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TITLE:

A Mini-Invasive Internal Fixation Technique for Studying Immobilization-Induced Knee Flexion Contracture in Rats

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KEYWORDS:

Joint contractures; knee joint; immobility; rat model; mini-invasive; internal fixation

SUMMARY:

Here, we present a protocol to describe a minimally invasive technique for knee joint immobilization in a rat model. This reproducible protocol, basing on muscle-gap separation modus and the mini-incision skill, is suitable for studying the underlying molecular mechanism of acquired joint contracture.

ABSTRACT:

Joint contracture, resulting from a prolonged joint immobilization, is a common complication in orthopedics. Currently, utilizing an internal fixation to restrict knee joint mobility is a widely accepted model to generate experimental contracture. However, implanting application will inevitably cause surgical trauma to the animals. Aiming to develop a less invasive approach, we combined a muscle-gap separation modus with a previously reported mini-incision skill during the surgical procedure: Two mini skin incisions were made on the lateral thigh and leg, followed by performing muscle-gap separation to expose the bone surface. The rat knee joint was gradually immobilized by a preconstructed internal fixation at approximately 135° knee flexion

without interfering essential nerves or blood vessels. As expected, this simple technique permits rapid postoperative rehabilitation in animals. The correct position of the internal fixation was confirmed by an x-ray or micro-CT scanning analysis. The range of motion was significantly restricted in the immobilized knee joint than that observed in the contralateral knee joint demonstrating the effectiveness of this model. Besides, histological analysis revealed the development of fibrous deposition and adhesion in the posterior-superior knee joint capsule over time. Thus, this mini-invasive model may be suitable for mimicking the development of immobilized knee joint contracture.

INTRODUCTION:

Joint contractures are defined as a restriction in the passive range of motion (ROM) of a diarthrodial joint[1,2]. The current therapies aiming to prevent and treat joint contracture have achieved some success[3,4]. However, the underlying molecular mechanism of acquired joint contracture remains largely unknown[5]. The etiology of joint contractures in different social communities is highly diverse and includes genetic factors, posttraumatic states, chronic diseases, and prolonged immobility[6]. It is widely accepted that immobility is a critical issue in the development of acquired joint contracture[7]. People who suffer from major joint contracture may ultimately result in physical disability[8]. Thus, a stable and reproducible animal model is necessary for investigating the potential pathophysiological mechanisms of acquired joint contracture.

The currently built immobilization-induced knee joint contracture models are mostly achieved by utilizing non-invasive plaster casts, external fixations, and internal fixations. Watanabe et al. reported the possibility of the use of plaster cast immobilization on rat knee joints[9]. By wearing a special jacket, one side of the lower limb joint of the rat is immobilized by a cast. The rat knee joint can remain fully flexed without any surgical trauma[10,11]. However, both the hip and ankle joint movements are also affected by this form of immobilization, which may increase the degree of muscle atrophy in *quadriceps femoris* or *gastrocnemius*[12]. In addition, edema and congestion of the hind limbs must be avoided by replacing the cast at set time points, which may affect the continuity of immobility. Another accepted method for the establishment of a knee joint contracture model is using external surgical fixation. Nagai et al. combined Kirschner wire and steel wire into an external fixator, which immobilized the knee joint to approximately 140° of flexion[13]. In this method, a resin is used to cover the surface to prevent skin scratches. Although external fixation immobilization is robust and reliable[14,15], percutaneous Kirschner wire pin tracks may increase the risk of infection[16]. In our own experience, using the external fixation technique may reduce the daily activity of rats due to an increase in the conditioned lick behavior.

Alternatively, Trudel et al. described a well-accepted model of joint contracture in the rat knee joint based on a surgical internal fixation[17] (this method was modified from the one used by Evans and colleagues[18]). Notably, this method highlights the importance of utilizing a mini-incision technique to minimize the surgical wounds. The efficient development of joint contracture has been proved in this model[19]. However, the protocol on how to perform a minimal dissection to expose the bone surface is still unclear[20]. Also, the precise position where the screw is drilling is not fully understood. The implantation of the internal fixation through a subcutaneous or submuscular way is still controversial[21]. To solve these problems, we have modified this method

by including an appropriate muscle-gap separation modus, which allows a mini-invasive exposure of the bone surface and the placement of the implantation through a submuscular channel. This protocol led to rapid postoperative rehabilitation in rats after surgery. Animals developed a limited joint range of motion after joint immobilization, which was consistent with morphological changes of capsular adhesion obtained from the histological analysis. We also describe an exact possible location of the drilled screws as confirmed by X-ray analysis or micro-CT analysis. Thus, this study aimed to describe in detail a minimal-invasive technique in a knee joint contracture model that was established by a muscle-gap separation modus combined with a mini-incision method. We believe that minimally invasive techniques can both reduce animal trauma and effectively mimic the pathological process of joint flexion contracture.

PROTOCOL:

All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by The Third Affiliated Hospital of Sun Yat-sen University institutional animal care and use committee (permission number: 02-165-01). All the animal experiments were performed according to the ARRIVE guidelines.

1. Preoperative preparation

NOTE: **Figure 1** shows the design of the surgical procedure.

1.1. Rigidly immobilize the knee joint with a plastic plate and two metal screws at approximately 135° flexion.

NOTE: Perform the surgery at the proximal femur and the distal tibia without violating the joint component.

1.2. Prepare materials and instruments for internal fixation.

1.2.1 Construct a medical grade polypropylene plastic plates by cutting a 5 mL syringe (**Figure 2a**) using a surgical scissor to fit the following dimensions: length, 25 mm; width, 10 mm; thickness, 1 mm (**Figure 2b**). Smooth the perimeter of the plate with a scalpel vertically. Rinse the plate with sterile saline to wash off the debris by three times.

1.2.1.1. Sterilize with 75% ethanol for 4 h followed by irradiating with ultraviolet light for 3 h.

1.2.2. Pre-drilling holes in the plastic plate: Prepare a hand-held low-speed electric drill with a speed of about 0-4000 rpm (**Figure 2c**). Drill two holes at both ends of the plate, diameters are 1 mm and 0.9 mm, respectively (**Figure 2d**). Match both ends of the plate with M 1.4 mm x 8 mm and M 1.2 mm x 6 mm steel screws, respectively (**Figure 2e**).

1.2.2.1. Wipe with 75% ethanol and sterilize with UV light for 3 h before use.

1.3. Prepare surgical instruments: 1 straight Mosquito-Type hemostatic clamp, 1 smooth curved forceps, 2 eyelid retractors, 1 needle-holder, 1 tissue forceps, 1 suture scissor, 1 micro tissue scissor and 1 scalpel (**Figure 2f**). Sterilize the surgical instruments by autoclaving at 121.3 °C for 20 min and drying.

1.4. Experimental animals

1.4.1. Use Specific Pathogen Free (SPF) grade skeletally mature male Sprague-Dawley (or Wistar) rats, weighing between 250 - 350 g in the experiment.

NOTE: Choose either female or male rats for the experiment.

1.4.2. Place the rats in cages and keep in a 12 h light/12 h dark cycle-controlled laboratory room. Provide adequate food and water.

2. Surgery

2.1. Adjust the temperature. Place a warming pad on a surgical platform in a thermostatic operating room.

2.2. Anesthesia and skin preparation

2.2.1. Weigh the rat with an electronic scale and record.

2.2.2. Place the rats into inhalational anesthetic machines to induced anesthesia. Restrain the rat and perform an intraperitoneal injection of sodium pentobarbital (30 mg/kg). Confirm that the animal is sufficiently anesthetized loss of its righting reflex[22]. Cover the eyes with gauze to protect from drying.

2.2.3. Shave the lower body of the rat including the two hind limbs with an electric clipper and disinfect with a tincture of povidone iodine twice and 75% ethanol three times.

2.2.4. Place the rat laterally, and cover with the surgical drape exposing one side hind leg and hip.

2.2.5. Disinfect the surgical area again with povidone iodine.

2.3. Immobilize the knee joint with internal fixation using a mini-invasive technique.

NOTE: Keep the incision properly moist with sterile saline during the operation. The surgery usually requires two surgeons.

2.3.1 Mark the direction of skin incision. At the distal end of the femur greater trochanter, draw a line along the body surface projection of the muscle gap between the *vastus lateralis* and *biceps femoris* (**Figure 3a**). Incise the epidermis skin along the drawing line approximate 1.5 cm (**Figure**

3b).

2.3.2. Bluntly dissect the muscle gap between *vastus lateralis* and *biceps femoris* with a tissue forceps until the femoral shaft is exposed approximately 1 cm in length (**Figure 3c**). Use the retractor to facilitate continuous separation of the muscle gap.

2.3.3. Incise the epidermis skin approximate 1 cm along the body surface projection of the muscle gap between the *tibialis anterior* and *fibularis longus* on the distal lower extremity (**Figure 3d**). Bluntly dissect the muscle gap until the tibia is exposed approximately 1 cm in length (**Figure 3e**).

2.3.4. Separate the soft tissues by the retractor and the smooth forceps, keep perpendicular and drill one 1.0 mm diameter hole into the femoral shaft at a speed of 1,500 rpm using an electric drill (**Figure 3f**). The proper drilling position is approximate 8 mm below the lower edge of the greater trochanter. Quickly press the wound to stop bleeding.

NOTE: Proper drilling diameter can avoid intraoperative fractures.

2.3.5. Drill one 0.9 mm diameter hole into the tibia approximate 4 mm below the edge of the tibiofibular fusion (**Figure 4a**). Perform the drilling carefully to prevent the crushing of muscles or tendons.

2.3.6. Use the straight Mosquito-Type hemostatic clamp to form a submuscular course from the tibia hole to femur hole. The submuscular tunnel passes below the *gastrocnemius* in the tibia end and above the *gluteus medius*, below the *biceps femoris* in the femur end.

2.3.7. Use one M 1.4 mm x 8 mm steel screw to secure one end of the plastic plate (with the 1.0 mm diameter hole) in the proximal femur (**Figure 4b**). Use one M 1.2 mm x 6 mm steel screw to secure another end of the plastic plate (with the 0.9 mm diameter hole) in the distal tibia (**Figure 4c**). Ensure the knee joint without varus deformity.

2.4. Close the wound: Suture the myofascia, deep fasciae, and subcutaneous tissue using 4-0 absorbable sutures (**Figure 4d**). Close the skin with silk sutures (**Figure 4f**).

3. Postoperative management

3.1. Apply postoperative analgesia through intravenous injection of Flurbiprofen at 12.5 mg/kg. Add 5 mg/mL Neomycin into drinking water for 5 days after the surgery.

3.2. Apply Flumazenil (0.2 mg/kg) and Atipamezole (1 mg/kg) through subcutaneous injection to antagonize the anesthesia.

3.3. Check whether the hind limb had over-edema in case of vascular injury. Made sure that the rats can walk normally in the case of nerve injury during surgery.

4. Postoperative examination

4.1. Observe the healing of the surgical incision and physically examine the knee joint to evaluate early signs of infection every other day postoperatively. Check the degree of swelling of the ankle and metacarpophalangeal joint in case of continuous edema.

NOTE: Early postoperative infection can cause wound exudate, leg swelling, and delayed wound healing.

4.2. Perform X-ray imaging of the hindlimb to ensure that correctly placed the screws on the first postoperative day.

NOTE: A Micro-CT scan analysis is another alternative option to display the proper location and the direction of the steel screws.

4.3. Measure the passive range of motion (ROM) to evaluate the development of contracture. Take a knee joint ROM measurement at different time cohorts postoperatively as described previously[20].

4.3.1. In brief, euthanize the rats and skin the hindlimbs. Remove the immobilizer and measure the knee joint angle using a mechanical arthrometer at two torques (667 or 1,060 g/cm)[23].

4.3.2. Calculate the ROM as a result of the total contracture, the myogenic contracture, and the arthrogenic contracture separately based on the investigation objectives[24].

NOTE: Set different time cohorts (i.e., 1, 2, 4, 8, 16, and 32 weeks) according to the research objectives. The contralateral knee joint (non-operative or sham-operated) can serve as a control[2].

4.4. Histological analysis of the posterior knee joint capsules.

4.4.1. Prepare the joint tissues. Dissect the knee joint tissue and fix it with 4% paraformaldehyde. Decalcify and embed it in paraffin as previously reported[25]. Cut the sections (5 µm) at the medial midcondylar level in the sagittal plane.

NOTE: Choose to perform different evaluating staining including HE, aldehyde-fuchsin-Masson Goldner (AFMG), Elastica–Masson, or Immunohistochemistry staining for histological study in the joint capsule based on your study objectives[15,26].

4.4.2. Observe histomorphometric changes in the posterior knee joint capsules. Photograph the posterior region of the knee joint. Observe fibrous deposition and adhesion changes between the diaphysis-synovium junction and the meniscus[6].

NOTE: Pathological changes of joint capsule are considered to be a pathogenic factor for knee joint contracture. Measure the length, the thickness, and the capsular areas of the posterior

capsule as previously described according to the research content[27].

REPRESENTATIVE RESULTS:

We observed that rats received minimally invasive surgery can return to the regular diet just one day postoperatively. In particular, the surgical incision has scarred without exudate (**Figure 5a**). The swelling of the ankle and metacarpophalangeal joints in the operative hindlimb has almost wholly disappeared two days postoperatively (**Figure 5b**) when compared with the contralateral side (**Figure 5c**). None of the signs of early infection were found in the rats. Rats can stand and exercise regularly (**Figure 5d**). The surgical wounds had healed entirely on day twelve postoperatively (**Figure 5**).

Visually, the immobilized knee joint was contracted after four weeks of immobilization, while the mini-invasive surgery had no visible effect on the contralateral limb (**Figure 6a**). The X-ray image shows the correct placement of the steel screws in the femur or the tibia (**Figure 6b**), although it did not show the location of the plastic plate. We also employed a high-resolution micro-CT scanner to image the immobilized lower limb. The 3D reconstruction analysis demonstrated that the screws were drilled laterally (**Figure 6c**). The drilling position is approximate 8 mm below the lower edge of the greater trochanter at the proximal femur and just (approximate 4 mm) below the edge of the tibiofibular fusion at the distal tibia (**Figure 6c**).

We measured six rats at the end of two times (28 days and 56 days), respectively, to compare the arthrogenic ROM deficits on the immobilized knee joint and the contralateral side after myotomies of the transarticular muscles[20]. The contralateral knee joint (non-operative) serves as a control. After 28 days of immobilization, the average arthrogenic deficits in extension ROM was 29.4 ± 3.3° for the immobilized knee joint, significantly higher than that in control (4.8 ± 2.8°, $P<$ 0.05). The arthrogenic deficits in ROM increased during immobilization in a time-dependent manner, demonstrated by the average arthrogenic deficits of 40.7 ± 4.3° for the immobilized knee joint, significantly greater than that in control, 11.2 ± 3.8° on the 56 days of immobilization ($p < 0.05$) (**Figure 7**).

Using Elastica–Masson-Staining, we analyzed the posterior-superior knee joint capsule at three-time points. On day one immobilization, no adhesion was observed in the joint space between the postero-superior joint capsule and the femur in the immobilized or the contralateral side knee joint (**Figure 8a,d**). However, we observed that there was fibro-adipose tissue deposited and adhesion had developed in the joint space after 28 days of immobilization (**Figure 8e**). The fibrous tissues even partially replaced this deposition after 56 days of immobilization (**Figure 8f**) while this type of adhesion was not observed in the contralateral side at different time points (**Figure 8 a,b,c**).

FIGURE AND TABLE LEGENDS:

Figure 1: **Graphical illustration of a lateral view of the knee joint immobilized with an internal fixation at 135° of flexion.**

Figure 2: **Design the polypropylene plastic plate into an internal fixation.** (**a-b**) A polypropylene

plastic plate was cleaved from the syringe. The dotted lines represent the approximate plate range. The plate has the following dimensions: length, 25 mm; width, 10 mm; thickness, 1 mm. (**c**) Photograph of the hand-held electric drill. (**d**) Drills with the 0.9 mm and 1.0 mm diameter at each end of the plate. The specification of the screw is 1.4 x 8 mm and 1.2 x 6 mm respectively. (**e**) The final form of a preconstructed internal fixation. (**f**) The surgical instruments.

Figure 3: **Macrographs of surgical exposure the middle femur and the distal tibia using the mini-invasive technique.** (**a**) A black line indicates the skin incision between the *vastus lateralis* (upper marked area) and *biceps femoris* (lower marked area). The dotted lines represent the approximate muscle range. (**b**) The surgical incision between the muscles is illustrated. The incision is away from the *sciatic nerve*. The black line represents the orientation of the sciatic nerve. (**c**) The exposure of the femoral midshaft by muscle-gap separation with the *vastus lateralis* and *capput vertebralis* indicated. (**d-e**) The exposure of the tibia is shown in relation to the *fibularis longus*. (**f**) The drill hole in the femoral shaft is illustrated with the *vastus lateralis,* and *capput vertebralis* indicated.

Figure 4: **Implantation of internal fixation.** (**a**) The hole made in the tibia is illustrated with the *fibularis longus,* and the *flexor digitorum profundis* indicated. (**b-c**) The plastic plate screwed into the drill hole is illustrated in relation to the *caput vertebralis* (**b**) and the *fibularis longus* (**c**). (**d-e**) Wound closure using vicryl suture. The dotted line (**e**) represents the approximate plastic plate range. (**f**) Postoperative overall view of the mini-incision.

Figure 5: **Observation of surgical incision healing.** (**a**) The surgical incision has scarred two days postoperatively. (**b-c**) The swelling of the ankle and metacarpophalangeal joints in the postsurgical limb (**b**) has almost completely disappeared two days postoperatively. Arrowheads indicate the ankle joints. (**d**) A rat can stand normally. (**e-f**) The wound has completely healed twelve days postoperatively. Black arrows indicate surgical healing incision.

Figure 6: **Evaluation of knee joint immobilization**. (**a**) The macroscopic image illustrates a contraction of the left knee joint after four weeks of immobilization. (**b**) Overall x-ray image shows the placement of the screws. (**c**) Microcomputed tomography analysis of the immobilized knee joint. The white arrows represent the fixed screws.

Figure 7: Analysis of arthrogenic deficits in joint extension range of motion (ROM). Data are presented as mean ± SEM (n = 6 per group). The arthrogenic deficits in extension ROM of the immobilized knee joints are significantly higher than that of the contralateral, nonoperative side (serve as a control group). Limitation in ROM represents joint immobilization induced a typical knee flexion contracture. Statistical analysis: The Equality of Variances was performed using Levene's Test, ROM differences between the contralateral and immobilized groups were compared at two-time point (28 and 56 days) by two tails Student's *t* test. Significance difference was determined by $*P < 0.05$ from the control.

Figure 8: **Histological changes in the posterior-superior knee joint capsule analyzed by Elastica–Masson-Staining at different time points.** Representative images of the posterior-superior joint

capsule in the contralateral knee joint (non-operative, upper panels), and the immobilized knee joint (operative, lower panels) on day 1, 28, and 56 during joint immobilization. After a day of immobilization, synovium was thick, and no adhesion was observed in the joint space between the postero-superior joint capsule and the femur (indicated by asterisks in a left row). After 28 days of immobilization, there was fibro-adipose tissue deposited in the joint space and adhesion had developed between postero-superior joint capsule and the femur (indicated by arrowhead). On days 56 of immobilization, the deposits still existed, and there was fibrous tissue increasingly appeared (indicated by arrow). The black border in the bottom left corner represents the magnified image of the joint space between the postero-superior joint capsule and the femur. F: femur; T: tibia; M: meniscus, the posterior horn; JS: joint space. Scale bar = 50 μm.

DISCUSSION:

This study aimed to elucidate a step-by-step knee joint immobilization method using a mini-invasive technique that permits rapid postoperative rehabilitation in animals after surgery. Conventionally, the muscle-gap separation approach is thought to be a minimally invasive technique in orthopedic surgery. As expected, we found that rats can return to a normal diet and activities just one day postoperatively, which was consistent with the previous study. Moreover, no artery or nerve injury occurred after the surgery, evidence that the muscle-gap separation modus ensured an adequate and safe bone exposure method. Although the invasive surgical effects can be reduced by using plaster casts, the possibility of edema occurrence in the hind limbs may affect the continuity of immobility. In this study, the ankle or toe swelling caused by surgical procedures disappeared entirely after two days postoperatively. These results highlight a reliable and stable joint immobilization model created by a mini-invasive technique aligned with the principle of rapid recovery. Clinically, the flexion contracture that is caused by immobilization is closer to a non-inflammatory course[6]. Edema can lead to the release of inflammatory mediators[4]. Therefore, using plaster casts to induced joint contracture cannot indeed be harmless. In the present study, two separate small incisions (of 1-1.5 cm) were performed on the femoral and tibial sides, respectively. The incision lengths were similar to the size of the incision that is required for K-wire drilling. Therefore, the mini-invasive effect of this method is more conducive to reducing trauma to that of external fixation. Besides, a previous randomized controlled trial demonstrated a possible correlation between the application of external fixation (percutaneously) and the increased risk of infection in the limb[16]. Considering there no rats had an early infection sign in the research, we assumed that the muscle gap separation technique is the key to this model because it can reduce bleeding and unnecessary cutting. Also, the internal fixator was trimmed down from the syringe, it is low cost and most importantly, non-toxic to animals. Although both the lateral and medial surgical approaches can establish an effective rat model of knee flexion contracture[28], this small-invasive technique, however, may only be implemented using the lateral approach rather than using the medial approach.

To our best knowledge, the precise screw drilling position at the proximal femur or distal tibia is not fully understood. Choosing to drill a hole in the middle section of the tibia may affect the blood supply in the tibia. The results obtained from the micro-CT analysis indicated that the proper drilling position is approximate 8 mm below the lower edge of the greater trochanter and approximate 4 mm below the edge of the tibiofibular fusion. The proper drilling position can help

avoid effects on the joint component or blood supply. However, the implantation of the internal fixation through a subcutaneous or submuscular way is still controversial. Interestingly, performing the muscle-gap separation technique is convenient for placing the implantation through a submuscular channel to a certain extent.

The results from the joint angle measurement were consistent with the histological analysis, demonstrating that knee joint contracture was successfully induced in the immobilized hindlimb. The average arthrogenic deficits in extension ROM was 29.4 ± 3.3°, 40.7 ± 4.3° on the immobilized knee joint at the end of 28 days and 56 days of immobilization, respectively, which were significantly higher than that in control ($P < 0.05$). We also found that typical adhesion had developed between in the joint space between the postero-superior joint capsule and the femur in the immobilized side knee joint (**Figure 8e,f**), which indicates that using the mini-invasive technique will not interfere with the occurrence of joint contracture. Taken together, the research indicates that this mini-invasive model produces stable results and is effective in inducing acquired joint flexion contracture.

This mini-invasive model still has some limitations. First, the tibia side screw will inevitably irritate the nearby tendons, including the *fibularis longus*. Second, drilling into the cortical bone may cause fractures. Third, there is still a chance of fixation failure. We believe that the use of 3D-built individualized splints is a possible option for building a non-invasive knee joint contracture model in the future[29].

In conclusion, the present study describes a mini-invasive knee joint contracture model that is based on a combination of the muscle gap separation modus and the mini-incision method. Given that internal surgical fixations can produce a well-accepted model of joint contracture, this mini-invasive technique may be useful in the study of immobilization-induced knee flexion contracture.

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DISCLOSURES:

The authors have nothing to disclose.

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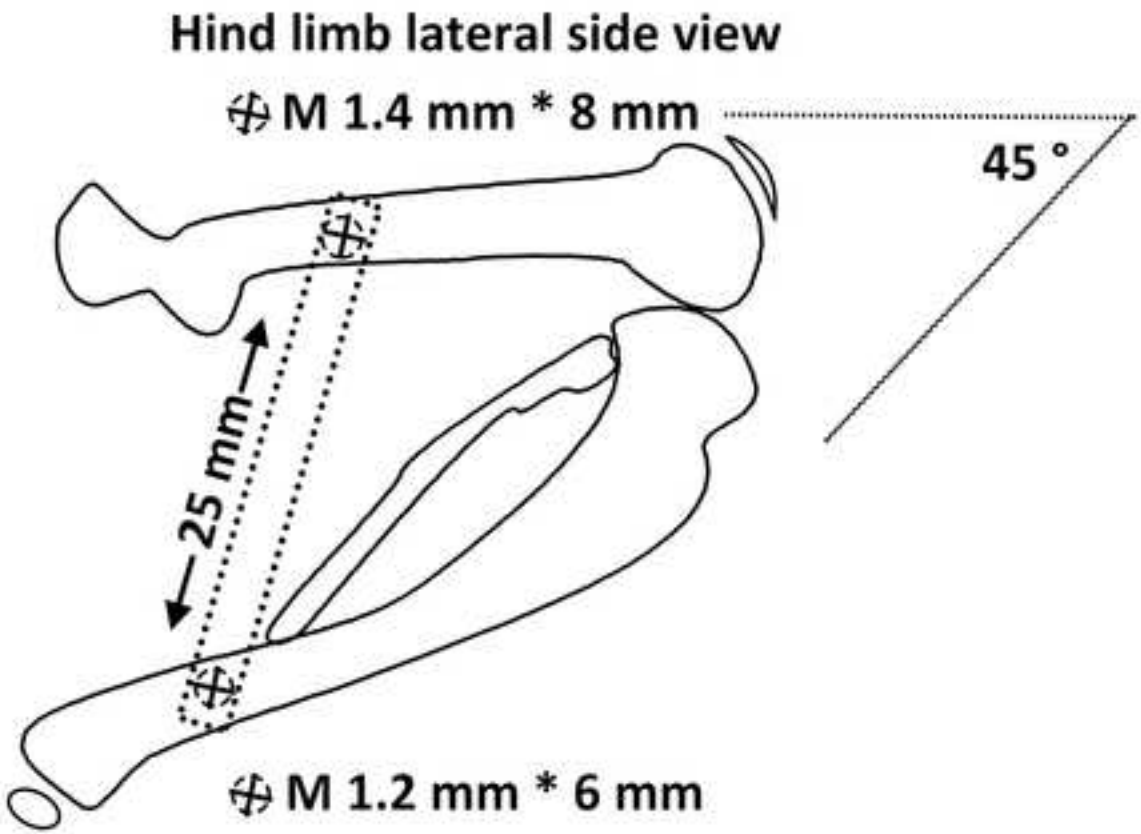

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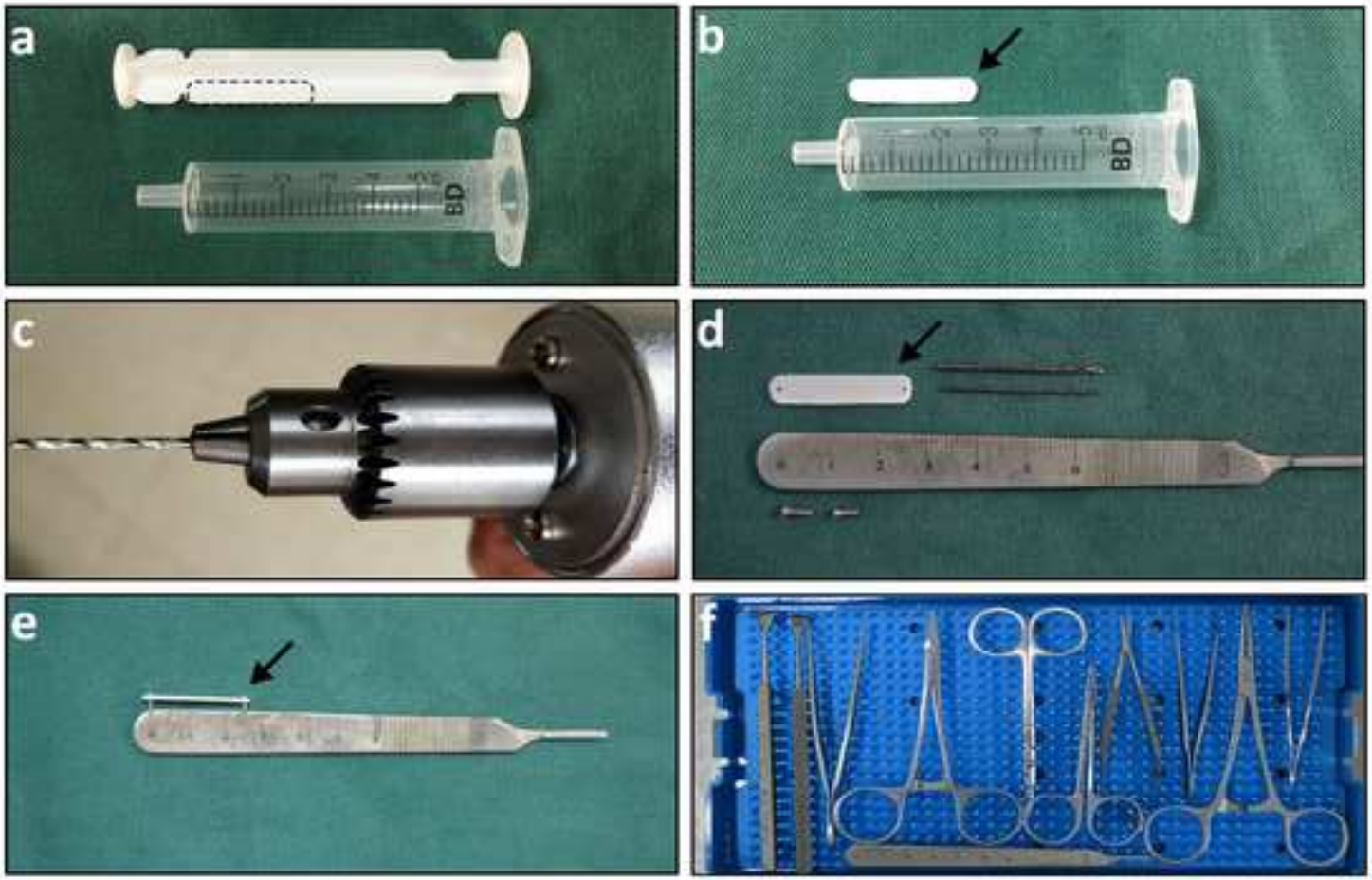

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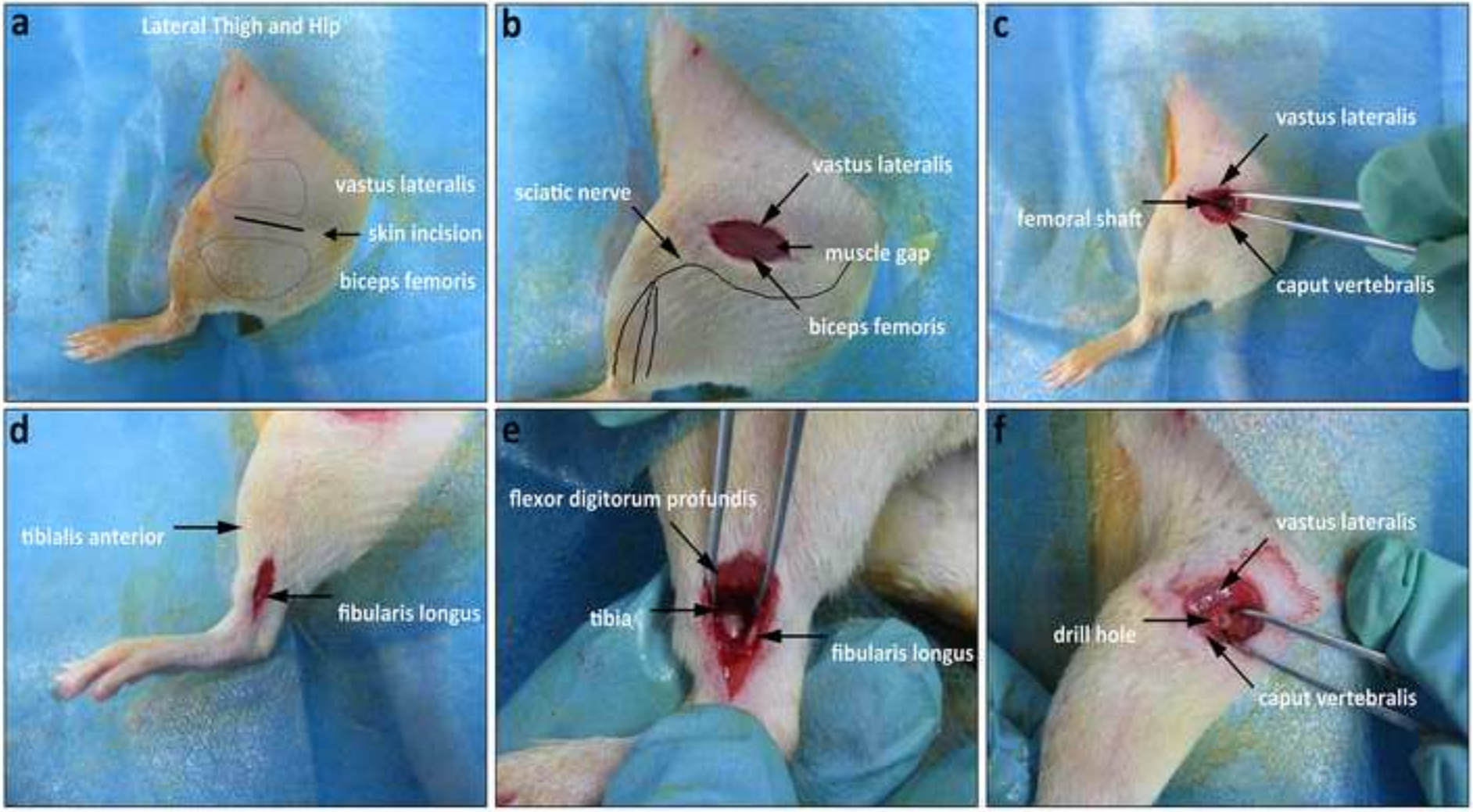

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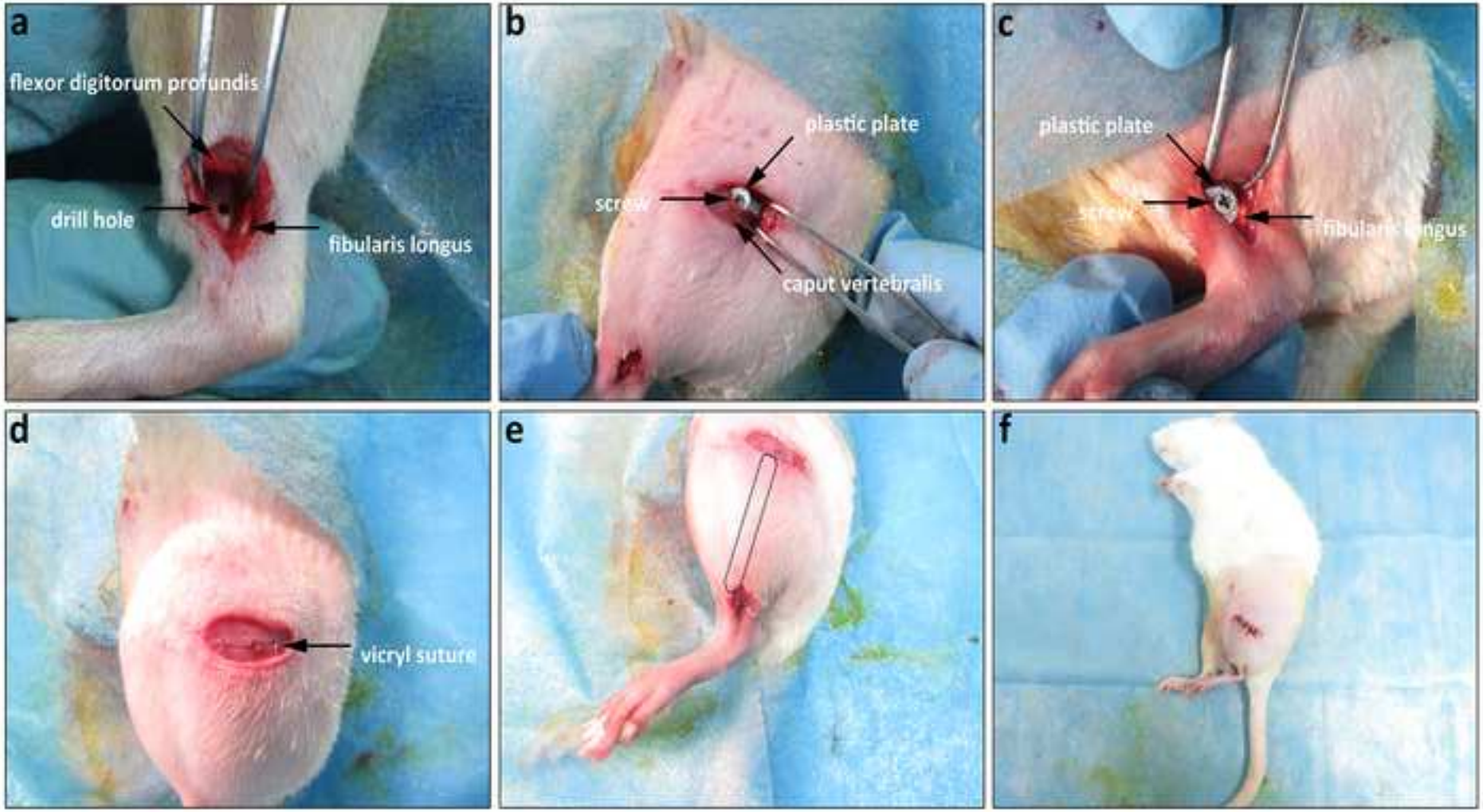

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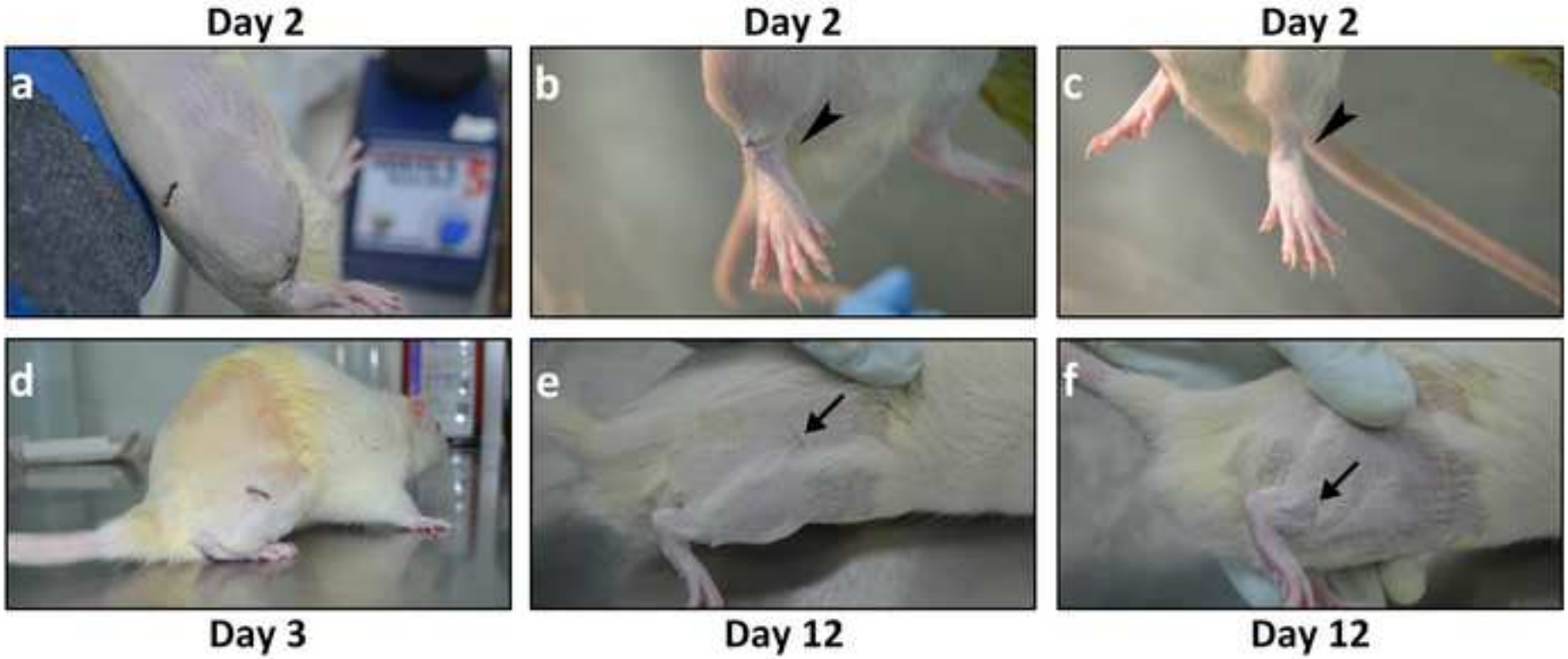

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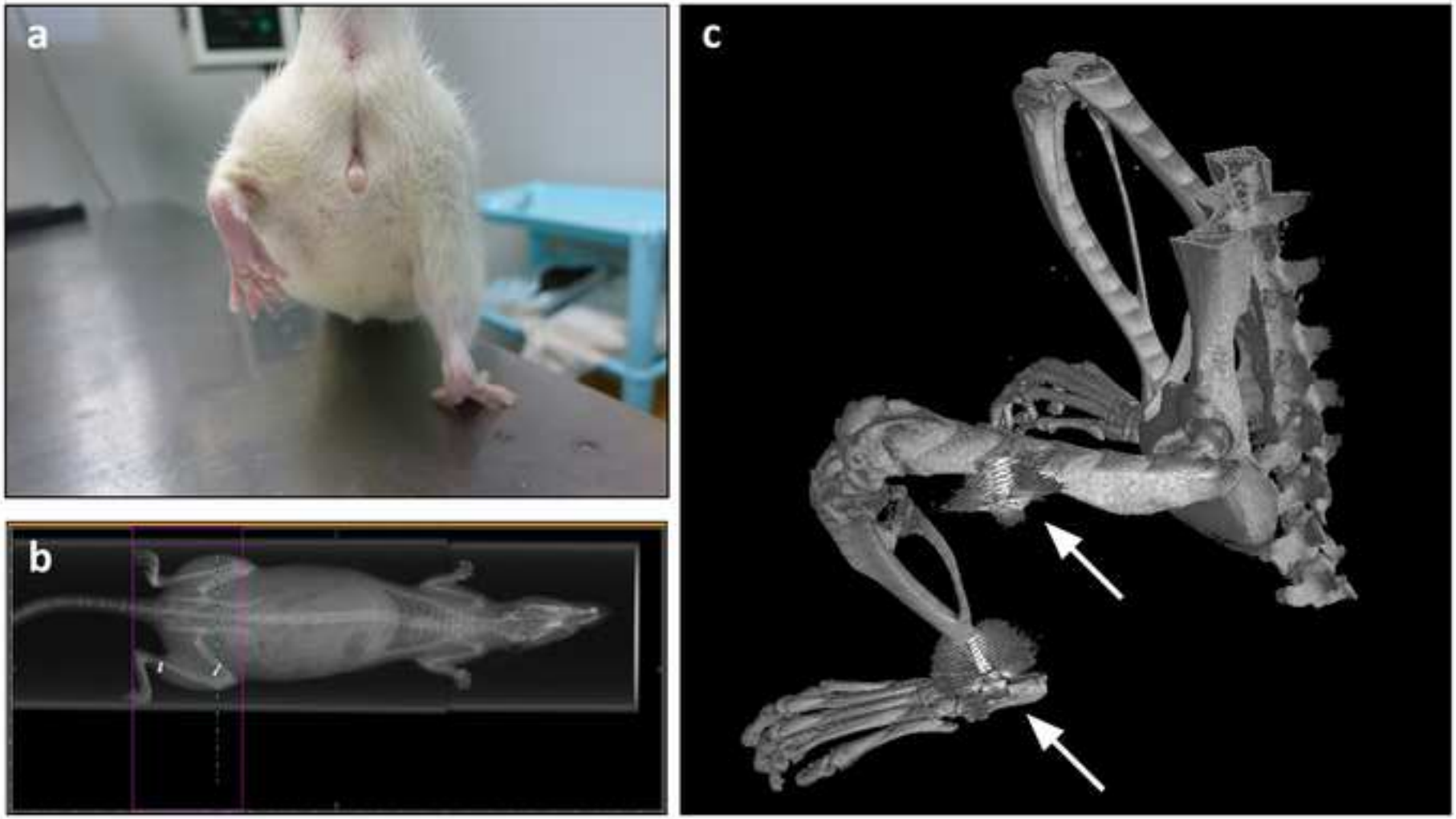

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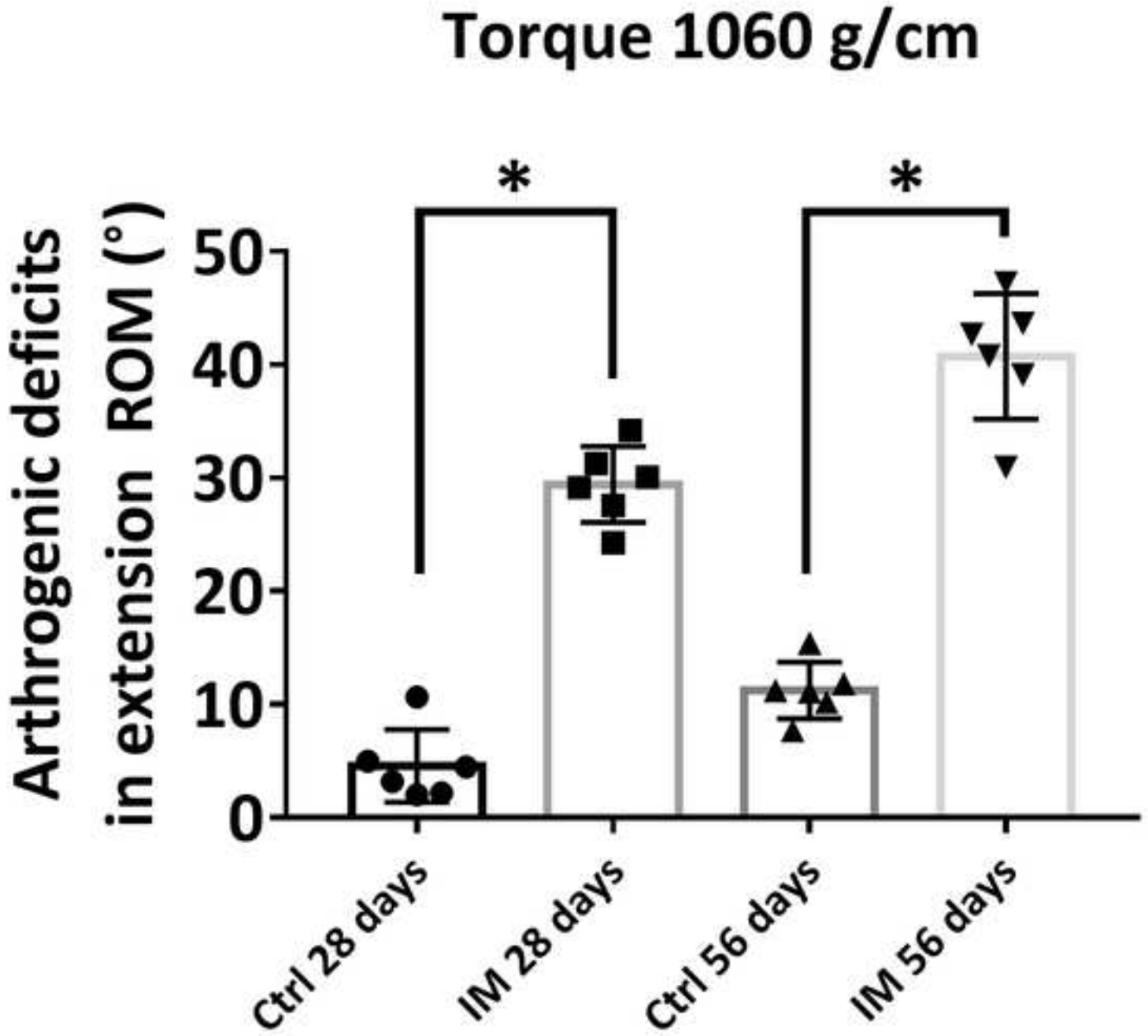

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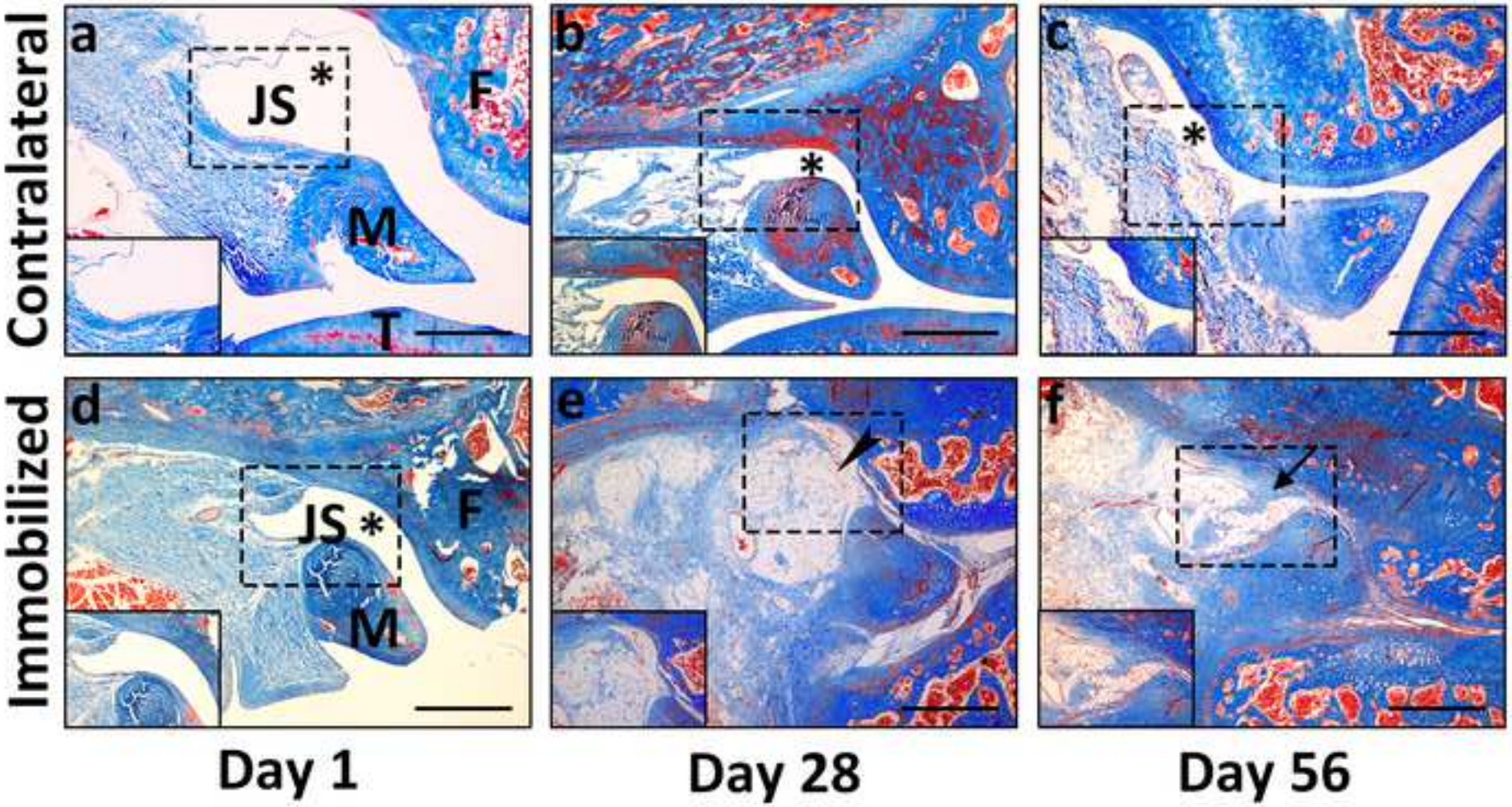

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anerdian	Shanghai Likang Ltd.	310173	antibacterial
Atipamezole	MCE	HY-12380A	antagonist anesthesia
Cross screwdriver	STANLEY	PH0*125mm	tighten the screws
Electric drill	WEGO	185	drill hole(with stainless steel drill 0.9mm;1.0mm)
Flumazenil	MCE	HY-B0009	antagonist anesthesia
Flurbiprofen	MCE	HY-10582	alleviate pain
Isoflurane	RWD	R510-22	start anaesthetize
Microsurgical instruments	RWD	/	Orthopaedic surgical instruments for animals
Neomycin	Sigma	N6386	antibacterial
Sodium pentobarbital	Sigma	P3761	anaesthetize
Stainless Steel screws	WEGO	m1.4*8; m1.2*6	screw(part of internal fixation)
Syringe	WEGO	3151474	use for plastic plate(part of internal fixation)
μ-CT	ALOKA	Latheta LCT-200	in vivo CT scan

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Title of Article: A mini-incision technique with internal fixation rat model for studying immobilization-induced knee flexion contracture

Author(s): Shihai Jiang, Xiaoyou Yi, Yuansen Luo, Dongjie Yu, Yuangao Liu, Lei Zhu, Kun Wang

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TITLE:

A Mini-Invasive Internal Fixation Technique for Studying Immobilization-Induced Knee Flexion Contracture in Rats

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KEYWORDS:

Joint contractures; knee joint; immobility; rat model; mini-invasive; internal fixation

SUMMARY:

Here, we present a protocol to describe a minimally invasive technique for knee joint immobilization in a rat model. This reproducible protocol, basing on muscle-gap separation modus and the mini-incision skill, is suitable for studying the underlying molecular mechanism of acquired joint contracture.

ABSTRACT:

Joint contracture, resulting from a prolonged joint immobilization, is a common complication in orthopedics. Currently, utilizing an internal fixation to restrict knee joint mobility is a widely accepted model to generate experimental contracture. However, implanting application will inevitably cause surgical trauma to the animals. Aiming to develop a less invasive approach, we combined a muscle-gap separation modus with a previously reported mini-incision skill during the surgical procedure: Two mini skin incisions were made on the lateral thigh and leg, followed by performing muscle-gap separation to expose the bone surface. The rat knee joint was gradually immobilized by a preconstructed internal fixation at approximately 135° knee flexion

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without interfering essential nerves or blood vessels. As expected, this simple technique permits rapid postoperative rehabilitation in animals. The correct position of the internal fixation was confirmed by an x-ray or micro-CT scanning analysis. The range of motion was significantly restricted in the immobilized knee joint than that observed in the contralateral knee joint demonstrating the effectiveness of this model. Besides, histological analysis revealed the development of fibrous deposition and adhesion in the posterior-superior knee joint capsule over time. Thus, this mini-invasive model may be suitable for mimicking the development of immobilized knee joint contracture.

INTRODUCTION:

Joint contractures are defined as a restriction in the passive range of motion (ROM) of a diarthrodial joint[1,2]. The current therapies aiming to prevent and treat joint contracture have achieved some success[3,4]. However, the underlying molecular mechanism of acquired joint contracture remains largely unknown[5]. The etiology of joint contractures in different social communities is highly diverse and includes genetic factors, posttraumatic states, chronic diseases, and prolonged immobility[6]. It is widely accepted that immobility is a critical issue in the development of acquired joint contracture[7]. People who suffer from major joint contracture may ultimately result in physical disability[8]. Thus, a stable and reproducible animal model is necessary for investigating the potential pathophysiological mechanisms of acquired joint contracture.

The currently build immobilization-induced knee joint contracture models are mostly achieved by utilizing non-invasive plaster casts, external fixations, and internal fixations. Watanabe *et al*. reported the possibility of the use of plaster cast immobilization on rat knee joints[9]. By wearing a special jacket, one side of the lower limb joint of the rat is immobilized by a cast. The rat knee joint can remain fully flexed without any surgical trauma[10,11]. However, both the hip and ankle joint movements are also affected by this form of immobilization, which may increase the degree of muscle atrophy in *quadriceps femoris* or *gastrocnemius*[12]. In addition, edema and congestion of the hind limbs must be avoided by replacing the cast at set time points, which may affect the continuity of immobility. Another accepted method for the establishment of a knee joint contracture model is using external surgical fixation. Nagai *et al*. combined Kirschner wire and steel wire into an external fixator, which immobilized the knee joint to approximately 140° of flexion[13]. In this method, a resin is used to cover the surface to prevent skin scratches. Although external fixation immobilization is robust and reliable[14,15], percutaneous Kirschner wire pin tracks may increase the risk of infection[16]. In our own experience, using the external fixation technique may reduce the daily activity of rats due to an increase in the conditioned lick behavior.

Alternatively, Trudel *et al*. described a well-accepted model of joint contracture in the rat knee joint based on a surgical internal fixation[17] (this method was modified from the one used by Evans and colleagues[18]). Notably, this method highlights the importance of utilizing a mini-incision technique to minimize the surgical wounds. It has been proved that the efficient development of joint contracture in this model[19]. However, the protocol on how to perform a minimal dissection to expose the bone surface is still unclear[20]. Also, the precise position where the screw is drilling is not fully understood. The implantation of the internal fixation through a subcutaneous or submuscular way is still controversial[21]. To solve these problems, we have modified this method

by including an appropriate muscle-gap separation modus, which allows a mini-invasive expose of the bone surface and to place the implantation through a submuscular channel. This protocol led to rapid postoperative rehabilitation in rats after surgery. Animals have developed a limited joint range of motion after joint immobilization which was consistent with morphological changes of capsular adhesion obtained from the histological analysis. We also describe an exact possible location of the drilled screws that confirmed by X-ray analysis or micro-CT analysis. Thus, this study aimed to describe in detail, in a knee joint contracture model, a minimal-invasive technique that was established by a muscle-gap separation modus combined with a mini-incision method. We believe that minimally invasive techniques can both reduce animal trauma and effectively mimic the pathological process of joint flexion contracture.

PROTOCOL:

All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by The Third Affiliated Hospital of Sun Yat-sen University institutional animal care and use committee (permission number: 02-165-01). All the animal experiments were performed according to the ARRIVE guidelines.

1. Preoperative Preparation

1.1. Design of the surgical procedure (**Figure 1**). Rigidly immobilize the knee joint with a plastic plate and two metal screws at approximately 135° flexion.

NOTE: Perform the surgery at the proximal femur and the distal tibia without violating the joint component.

1.2. Prepare materials and instruments for internal fixation.

1.2.1 Construct a medical grade polypropylene plastic plates by cutting a 5mL syringe (**Figure 2a**) using a surgical scissor to fit the following dimensions: length, 25 mm; width, 10 mm; thickness, 1 mm (**Figure 2b**). Smooth the perimeter of the plate with a scalpel vertically. Rinse the plate with sterile saline to wash off the debris by three times. Sterilize with 75 % ethanol for 4 h followed by irradiating with ultraviolet light for 3 h.

1.2.2. Pre-drilling holes in the plastic plate: Prepare a hand-held low-speed electric drill with a speed of about 0-4000 *rpm* (**Figure 2c**). Drill two holes at both ends of the plate, diameters are 1 mm and 0.9 mm, respectively (**Figure 2d**). Mach both ends of the plate with M 1.4 mm * 8 mm and M 1.2 mm * 6 mm steel screw, respectively (**Figure 2e**). Wipe with 75 % ethanol and sterilize with UV light for 3 h before use.

1.3. Prepare surgical instruments: 1 straight Mosquito-Type hemostatic clamp, 1 smooth curved forceps, 2 eyelid retractors, 1 needle-holder, 1 tissue forceps, 1 suture scissor, 1 micro tissue scissor and 1 scalpel (**Figure 2f**). Sterilize the surgical instruments by autoclaving at 121.3 °C for 20 min and drying.

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1.4. Experimental Animals.

1.4.1. Use Specific Pathogen Free (SPF) grade skeletally mature male Sprague-Dawley (or Wistar) rats, weighing between 250 - 350 g in the experiment.

NOTE: Choose either female or male rats for the experiment.

1.4.2. Place the rats in cages and keep in a 12 h light/12 h dark cycle-controlled laboratory room. Provide adequate food and water.

2. Surgery process

2.1. Adjust the temperature. Place a warming pad on a surgical platform in a thermostatic operating room.

2.2. Anesthesia and skin preparation.

2.2.1. Weight the rat with an electronic scale and record.

2.2.2. Place the rats into inhalational anesthetic machines to induced anesthesia. Restrain the rat and perform an intraperitoneal injection of sodium pentobarbital (30 mg/kg). Confirmed the animal is sufficiently anesthetized as determined by losing its righting reflex[22]. Cover the eyes with gauze to protect from drying.

2.2.3. Shave the lower body of the rat including the two hind limbs with an electric clipper and disinfect with a tincture of iodine twice and 75% ethanol three times.

2.2.4. Place the rat laterally, cover with the surgical drape exposing one side hind leg and hip.

2.2.5. Disinfect the surgical area again with Povidone Iodine.

2.3. Immobilize of the knee joint with internal fixation using a mini-invasive technique.

NOTE: Keep the incision properly moist with sterile saline during the operation. The surgery usually requires two surgeons.

2.3.1 Mark the direction of skin incision. At the distal end of the femur greater trochanter, draw a line along the body surface projection of the muscle gap between the *vastus lateralis* and *biceps femoris* (**Figure 3a**). Incise the epidermis skin along the drawing line approximate 1.5 cm (**Figure 3b**).

2.3.2. Bluntly dissect the muscle gap between *vastus lateralis* and *biceps femoris* with a tissue forceps until the femoral shaft is exposed approximately 1 cm in length (**Figure 3c**). Use the retractor to facilitate continuous separation of the muscle gap.

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2.3.3. Incise the epidermis skin approximate 1 cm along the body surface projection of the muscle gap between the *tibialis anterior* and *fibularis longus* on the distal lower extremity (**Figure 3d**). Bluntly dissect the muscle gap until the tibia is exposed approximately 1 cm in length (**Figure 3e**).

2.3.4. Separate the soft tissues by the retractor and the smooth forceps, keep perpendicular and drill one 1.0 mm diameter hole into the femoral shaft at a speed of 1,500 *rpm* using an electric drill (**Figure 3f**). The proper drilling position is approximate 8 mm below the lower edge of the greater trochanter. Quickly press the wound to stop bleeding.

NOTE: Proper drilling diameter can avoid intraoperative fractures.

2.3.5. Again, drill one 0.9 mm diameter hole into the tibia approximate 4 mm below the edge of the tibiofibular fusion (**Figure 4a**). Perform the drilling carefully to prevent the crushing of muscles or tendons.

2.3.6. Use the straight Mosquito-Type hemostatic clamp to form a submuscular course from the tibia hole to femur hole. The submuscular tunnel passes below the *gastrocnemius* in the tibia end and above the *gluteus medius*, below the *biceps femoris* in the femur end.

2.3.7. Use one M 1.4 mm * 8 mm steel screw to secure one end of the plastic plate (with the 1.0 mm diameter hole) in the proximal femur (**Figure 4b**). Use one M 1.2 mm * 6 mm steel screw to secure another end of the plastic plate (with the 0.9 mm diameter hole) in the distal tibia (**Figure 4c**). Ensure the knee joint without varus deformity.

2.4. Close of the wound: Suture the myofascia, deep fasciae, and subcutaneous tissue using 4-0 absorbable sutures (**Figure 4d**). Close the skin with silk sutures (**Figure 4f**).

3. Postoperative management

3.1. Apply postoperative analgesia through intravenous injection of Flurbiprofen at 12.5 mg/kg. Add Neomycin 5 mg/mL into drinking water for five days after the surgery.

3.2. Apply Flumazenil (0.2 mg/kg) and Atipamezole (1 mg/kg) through subcutaneous injection to antagonize the anesthesia.

3.3. Check whether the hind limb had over-edema in case of vascular injury. Made sure that the rats were able to walk normally in the case of nerve injury during surgery.

4. Postoperative examination

4.1. Observe the healing of the surgical incision and physical examine the knee joint to evaluate early signs of infection every other day postoperatively. Check the degree of swelling of the ankle and metacarpophalangeal joint in case of continuous edema.

Note: Early postoperative infection can cause wound exudate, leg swelling, and delayed wound healing.

4.2. Perform X-ray imaging of the hindlimb to ensure that correctly placed the screws on the first postoperative day.

NOTE: A Micro-CT scan analysis is another alternative option to display the proper location and the direction of the steel screws.

4.3. Measure the passive range of motion (ROM) to evaluate the development of contracture. Take a knee joint ROM measurement at different time cohorts postoperatively as described previously[20]. In brief, euthanasia the rats and skin the hindlimbs. Remove the immobilizer and measure the knee joint angle using a mechanical arthrometer at two torques (667 or 1,060 g/cm)[23]. Calculate the ROM as a result of the total contracture, the myogenic contracture, and the arthrogenic contracture separately based on the investigation objectives[24].

NOTE: Set different time cohorts (i.e., 1, 2, 4, 8, 16, and 32 weeks) according to your research objectives. The contralateral knee joint (non-operative or sham-operated) can serve as a control[2].

4.4. Histological analysis of the posterior knee joint capsules.

4.4.1. Prepare the joint tissues. Dissect the knee joint tissue and fix it with 4 % paraformaldehyde. Decalcify and embed it in paraffin as previously reported[25]. Cut the sections (5-µm) at the medial midcondylar level in the sagittal plane.

Note: Choose to perform different evaluating staining including HE, aldehyde-fuchsin-Masson Goldner (AFMG), Elastica–Masson, or Immunohistochemistry staining for histological study in the joint capsule based on your study objectives [15,26].

4.4.2. Observe histomorphometric changes in the posterior knee joint capsules. Photograph the posterior region of the knee joint. Observe fibrous deposition and adhesion changes between the diaphysis-synovium junction and the meniscus[6].

NOTE: Pathological changes of joint capsule are considered to be a pathogenic factor for knee joint contracture. Measure the length, the thickness, and the capsular areas of the posterior capsule as previously described according to the research content[27].

REPRESENTATIVE RESULTS:

We observed that rats received minimally invasive surgery can return to the regular diet just one day postoperatively. In particular, the surgical incision has scarred without exudate (**Figure 5a**). The swelling of the ankle and metacarpophalangeal joints in the operative hindlimb has almost wholly disappeared two days postoperatively (**Figure 5b**) when compared with the contralateral side (**Figure 5c**). None of the signs of early infection were found in the rats. Rats can stand and

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exercise regularly (**Figure 5d**). The surgical wounds had healed entirely on day twelve postoperatively (**Figure 5**).

Visually, the immobilized knee joint was contracted after four weeks of immobilization, while the mini-invasive surgery had no visible effect on the contralateral limb (**Figure 6a**). The X-ray image shows the correct placement of the steel screws in the femur or the tibia (**Figure 6b**), although it did not show the location of the plastic plate. We also employed a high-resolution micro-CT scanner to image the immobilized lower limb. The 3D reconstruction analysis demonstrated that the screws were drilled laterally (**Figure 6c**). The drilling position is approximate 8 mm below the lower edge of the greater trochanter at the proximal femur and just (approximate 4 mm) below the edge of the tibiofibular fusion at the distal tibia (**Figure 6c**).

We hired six rats at the end of two times (28 days and 56 days), respectively, to compare the arthrogenic ROM deficits on the immobilized knee joint and the contralateral side after myotomies of the transarticular muscles[20]. The contralateral knee joint (non-operative) serves as a control. After 28 days immobilization, the average arthrogenic deficits in extension ROM was 29.4 ± 3.3° for the immobilized knee joint, significantly higher than that in control (4.8 ± 2.8°, $P< 0.05$). The arthrogenic deficits in ROM increased during immobilization in a time-dependent manner, demonstrating by the average arthrogenic deficits was 40.7 ± 4.3° for the immobilized knee joint, significantly greater than that in control, 11.2 ± 3.8° on the 56 days immobilization ($p < 0.05$) (**Figure 7**).

Using Elastica–Masson-Staining, we analyzed the posterior-superior knee joint capsule at three-time points. On day one immobilization, no adhesion was observed in the joint space between the postero-superior joint capsule and the femur in the immobilized or the contralateral side knee joint (**Figure 8a, d**). However, we observed that there was fibro-adipose tissue deposited and adhesion had developed in the joint space after 28 days immobilization (**Figure 8e**). The fibrous tissues even partially replaced this deposition after 56 days immobilization (**Figure 8f**) while this type of adhesion was not observed in the contralateral side at different time points (**Figure 8 a,b, c**).

FIGURE AND TABLE LEGENDS:

Figure 1: **Graphical illustration of a lateral view of the knee joint immobilized with an internal fixation at 135° of flexion.**

Figure 2: **Design the polypropylene plastic plate into an internal fixation.** (a-b) A polypropylene plastic plate was cleaved from the syringe. The dotted lines represent the approximate plate range. The plate has the following dimensions: length, 25 mm; width, 10 mm; thickness, 1 mm. (c) Photograph of the hand-held electric drill. (d) Drills with the 0.9 mm and 1.0 mm diameter at each end of the plate. The specification of the screw is 1.4 * 8 mm and 1.2 * 6 mm respectively. (e) The final form of a preconstructed internal fixation. (f) The surgical instruments.

Figure 3: **Macrographs of surgical exposure the middle femur and the distal tibia using the mini-invasive technique.** (a) A black line indicates the skin incision between the *vastus lateralis* (upper

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2. Arthrogenic ROM deficits = 180° - arthrogenic ROM.

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marked area) and *biceps femoris* (lower marked area). The dotted lines represent the approximate muscle range. (b) The surgical incision between the muscles is illustrated. The incision is away from the *sciatic nerve*. The black line represents the orientation of the sciatic nerve. (c) The exposure of the femoral midshaft by muscle-gap separation with the *vastus lateralis* and *capput vertebralis* indicated. (d-e) The exposure of the tibia is shown in relation to the *fibularis longus*. (f) The drill hole in the femoral shaft is illustrated with the *vastus lateralis,* and *capput vertebralis* indicated.

Figure 4: **Implantation of internal fixation.** (a) The hole made in the tibia is illustrated with the *fibularis longus,* and the *flexor digitorum profundis* indicated. (b-c) Screwe the plastic plate screwed into the drill hole is illustrated in relation to the *caput vertebralis* (b) and the *fibularis longus* (c). (d-e) Wound closure using vicryl suture. The dotted line (e) represents the approximate plastic plate range. (f) Postoperative overall view of the mini-incision.

Figure 5: **Observation of surgical incision healing.** (a) The surgical incision has been scarred two days postoperatively. (b-c) The swelling of the ankle and metacarpophalangeal joints in the postsurgical limb (b) has almost completely disappeared two days postoperatively. Arrowheads indicate the ankle joints. (d) A rat can stand normally. (e-f) The wound has completely healed twelve days postoperatively. Black arrows indicate surgical healing incision.

Figure 6: **Evaluation of knee joint immobilization**. (a) The macroscopic image illustrates a contraction of the left knee joint after four weeks of immobilization. (b) Overall x-ray image shows the placement of the screws. (c) Microcomputed tomography analysis of the immobilized knee joint. The white arrows represent the fixed screws.

Figure 7: Analysis of arthrogenic deficits in joint extension range of motion (ROM). Data are presented as mean ± SEM (n = 6 per group). The arthrogenic deficits in extension ROM of the immobilized knee joints are significantly higher than that of the contralateral, nonoperative side (serve as a control group). Limitation in ROM represents joint immobilization induced a typical knee flexion contracture. Statistical analysis: The Equality of Variances was performed using Levene's Test, ROM differences between the contralateral and immobilized groups were compared at two-time point (28 and 56 days) by two tails Student's *t* test. Significance difference was determined by $*P < 0.05$ from the control.

Figure 8: **Histological changes in the posterior-superior knee joint capsule analyzed by Elastica–Masson-Staining at different time points.** Representative images of the posterior-superior joint capsule in the contralateral knee joint (non-operative, upper panels), and the immobilized knee joint (operative, lower panels) on day 1, 28, and 56 during joint immobilization. After a day of immobilization, synovium was thick, and no adhesion was observed in the joint space between the postero-superior joint capsule and the femur (indicated by asterisks in a left row). After 28 days of immobilization, there was fibro-adipose tissue deposited in the joint space and adhesion had developed between postero-superior joint capsule and the femur (indicated by arrowhead). On days 56 of immobilization, the deposits still existed, and there was fibrous tissue increasingly appeared (indicated by arrow). The black border in the bottom left corner represents the

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magnified image of the joint space between the postero-superior joint capsule and the femur. F: femur; T: tibia; M: meniscus, the posterior horn; JS: joint space. Scale bar = 50 μm.

DISCUSSION:

This study aimed to elucidate a step-by-step knee joint immobilization method using a mini-invasive technique that permits rapid postoperative rehabilitation in animals after surgery. Conventionally, muscle-gap separation approach is thought to be a minimally invasive technique in orthopedic surgery. As expected, we found rats can return to normal diet and activities just one day postoperatively, which were consistent with the previous study. Moreover, no artery or nerve injury was occurred after the surgery, evidence that the muscle-gap separation modus ensured an adequate and safety bone exposure method. Although the invasive surgical effects can be reduced by using plaster casts, the possibility of edema occurrence in the hind limbs may affect the continuity of immobility. In our study, the ankle or toe swelling caused by surgical procedures disappeared entirely after two days postoperatively. These results highlight a reliable and stable joint immobilization model created by a mini-invasive technique in lines with the principle of rapid recovery. Clinically, the flexion contracture that is caused by immobilization is closer to a non-inflammatory course[6]. Edema can lead to the release of inflammatory mediators[4]. Therefore, using plaster casts to induced joint contracture cannot indeed be harmless. In the present study, two separate small incisions (of 1-1.5 cm) were performed on the femoral and tibial sides, respectively. The incision lengths were similar to the size of the incision that is required for K-wire drilling. Therefore, the mini-invasive effect of this method is more conducive to reducing trauma to that of external fixation. Besides, a previous randomized controlled trial demonstrated a possible correlation between the application of external fixation (percutaneously) and the increased risk of infection in the limb[16]. Considering there were no rats have an early infection sign in our research, we assumed that the muscle gap separation technique is the key to this model because it can reduce bleeding and unnecessary cutting. Also, the internal fixator was trimmed down from the syringe, it is low cost and most importantly, non-toxic to animals. Although both the lateral and medial surgical approaches can establish an effective rat model of knee flexion contracture[28], this small-invasive technique, however, may only be implemented using the lateral approach rather than using the medial approach.

To our best knowledge, where the precise screw drilling position at the proximal femur or distal tibia is not fully understood. Choosing to drill a hole in the middle section of the tibia may affecting the blood supply in Tibia. Our results obtained from Micro-ct analysis indicate that the proper drilling position is approximate 8 mm below the lower edge of the greater trochanter and approximate 4 mm below the edge of the tibiofibular fusion. The proper drilling position leads to avoid affecting the joint component or blood supply. Somehow, the implantation of the internal fixation through a subcutaneous or submuscular way is still controversial. Interestingly, performing the muscle-gap separation technique is convenient for placing the implantation through a submuscular channel to a certain extent.

The results from the joint angle measurement were consistent with the histological analysis,

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demonstrating that knee joint contracture was successfully induced in the immobilized hindlimb. The average arthrogenic deficits in extension ROM was 29.4 ± 3.3°, 40.7 ± 4.3° on the immobilized knee joint at the end of 28 days and 56 days immobilization respectively, which were significantly higher than that in control ($P < 0.05$). We also found that typical adhesion had developed between in the joint space between the postero-superior joint capsule and the femur in the immobilized side knee joint (**figure 8 e,f**), which indicates that using mini-invasive technique will not interfere with the occurrence of joint contracture. Taken together, our research indicates that this mini-invasive model produces stable results and is effective in inducing acquired joint flexion contracture.

This mini-invasive model still has some limitations. First, the tibia side screw will inevitably irritate the nearby tendons, including the *fibularis longus*. Second, drilling into the cortical bone may cause fractures. Third, there is still a chance of fixation failure. We believe that the use of 3D-built individualized splints is a possible option for building a non-invasive knee joint contracture model in the future[29].

In conclusion, the present study describes a mini-invasive knee joint contracture model that is based on a combination of the muscle gap separation modus and the mini-incision method. Given that internal surgical fixations can produce a well-accepted model of joint contracture, this mini-invasive technique may be useful in the study of immobilization-induced knee flexion contracture.

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DISCLOSURES:

The authors have nothing to disclose.

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