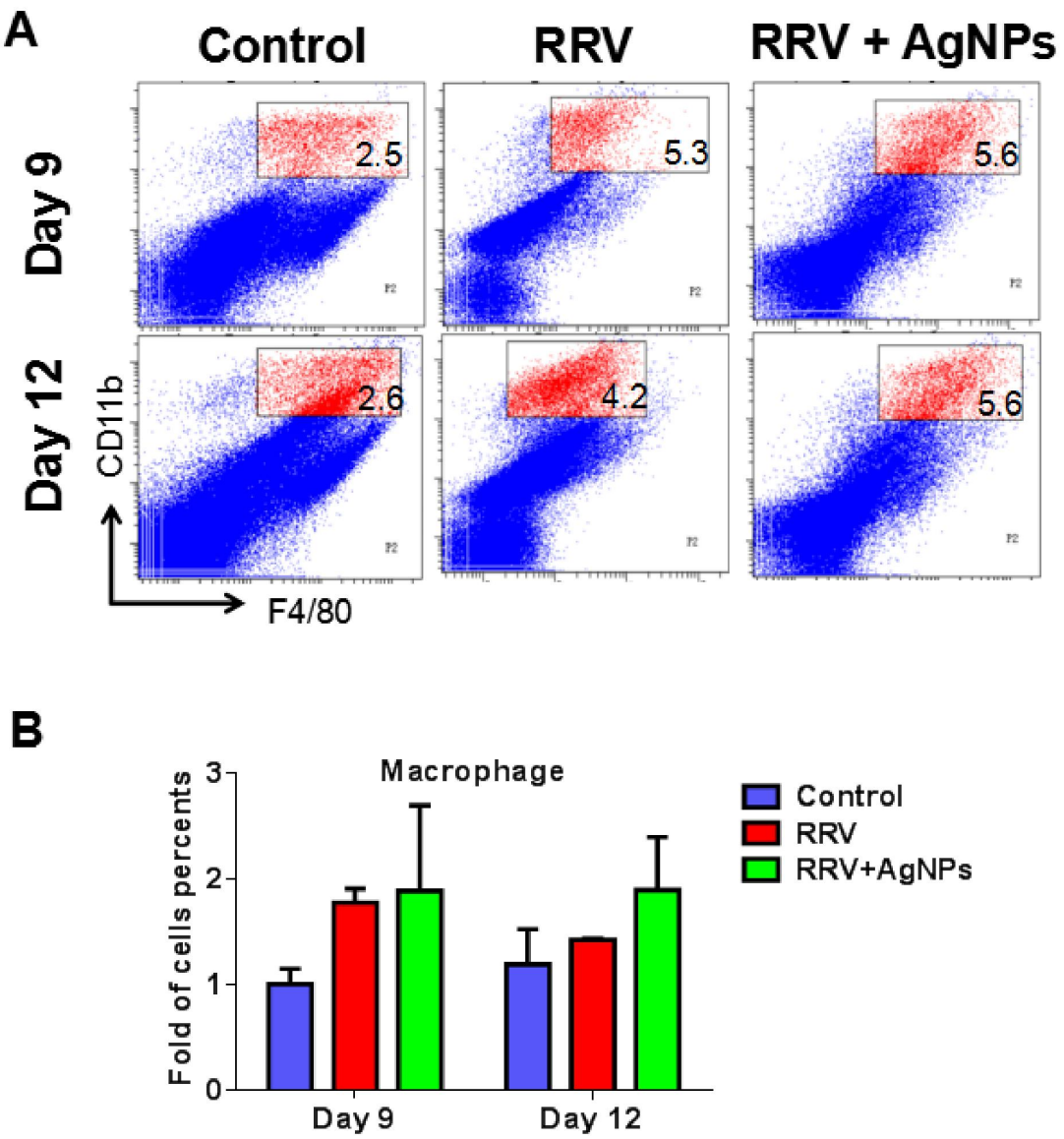

Figure B. Percentage of macrophages in the liver tissue. Mice were sacrificed on days 9 and 12, and the livers were processed into cell suspensions, and the proportion of macrophages ($CD11b^{+}F4/80^{+}$) was detected by flow cytometry. (A) Percentage of macrophages of each group at different time points after injection with AgNps was recorded; y-axis indicated the fold percentage of macrophages (B), which was calculated relative to the percentage of the control group at day 9. n = 10 in each group.

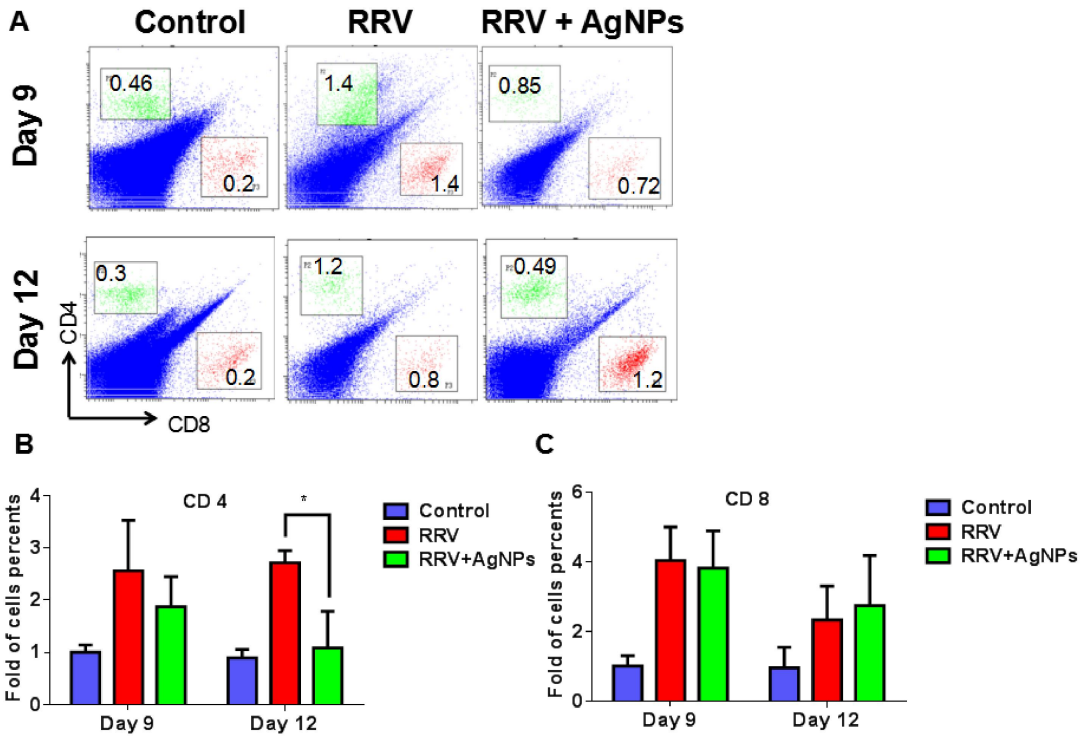

Figure C. Percentage of CD4⁺ and CD8⁺ T cells in the liver tissue. Mice were sacrificed on days 9 and 12, and the livers were processed into cell suspensions, and the proportion of CD4⁺ and CD8⁺T was detected by flow cytometry. (A) Percentage of macrophages of each group at different time points after injection with AgNps was recorded; y-axis indicated the fold percentage of CD4⁺ (B) and CD8⁺ (C) T cells, which was calculated relative to the percentage of the control group at day 9. *P＜0.05, n = 10 in each group.

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If I may be of further assistance, please let me know.

Best Wishes,
Laura

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