Bone & Joint Research Lab

Mashhad University of Medical Sciences

# Animal Studies Protocol of Subcutaneous Implantation in a Rat Model

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## 1.Introduction

Due to the importance of compliance standards in research, such as animal reproduction, breeding, and studies, they play a crucial role in improving the quality of research projects. This protocol has been prepared by esteemed researchers at the Orthopedics Research Center (ORC) of Mashhad University of Medical Sciences (MUMS).

Mice and rats have traditionally been the preferred species for biomedical and psychology research animal models, owing to their similarities in anatomy, physiology, and genetics to humans. In the following sections, we will discuss various injection methods and subcutaneous implant techniques.

## 2. Ethical Considerations in Mouse Experiments for Using Animals in Research

### 2.1. Type of Mouse

#### 2.1.1. Syrian Mouse (Small Laboratory)

Due to their short reproduction time, high reproduction ability, and affordable breeding and maintenance costs, syrian mouse is widely used for genetic research and experimental animal model. Also, relatively short life span has made these animals suitable for pharmacology, radiobiology and toxicology experiments. Advantages of rodents include their small size, ease of maintenance, short life cycle, and abundant genetic resources.

### 2.1.2. Rat Mouse (Large Laboratory Mouse)

After syrian, rats are the most commonly used experimental animals. Rats are mainly used in medical and veterinary research such as oncology and drug evaluation, research related to nutrition, behavior and toxicology.

## 2.2. Physical Movement of Mouse

One of the most common methods for moving either an adult mouse or rat is by lifting the animal by its tail. but it should be noted that the tail is taken from the base of tail. But it should be noted that the tail is taken from the base of tail. In order to safety keeping of mouse, one hand placed under or around their chest shoulder. Then index and thumbs finger are placed under the hands of the mouse and the mouse's hands are stretched from the back towards the spine of the animal.

## 2.3. Recommended or Prescription of Drugs

### 2.3.1. Recommended or Prescription Drugs

The administration route for medication is often classified based on the location at which the drug is given, such as oral or non-oral routes. Oral administration involves taking a substance through the mouth. In non-oral methods, it is advisable to use the smallest needle size possible to minimize pain and tissue damage. Table 1 below provides information on needle sizes and injection volumes for each method. Non-oral prescription of drugs includes subcutaneous injection, intraperitoneal injection (IP), intramuscular injection, and intravenous injection (IV). Subcutaneous administration typically causes minimal pain or discomfort. The preferred injection site is usually over the shoulders or in the loose skin over the neck, although other areas with loose folds of skin can also be used. However, in small rodents like rats, the small

muscle mass makes intramuscular administration technically difficult and painful for the animal due to muscle distension. If intramuscular injections are necessary, they can be made into the front or back of the thigh in all small rodents. In rats, the muscle mass is usually sufficient for accurately administering small volumes of material, ideally 0.05ml or less.

Administering drugs intravenously can be technically challenging, often requiring the use of restraining devices. These devices should be chosen carefully to ensure they are an appropriate size for the animal being injected. The primary IV sites for mice include the right and left lateral tail veins, lateral saphenous vein, and dorsal metatarsal vein. When conducting animal studies on rats, appropriate anesthesia is necessary. It is crucial to choose and use drugs according to ethical guidelines for animal research. Generally, there are two methods for inducing unconsciousness in rats: inhalation and intravenous injection. Inhalation anesthetics (such as nitrous oxide, halothane, isoflurane, desflurane, and sevoflurane, which are commonly used in practice) are employed for both induction and maintenance of general anesthesia in the operating room. For intravenous injection, drugs such as diazepam (5 mg per kg), pentobarbital (50-60 mg per kg), or ketamine hydrochloride (75 mg) can be used depending on the required duration of unconsciousness.

Although ketamine-xylazine (KX) anesthesia is frequently used in rats, many reports indicate inconsistent anesthetic effects, long induction time, insufficient anesthesia levels, or too short sleep duration. The duration of anesthesia with KX typically ranges from 20 to 60 minutes. In this particular study, IV administration was used to anesthetize rats, as described below. Intraperitoneal injection is a commonly used technique to administer substances into the peritoneal cavity of rats, although it can induce high levels of stress in animals. It is an important method for delivering drugs, including anesthetics, in rodents due to the large size

of the peritoneal area. When performing an intraperitoneal injection on a rat, it is advisable to target the lower right or left quadrant of the abdomen, while avoiding the bladder, liver, and other internal organs. This area is rich in blood vessels, allowing the drug to quickly enter the bloodstream after injection. To minimize the risk of damaging the urinary bladder, cecum, and other abdominal organs, it is recommended to insert the needle at a 10-20˚ angle to the horizontal plane of the animal, with the bevel facing upwards (as shown in Figure 1).

Table 1. needle size and volume injection for each injection in type of mouse

| **Intravenous injection** | | **Intramuscular injection** | | **Intraperitoneal injection** | | **Subcutaneous injection** | | **Injection method** |
|---|---|---|---|---|---|---|---|---|
| volume(ml) | Needle size | volume (ml) | Needle size | volume(ml) | Needle size | volume(ml) | Needle size | Type |
| 25 per kg | 26 | 0.05 per site | 27 | 0.5 | 25 | 0.25 | 25 | Syrian |
| 20 per kg | 25 | 0.01 per site | 25 | 2 | 25 | 1 | 25 | Rat |

Figure1. Landmarks for IP Injection

### 3. Subcutaneous Implantation in Rats

In this study, the laboratory rat was chosen as the primary animal model for experimentation. Anesthesia using appropriate sedative drugs was administered to ensure unconsciousness during the procedure. This section outlines the necessary equipment requirements, anesthetic method, and surgical technique for subcutaneous implantation in rats.

### 3.1. Materials and Equipment for Subcutaneous Implantation in Rat

Table 2 illustrates the required equipment and materials for successful subcutaneous implantation.

Table 2. materials and equipment requirement for subcutaneous implant in rat

| List of Equipment and Materials | | | | | |
|---|---|---|---|---|---|
| **Number** | **Name of material** | **Material Type** | | **Sterilization** | **Application** |
| | | Non-Consumable Materials | Consumable Materials | | |
| 1 | Insulin syringe | | • | | Injection of drugs |
| 2 | Syringe (5,10cc) | | | | Injection of drugs |
| 3 | Surgical gloves (size: 7, 7.5, 8) | | • | | Surgery |
| 4 | Single use, Latex gloves | | • | | Surgery |
| 5 | Kleenex | | • | | Surgery |
| 6 | Gauze | | • | • | Surgery |
| 7 | Surgical instruments set (Scalpels, scissors, and saws, forceps, clamps, needle, sutures and retractors) | • | | • | Surgery |
| 8 | Betadine (Povidone-iodine) | | • | | Topical antiseptic |
| 9 | Ketamine | | • | | Unconsciousness drugs |
| 10 | Xylazine | | • | | Unconsciousness drugs |
| 11 | Normal Saline | | • | | Washing |
| 12 | Intravenous Sugar Solution | | • | | Water supply |
| 13 | Tetracycline | | • | | Antibiotics |
| 14 | Deionize water | | • | | Washing |
| 15 | Saline dextrose | | • | | Water supply |
| 16 | Alcohol 70% | | • | | Antiseptic, Disinfectant and Antidote |

### 3.2. Step of Subcutaneous Implant

Subcutaneous implantation is utilized in rats to investigate immune system reactions and perform pathology tests on samples. Pathology tests aim to examine the local effects of various biomaterials for medical applications. In this study conducted at Mashhad University of Medical Sciences, the pathological behavior of fabricated scaffolds for tissue engineering applications was evaluated through subcutaneous implantation.

The steps involved in subcutaneous implantation are as follows:

1. Preparing rats prior to anesthesia
2. Administering anesthesia to rats using a ketamine/xylazine combination
3. Preparing rats before surgery
4. Identifying and marking the location for subcutaneous implantation, followed by making an incision
5. Performing the suturing process
6. Administering antibiotic drugs
7. Maintaining proper conditions for the rats after surgery
8. Conducting staining tests and fixing the tissue
9. Evaluating pathology parameters

#### 3.2.1. First Step: Preparation of Rats Prior to Anesthesia

The purpose of this protocol is to establish ethical guidelines for researchers and individuals involved in animal experimentation. It is crucial to consider the well-being of laboratory rats to ensure accurate and reliable results. One important aspect is maintaining proper light-dark cycles to regulate the animals' sleep-wake patterns, which should follow a 12-hour on and 12-hour off schedule. Additionally, the air circulation within the animal laboratory should be

maintained using a blower to continuously introduce fresh air, while a vacuum system removes stale air. The temperature should be maintained between approximately 22-25 degrees Celsius, and the recommended air humidity range is 30% to 60%. The covers of the animal boxes must be replaced every two days with clean covers, autoclaved if necessary. Animals should have unrestricted access to food and water. Table 3 provides specific nutritional requirements for rats and Syrian hamsters. It is imperative that laboratory conditions, including temperature, humidity, and light-dark cycles, adhere to standardized ranges. Furthermore, animals with similar characteristics such as weight and age should be carefully selected for each study.

Table 3. Suitable feeding conditions for Syrian and rats

| Species | Weight (gr) | | Amount of Food Needed per Day | Amount of Water Needed per Day |
|---|---|---|---|---|
| | female | male | | |
| Syrian | 18-40 | 20-40 | 15 gr per 100gr weight | 15 ml per 100 gr weight |
| Rat | 250-300 | 300-400 | 100 gr per 1 kg weight | 80-110 ml per 1 kg weight |

### 3.2.2. Second Step: Administering Anesthesia to Rats

To induce reversible loss of sensation and consciousness during rat surgeries and subcutaneous implantation, a combination of ketamine hydrochloride (75 mg per kg) with xylazine (5-10 mg per kg) or acepromazine (2.5 mg per kg) is administered intraperitoneally (IP). The specific dosage for anesthetizing rats involves a ratio of 2.5 parts ketamine to 1.5 parts xylazine. Generally, approximately four units of the anesthetic drugs are required for rats weighing between 200-250 grams. The duration of anesthesia typically ranges from 20 to 60 minutes. Ketamine acts on the N-Methyl-D-aspartate (NMDA) receptors in the nerves, while xylazine stimulates alpha-2 adrenergic receptors within the central nervous system, thereby reducing the

release of epinephrine in the nerves. As a result, anesthesia occurs in rats approximately 15 minutes after injection

### 3.2.3. Third Step: Preparation of Rats before Surgery

Performance evaluation in rats is a crucial aspect before and during anesthesia. Prior to undergoing surgery, you will meet with an anesthetist, a specialist doctor, to discuss the most appropriate anesthetic for you. Additionally, it is essential to adhere to the standard post-anesthesia care protocols for animals. In order to ensure proper subcutaneous implantation, the hair in the specific area of the rat's body is shaved using either Gillet or an experimental animal shaver. Once the preparation is complete, the rats are positioned in a suitable surgical location. It is important to have replicate samples, with a minimum of five replicates per condition for all animal experiments.

### 3.2.4. Fourth Step: Determining the Location of Subcutaneous Implantation and Performing the Incision

To initiate the surgical stage, prepare gloves, general surgical instruments, sterile gauze, and materials for the animal study. Figure 2 illustrates the site where the subcutaneous implantation will take place in a rat. First, clean the area with 70% alcohol and Betadine, which act as disinfectants and antiseptics to treat contaminated wounds and prepare the rat's skin before the operation.

The size of the scaffolds determines the length of the incision, which is typically 1-2 cm to allow placement of the scaffold beneath the rat's skin. However, the incision may need to be larger, depending on the specific surgery and the severity of the problem. After creating the incision site, use tissue forceps and a scalpel (10 blade) to carefully position the sterile samples

with their designated codes under the designated location in the rat. All samples must undergo sterilization using the plasma method.

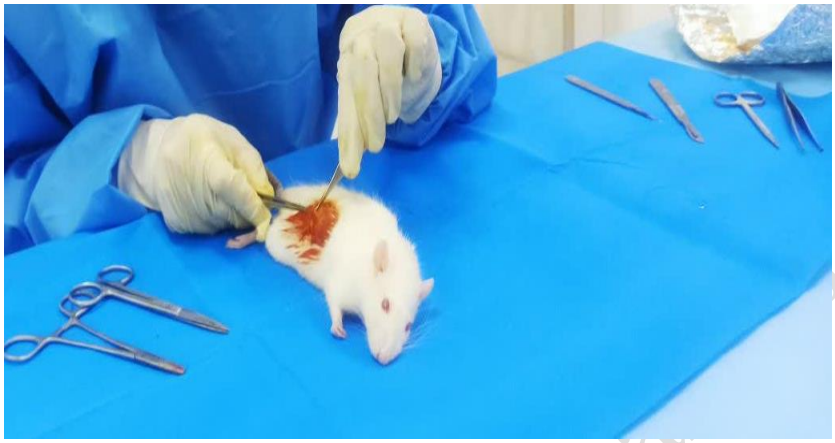

Figure 2. site of subcutaneous implantation

### 3.2.5. Fifth Step: Suturing Process

The samples are carefully positioned in the appropriate location. Subsequently, a suture needle and silk suture (4-0) are employed to suture the incision site. Figure 3 illustrates the rat suturing method.

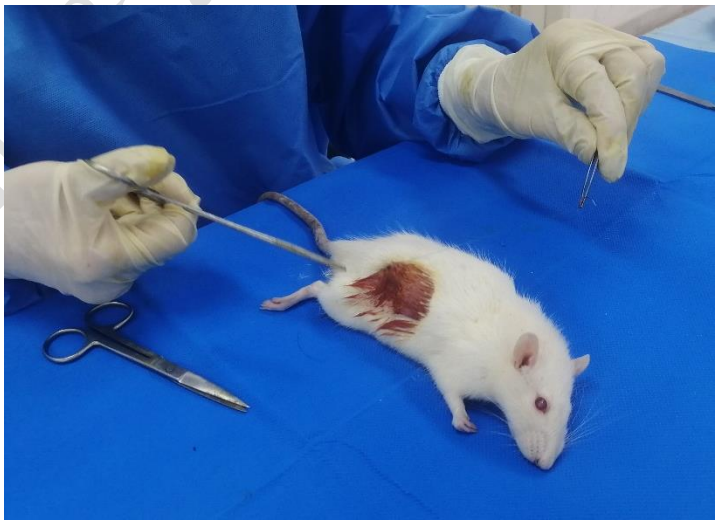

Figure 3. method of suture in rat

### 3.2.6. Sixth Step: Administering Antibiotic Drugs

After the surgery, rats with specific codes are administered antibiotic drugs and intravenous sugar solution. The rats are placed in boxes with paper covering the bottom. This is done to prevent the rats from consuming straw immediately after surgery when they are still weak. The intravenous sugar solution, also known as dextrose solution, is a mixture of glucose and water. It is utilized to address low blood sugar levels or water loss without electrolyte depletion (1ml). Furthermore, tetracycline (tetracycline, doxycycline, minocycline, tigecycline) belongs to a class of medications used for the management and treatment of various bacterial infection.

### 3.2.7. Seventh Step: Maintaining Proper Conditions for the Rats after Surgery

After 24 hours of surgery, it is crucial to assess the general condition of the rats and inspect the surgical site. Additionally, the paper lining the floor of the boxes should be replaced with clean straw, and an ample supply of food and water should be provided to the rats.

### 3.2.9. Eighth Step: Conducting Staining Tests and Fixing the Tissue

Fixation is the initial step in preserving tissues for pathological diagnosis. Its primary purpose is to halt autolysis and putrefaction, coagulate soluble and structural proteins, reinforce tissues against subsequent processing damage, and aid in staining. Fixation comprises two main steps: cessation of normal tissue functions (killing) and stabilization of tissue structure (preservation). The overall aim of tissue fixation is to maintain cells and tissue components as close to their natural state as possible, enabling the preparation of thin, stained sections. Fixation is a physiochemical process involving the chemical fixation of cells or tissues. Fixatives serve multiple functions, including preventing autolysis and tissue putrefaction. Common fixative agents include formaldehyde, glutaraldehyde, osmium tetroxide, glyoxalin, picric acid, among

others. These methods have primarily evolved since formalin 10% was established as a fixative capable of quickly and permanently preserving relatively large tissue specimens. Fixation is a physiochemical process that can be achieved through either chemical or physical means. Physical methods encompass techniques such as heating, microwaving, and cryopreservation (freeze-drying). Chemical fixation commonly employs immersion fixation, where cells or tissues are directly immersed in a fixation solution.

In this study, the immersion method was used to fixate the tissue. Consequently, the sample, along with the surrounding tissue, was fully submerged in 10% formalin for 48 hours. Subsequently, the samples were washed at least three times with distilled water and stored in 70% alcohol to prepare for the staining stage.

### 3.2.10. Ninth Step: Evaluating Pathology Parameters

The subcutaneous tissue is gently spread using forceps to obtain a sample for the pathology test. Following tissue fixation, the samples undergo evaluation for various characteristics such as edema or swelling, necrosis, foreign body granulomatous, inflammation, calcification, and fibroblastic changes after a period of 30 days. The steps of the test are outlined in Table 4.

Table 4. tissue processing and staining steps

| H & E staining steps | | Tissue processing steps | |
|---|---|---|---|
| 5-10 minute | Xylene I | 2hr | 70% Alcohol |
| 5-10 minute | Xylene II | 2hr | 80% Alcohol |
| 5-10 minute | Xylene III | 2hr | 90% Alcohol |
| 5 minute | 100% Alcohol | 2hr | 95% Alcohol |
| 3 minute | 90% Alcohol | 4hr | 100% Alcohol |
| 3 minute | 70% Alcohol | 2hr | Xylene I |
| 3 minute | DI water | 2hr | Xylene II |
| 3-5 minutes | Hematoxylin | 4hr | Wax bath |
| until the color of the solution becomes clear | Tap water | Paraffin blocks preparation followed by sectioning by microtome and then staining | |
| 1-2 dip | 1% Acid Alcohol | | |
| 1-2 dip | Tap water | | |
| 1-2 dip | DI water | | |
| 3-4 minute | Eosin | | |
| 1-2 dip | DI water | | |
| 1-2 dip | 70% Alcohol | | |
| 1-2 dip | 90% Alcohol | | |
| 5 minute | 100% Alcohol | | |
| 3-5 minutes | Xylene IV | | |
| 3-5 minutes | Xylene V | | |
| Mounting | Entelan | | |



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