Journal of Visualized Experiments

Preparation of a Naringenin compound solution for in vivo application

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62736R5
Full Title:	Preparation of a Naringenin compound solution for in vivo application
Corresponding Author:	Qin Bian Longhua Hospital, Shanghai University of Traditional Chinese Medicine Shanghai, Shanghai CHINA
Corresponding Author's Institution:	Longhua Hospital, Shanghai University of Traditional Chinese Medicine
Corresponding Author E-Mail:	bianqin213@126.com
Order of Authors:	Shufen Liu
	Jingcheng Dong
	Qin Bian
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Shanghai, China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Preparation of Naringenin Solution for *In Vivo* Application

AUTHORS AND AFFILIATIONS:

Shufen Liu[1], Jingcheng Dong[2], Qin Bian[2]

[1]Spine Research Institute, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

[2]Department of Integrative Medicine, Huashan Hospital, Fudan University, Shanghai, China

Email addresses of co-authors:

Shufen Liu (xiaoliufenfen@163.com)

Jingcheng Dong (jcdong2004@126.com)

Qin Bian (bianqin213@126.com)

Corresponding authors:

Qin Bian (bianqin213@126.com)

Shufen Liu (xiaoliufenfen@163.com)

KEYWORDS:

compound, solvent, ethanol, DMSO, Tween 80, naringenin, diabetic osteoporosis, blood glucose test, TRAP staining, ELISA assay, mouse, *in vivo*

SUMMARY:

Here, the protocol presents the preparation of naringenin solution for *in vivo* intraperitoneal administration. Naringenin is fully dissolved in a mixture of dimethylsulfoxide, Tween 80, and saline. The antidiabetic osteoporotic effects of naringenin were assessed by blood glucose testing, tartrate-resistant acid phosphatase staining, and enzyme-linked immunosorbent assay.

ABSTRACT:

The preparation of a compound (phytochemical) solution is an overlooked but critical step prior to its application in studies such as drug screening. The complete solubilization of the compound is necessary for its safe use and relatively stable results. Here, a protocol for preparing naringenin solution and its intraperitoneal administration in a high-fat diet and streptozotocin (STZ)-induced diabetic model is demonstrated as an example. A small amount of naringenin (3.52–6.69 mg) was used to test its solubilization in solvents, including ethanol, dimethylsulfoxide (DMSO), and DMSO plus Tween 80 reconstituted in physiological saline (PS), respectively. Complete solubilization of the compound is determined by observing the color of the solution, the presence of precipitates after centrifugation (2000 x *g* for 30 s), or allowing the solution to stand for 2 h at room temperature (RT). After obtaining a stable compound/phytochemical solution, the final concentration/amount of the compound required for *in vivo* studies can be prepared in a solvent-only (no PS) stock solution, and then diluted/mixed with PS as desired. The antidiabetic osteoporotic effects of naringenin in mice (intraperitoneal administration at 2 mg/mL) were assessed by measuring blood glucose, bone mass (micro-CT), and bone resorption rate (TRAP

staining and ELISA). Researchers looking for detailed organic/phytochemical solution preparations will benefit from this technique.

INTRODUCTION:

With increasing studies concerning the use of phytochemical compounds for drug screening, approaches to prepare phytochemical solutions to evaluate their optimal effects are worth giving attention to. Many aspects such as the dissolution methodology, dosage, and concentration are to be considered when preparing the compound[1].

Solvent-based dissolution is widely used for organic compound preparation[1]. The commonly used solvents include water, oil, dimethyl sulfoxide (DMSO), methanol, ethanol, formic acid, Tween, glycerin, etc[2]. Although a suspension with undissolved substances is acceptable when the compound is intragastrically administered, a fully dissolved solute is critical for intravenous administration. Since oil solution, suspension, and emulsion can cause capillary embolisms, an aqueous solution for compound preparation is suggested, especially when administering intravenous, intramuscular, and intraperitoneal injections[3].

The effective dose range varies among compounds and even among diseases treated with the same compound. Determinations of the effective and the safe dose and the concentration are dependant on literature and preliminary experiments[4]. Here, the preparation of the compound naringenin is demonstrated as an example.

Naringenin (4,5,7-trihydroxy-flavanone), a polyphenolic compound, has been studied in disease treatment for its hepatoprotective[5], antidiabetic[6], anti-inflammatory[7], and anti-oxidant activities[8]. For *in vivo* applications, the oral administration of naringenin is commonly used. Previous studies reported preparing naringenin solution in 0.5%–1% carboxymethyl cellulose, 0.5% methylcellulose dose, 0.01% DMSO, and physiological saline (PS) at 50–100 mg/kg, administered by oral gavage[9–12]. Besides, other studies have reported supplementing naringenin with chow at 3% (wt/wt) for oral intake at a dose of 3.6 g/kg/d[13,14]. Studies have also reported using ethanol (0.5% v/v), PS, and DMSO to dissolve naringenin for intraperitoneal injection at 10–50 mg/kg[15–18]. In a study of temporal lobe epilepsy, mice received an injection of naringenin suspended in 0.25% carboxymethyl cellulose dissolved in PS[19]. Though these studies report the use of different solvents to prepare naringenin solutions, further details, such as dissolving status and animal response, have not been reported.

This protocol introduces a procedure for preparing naringenin solution for *in vivo* application in diabetic-induced osteoporosis. The preparation of the injection solution includes preparing solvents and compounds, dosage estimation, dissolution process, and filtration. The dosage was determined based on literature research and preliminary experiments by monitoring mice after administering injections every day for 3 days and modifying the dosage according to mouse behaviors. The final chosen concentration (20 mg/kg b.w.) was administered intraperitoneally 5 days per week for 8 weeks in a high-fat diet and streptozotocin (STZ)-induced diabetic mice[20,21]. The effects of naringenin in diabetic osteoporosis were evaluated by blood glucose testing, micro-

CT, tartrate-resistant acid phosphatase (TRAP) staining, and enzyme-linked immunosorbent assay (ELISA).

Overall, it was observed that naringenin at a concentration range of 40–400 mg/mL did not completely dissolve in either ethanol or DMSO or 5% (ethanol or DMSO) plus 95% PS (v/v). However, naringenin dissolved completely in a mixture of 3.52% DMSO, 3.52% Tween 80, and 92.96% PS. The detailed procedure will help researchers to prepare the compound as an injection solution for *in vivo* application.

PROTOCOL:

The investigations described conformed to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Shanghai University of Traditional Chinese Medicine Animal Care and Use Committee.

1. Preparation of solvents and estimation of naringenin required for *in vivo* application

1.1 Prepare the following solvents: Tween-80 (final concentration range: 0.5%–1%), DMSO, glycerin (final concentration range: 15%–20%), ethanol (final concentration range: 12% for intramuscular injection)[22], and 0.9% PS.

1.2 Estimate the amount of naringenin required based on the dose, number of mice, and injection frequency.

1.2.1 Order 10 mice (C57/BL/6, male, 5 weeks old, SPF) to administer naringenin 5 days a week for 8 weeks.

NOTE: The dose required for intraperitoneal injection is 20 mg/kg b.w.[23].

1.2.2 Weigh 160 mg of naringenin based on the following calculation: 20 mg/kg x 0.02 kg/mouse x 10 mice x 5 days/week x 8 weeks = 160 mg.

1.3 Calculate the concentration of naringenin to be injected *in vivo*.

1.3.1 Prepare the recommended volume based on the bodyweight of the mice. The recommended volume of naringenin applied to each mouse is 1% of body weight (0.3 mL). In this experiment, 0.2 mL per mouse was used.

1.3.2 Calculate the total volume: 0.2 mL per mouse x 10 mice x 5 days/week x 8 weeks = 80 mL.

1.3.3 Calculate the concentration of the Naringenin in stock: 160 mg/80 mL solvents = 2 mg/mL.

1.3.4 Calculate the volume for each day: 0.2 mL per mouse x 10 mice = 2 mL.

2. Dissolution

2.1 Ethanol solution

2.1.1 To prepare naringenin solution at 2 mg/mL, weigh 3.52 mg of naringenin and add it into a 2.0 mL tube.

NOTE: To achieve a concentration of 2 mg/mL, the total volume required will be 1760 µL (calculation: 3.52 mg/1760 µL = 2 mg/mL).

2.1.2 Quickly spin down (2000 x *g* for 30 s) to make the naringenin powder settle at the bottom of the tube (**Figure 1A**).

2.1.3 Add 8.8 µL of 100% ethanol to the tube to prepare a 0.5% (v/v) solution with respect to the total volume required[2]. Naringenin does not dissolve completely (**Figure 1B**).

2.1.4 Continue to add 79.2 µL of 100% ethanol to the tube to prepare a 5% (v/v) solution with respect to the total volume required (calculation: (79.2 µL + 8.8 µL) / 1760 µL x 100% = 5%). Naringenin does not dissolve completely (**Figure 1B**).

2.1.5 Add 1672 µL of 0.9% PS to the tube containing 5% ethanol as described in step 2.1.4. This will produce an emulsion (**Figure 1D**). Centrifuge (2000 x *g* for 30 s) the solution to check whether naringenin is completely dissolved in the solution. White precipitates of undissolved naringenin appear in the solution (**Figure 1E**).

2.2 DMSO solution

2.2.1 To prepare naringenin solution at 2 mg/mL, weigh 3.95 mg of naringenin and add into a 2.0 mL tube.

NOTE: To achieve a concentration of 2 mg/mL, the total volume required will be 1975 µL (calculation: 3.95 mg/1975 µL = 2 mg/mL).

2.2.2 Quickly spin down (2000 x *g* for 30 s) to make the naringenin powder settle at the bottom of the tube.

2.2.3 Add 9.8 µL of DMSO to the tube to prepare a 0.5% (v/v) solution (calculation: 9.8 µL/1975 µL x 100% = 5%). Naringenin dissolves completely (**Figure 2A**).

2.2.4 Add 88.2 µL of DMSO to the tube to prepare a 5% (v/v) solution with respect to the total volume required (calculation: (88.2 µL + 9.8 µL) /1975 µL x 100% = 5%). Naringenin dissolves completely (**Figure 2B**).

2.2.5 Add 1877 µL of 0.9% PS (v/v percent of 95%) to the solution prepared in step 2.2.4. An emulsion is produced (**Figure 1C**). Centrifuge (2000 x *g* for 30 s) the solution to check whether naringenin is completely dissolved in the solution. White precipitates of undissolved naringenin appear in the solution (**Figure 1D**).

2.3 Tween-80 and DMSO solution

2.3.1 To prepare the naringenin solution at 2 mg/mL, weigh 6.69 mg of naringenin and add into a 5.0 mL tube.

NOTE: To achieve the final concentration of 2 mg/mL, the total solution volume required will be 3345 µL (calculation: 6.69 mg/3345 µL = 2 mg/mL).

2.3.2 Quickly spin down (2000 x *g* for 30 s) to make the naringenin powder settle at the bottom of the tube.

2.3.3 Add 117.7 µL of DMSO to prepare a 3.5% (v/v) solution with respect to the total volume required (calculation: 117.7 µL /3345 µL x 100% = 3.5%). Naringenin dissolves completely (**Figure 3A**)

2.3.4 Add 117.7 µL of Tween 80 to the solution prepared in step 2.3.3 to attain 3.5% (v/v) Tween 80 and 3.5% (v/v) DMSO. Observe the complete dissolution of naringenin (**Figure 3B**)

2.3.5 Slowly add the solution prepared in step 2.3.4 to a 5.0 mL tube containing 3109.6 µL of 0.9% PS (v/v percent of 93%) and shake well to obtain an apparent naringenin solution (**Figure 3C**).

2.3.6 Let the solution prepared in step 2.3.5 stay at room temperature (RT) for 2 h. The solution is still apparent without any visible precipitates (**Figure 3D**).

2.4 Preparation of naringenin solution for *in vivo* administration

2.4.1 According to step 1.2.2, weigh 160 mg of naringenin (160 mg / 2 mg/mL = 80 mL).

2.4.2 Add 2.8 mL of DMSO to attain 3.5% (v/v) solution (calculation: 2.8 mL / 80 mL x 100% = 3.5%)

2.4.3 Then, add 2.8 mL of Tween 80 to the solution prepared in step 2.4.2 to attain 3.5% (v/v) Tween 80 and 3.5% (v/v) DMSO.

2.4.4 Aliquot the solution prepared in step 2.4.3 into four tubes, 1.4 mL per tube [(2.8 mL + 2.8 mL) / 4 = 1.4 mL].

2.4.5 Aliquot 18.6 mL of 0.9% PS to five 15 mL tubes.

2.4.6 Store the solution prepared in step 2.4.3 (stock solution) and 2.4.5 at 4 °C（2.8 mL + 2.8 mL + 18.6 mL x 4 = 80 mL).

2.4.7 Take 140 µL of the stock solution (step 2.4.3) and mix it with 1860 µL of 0.9% PS to prepare 2 mL of naringenin solution for 1 day of administeration.

2.4.8 Filter the solution through a 0.2 µm filter.

3 Naringenin solution administration

3.1 Handling and restraint

3.1.1 Lift the mouse by the base of the tail and place it on a solid surface to position its tail gently back.

3.1.2 Grasp the scruff of the neck behind the ears with the left thumb and index finger and position the tail between the little and the ring finger. Keep the mouse in a supine position with its posterior end slightly elevated.

3.2 Injection

3.2.1 Grasp the back skin of the mouse so that the abdominal skin is taut.

3.2.2 Push the needle (insulin syringe) in at an angle of 10° between the needle and the abdominal surface, approximately 1 cm lateral to the intersection of the root line of both thighs and the anterior midline.

3.2.3 Run the needle subcutaneously in a cranial direction for 3–5 mm, and then insert it at a 45° angle into the abdominal cavity.

3.2.4 When the needle passes through the abdominal wall, the resistance disappears, and no reflux material is withdrawn. Slowly push the solution.

3.2.5 After the injection, slowly pull the needle out and rotate it slightly to prevent leakage. The recommended volume is 5–10 µL/10 g.

4 Blood glucose test

NOTE: Test the blood glucose 1 day prior to the injection and 1 and 2 months after the injection.

4.1 Fast the mice for 15 h before the blood glucose test.

4.2 Open the test strips and mark the date. Once opened, use the test strips within 3 months. Store the test strips at 2–30 °C. Tightly close the lid after removing the strip to prevent the formation of moisture.

4.3 Wipe the scissors with cotton ball/swabs soaked in 70%–75% alcohol.

4.4 Make a cut in the tip of the tail with the scissors and squeeze out a drop of blood.

4.5 Wipe the drop of blood with a tissue paper.

4.6 Squeeze out another drop of blood and collect it on the edge of a test strip.

4.7 Read and record the result displayed on the glucose meter.

4.8 To stop the bleeding, pinch the tail of the mouse with a sterile gauze and wipe the area with 75% alcohol.

5. TRAP staining

5.1 Slides preparation

5.1.1 Perform fasting blood glucose tests on the mice once a week. When the blood glucose levels are ≥ 11.1 mmol/L (indicative of successful type II diabetes mice model[24]), euthanize the mice using CO2 and collect the Lumbar 4^{th}–6^{th} (L4–L6) (no euthanasia is performed while fasting blood glucose test).

5.1.2 Fix the L4–L6 samples with 4% paraformaldehyde for 24 h (ensure that the volume of paraformaldehyde is >20x the volume of the tissue), and then wash for 2 h in continuous flow of tap water.

5.1.3 To decalcify the samples, immerse them in a 10% ethylenediaminetetraacetic acid (EDTA) solution for 2 weeks at RT in static condition until the samples are softened. Ensure that the volume of the decalcifying solution is 20–30 times the volume of the tissue/sample. Change the EDTA solution every other day.

5.1.4 Dehydrate the sample using a dehydrator.

5.1.4.1 Place the specimens in tissue processing embedding cassettes. Number the cassette using a pencil.

5.1.4.2 Set the dehydrator program as follows: 75% alcohol for 2 h, 85% alcohol for 1 h, 95% alcohol for 1 h, 95% alcohol for 2 h, anhydrous ethanol (I) for 2 h, anhydrous ethanol (II) for 2 h, anhydrous ethanol (III) for 2 h, xylene (I) for 1 h, xylene (II) for 1 h, xylene (III) for 1 h, paraffin wax (I) for 2 h, and paraffin wax (II) for 2 h, at RT.

5.1.5 Embed the samples in paraffin wax.

5.1.5.1 Add paraffin wax to the cassette tray of the paraffin embedding station and heat to 60 °C. Immerse the dehydrated specimens for at least 2 h.

5.1.5.2 Place the tissue cassette in the cassette tray and preheat.

5.1.5.3 Add paraffin wax to the paraffin reservoir and heat to 60 °C.

5.1.5.4 After 2 h, take both tissue cassette and specimens to the work area. Pour the preheated paraffin wax from the paraffin reservoir into the tissue cassette. Place the specimen into the paraffin wax, ensure that the paraffin wax completely covers the tissue, and then immediately move the cassette onto an icing station.

5.1.6 Use a microtome to cut the paraffin-embedded samples into 5–6 µm sections. Unfold the sections in 40 °C warm water for less than 10 s. Collect the sections on APS (amino silane) coated glass slides. Dry the slides at RT for 1 h, and then move the slides into an oven set at 60 °C for overnight.

5.2 TRAP reagent preparation

5.2.1 Prepare basic stock incubation solution: Dissolve 9.2 g of sodium acetate anhydrous, 11.4 g of L-(+) tartaric acid, and 2.8 mL of glacial acid in 1000 mL of distilled water. Adjust pH to 4.7–5.0 and store at RT for up to 6 months.

5.2.2 Prepare Naphthol-ether solution: Dissolve 0.1 g of naphthol AS-BI phosphate in 5 mL of ethylene glycol monoethyl ether. Store at 4 °C for up to 5 weeks.

5.2.3 Prepare Sodium nitrite solution: Dissolve 1 g of sodium nitrite in 25 mL of distilled water. Store at 4 °C.

5.2.4 Prepare Pararosaniline dye: Add 1 g of pararosaniline base to 20 mL of 2N HCl (83 mL of HCl in 417 mL of water). Use a stir plate to dissolve the base and filter it before use.

5.3 TRAP staining

5.3.1 Fill two Coplin jars with 50 mL of basic stock incubation solution and place in a 37 °C oven for 2 h.

5.3.2 Take one Coplin jar and add 0.5 mL of naptol-ether solution.

5.3.3 Place the slides in the Coplin jar and incubate at 37 °C for 1 h.

NOTE: Prepare atleast three slides for each group.

5.3.4 A few minutes before the incubation time is over, add 1 mL of sodium nitrite solution and 1 mL of pararosaniline dye. Mix gently for 30 s and leave it for 2 min.

5.3.5 Add the solution prepared in step 5.2.2 to the other preheated Coplin jar containing the basic stock solution. Mix the solution well and insert the slides from the Coplin jar in step 5.3.3.

5.3.6 Incubate for 15–20 min at RT.

5.3.7 Rinse the slices in another Coplin jar with 200 mL of PBS for 5 min.

5.3.8 Counter-stain the slices with 100% hematoxylin for 30 s in a Coplin jar.

5.3.9 Dehydrate the slices with 85%, 95%, and 100% alcohol (200 mL) for 2 min each and treat in xylene (200 mL) for 2 min for 3x. Use Coplin jars to perform each step.

5.3.10 Secure the section on the cover glass with a coverslip using resin. Ensure to avoid the trapping of air bubbles.

6. ELISA

6.1 Sample preparation

6.1.1 Remove the soft tissues from the femur and the tibia of the mouse. Clean the bones with gauze.

6.1.2 Place the bone samples into a 1 mL microcentrifuge tube and store the sample tubes at -80 °C.

NOTE: The samples can also be stored in a liquid nitrogen tank for no more than 6 months.

6.1.3 Weigh the bone sample. Dilute with 0.9% PS at a ratio of 1:10. For example, dilute 0.1 g of bone with 1 mL of PS.

6.1.4 Add 3 mm zirconia beads into the tube and grind the samples 3x at 70 Hz for 30 s with 20 s rest in between.

6.1.5 Centrifuge the samples at 4 °C and 12,000 x *g* for 5 min. Collect the supernatant.

6.2 ELISA assay kit

NOTE: Perform ELISA according to the assay protocol specified by the manufacturer.

6.2.1 Add 100 µL of each dilution of standard, blank, and sample into the appropriate wells. Incubate for 90 min at 37 °C.

6.2.2 Decant the liquid from each well and add 100 µL of biotinylated detection antibody working solution. Incubate for 1 h at 37 °C.

6.2.3 Decant the solution, add 350 µL of wash buffer to each well. Soak for 1 min, repeat 3x.

6.2.4 Add 100 µL of HRP conjugate working solution to each well. Incubate for 30 min at 37 °C.

6.2.5 Decant the solution. Repeat the wash process 5x as described in step 6.2.3.

6.2.6 Add 90 µL of the substrate reagent. Incubate for 15 min at 37 °C protected from light.

6.2.7 Add 50 µL of the stop solution.

6.3 Use a plate reader to record the absorbance of each well at 450 nm.

6.4 Standard curve generation

6.4.1 Plot the respective mean absorbance values against the serially diluted protein concentrations.

6.4.2 Join the points to create the best fit curve. Use any appropriate computer application (spreadsheet) to generate the standard curve equation.

6.5 Substitute the absorbance value of each sample in the standard curve equation to obtain the concentration of the respective sample.

REPRESENTATIVE RESULTS:

The bodyweight of the high-fat diet-fed and STZ-induced diabetic mice was found to decrease when compared with that of the control groups from 0–8 weeks after STZ treatment. The weight loss of naringenin-treated mice was significant compared to the nontreated mice (STZ group) at week 4. The control and STZ groups were administered with the same volume of PS (**Table 1**). The blood glucose level in diabetic mice dramatically increased within 1 month after STZ induction. It then automatically decreased to a level observed 2 months ago when the animal model was established. Naringenin treatment lowered the blood glucose levels by 51.8% and 34.8% at 1 and 2 months, respectively (**Table 2**). STZ-induced diabetic mice exhibited bone loss, as indicated by the decrease in the bone volume/tissue volume (BV/TV) (30.97%) and the number of trabeculae (Tb.N) (11.4%), respectively. The changes in the values of these two parameters suggest that naringenin treatment significantly rescued the bone loss (**Table 3**). Osteoclast activity as indicated by N.oc/Tb.Ar (osteoclast number per trabecular bone area)was increased in high-fat diet and STZ-induced diabetic mice, although no statistical significance was observed between the control and the disease models. Naringenin treatment significantly decreased

osteoclast activities, as shown in **Figure 4** and **Table 4**. The C-terminal telopeptide of type I collagen (CTIX) and N-terminal propeptide of type I procollagen (PINP) were elevated by 68.09% and 204.88% in diabetic animals, respectively, indicating a dramatic increase in the bone resorption rate. Naringenin significantly decreased both indicators of the bone resorption rate (**Table 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Dissolving naringenin in ethanol. (**A**) Naringenin powder in the tube after spin down. (**B**) Naringenin + ethanol (400 mg/mL - 3.52 mg of naringenin in 8.8 µL of ethanol). (**C**) Naringenin + ethanol (40 mg/mL - 3.52 mg of naringenin in 8.8 µL of ethanol) (**D**) Naringenin in 5% (v/v) ethanol and 95% PS (0.9%). (**E**) Precipitates in **D** after spin down. (**F**) Measurement for obtaining scale bar for **Figure 1**, **Figure 2**, and **Figure 3**. Nar: Naringenin. Scale bar = 1 cm.

Figure 2: Dissolving naringenin in DMSO. (**A**) Naringenin + DMSO (400 mg/mL - 3.95 mg of naringenin in 9.8 µL of DMSO). (**B**) Naringenin + DMSO (40 mg / mL- 3.95 mg of naringenin in 98 µL of DMSO). (**C**) Naringenin in 5% (v/v) DMSO and 95% PS (0.9%). (**D**) Precipitates in **C** after spin down. Nar: Naringenin. Scale bar = 1 cm.

Figure 3: Dissolving naringenin in DMSO and Tween 80. (**A**) Naringenin + DMSO (57.2 mg/mL - 6.69 mg of naringenin in 117.7 µL of DMSO). (**B**) Naringenin + DMSO + Tween (57.2 mg/mL - 6.69 mg of naringenin in 117.7 µL of DMSO and 117.7 µL of Tween 80). (**C**) Naringenin in the mixture of 3.5% (v/v) DMSO, 3.5% (v/v) Tween 80, and 93% PS (0.9%). (**D**) No precipitates in **C** after spin down. Nar: Naringenin. Scale bar = 1 cm.

Figure 4: The effect of naringenin on the osteoclast activity of the high-fat diet-fed and STZ-injected (STZ) mice. TRAP staining of trabecular bone and osteoclasts of L4 vertebrae. Triangles indicated osteoclasts. Scale bar = 100 µm. This figure has been modified from Liu et al.[25].

Table 1: Bodyweight of high-fat diet-fed and STZ-injected (STZ) mice across groups and periods. Data are shown as the mean ± s.d. ** $p < 0.01$ vs. Control, ΔΔ $p < 0.01$ vs. STZ.

Table 2: Fasting blood glucose of STZ mice across groups and periods. Data are shown as the mean ± s.d. * $p < 0.05$ ** $p < 0.01$ vs. Control, ΔΔ $p < 0.01$ vs. STZ.

Table 3: Bone mass related parameters of STZ mice across groups. Data are shown as the mean ± s.d. * $p < 0.05$ vs. Control, Δ $p < 0.05$, ΔΔ $p < 0.01$ vs. STZ.

Table 4: Osteoclast activity of STZ mice across groups. Data are shown as the mean ± s.d. ΔΔ $p < 0.01$ vs. STZ.

Table 5: Bone resorption rate of STZ mice across groups. Data are shown as the mean ± s.d. * $p < 0.05$ vs. Control, ΔΔ $p < 0.01$ vs. STZ.

DISCUSSION:

The preparation of phytochemical solution is the basis for its application *in vivo*. In this protocol, the preparation of naringenin solution was demonstrated by using different solvents, such as ethanol, DMSO, Tween 80, and 0.9% PS. The solution in completely dissolved status needs to be further monitored by allowing it to remain at room temperature for some extended hours, and then filtered before being used *in vivo*.

Solvent determination is a critical step in this protocol. There are many solvent options for dissolving compounds, of which ethanol, DMSO, and PS are the most widely used. Ethanol can dissolve many water-insoluble compounds because of its highly polar properties, allowing hydrogen bonding and thus dissolving both polar and nonpolar substances. Moreover, the concentration of ethanol may determine the properties of the compound/phytochemical. For example, 75 wt.% ethanol/water solvent is considered the best for extracting the highest yield of polyphenols and has the strongest anti-oxidant properties[26]. Another study found that ethanol concentration could be lowered to 32.5% at 150 °C for polyphenol extracts to express anti-oxidant property[27]. However, a high concentration of ethanol may cause neurotoxicity and hepatotoxicity[28]. Ethanol injection (i.p.) in a range of concentrations from 8%–32% v/v is commonly used for behavioral evaluation and may cause conditional taste aversion and hypothermia[29]. DMSO is a dipolar aprotic solvent of high polarity and is used as a solvent to dissolve numerous organic compounds. A comparative study indicated that DMSO/methanol (50:50 v/v) resulted in the optimum yield of phenolic acids in citrus rinds[30]. However, dose, concentration, and frequency are not ignorable factors when DMSO is delivered to animals. A 17.7 g/kg dose given intraperitoneally in mice attained LD50 while lowering the dose to 2.5 g/kg for 6 weeks in mice did not cause observable adverse effects[31]. Although the suggested DMSO concentration is 0.5%–5%, DMSO is not capable of dissolving many compounds. Colucci et al. tested the effects of DMSO and DMSO-containing saline at different concentrations by intracerebroventricular and oral administration in mice. The study demonstrated that a solution of 25% DMSO in saline did not change animal behavioral responses[32]. Tween 80 is a nonionic surfactant and is widely used as a co-solvent to increase the solubility of poorly soluble drugs and enhance pharmacokinetic features[33]. A concentration of 1% Tween 80 was chosen considering safety[33]. Thus, the above solvents and surfactants at different concentrations were used to fully solubilize naringenin for intraperitoneal administration.

Some suggestions are listed here for consideration. First, we suggest starting from a small amount of phytochemical compound for preliminary experiments considering consumption cost. Second, it is necessary to perform comprehensive literature research, especially close studies regarding animal species, diseases, administration routes, and frequency, before preparing the solution. Third, the concentration ranges of solvents and co-solvents such as surfactants are dependent on the available literature, preliminary experiments, and the purpose of the study design. Fourth, using an insulin syringe instead of a regular syringe is recommended to reduce the injection injury from a relatively high frequency of administration. Fifth, to maintain sterile conditions, it is recommended to sterilize the solution using a 0.2 μm filter and use sterile syringes and cotton swabs soaked in alcohol when dealing with live animals.

The advantages of the protocol are its simple operation and low cost. In summary, the protocol demonstrates the preparation of a phytochemical solution for intraperitoneal administration in mice, with naringenin as an example. The protocol will benefit the researchers dealing with drug screening or pharmacology.

ACKNOWLEDGMENTS:

This work was supported by the National Natural Science Foundation of China (81973607 and 81573992).

DISCLOSURES:

The authors have nothing to disclose.

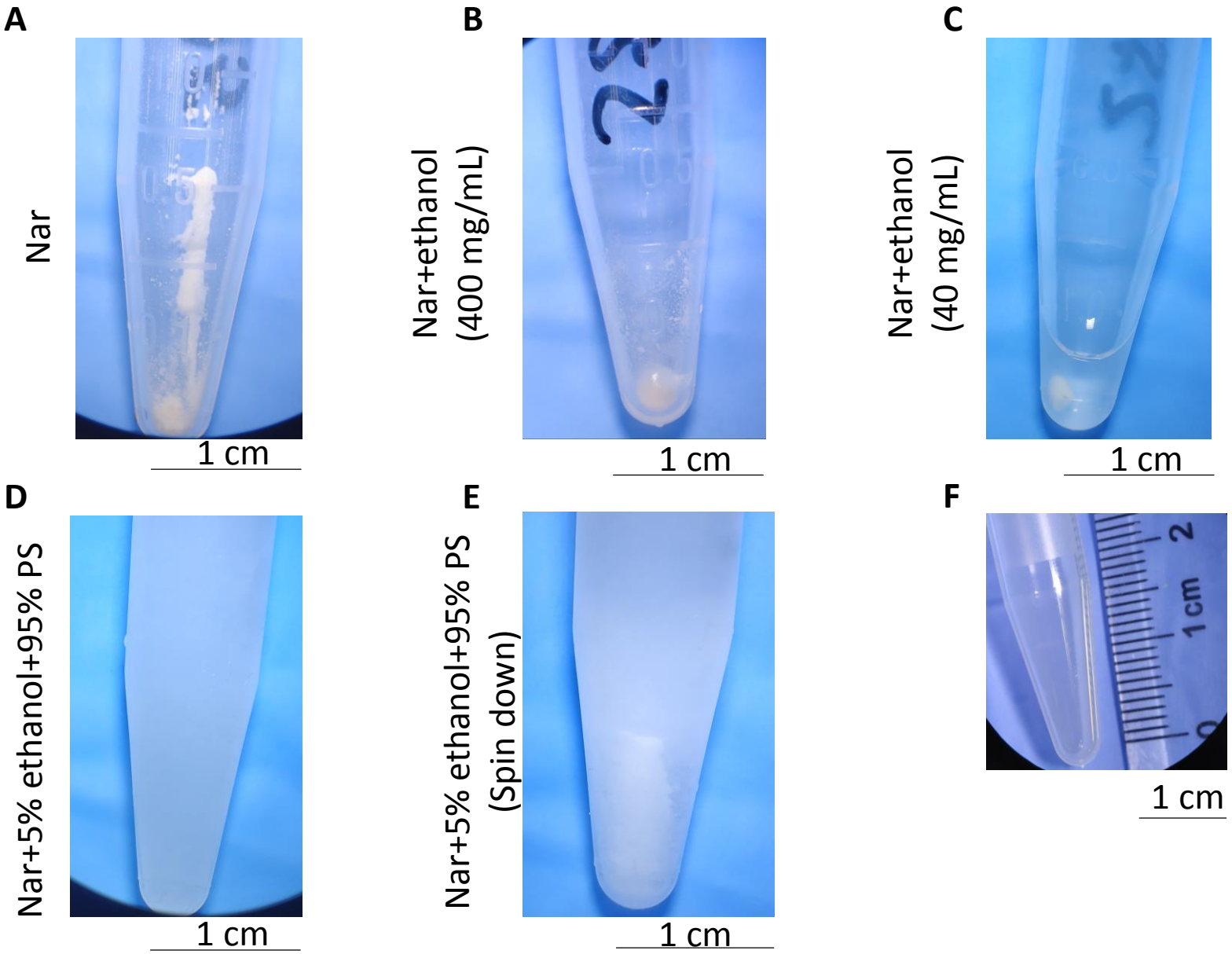
REFERENCES:

1. Stoye, D. Solvents. *Ullmann's Encyclopedia of Industrial Chemistry*. Wiley Online Library (2000).
2. Bouchard, D. et al. Optimization of the solvent-based dissolution method to sample volatile organic compound vapors for compound-specific isotope analysis. *Journal of Chromatography A*. **1520**, 23–34 (2017).
3. Turner, P. V., Brabb, T., Pekow, C., Vasbinder, M. A. Administration of substances to laboratory animals: routes of administration and factors to consider. *Journal of the American Association for Laboratory Animal Science: JAALAS*. **50** (5), 600–613 (2011).
4. Vandenberg, L. N. et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*. **33** (3), 378–455 (2012).
5. Hernández-Aquino, E., Muriel, P. Beneficial effects of naringenin in liver diseases: Molecular mechanisms. *World Journal of Gastroenterology*. **24** (16), 1679–1707 (2018).
6. Den Hartogh, D. J., Tsiani, E. Antidiabetic properties of naringenin: A citrus fruit polyphenol. *Biomolecules*. **9** (3) (2019).
7. Tutunchi, H., Naeini, F., Ostadrahimi, A., Hosseinzadeh-Attar, M. J. Naringenin, a flavanone with antiviral and anti-inflammatory effects: A promising treatment strategy against COVID-19. *Phytotherapy Research*. **34** (12), 3137–3147 (2020).
8. Zaidun, N. H., Thent, Z. C., Latiff, A. A. Combating oxidative stress disorders with citrus flavonoid: Naringenin. *Life Sciences*. **208**, 111–122 (2018).
9. Tsuhako, R., Yoshida, H., Sugita, C., Kurokawa, M. Naringenin suppresses neutrophil infiltration into adipose tissue in high-fat diet-induced obese mice. *Journal of Natural Medicines*. **74** (1), 229–237 (2020).
10. Annadurai, T. et al. Antihyperglycemic and anti-oxidant effects of a flavanone, naringenin, in streptozotocin-nicotinamide-induced experimental diabetic rats. *Journal of Physiology and Biochemistry*. **68** (3), 307–318 (2012).
11. Li, S. et al. Naringenin improves insulin sensitivity in gestational diabetes mellitus mice through AMPK. *Nutrition & Diabetes*. **9** (1), 28 (2019).
12. Devan, S., Janardhanam, V. A. Effect of Naringenin on metabolic markers, lipid profile and expression of GFAP in C6 glioma cells implanted rat's brain. *Annals of Neurosciences*. **18** (4), 151–155 (2011).

13. Burke, A. C. et al. Naringenin enhances the regression of atherosclerosis induced by a chow diet in Ldlr-/- mice. *Atherosclerosis*. **286**, 60–70 (2019).
14. Assini, J. M. et al. Naringenin prevents obesity, hepatic steatosis, and glucose intolerance in male mice independent of fibroblast growth factor 21. *Endocrinology*. **156** (6), 2087–2102 (2015).
15. Alifarsangi, A., Esmaeili-Mahani, S., Sheibani, V., Abbasnejad, M. The citrus flavanone naringenin prevents the development of morphine analgesic tolerance and conditioned place preference in male rats. *The American Journal of Drug and Alcohol Abuse*. **47** (1), 43–51 (2020).
16. Sirovina, D., Oršolić, N., Gregorović, G., Končić, M. Z. Naringenin ameliorates pathological changes in liver and kidney of diabetic mice: a preliminary study. *Archives of Occupational Hygiene and Toxicology*. **67** (1), 19–24 (2016).
17. Nguyen-Ngo, C., Willcox, J. C., Lappas, M. Anti-diabetic, anti-inflammatory, and anti-oxidant effects of Naringenin in an In vitro human model and an in vivo murine model of gestational diabetes mellitus. *Molecular Nutrition & Food Research*. **63** (19), e1900224 (2019).
18. Ahmed, L. A., Obaid, A. A. Z., Zaki, H. F., Agha, A. M. Naringenin adds to the protective effect of L-arginine in monocrotaline-induced pulmonary hypertension in rats: favorable modulation of oxidative stress, inflammation and nitric oxide. *European Journal of Pharmaceutical Sciences*. **62**, 161–170 (2014).
19. Park, J. et al. Naringenin ameliorates kainic acid-induced morphological alterations in the dentate gyrus in a mouse model of temporal lobe epilepsy. *Neuroreport*. **27** (15), 1182–1189 (2016).
20. Matsui, H. et al. Early-stage Type 2 diabetes mellitus impairs erectile function and neurite outgrowth from the major pelvic ganglion and downregulates the gene expression of neurotrophic factors. *Urology*. **99**, 287.e1–287.e7 (2017).
21. Sharma, G., Ashhar, M. U., Aeri, V., Katare, D. P. Development and characterization of late-stage diabetes mellitus and -associated vascular complications. *Life Sciences*. **216**, 295–304 (2019).
22. Rowe, R. C., Sheskey, P. J., Owen, S. C. *Handbook of Pharmaceutical Excipients*. Pharmaceutical Press, London, Chicago (2003).
23. Fallahi, F., Roghani, M., Moghadami, S. Citrus flavonoid naringenin improves aortic reactivity in streptozotocin-diabetic rats. *Indian Journal of Pharmacology*. **44** (3), 382–386 (2012).
24. Liu, F., Yang, H., Zhou, W. Comparison of the characteristics of induced and spontaneous db/db mouse models of type 2 diabetes mellitus. *Acta Laboratorium Animalis Scientia Sinica.* **22** (6), 54–59,74 (2014).
25. Liu, S., Dong, J., Bian, Q. A dual regulatory effect of naringenin on bone homeostasis in two diabetic mice models. *Traditional Medicine and Modern Medicine*. **03** (02), 101–108 (2020).
26. Sun, C., Wu, Z., Wang, Z., Zhang, H. Effect of ethanol/water solvents on phenolic profiles and anti-oxidant properties of beijing propolis extracts. *Evidence-Based Complementary and Alternative Medicine*. **2015**, 595393 (2015).
27. Huaman-Castilla, N. L. et al. The impact of temperature and ethanol concentration on the global recovery of specific polyphenols in an integrated HPLE/RP process on carménère pomace extracts. *Molecules (Basel, Switzerland)*. **24** (17) (2019).
28. van Thriel, C. Toxicology of solvents (including alcohol). *Reference Module in Biomedical Sciences*. (2014).

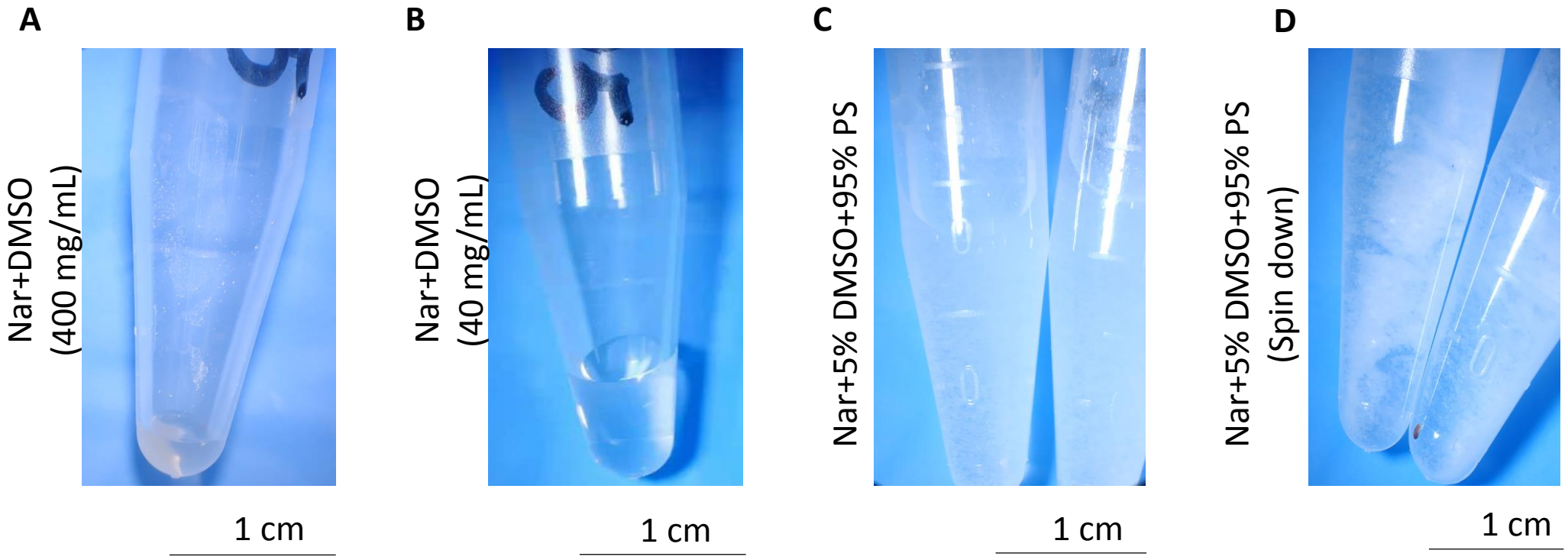
29. Linakis, J. G., Cunningham, C. L. Effects of concentration of ethanol injected intraperitoneally on taste aversion, body temperature, and activity. *Psychopharmacology*. **64** (1), 61–65 (1979).
30. Magwaza, L. S. et al. Rapid methods for extracting and quantifying phenolic compounds in citrus rinds. *Food Science & Nutrition*. **4** (1), 4–10 (2016).
31. Smith, E. R., Hadidian, Z., Mason, M. M. The single--and repeated--dose toxicity of dimethyl sulfoxide. *Annals of the New York Academy of Sciences*. **141** (1), 96–109 (1967).
32. Colucci, M. et al. New insights of dimethyl sulphoxide effects (DMSO) on experimental in vivo models of nociception and inflammation. *Pharmacological Research*. **57** (6), 419–425 (2008).
33. Kawakami, K., Oda, N., Miyoshi, K., Funaki, T., Ida, Y. Solubilization behavior of a poorly soluble drug under combined use of surfactants and co-solvents. *European Journal of Pharmaceutical Sciences*. **28** (1–2), 7–14 (2006).

Figure 1



Nar: Naringenin

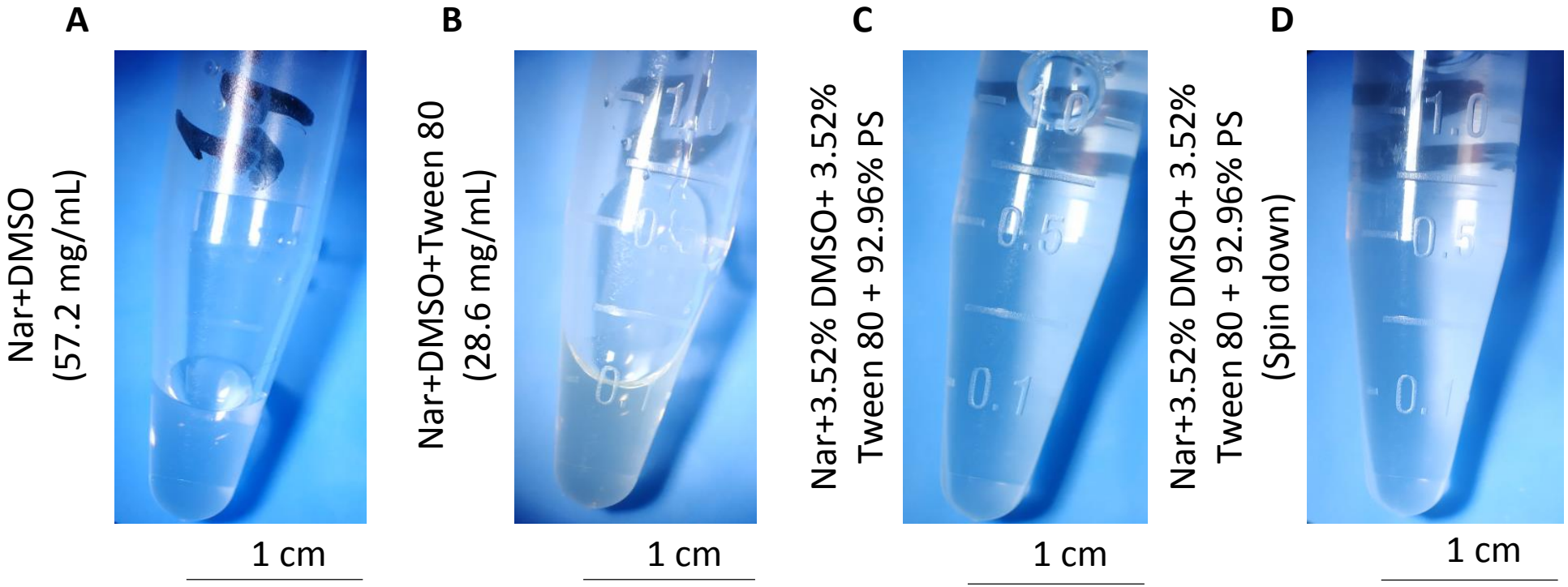
Figure 2



Nar: Naringenin

Figure

Figure 3



Nar: Naringenin

Figure

Figure 4

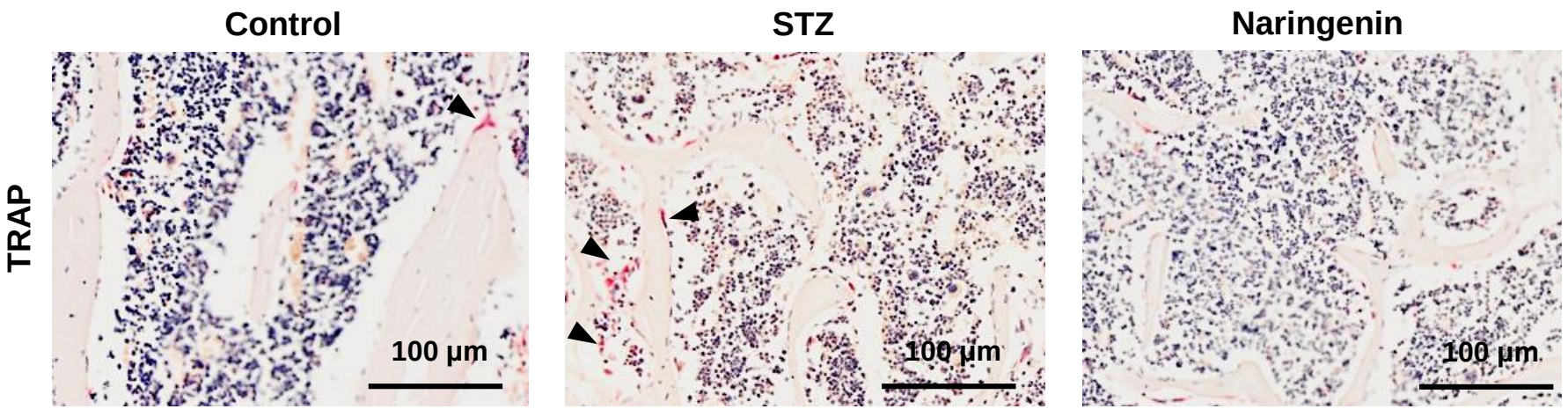


Table 1

(g)	0 week	1 week	2 weeks	4 weeks	5 weeks
Control	23.7 ± 0.2	25.1 ± 1.3	26.2 ± 1.0	27.7 ± 0.5	31.1 ± 0.7
STZ	16.8 ± 1.7**	18.2 ± 2.5**	18.6 ± 2.5**	18.2 ± 1.4**	21.3 ± 1.6**
Naringenin	16.6 ± 1.1**	17.6 ± 1.5**	17.4 ± 1.7**	15.6 ± 1.4**ΔΔ	18.4 ± 1.5**ΔΔ

** p < 0.01 vs. Control
ΔΔ p < 0.01 vs. STZ

6 weeks	8 weeks
31.7 ± 0.8	32.7 ± 1.3
22.0 ± 1.4**	20.8 ± 1.4**
17.7 ± 1.4**ΔΔ	15.5 ± 1.0**ΔΔ

Table 2

(mmol/L)	0 month	1 month
Control	4.9 ± 0.9	8.4 ± 0.7
STZ	12.8 ± 4.2**	22.8 ± 4.3**
Naringenin	13.2 ± 3.5**	11.0 ± 1.9ΔΔ

* $p < 0.05$, ** $p < 0.01$ vs. Control

ΔΔ $p < 0.01$ vs. STZ

2 months
8.3 ± 0.5
15.5 ± 2.7*
10.1 ± 5.3ΔΔ

Table 3

	BV/TV (%)
Control	0.268 ± 0.046
STZ	0.185 ± 0.081*
Naringenin	0.241 ± 0.032Δ

* p < 0.05 vs. Control
Δ p < 0.05, ΔΔ p < 0.01 vs. STZ

Tb.N (1/mm)
5.35 ± 0.31
4.74 ± 0.77*
5.47 ± 0.19ΔΔ

Table 4

$1/\mu m^2$	**N.oc/T.Ar**
Control	0.000182 ± 8.84E-05
STZ	0.00024 ± 2.06E-05
Naringenin	0.000156 ± 3.88E-05ΔΔ

ΔΔ $p < 0.01$ *vs.* STZ

Table 5

ng/mL	**CTIX**	**PINP**
Control	22 ± 8.98	1.64 ± 0.95
STZ	36.98 ± 22.57	5 ± 2.33 *
Naringenin	5.31 ± 2.09 ΔΔ	0.85 ± 0.02 ΔΔ

* $p < 0.05$ *vs.* Control
ΔΔ $p < 0.01$ *vs.* STZ

Editorial comments:

1. Please employ professional copyediting services as there are many grammatical errors in the manuscript that significantly affects the comprehension of the manuscript.

We ordered professional copyediting services from AJE company. The invoice is attached.

2. Please revise the following lines to avoid previously published work: 395-396.

Completed.

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

Completed.

4. Line 87: Please provide a reference for the development of STZ induced diabetic mice as the diabetic induction is not discussed in the protocol section.

Completed.

5. Line 105-107: How are the solvents prepared? What is used along with the solvents for obtaining the final concentration range, as mentioned? Please revise the lines as steps to direct the reader to do something.

The final concentration range is a recommendation here. The preparation of the solvents for obtaining the final concentration is introduced in the following protocol.

6. Line 111-127: The Protocol should contain only action items that direct the reader to do something. 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. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Completed.

7. Line 133-134: Please specify whether the total volume mentioned is the volume of 100% Ethanol or the final solution after dilution of ethanol?

Completed.

8. Line 139-140: Is it v/v with respect to the total volume mentioned in step 2.1.1? If so, please rephrase the sentence to make it clear. Why is it necessary for the v/v % to be 0.5%? Is this because the solubility was checked for a range of values?

The concentration is recommended by the literature and we added it.

9. Line142-143: How does adding 88 µL of ethanol change the v/v percentage to 5%? Is the percentage correct? If 88 µL of ethanol is added to the tube, the total volume in the

tube becomes 96.8 µL, and the resulting v/v% would be 5.5%. Please clarify.

Added calculation to clarify.

10. Lines 136-146: The total volume of ethanol and 0.9% PS comes to 1768.8 µL, which does not match 1760 µL mentioned in step 2.1.1, ultimately reducing the concentration. Please comment.

Corrected and added calculation.

11. Line 150-151: Please mention whether DMSO or 5% DMSO is used?

It is DMSO.

12. Line 156-157: If the v/v percentage (0.5%) mentioned in the step is with respect to the total volume in step 2.2.1, please specify it in the step. Please clarify the steps.

Added calculation.

13. Line 159-160: How does the v/v percentage increase from 0.5% to 5% with the addition of 98 µL DMSO? Is it with respect to the total volume in step 2.2.1? Why are both 2.2.3 and 2.2.4 steps required if naringenin is fully dissolved? Can one directly add 107.8 µL of DMSO to 3.95 mg of Naringenin?

We added calculation to clarify the first and second questions. DMSO is toxic, so the concentration of DMSO is the lower the better. When the initial concentration of 5% is unable to reach the full solubility, higher concentration can be tried.

14. Line 162-163: The total volume becomes 1877 + 98+ 9.8 = 1984.8 µL, which is different from 1975 µL mentioned in 151. Please comment.

Corrected.

15. Line 167-168: Please specify the solution used to attain the total volume. Is it the equal mixture of DMAO and Tween 80?

Yes.

16. Line 173-177/176-177: Adding 117 µL of DMSO/Tween 80 will result in 3.49% v/v. Please clarify how is 3.52% obtained as mentioned in steps 2.3.3 and 2.3.4.

Added calculation.

17. Line 179-180: Please specify the type of tube is used? 5 mL or 15 mL?

Completed.

18. Line 190-193: Please specify how is 3.52% obtained. With respect to what volume?

Corrected.

19. Line199: Please check whether the step numbers specified are correct. Are the stock solutions from 2.4.3 mixed with 0.9% PS in 2.4.5?

Corrected and added calculation.

20. Line 201-201: Please specify from where/which step is 140.8 µL of stock solution taken.

Corrected.

21. Line 245: Does “alcohol cotton ball” refer to cotton ball/swabs soaked in 70-75% alcohol?

Yes and corrected.

22. Line 261: Please specify at what time point are the mice sacrificed? Please specify the euthanasia method.

Completed.

23. Line 263: Please specify the volume of paraformaldehyde used to fix the Lumbar. Was a continuous flow of tap water used for 2 h of washing, or was the tissue immersed in a specific volume of tap water for 2 h. Please clarify.

Completed.

24. Line 265-266: Please specify the volume of EDTA. Were the samples kept in static condition? Was any specific temperature maintained?

Completed.

25. Line 287-289: How long are the tissues incubated in paraffin before proceeding to

sectioning?

Added the missing information.

26. Line 291: Please mention how the sections are collected? Are glass slides used for collecting sections? Were the slides coated?

APS (Amino Silane) coated glass slide

Completed.

27. Line 314: Please specify the number of slides placed.

Completed.

28. Line 319-320: What is 3.9.2.4? Please add the necessary details to add more clarity to the step.

Corrected.

29. Line 324: Please mention what is rinsed. Slides? How is it performed? Are the slides rinsed in the Coplin jar?

Yes. Added.

30. Line 326-329: Are the steps performed with the slides in the Coplin jar?

Yes, added.

31. Line 331: Are the coverslips placed on the sections on the cover glass?

Yes. Corrected.

32. Line 339: Store in liquid nitrogen tank for how long?

No more than six months. Added the information.

33. Please specify the details of control groups used in the study for evaluating the effect of naringenin. What was injected into these animals?

Added in Representative results, first paragraph.

34. Figure 1: Please ensure that the figure labels match the details provided in the protocol. Consider adding the details mentioned in the protocol steps to the figure labels. For example, revise the label of Figure 1A to “Nar + ethanol (400 mg/mL- 3.52 mg of naringenin in 8.8 µL of ethanol)”. Maintain a single space between the number and the unit.

Added.

35. Figure 2: Consider adding the details mentioned in the protocol steps to the figure labels. For example, revise the label of Figure 2A to “Nar + DMSO (400 mg/mL- 3.95 mg of naringenin in 9.8 µL of DMSO)”.

Added.

36. Figure 3: Consider adding the details mentioned in the protocol steps to the figure labels. For example, revise the label of Figure 3A to “Nar + DMSO (57.2 mg/mL- 6.69 mg of naringenin in 117 µL of DMSO)”.

Added.

From: **Xiao Ling** lxiao@wspc.com
Subject: Re: Copyright for article : Feedback on Journal TMMM
Date: June 14, 2021 at 11:13 PM
To: bianqin213@126.com
Cc: tmmm_MEAsst_zhang 69822467@qq.com, Feng Liu TMMM ahchlf@126.com

XL

Dear Dr. Bian,

Your article in TMMM has been published as an open access article with CC BY NC licence. You are free to reproduce the figures in your next paper, provided that you cite the original TMMM article.

Best regards,
Ling

Ms XIAO Ling
Editorial Department
World Scientific Publishing
5 Toh Tuck Link, Singapore 596224
Tel: 65-6466-5775 ext 430
Email: lxiao@wspc.com

From: Editor SG <editor@wspc.com.sg>
Sent: 14 June 2021 14:15
To: Xiao Ling <lxiao@wspc.com>
Subject: FW: Copyright for article : Feedback on Journal TMMM

From: World Scientific Publishing Company <customercare@wspc.com>
Sent: Friday, 11 June 2021 11:40 pm
To: Editor SG <editor@wspc.com.sg>
Subject: Copyright for article : Feedback on Journal TMMM

Form Fields:

a_name: Qin Bian
b_sender_email: bianqin213@126.com
checkbot: 11
d_message: Dear Editor,

I am submitting a manuscript about the protocol focusing on how to prepare naringenin compound and its application for in vivo study. I am going to re-use and modify some of the Figures in the article "A dual regulatory effect of naringenin on bone homeostasis in two diabetic mice models" published in Traditional Medicine and Modern Medicine (Vol.

3, No. 2 (2020) 1–8, DOI: 10.1142/S2575900020500093). Can I ask for copyright release?

Thank you & Best regards,
Qin Bian
emailSubject: Copyright for article : Feedback on Journal TMMM
emailTo: editor@wspc.com.sg
submit: Submit

Customer Service Department World Scientific Publishing Co. Pte. Ltd.
5 Toh Tuck Link, Singapore 596224
customercare@wspc.com
Tel: 65-6466-5775
Fax: 65-6467-7667

INVOICE

Invoice# SSM34Y6DJ

Balance Due
$268.20

601 W Main St, Ste 102
Durham, North Carolina, 27701, USA
Tax ID: 412141424

Invoice Date :	28 Jun 2021
Submission :	SM34Y6DJ
Group Code :	Springer
Word Count :	4279
Title :	Preparation of a Naringenin Compound Solution for In Vivo Application
Discounts Applied :	10% (E25F43AE114EAAB)

Bill To
Qin Bian
Fudan University
12 Middle Urumqi Road
Shanghai
200040
China

#	Item & Description	Base Price	Discount	Amount
1	Standard Editing Standard Editing	298.00	29.80	268.20
			Sub Total	268.20
			Total	**$268.20**
			Balance Due	**$268.20**

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Preparation of compound solution for in vivo application: an example of Naringenin
Author(s):	Shufen Liu, Jingcheng Dong, Qin Bian

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[X] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.