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Current Protocols in Mouse Biology

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Abstract

Bone is an essential organ that not only confers structural stability to the organism, but also serves as a reservoir for hematopoietic elements and is thought to affect systemic homeostasis through the release of endocrine factors as well as calcium. The loss of bone mass due to an uncoupling of bone formation and bone resorption leads to increased fragility that can result in devastating fractures. Further understanding of the effects of environmental stimuli on the development of bone disease in humans is needed, and can be studied using animal models. In this chapter, we present established and novel methods for the induction of bone loss in mice, including manipulation of diet and environment, drug administration, irradiation, and hormone deficiency. All of these models are directly related to human cases, and can thus be used to investigate the causes of bone loss resulting from these interventions.

Keywords

Bone loss; mouse models; calorie restriction; high fat diet; rosiglitazone; cold exposure; irradiation; ovariectomy

Introduction

Bone is an important organ that provides structural support, houses hematopoietic elements, and can act as an endocrine organ to affect systemic homeostasis and calcium balance (Karsenty & Oury 2012). Osteopenia, a state of low bone mass, is characteristic of bone diseases such as osteoporosis, and is a result of the uncoupling of bone remodeling. When bone formation and resorption are uncoupled, the net loss of bone mass increases fragility and the risk of fracture, which has become a costly healthcare issue (Office of the Surgeon General 2004). The causes of low bone mass in humans are not fully understood, although it is known that bone density decreases with age and diseases such as osteoporosis are more prevalent in post-menopausal women. Many models of age-related bone loss require aging of the mice, which can be costly and time consuming. To study the mechanisms affecting

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bone diseases such as osteoporosis, it is important to investigate hormonal, nutritional, and environmental factors that may alter bone homeostasis.

A number of genetically-manipulated mouse models have been developed to study bone disease, but these do not necessarily reflect upon the effects of environment, drugs, and diet, which are known effectors of bone remodeling in humans. Thus, we have presented here a number of inducible models of bone loss to study bone homeostasis and the mechanistic changes brought on by these effectors. These protocols allow for the in-depth study of the effects of temperature, diet, drugs, hormones, and radiation on bone, all of which are all applicable to human cases of reduced bone density.

Basic protocols 1 and 2 allow the user to study the effect of cold exposure in mice. This environmental stress is known to activate sympathetic nervous system (SNS) signaling, which is responsible for the fight-or-flight response. Recent studies have also suggested that SNS signaling has a direct negative effect on bone (Nagao et al. 2011). Basic protocols 3 and 4 focus on induction of calorie insufficiency and calorie excess, respectively, allowing the user to test the effects of very different diets with potentially similar negative effects on bone. Basic protocol 5 presents the use of a drug, Rosiglitazone, to induce bone loss, and allows for the study of insulin sensitization and its downstream effects on bone. Basic protocol 6 highlights irradiation as a cause of bone loss, which is important to consider in the study of cancer treatment effects on the skeleton and studies using bone marrow transplant as a model for hematopoietic repopulation. Basic protocol 7 presents the surgical method of ovariectomy, which causes a reduction in systemic estrogen levels and has a known negative effect on bone. Downstream applications for these protocols can include the elucidation of mechanisms affecting bone loss, and the potential for the development of treatments to reverse the negative effects of these models.

While these protocols give a detailed outline of methodology, the careful consideration of longitudinal and endpoint analyses when planning any experiment is critical. It is strongly recommended that the scientist perform longitudinal body composition analysis by dual-energy x-ray absorptiometry (DEXA) or *in vivo* micro-computed tomography (μCT) in 3-week intervals to monitor the effects of these studies in real time. These methods provide valuable data points and can highlight important changes in body composition that may otherwise go unnoticed with endpoint analyses alone. Tissue and blood collection for endpoint analyses such as *ex vivo* μCT, histology, histomorphometry, biomechanics, and serum biochemistry should be considered prior to sacrifice so as to maximize the output of each animal. Please refer to (Esapa et al. 2012) for additional time considerations and methodology for these techniques.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Basic Protocol 1

Title: Chronic Sympathetic Nervous System Activation by Long-Term Cold Exposure

Introduction—Allen's rule, first posited in 1877 by Joel Asaph Allen, states that animals living in colder climates tend to have shorter appendages than similar species living in warmer climates. This is supported by recent studies in which mice raised in cold temperatures have shorter bones than littermates raised at room temperature (Serrat et al. 2008). Previous studies have also shown that bone mineral density in mice is decreased during the winter months as compared to summer months (Delahunty et al. 2009). Signaling through the sympathetic nervous system (SNS), which controls the fight-or-flight response, is thought to negatively regulate bone mass through β_2-adrenergic receptors to inhibit osteoblasts and stimulate bone resorption (Elefteriou et al. 2005). Since exposure to cold is a stimulus for SNS activity, the protocol below describes a method for chronic SNS activation by 4°C cold exposure

Materials

Reagents and solutions: Lubricant

Equipment: Appropriate housing facility for mice which includes:

Cages containing appropriate bedding, food, and water

Room held at 18°C on an appropriate light cycle

Room held at 4°C on an appropriate light cycle

Room held at room temperature (22°C) on an appropriate light cycle

Optional: room held at 30°C on an appropriate light cycle

Rectal thermometer

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Randomly assign gender- and age-matched littermate controls to either control (room temperature) or experimental (cold exposure) groups
    a. Note: a group held at thermoneutrality (30°C) can also be included
2. Place appropriate number of cages containing bedding, food, and water in cold room set to 18°C
    a. Note: prepare one cage per mouse, as mice will be housed individually
3. Allow cages to cool overnight
4. Take baseline temperature reading on all mice using rectal thermometer
5. Place experimental (cold exposure) mice in labeled cages in room set to 18°C

6. Keep room temperature control and thermoneutral groups at 22°C
7. Visually monitor disposition of experimental mice to ensure that they are not distressed
    a. Note: signs of distress include hunched posture, labored breathing, and lethargy; mice displaying these symptoms should be removed from the study immediately
8. Allow experimental mice to remain in cold room set to 18°C for one week, measuring core temperature of all mice daily
9. After one week, move experimental mice to cold room set at 4°C
    a. Note: if thermoneutral group is included, place this group of mice into a room set at 30°C on same day
10. Monitor disposition of experimental mice to ensure that they are not distressed
11. Maintain mice in assigned rooms for an additional three weeks, measuring core temperature and disposition of all mice daily
12. After final temperature reading, euthanize mice and collect tissues for further analysis as described above

Basic Protocol 2

Title: Acute Sympathetic Nervous System Activation by Short-Term Cold Exposure

Introduction—As opposed to chronic SNS activation during long-term cold exposure, acute cold exposure can be used to test the immediate effects of environmental stress. This model provides a rapid method of testing the initial systemic response to cold. Although this protocol provides a method for 6-hour cold exposure, total time can be adjusted as needed.

Materials

Reagents and solutions: Lubricant

Equipment: Cages (with bedding, food, and water)

Cold room at 4°C

Room held at room temperature (24°C)

Rectal thermometer

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Randomly assign gender- and age-matched littermate controls to either control (room temperature) or experimental (cold exposure) groups
2. Place cages containing bedding, food, and water in cold room set to 4°C

a. Note: prepare one cage for each mouse, as mice will be housed individually

3. Allow cages to cool overnight
4. Take baseline temperature reading on each mouse using rectal thermometer
5. Place experimental (cold exposure) mice in labeled cages in room set to 4°C
6. Room temperature control mice remain at 22°C
7. Visually monitor disposition of mice every 15 minutes
   a. Note: signs of distress include hunched posture, labored breathing, and lethargy; mice displaying these symptoms should be removed from the study immediately
8. Measure core temperature every 60 minutes by rectal thermometer
   a. Note: mice found to be hypothermic (core temperature ≤30°C) or showing signs of distress should be immediately removed from cold room
9. Repeat steps 7–8 for 6 hours
10. After final temperature reading, euthanize mice and collect tissues for further analysis as described above

Alternate Protocol 1

Alternate Protocol Title: Acute Sympathetic Nervous System Activation by BRL-37344 Administration

Introduction—An alternative to acute cold exposure, BRL-37344 is a preferential β_3-adrenergic receptor agonist that activates SNS signaling. This injectable compound can be administered 3 hours prior to tissue collection, which allows for a quick study on the effects of acute SNS activation.

Materials

Reagents and Solutions: BRL-37344 sodium salt hydrate (Sigma B169)

Concentration of 0.1mg/mL in PBS

Equipment: 25 gauge needle

Syringe

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Firmly scruff mouse and hold the tail between your lower fingers
2. Position the mouse so that the head is tilted down and its ventral side is facing up

3. Administer BRL-37344 solution at a dose of 1mg/kg body weight (0.1mL/10g body weight) by intraperitoneal injection using a 25gauge needle
4. Wait 3 hours
5. Euthanize mice and collect tissues for further analysis as described above

Basic Protocol 3

Title: 30% Calorie Restriction as a Partial Model of Anorexia Nervosa

Introduction—In humans, anorexia nervosa is known to cause weight loss and dramatic loss of bone mineral density. A 30% calorie restriction diet can be used in mice to partially model anorexia and has similar negative effects on bone mass in adolescent mice, although this caloric restriction is less dramatic than that seen in humans. Restriction of calories during peak bone acquisition in mice from 3–12 weeks of age closely models the context-specific nature of adolescent anorexia in humans. The following protocol uses a diet that is enriched in its vitamin and mineral content so that mice are provided with full nutritional value when the experimental diet is fed at 70% of *ad libitum* control diet by weight, resulting in a 30% calorie restriction without nutritional deprivation.

Materials

Reagents and Solutions: Control diet (10% kcal from fat – Research Diets D12450B, Research Diets Inc., New Brunswick, NJ, USA)

30% CR Diet (CR, 30% kcal caloric and fiber restriction – Research Diets D10012703)

Equipment: Appropriate housing facility for mice

Cages (with bedding, food, and water)

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Randomly assign gender- and age-matched littermate controls to either control or experimental (calorie-restricted) groups
2. Place mice assigned to the calorie-restricted group into individual cages
3. Take baseline weight and body composition readings on each mouse using DEXA or *in vivo* μCT
4. Measure amount of diet (fed *ad libitum*) consumed daily by all control mice
5. Take average amount of control diet consumed (g/mouse/day), then calculate 70% of this average to determine the appropriate amount of 30% CR diet
6. Place appropriate amount of 30%CR diet into each cage housing experimental mice
7. Repeat steps 4–6 daily

8. Maintain mice on appropriate diets for desired amount of time, typically 10–15 weeks, then euthanize mice and collect tissues for further analysis as described above

Basic Protocol 4

Title: High-fat Diet Administration as a Model of Diet-Induced Obesity

Introduction—The effects of high-fat diet and obesity on bone density are controversial; however, recent animal studies suggest that factors secreted by adipocytes, such as inflammatory markers, may have a negative effect on bone (Gautam et al. 2014). Human studies also suggest that increased visceral fat, as opposed to subcutaneous fat, is negatively correlated with bone parameters (Gilsanz et al. 2009; Cohen et al. 2013). The protocol below describes administration of a 60% very high-fat diet, but could be adapted for use with a 45% high fat diet; however, fat content must be taken into careful consideration, as recent studies describe differential effects of 45% and 60% high-fat diets on bone compartments (Cao et al. 2009; Ionova-Martin et al. 2011).

Materials

Reagents and Solutions: Control diet (10% kcal from fat – Research Diets D12450B, Research Diets Inc., New Brunswick, NJ, USA)

Very High-fat Diet (VHFD, 60% kcal from fat – Research Diets D12492)

Equipment: Appropriate housing facility for mice

Cages (with bedding, food, and water)

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Randomly assign gender- and age-matched littermate controls to either control or experimental (very high fat diet) groups
2. Take baseline weight and body composition readings on each mouse by DEXA or *in vivo* μCT
3. Feed both control and experimental groups *ad libitum*
4. Replace food in experimental group cages every other day
5. Maintain mice on appropriate diets for desired amount of time, typically 10–15 weeks, then euthanize mice and collect tissues for further analysis as described above.

Note: the high fat content of the diet decreases the solidification of the pellets. The diet should be stored at −20C so as to retain its form, as recommended by the manufacturer. We recommend replacing the diet in the cage every other day so as to avoid the pellets becoming rancid.

Basic Protocol 5

Title: Rosiglitazone Administration in the Diet as a Model of Thiazolidinedione-Induced Bone Loss

Introduction—Rosiglitazone, a member of the thiazolidinedione (TZD) class of anti-diabetic drugs, increases insulin sensitivity by activating peroxisome proliferator-activated receptor-gamma (PPARγ). Previous studies have shown, however, that Rosiglitazone treatment has negative effects on bone by increasing bone resorption and suppressing bone formation (Liu et al. 2013; Bilezikian et al. 2013). The protocol below details methods of Rosiglitazone feeding, however, administration of this compound by oral gavage should be considered if precise dosing is desired.

Materials

Reagents and Solutions: Control Diet (Research Diets D12450B)

Rosiglitazone Diet

High-fat diet containing 20mg/kg rosiglitazone maleate (Cayman Chemicals, Ann Arbor, MI), custom made by Research Diets

Equipment: Appropriate housing facility for mice

Cages (with bedding, food, and water)

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Randomly assign gender- and age-matched littermate controls to either control or experimental (rosiglitazone) groups
2. Take baseline weight and body composition readings on each mouse by DEXA or *in vivo* μCT
3. Feed both control and experimental groups *ad libitum*
4. Maintain mice on appropriate diets for desired amount of time, then euthanize mice and collect tissues

Note: the rosiglitazone diet should be stored at −20°C, as recommended by the manufacturer. We recommend replacing the diet in the cage every other day so as to avoid degradation of the compound.

Alternate Protocol 2

Alternate Protocol Title: Rosiglitazone Administration by Gavage as a Model of Thiazolidinedione-Induced Bone Loss

Introduction—As Rosiglitazone feeding may not provide consistent dosing results, Rosiglitazone administration by gavage may be considered as an alternative. Since mice lack

the ability to regurgitate, the administration of Rosiglitazone by way of gavage ensures that the drug will be administered in the appropriate dose. Unlike intraperitoneal injection, oral gavage also mimics oral dosing commonly used for humans.

Materials

Reagents and Solutions: Rosiglitazone maleate (2 mM in 5% BSA and 5% dimethly sulfoxide) diluted to the final concentration of 2 μM as described in (Im et al. 2005)

Equipment: 18–20 gauge feeding tubes with rounded tip

Appropriate housing facility for mice

Cages (with bedding, food, and water)

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Measure distance from the tip of the animal's head to last rib
2. Mark this distance on the gavage tube

   Note: it is imperative that the stomach not be perforated during this procedure; if the tube is inserted into the animal past this demarcation, the procedure will likely be fatal, thus we recommend training prior to performing gavage.
3. Firmly scruff mouse and hold the tail between your lower fingers

   Note: it is important that the mouse be immobilized during this procedure so as to avoid injury
4. Gently tilt the head back to create a straight line down through the esophagus
5. Gently place tube into the mouth, over the tongue
6. Advance the tube while gently pressing the rounded tip to the roof of the mouth
7. Allow the tube to slide into the esophagus in one, smooth motion

   Note: this step should not be forced; if the tube encounters resistance, remove it and repeat the procedure as needed
8. Administer appropriate volume of solution
9. Gently remove the tube from the mouse, following the same line as the insertion
10. Monitor the animal for signs of distress for 5–10 minutes after dosing

    Note: signs of distress include hunched posture, labored breathing, and lethargy. Uncontrolled bleeding may occur if the internal organs have been perforated. Any mouse exhibiting these symptoms should be euthanized immediately
11. Monitor again 12–24 hours after dosing to ensure that animal is not in distress

12. Repeat administration by oral gavage daily for desired treatment period

Basic Protocol 6

Title: Irradiation in Mice as a Model of Radiation-Induced Bone Loss

Introduction—Irradiation is known to have a negative effect on the bone marrow microenvironment, leading to a reduction in bone mass post-irradiation (Green & Rubin 2014). This is a straightforward method for the induction of bone loss, but systemic radiation effects must also be considered when performing the experiment. The protocol below describes a method of lethal irradiation, which is described as such because the procedure is fatal within two weeks if the mouse is not transplanted with bone marrow donor cells. If bone marrow transplantation is desired, we recommend a comprehensive understanding of transplant procedures prior to performing irradiation experiments (Lundberg & Skoda 2011).

Materials

Reagents and Solutions: Medicated water (pH ~ 2.5) containing 2mg/mL neomycin sulfate

Equipment: Irradiator

Appropriate housing facility for mice

Cages (with bedding, food, and water)

Mice (gender- and age-matched, littermate controls), at least 10 per group for statistical analysis

Protocol Steps

1. Prepare medicated water two weeks prior to irradiation
2. Replace water in cages with medicated water
    a. Note: mice should be maintained on medicated water for two weeks prior to bone marrow transplant and two weeks after bone marrow transplant
3. On day of experiment, separate radiation doses into two sub-lethal doses at least 3 hours apart
    a. Note: in order to reduce stress on the animal, it is recommended that two sub-lethal doses of radiation be administered on the same day. The total dose should equal approximately 1000 rads (10Gy)
4. Irradiate mouse using directions provided with your irradiator model
    a. Note: dose of radiation is dependent upon time inside the machine and strength of the radiation source. Your apparatus should have a time/rad conversion chart that will display the time required for appropriate radiation exposure.
5. Maintain mice on medicated water for two weeks

Basic Protocol 7

Title: Ovariectomy in Mice as a Model of Postmenopausal Bone Loss

Introduction—Removal of the ovaries by ovariectomy (OVX) results in estrogen deficiency and is used as a model for postmenopausal bone loss. As it is difficult to describe surgical procedures in exact detail, please consult a trained animal surgeon and refer to a surgical manual (Lyons & Waterman 2012) for proper consideration of anesthetic and familiarization with surgical technique prior to attempting this protocol. We also recommend surgical courses, such as the Workshop on Surgical Techniques in the Laboratory Mouse at The Jackson Laboratory.

Materials

Reagents and Solutions: Isofluorane

Alternative: Tribromoethanol (Avertin) by intraperitoneal injection at 0.2 ml/10g body weight

Betadine solution (10%)

Equipment: Nose cone for administration of isofluorane

Electric razor (fur clipper)

Gauze

Dissecting scope (optional)

Forceps (2)

Scissors

Absorbable sutures

Wound clips

Heating pad

Protocol Steps

1. Place Isofluorane nose cone on the mouse and wait until the animal is immobilized (~2 minutes)
2. Shave fur off of the animal from just below ribs to just above hips
3. Position animal so that it is laying on its right side
4. Disinfect skin with Betadine solution
5. Make an incision in the skin parallel to the spine and halfway between the last rib and the top of the hip
6. Use forceps to lift the body wall and make a second, identical incision

7. Use forceps to lift the skin and body wall while spreading open the incision with a second pair of forceps
8. Using this technique to create a window, look inside the body cavity to find the ovarian fat pad
9. Pull ovarian fat pad out of the incision using forceps
10. Using a second pair of forceps, tightly clamp between the uterine horn and the oviduct
11. Release the fat pad and clamp tightly just above the forceps in step 10
12. While maintaining pressure on forceps in step 10, tear away the ovarian fat pad, ovary, and oviduct with forceps in step 11
13. Maintain pressure on forceps from step 10 for 15 seconds to prevent bleeding
14. Place externalized tissues back into the body cavity
15. Bring edges of abdominal wall incision together and close with absorbable sutures
16. Bring skin incision together and close with wound clips
17. Repeat steps 4–15 on right side
18. For sham operations, complete steps 1–8, then 13–15 on both sides
19. Return mouse to cage that has been placed on a heating pad
    a. Mice are not able to regulate body temperature as well while under anesthesia, so heating pad must be used to keep animals warm
20. Monitor mice while coming out of anesthesia
21. Monitor wound clips daily for signs of infection
22. After approximately 7days, remove wound clips

Reagents and Solutions

Medicated water: add approximately 2.6 ml of 20% HCl into 2L of water (final pH of ~2.5). Autoclave to sterilize, then add neomycin sulfate at a concentration of 2mg/ml (Calbiochem Cat # 4801). Store at room temperature and protect from light.

Commentary

Background Information

The protocols described here provide diverse methods for the induction of bone loss; these protocols are ideal for investigators who would like to study the effects of environment affecting bone density, or who may not have access to genetically manipulated mouse models. These methods are relatively inexpensive and most require only basic training in mouse handling.

Critical parameters and Troubleshooting

As longitudinal and end point analyses are important to consider when carrying out these experiments, it is strongly recommended that you familiarize yourself with techniques for the analysis of bone mineral content and density prior to performing the protocols in this manuscript (Esapa et al. 2012). Please note that all studies involving live animals should conform to the ethical regulations set forth by governmental and Institutional Animal Care and Use Committees (IACUC), and must also be reviewed and approved by these organizations prior to attempting the procedures included here. Surgical procedures described should only be attempted by qualified animal surgeons and it is recommended that all users refer to protocols related to basic mouse survival surgery (Lyons & Waterman 2012).

Long-Term Cold Exposure—Seasonal changes in bone mineral density may affect the outcome of the experiment, so it is important to consider timing when preparing the experiments. Mouse strain should also be considered; wildtype strains, such as C57BL/6J, are capable of adjusting to 18°C and 4°C temperatures quickly, while other strains, such as *Ucp1*−/−, may require longer adaptation times at 18°C. A pilot experiment is recommended for strains with unknown responses to reduced ambient temperature. All mice should be held in individual cages so as to remove huddling as a factor. To adapt mice to being housed individually, it is recommended that they be separated prior to the start of the experiment in order to remove any behavioral changes that may occur in the early stages of isolation. Cages should have minimal bedding and no additional warming materials, such as nestlets, should be used. When performing the experiments, stress response to handling and rectal probing for core temperature should also be considered. It is recommended that the mice be adapted to handling and rectal probing prior to the start of the experiment.

Short-Term Cold Exposure—As above, temporal considerations should be made to minimize variation. As mouse activity is increased at night, experimental timing should be adjusted depending upon desired time points. Due to the short nature of the experiment, adaptations should be made prior to the start of the experiment to minimize the input of acute stress responses such as isolation and handling.

BRL Treatment—To reduce the amount of stress to the animal, it is important to be properly trained in mouse handling techniques. As above, time of day should be considered when planning the experiment.

30% Calorie Restriction—It is very important that mice on a calorie-restricted diet be held in individual cages so as to ensure precise feeding. Cages should have appropriate warming materials to account for the loss of huddling behavior. Control mice can be group-housed, however, individual housing for all mice is ideal to control for changes in social behavior and temperature. It is also important to consider the age of the mice at the start of calorie restriction; 30% CR diet is known to have negative effects on BMD in mice when given for 9 weeks from 3–12 weeks of age (Devlin et al. 2010), however, some studies suggest that calorie restriction in older mice can increase longevity (Harrison & Archer 1987) and could be protective of the skeleton in some strains.

High Fat Diet—Previous studies have indicated strain-dependent effects of diet on bone density (Parhami et al. 2001), and unpublished results from our lab have also indicated strain-dependent, compartment-specific changes in bone density in response to a very high-fat diet. For this reason, mouse strain must be considered during planning of these experiments. As these compartment-specific changes will likely go unnoticed with procedures such as whole-body DEXA scanning, it is recommended that the bones be scanned by micro-computed tomography (μCT) to visualize the cortical and trabecular regions in greater detail (Esapa et al. 2012). Variability in the response to diet within each strain must also be considered, as recent evidence suggests that genetically identical C57BL/6J mice given a high fat diet display variable levels of weight gain, which is likely controlled by epigenetic mechanisms that may also affect bone responses (Koza et al. 2006).

Rosiglitazone Administration—Since food intake of mice on a Rosiglitazone diet may be different from control-fed mice, or food intake may vary within the Rosiglitazone-fed group, oral gavage should be strongly considered as an alternative. Intraperitoneal injection may also be considered, however, please note that oral routes of administration more closely mimic human treatment with Rosiglitazone.

Irradiation—Irradiator access should be monitored by a Radiation Safety Office or similar governing body within your institution. The hazards associated with use of an irradiator must also be properly understood by the user, and appropriate radiation safety training should be completed prior to use. As the dose of irradiation listed in this protocol is lethal if the mice are not reconstituted with bone marrow, it is important to carefully consider alternatives.

Ovariectomy—As there are strain-dependent variations in cancellous bone loss after OVX, it is important to consider the strain of mouse being used for these experiments (Iwaniec et al. 2006). Post-procedural care of these animals is critical; mice should be monitored frequently for signs of distress or infection, and proper care should be taken when administering analgesics. We recommend a strong background in animal surgery and a complete understanding of health evaluation methods (Burkholder et al. 2012).

Anticipated Results

Long-Term Cold Exposure

As noted above, previous studies have shown that exposure to cold in developing adolescent mice results in reduced bone length as compared to room temperature controls. In older mice, adaptive responses to cold in some strains may mask changes in BMD; in this case, the protocol can easily be modified to extend the length of cold treatment.

Short-Term Cold Exposure

Due to the short nature of this experiment, histomorphometry and μCT analyses are almost certainly not sensitive enough to detect immediate changes in bone parameters; however, serum biochemistry, gene expression, and histology may be able to shed light on immediate responses to cold exposure.

BRL Administration

Although activation of the sympathetic nervous system has been shown to cause reduced bone formation/increased resorption, for the reasons listed above, immediate changes in bone density are unlikely to be detected, however, changes in serum levels of bone turnover markers such as procollagen type I N-terminal propeptide (PINP), C-terminal telopeptide (CTx), and osteocalcin can be tested.

30% Calorie Restriction

As discussed in Critical Parameters, calorie restriction during adolescence leads to a reduction in BMD, but may have a positive effect on older mice. Unpublished results from our lab demonstrate that, in fact, protection from calorie restriction-induced bone loss is strain-dependent, with C3H/HeJ mice given a 30%CR diet from 40–52 weeks of age having a significant reduction in trabecular BV/TV while C57BL/6J mice are unaffected.

High Fat Diet

In mice, numerous studies indicate that high fat diet feeding results in reduced bone mineral density; however, as discussed in Critical Parameters above, the response to HFD can be strain-dependent. Other studies have also indicated that the response to HFD is age- and sex-dependent, as adolescent mice tend to be more susceptible than older mice (Inzana et al. 2013) and males have demonstrated greater levels of bone loss on HFD than females (Gautam et al. 2014).

Rosiglitazone Administration

Numerous studies have shown that Rosiglitazone treatment is deleterious to bone, as discussed above, and these effects seem to be independent of age (Lazarenko et al. 2007), making this a flexible model, although the insulin-sensitizing effects of this drug on systemic metabolism must be considered. It has also been demonstrated that the effects of Rosiglitazone may be more pronounced in C3H/HeJ mice as compared to C57BL/6J or A/J strains (Ackert-Bicknell et al. 2009).

Irradiation

It is understood that irradiation has a negative effect on bone; this has been studied in human cancer patients receiving radiation therapy, as well as in astronauts exposed to prolonged skeletal unloading and radiation in space (Lang et al. 2004). The above protocol presents a short-term model of irradiation in which bone loss is examined within two weeks post-irradiation. Longer studies have also demonstrated bone loss (Hamilton et al. 2006) and this protocol can be easily modified to examine the long-term effects of sub-lethal irradiation on bone, or to examine the effects of irradiation with bone marrow reconstitution.

Ovariectomy

Successful ovariectomy procedures should result in reduced uterine weight and significantly increased body weight as compared to controls. In addition, ovariectomy consistently results in significant loss of BMD as compared to sham-operated controls.

Time Considerations

At the end of each of the protocols listed, sacrifice is required for the harvesting and processing of tissues and blood for further analysis. It is recommended that you allow approximately 10 minutes per mouse for the collection of all relevant tissues; this may take longer if bone is to be collected for gene expression analysis or bone marrow stromal cell culture, as all muscle tissue must be removed prior to freezing or processing. Time for bench setup and tube labeling must also be considered; this can take up to 2 minutes per mouse depending upon the number of tissues and analyses desired.

Long-term Cold Exposure

Allow approximately two minutes per mouse for temperature measurements, as cage manipulation, probe lubrication, animal positioning, temperature reading/recording, and probe cleaning can be tedious. It is recommended that these readings be completed daily and at or around the same time to account for circadian rhythm. Cage changes should also be scheduled appropriately so as to cause the least amount of disruption to normal reading times, and should be noted in experimental records. General assessment of disposition should require approximately one minute per animal, although this may take longer if a mouse requires further analysis or is exhibiting signs of distress and needs to be removed from the study. As an n of 10 per group is recommended for statistical significance, the total daily time commitment can reach approximately 2 hours for 4 groups of mice. In addition, on the days when ambient room temperature is reduced (22°C to 18°C and 18°C to 4°C), it is recommended that experimental mice be monitored once per hour for 6–8 hours to ensure that they are adapting adequately and are not in distress.

Short-Term Cold Exposure

As above, allow up to two minutes per mouse for temperature measurements and at least one minute for general assessment of disposition. Due to the short nature of the experiment, with temperature readings taken hourly, it is recommended that these experiments be separated into two experiments with an n of 5 per group. As mice must be sacrificed on the same day, smaller groups also facilitate tissue harvest at the end of the experiment.

BRL Administration

Injection of BRL can take up to 1 minute per mouse, but one should consider the amount of time elapsed between each mouse during harvest, and stagger injections accordingly. Care should also be taken to sacrifice mice in the order in which they received injections.

30% Calorie Restriction

Average food intake calculations from control mice and subsequent measurement and feeding of the 30% CR diet can take up to 5 minutes per cage, including daily body weight measurements. As the 30% CR diet pellets are generally larger than what is needed, it is recommended that the diet be broken into smaller pieces to facilitate measurements, which can be done prior to the start of the experiment. Longitudinal DEXA body composition analysis requires approximately 10 minutes per mouse and, in addition to baseline readings, can be performed at 3-week intervals throughout the course of the experiment.

High Fat Diet

In addition to body weight measurements which require less than one minute per mouse, this protocol recommends changing the diet pellets in each cage every two days, which takes approximately 5–10 minutes. Longitudinal body composition measurements can also be taken as described above.

Rosiglitazone Administration

Administration of Rosiglitazone in the diet requires that the diet pellets in each cage be changed every other day, which takes 5–10 minutes. Daily gavage of Rosiglitazone takes approximately one minute per mouse, as well as a 5–10 minute monitoring period to ensure that the mouse has not been harmed during the procedure. Longitudinal body weight and composition measurements can be done as described above.

Irradiation

As the use of radiation sources is tightly regulated, be sure to allow appropriate time for application processing, background checks, and other administrative duties related to the use of irradiators. Additionally, two weeks prior to the start of the experiment, mice must be placed on medicated water, which should be replaced every 3 days. Preparation of medicated water will take at least 2 hours, as it must be autoclaved and cooled prior to the addition of neomycin sulfate. On the day of irradiation doses are separated by 3 hours, and dosing time is dependent upon the machine model and strength of the radiation source; please refer to your specific model for time considerations. Mice should remain on medicated water for two weeks following the procedure.

Ovariectomy

Allow approximately 20 minutes for preparation and sterilization of the surgical area and warming of the heating pad. Allow 10–15 minutes per mouse for administration of anesthesia, fur removal, and surgical procedure. Mice should be monitored for an additional 15 minutes after the procedure to ensure that they are not in distress once anesthesia has worn off. Daily post-procedure monitoring for infection requires at least one minute per mouse, which may be increased if complications arise. Longitudinal body weight and composition measurements can be done as described above.

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