Preface

In the past decade, adipose tissue has emerged as a dynamic organ, producing a wide spectrum of adipokines (e.g., hormones, growth factors, cytokines) and playing an important role in the pathogenesis of obesity and its associated metabolic disorders. Consequently, research into the biology and pathophysiology of adipose tissue has attained an unparalleled stature. Since the publication of the first edition of *Adipose Tissue Protocols*, the field has grown enormously, and the techniques and experimental approaches used have undergone radical expansion and revision, warranting a new edition of this text. The second edition combines updates of a selection of chapters from the first edition, with a large number of new contributions covering both conventional and state-of-the-art methodologies, as well as whole animal, cellular, and molecular approaches that have emerged or been extended to adipose tissue research during the past few years.

Unlike many other research areas in which the use of standard techniques and off-the-shelf kits is often sufficient and satisfactory, research involving adipose tissues and adipocytes requires considerable expertise. The methods compiled here are contributed by internationally recognized experts in their respective fields who have many years of experience not only in using these techniques, but also in troubleshooting and perfecting them. Consequently, each chapter contains a step-by-step description of the method and, more importantly, it provides invaluable tips and tricks to safeguard against potential mishaps and pitfalls. The chapters are arranged in a logical order, progressing from an overview of adipose tissue biology to detailed protocols covering all key aspects of adipose tissue research. *Adipose Tissue Protocols, Second Edition* will undoubtedly be an essential manual for newcomers to this burgeoning area, as well as a valuable addition to the laboratories and offices of more experienced researchers.

I would like to express my gratitude to all the authors who have made this book possible and thank Professor John Walker, the Series Editor, for the invitation to edit this second edition. My thanks are also due to Mr. Andrew Williams for his diligent assistance during the final stages of editing, proofreading, and collating. Last, but not least, a special thank you to Dr. Haiyan Guan for her help and support throughout the preparation of this book.

Kaiping Yang

Chapter 2
Choosing an Adipose Tissue Depot
for Sampling

Factors in Selection and Depot Specificity

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Summary The importance and the role of adipose tissues are now largely expanded not only because the very high occurrence of obesity but also because the emerging view that adipose tissue could be a reservoir of therapeutic cells. A critical examination of the adipose tissue features according to their location shows that sampling is not as easy as previously thought and needs special attention to heterogeneity and differences. We discussed here these different points and give precise protocols to sample the different adipose tissues and manipulate them.

Key words White adipose tissue; brown adipose tissue; plasticity; heterogeneity; preadipocytes; adipose-derived stroma cells.

1 Introduction

Adipose tissues (ATs) were long considered as negligible and as simple filling tissues. The increase in knowledge concerning their role in energy balance and the increased occurrence of metabolic disorders, such as obesity and syndrome X, have focused the attention of the scientific community on these tissues. This evolution has been speeded by both the discovery of leptin and the development of transgenic and knockout techniques *(1,2)*. The former and the following studies on adipokines emphasized the endocrine function and the involvement of adipose mass in most physiological functions *(3,4)*. The latter made it possible to test hypotheses elaborated from in vitro findings in the organisms, but these approaches also revealed unexpected results and findings concerning the development of AT *(2)*. More recently, the discovery that unexpected phenotypes can be obtained from stroma cells purified from adipose tissue has largely amplified the concept of adipose tissue plasticity *(5)*. Although previous reports demonstrated the capability of preadipocytes to exhibit an osteogenic potential, the consideration of adipose tissue as a reservoir of stem cells was really undertaken after a publication by Zuk et al. *(6–8)*. Such findings associated with the easy sampling of adipose tissue are rapidly

attracting many new investigators. This chapter aims to give a general picture of the ATs presently available in mammals and to describe their sampling.

1.1   Concept and Principles: How to Classify ATs?

Three functionally different types of adipose tissues can be classically described in mammals: brown adipose tissue (BAT), white adipose tissue (WAT), and bone marrow adipose tissue (BMAT). The role of BMAT is poorly investigated and seems related to the control of hematopoiesis and osteoblastogenesis by acting as energy stores, but also via their paracrine activities *(9,10)*. Beside these classic locations of adipose tissues, the emergence of adipocytes can be observed at unexpected locations, particularly in degenerative tissues. In this chapter, we will focus our discussions on white and brown fat tissues only.

Brown (BAT) and white adipose tissue (WAT) are characterized by different anatomical locations, morphological structures, functions, and regulation (*11–14*; *see also* **Subheadings 3.2–3.4.**). Both are called adipose because of the amount of fat stored in both types. BAT is so called because of its characteristic color, originating mostly from its abundant vascularization and cytochromes. Both ATs are able to store energy as triglycerides, although whereas white fat releases this energy according to the needs of the organism, brown fat converts it as heat. WAT is the main store of energy as lipids for the organism *(14)*. BAT plays an important role in the regulation of body temperature in hibernating, as well as in small and newborn mammals *(15)*. The developmental patterns of ATs are different and are species-dependent (*see* **Subheadings 3.2–3.4**). Adipocytes within a pad were long considered to belong to a single phenotype, i.e., either brown or white adipocytes.

Although studies on WAT were always concerned with energy metabolism, studies on BAT were first focused on nonshivering thermogenesis and thermoregulatory purposes *(15)*. Later, the involvement of BAT in diet-induced thermogenesis led researchers to also investigate its role in various conditions associated with changes of energy balance *(16–18)*.

1.2   Typical BAT

The main features specific to BAT are summarized in **Table 2.1** and are compared with WAT. Its thermogenic function is assumed by the numerous mitochondria and by the presence of mitochondrial protein, uncoupling protein 1 (UCP1), in the brown adipocytes. UCP1 is specifically expressed in these cells and is located in the inner mitochondrial membrane. It is able to uncouple the mitochondria and enables heat production (*17,19*). Recently, the homologous proteins, UCP2 and UCP3, have been cloned *(19)* and can also be detected in this tissue. Some biochemical or molecular makers, including nuclear factors more or less specific to brown fat, are available, and these are also given in **Table 2.1** *(20,21)*.

Table 2.1 Main features differentiating WAT and BAT in rodents

	WAT	BAT
Location of main depots	Inguinal, retroperitoneal, gonadal (compare with **Table 2.2**)	Interscapular, perineal, axillary, paravertebral
Color	Ivory or yellow	Brown
Vascular sytem	++	+++
Innervation	Sympathetic (++)	Sympathetic (+++)
Adipose cells	Unilocular cells	Multilocular cells
Functions	Storage of energy as triglycerides	Storage of energy as triglycerides
	Fatty acids and glycerol release	Heat production
	Secretory tissue	Secretory tissue
Immune cells	+++	+/–
UCPs	UCP2 (++)	UCP1, UCP2 (+), UCP3
Deiodase type II	+	+++
GMP reductase	–	+++
Leptin	+++	At birth, not in adult
α-, β-Adrenoceptors	β_3 (++),α_2 (+)	β_3 (+++)
PGC1	+	+++

In most mammals, BAT develops during gestation and perinatal life *(15)*. It is prominent in the newborn or in young mammals in which nonshivering thermogenesis is necessary to counteract heat loss associated with birth and atmospheric life. It is mostly located around arterial vessels and vital organs. One exception is the piglet, which displays no brown fat, and is subject to thermoregulation dysfunction *(22)*. The development and quantity of BAT are associated with the degree of nonshivering thermognesis required by the organism to maintain its body temperature. This need corresponds to the balance between heat produced by the metabolic body mass and heat loss, which is correlated to body surface and the adequacy of insulation. With increasing age, as the rate of heat loss per unit body weight decreases, the tissue becomes indistinguishable from white fat. This point is crucial in humans and the existence of scattered brown adipocytes in adult is always debated and will be considered in the next paragraph *(18)*. Nevertheless, in hibernators and in some other small mammals (mice, rats, and so on), it regresses only partially and remains identifiable throughout life. In these species, adipose precursor cells are latent in the tissue, and can be recruited as necessary. This general presentation must be modified according to species and to the developmental stage of the newborn, as summarized in **Table 2.2** *(15)*. The most studied typical brown adipose deposits are the interscapular (IBAT) and perirenal BATs in rodents and large mammals, respectively. IBAT is located subcutaneously between the shoulders, and can easily be dissected (*see* **Subheading 3**). It is the only fat pad distinguishable at birth in laboratory rodents. Perirenal BAT is brown in large mammals during the perinatal period, its weight is greater, and it is impossible to sample or remove the whole pad without removing the kidney.

One of the strongest inducers of this type of AT is cold exposure. Acute exposure induces marked changes in metabolism and gene regulation but also stimulates proliferation and differentiation of the precursors into brown adipocytes, leading to

Table 2.2 Evolution of typical BAT according to species

	Immature (hamster)	Altricial (mouse, rat)	Precocial (rabbit, guinea pig, ruminants, primates)
Newborn	Underdeveloped	Nest-dependent	Well-developed
Amount at birth	1–2% body wt	1–2% body wt	2–5% body wt
Several days after birth	Poorly developed	11–12% of body wt	Decreasing
Several weeks after birth	Developing	Partial regression	Transformation into white fat
Adult	Developed	Present	Absent

the development of this tissue in days or weeks after exposure. Catecholamines or β-adrenoceptor agonists mimic the majority of these effects *(14,23,24)*.

1.3   WAT

This is the most abundant tissue of fat mass, and may account for more than half of body weight in severe obesity. It was considered as less vascularized and innervated than brown fat, but various reviews have questioned this opinion *(25–27)*. The importance of white fat in energy balance via its metabolism is well known. Besides this classic view, the wide range of products secreted by adipose cells emphasizes its secretory function and opens interesting fields for the understanding of the established links between the increase of fat mass and various associated disorders, such as cardiovascular disease *(4,28–30)*. When white fat is compared with brown fat, it is noteworthy that no specific marker of white fat is presently available to positively identify it. In the adult, leptin could be a good marker for positive identification of white fat, but its strong expression at birth in brown fat makes this an open question *(31,32)*. Most of its development occurs after birth, and primarily results from hypertrophy of white adipose cells, which can reach 150 μm in diameter in some species. Nevertheless, a pool of preadipose cells is maintained throughout life in most species, including humans and can participate in this growth *(33)*.

1.4   Heterogeneity and Plasticity

The aforementioned classification must be qualified because of several findings: the presence of scattered brown adipocytes in white fat; the different properties of WATs according to location; and the putative conversion of one AT phenotype to the other.

1.4.1 Heterogeneity Within and Between Pads

Brown adipocytes have been observed in noncold-exposed rodents, as well as in several deposits considered as typical white fat in primates. The number of these cells can vary according to the location of fat pads and are most numerous in the periovarian fat of rodents, which can be compared with a patchwork of brown and white adipocytes *(18,34,35)*.

It has long been known that the location of the development of adipose deposits during obesity differs according to gender and genetic determinants *(36–38)*. Abdominal obesity is predominant in the male; subcutaneous (SC) fat mass is mostly involved in female obesity. Sex hormones play a major role in these differences *(37)*. Increased intra-abdominal body fat mass is considered as an independent risk factor for health problems linked to obesity and is positively correlated with increased overall morbidity and mortality *(39–41)*. These findings have been the basis for numerous investigations, including genetic approaches to differences of metabolic properties or precursor pools according to location of fat, and they reinforce the concept of heterogeneity but, in this case, between the fat pads. Taken together, these studies make it possible to distinguish SC from internal fat and upper from lower body fat.

However, this classification is not sufficiently clear and needs further definition: For instance, in humans, omental adipose fat is the most sensitive tissue in lipolysis, as well as in lipogenesis *(42–44)*. This heterogeneity exists whatever the species *(45,46)*. For example, abdominal pads also have greater interleukin 6 or plasminogen activator inhibitor secretion, in vitro differentiation capacity, thiazolidinedione sensitivity, and apoptosis than SC pads and a different redox metabolism *(47–51)*. One exception seems to be leptin expression, which is higher in SC tissue *(52,53)*. It is noteworthy that the regulation of this gene is depot-related *(54)*. These depot-specific properties are partly genetically determined *(38)*. Such heterogeneity also can be observed at molecular levels in rodents, as well as in humans *(55,56)*. This could be attributed to the developmental origin of the different fat pads *(57)*.

For a long time, adipose lineage cells have attracted most investigations and other cells constituting the whole adipose tissue were neglected. This view is changing because the emerging importance of the relationship between immune/inflammation and adipose tissue development *(30,58)*. Thus, adipose tissue also contains immune cells, i.e. macrophages and lymphocytes, the amount of which will vary according to deposits *(21)*.

1.4.2 Plasticity

Plasticity is the term which is used to indicate that some deposits are capable of converting from one type of AT to another. The transformation that has been described concerns the transformation of BAT into WAT-like AT, which takes place, as previously indicated, during postnatal development *(15)*. The reversibility of this process differs according to species *(18,34,59,60)*. The term of "convertible adipose tissue" was used

to describe the deposits able to be reversibly transformed *(59)*. In fact, the same results can be obtained with all deposits, but with different intensity levels *(61,62)*. Marked development of brown adipocytes occurs among fat considered and studied as typical white fat, i.e., periovarian fat in rats, inguinal fat in mice, and numerous white fat pads in dogs. The proportion of the two phenotypes of adipose cells changes according to physiological (cold exposure, development, gestation-lactation cycles), pharmacological (β3-adrenergic agonist treatment), and pathophysiological conditions and genetic background *(34,60,63,64)*. The cellular mechanisms involved in this plasticity are not clearly deciphered. Although the overexpression of co-activator of peroxisome proliferators-activated receptor (PPAR) γ, PGC-1, drives the emergence of brown adipocyte phenotype from white adipocyte, convergent data, including genetic manipulations allowing irreversible labeling of brown adipocytes, show the independence of both lineages *(65,66)*. Both hypotheses could be reconciled by considering that brown adipocytes could be transformed into a white adipocyte-like cells that we call dormant or masked brown adipocytes *(5)*. Whatever the answer, the data lead to conclusions about the heterogeneity within or between pads and the potential for transformation between the two phenotypes of adipose tissues, for which we have first proposed the term plasticity *(5)*. From these considerations, this notion is now extended. Indeed, it was recently established that preadipocytes can behave as endothelial- and macrophage-like cells and that adipose tissues host multipotent adipose-derived stroma cells (ADSCs) *(5,67,68)*. Again, these striking properties display site specific differences *(69,70)*.

The cell plasticity of adipose derived cells raises great hope raises great hope in regenerative medicine because adipose tissue can be easily harvested in adults and could represent an abundant source of regenerative cells. So far, when all data are carefully collected, it appears that adipose tissues can be considered as a subtle and complex mixture of cells, the differentiation potential of which can vary according to the deposit.

In any event, the investigator must be cautious and take into account this aspect of AT biology when: mice or rats are used as a model for humans; fat pads have to be pooled to obtain sufficient sample quantity; or it is only possible to remove an aliquot of AT to interpret the results as the index of the whole fat pad or the whole fat mass.

2   Materials

No specific materials are needed except sharpened and pointed surgery tools.

3   Methods

3.1   *Choice of Species*

The criteria of choice are numerous and are grouped here into three levels (**Table 2.3**). The first is the scientific aim, and the choice of species will be strictly dependent on it. In other cases, the decision is less clear, and each aspect may need discus-

Table 2.3 Parameters to be taken into account in the study of AT

	Parameters
Questions	Model for human physiology or pathology; BAT or WAT studies; physiological investigations; in vivo developmental studies; plasticity studies; transgenic model; photoperiod effect; in vitro studies.
Extrinsic	Species; breed; age; sex; diet; room temperature; photoperiod; gestation-lactation cycles
Intrinsic	Importance of metabolic pathways; BAT vs WAT phenotype; plasticity (*see* **Subheading 2.4.2.**); SC vs internal; accessibility of innervation or vascular system (depending on studies)

Table 2.4 Advisable species according to the investigations

Investigation fields	Advisable species
Model for humans	Primates, not rodents
BAT or WAT studies	All species
Physiological investigations	Rats
In vivo developmental studies	All species
Plasticity studies	Mouse, rat, rabbit
Transgenic and knockout models	Mouse
Photoperiodic effect	Sensitive species: hamster
Diet-obesity	Rats according to breed; dogs with high-fat diet; primates

Table 2.5 Main features differentiating ATs in rats, mice, and humans

	Rats and mice	Humans
Location of fat pad:		
Interscapular	+	−
Periovarian	+	−
Epididymal	+	
Persistence of brown fat in adults	+++	+/−
Convertible features	Mice > rats	
BAT → WAT	+	+++
WAT → BAT	+++	?
	Ing > PO > RP > Ep	
ADSC plasticity	Ing > PO > RP > Ep	
Main site of lipogenesis	AT	Liver
Glucose transport sensitive to insulin	+++	+
Catecholamine-stimulated lipolysis	β_1, β_2, β_3 (+)	β_1, β_2 (+) α_2 (−)

PO, periovarian; RP, retroperitoneal; Ep, epididymal.

sion. Nevertheless, an aid to decision can be suggested as illustrated in **Table 2.4**, which shows that rodents are valuable and convenient models in most cases, except for human studies. The chief reasons are given in **Table 2.5**. From these data, it is clear that classic laboratory rodents are not a good model for humans in metabolic or developmental studies. When metabolic features are considered,

no important difference exists between adipocytes from non-human and human primates (*23*). Therefore, the only physiological models available as human models are primates. For developmental studies, large animals and rabbits seem to present the same features and can be used at least until weaning. After this time, the great difference in metabolism excludes the use of these animal species as human models.

3.2   Choice of Pad

When the species has been decided upon, the location of the fat pad to be studied must be chosen. Two aspects must be considered: once again, the scientific aim and the amount of tissue needed. Both aspects are summarized in **Tables 2.6** and **2.7** for rats or mice. The coarse ratio between IBAT and the three other sites described in **Table 2.7** is quite different in these two closely related rodent species, which suggests that IBAT is relatively more important in the mouse, the species used for transgenic studies, than in the rat. Whatever the AT, fine dissection is required, because of the developed vascular system and numerous lymph nodes.

3.3   Sampling IBAT in Mice or Rats

1. After euthanasia (*71*), the animals are placed on the abdomen, the head toward the investigator.

Table 2.6   Choice of fat pads in the rat or mouse according to the aim of investigation

Aim	IBAT	PO	RP	Inguinal	Ep
Sc vs abdominal				+	+
Plasticity	+	Rat		Mouse	
Denervation	+	+	+		
Vascular system	+			+	+
Isolated adipocytes	+			+	+
Primary culture	+			+	+

IBAT, interscapular brown adipose tissue; PO, periovarian adipose tissue; RP, retroperitoneal adipose tissue; Inguinal, Inguinal adipose tissue; Ep, epididymal adipose tissue.

Table 2.7   Weights of major sites of AT in young adult rats (9- to 10-wk old) and mice (7- to 8-wk-old)

	IBAT	Inguinal (g)	Gonadal (g)	Retroperitoneal (g)	IBAT/WAT
Mice	0.14	0.35	0.2–0.4	Negligible	Approx 20%
Rats	0.3	2.5	1.2	1	Approx 7%

IBAT, interscapular brown adipose tissue; WAT, white adipose tissue.

2. The shoulder region is abundantly rinsed with 70% EtOH to wet the coat and to avoid having hairs on the samples.
3. The skin just behind the head is grasped with tongs, lifted, and incised with scissors.
4. The skin is widely incised from this point to the middle of the black, and the field is opened.
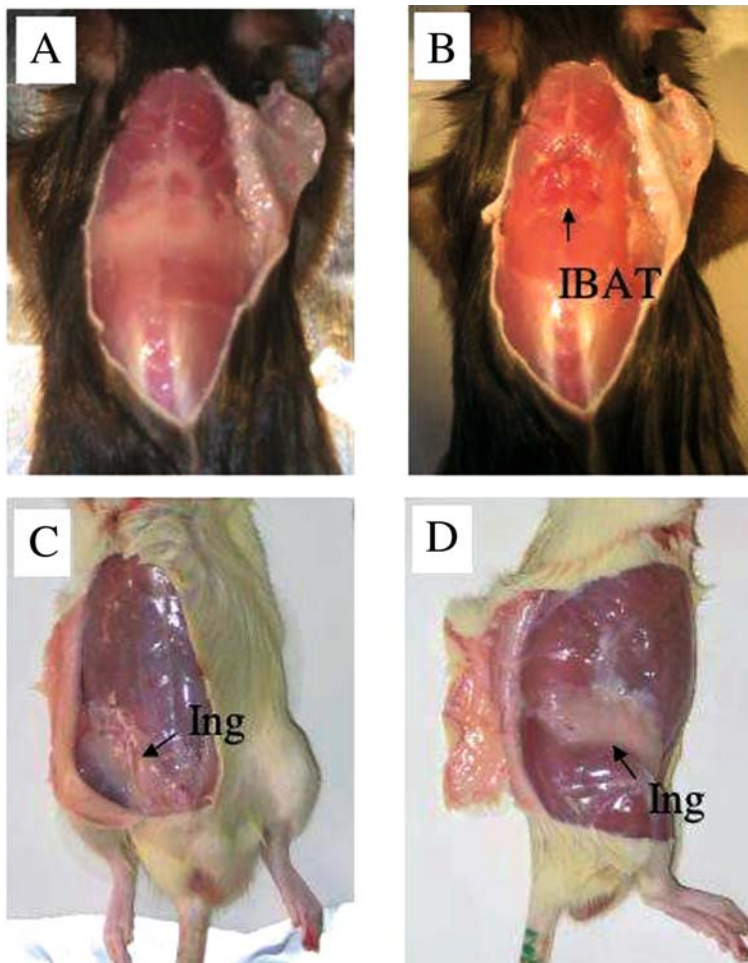5. The butterfly-shaped IBAT is revealed.

Fig. 2.1 IBAT and inguinal adipose tissue in rats. **(A)** Aspect of IBAT before removing the white part. **(B)** The white part of the pad has been carefully dissected. Brown fat appears as a butterfly between shoulders **(C,D)** Front and side views, respectively, of inguinal fat

6. Rub the fat pad with a paper tissue to discard the white part just above the IBAT and then carefully dissect the pad.
7. A binocular microscope can be used but, with some practice, this is not necessary; or remove the fat pad, and afterwards carefully dissect the butterfly of brown fat. In all cases, care must be taken to avoid the muscle closely associated with the brown fat.
8. The sample is ready and the parts of the pad can be separated as required. If RNA is to be extracted, freeze the tissue by immersion in liquid nitrogen and store at −80°C. It is better to freeze it at once, rather than to freeze it after putting it into a container, to prevent it sticking to the walls.

3.4   General Considerations for Sampling White Fat

WATs are organized in lobules, and the various pads can be found together within connective tissues, particularly in obese animals. Therefore, before cutting with scissors, it is sometimes better to separate the different parts by hand, taking care to remove only the whole fat of interest. AT can be frozen by immersion in liquid nitrogen, the same for brown fat. After freezing, the tissue can be reduced to powder to facilitate and homogenize the sample.

3.4.1   Sampling Inguinal AT in Mice or Rats

1. The procedure is the same as that previously described for IBAT but, in this case, the rodent is placed on its back with the tail toward the investigator.
2. The abdomen is rinsed with EtOH, and the skin is widely incised.
3. After removing the pad, dissect and discard the lymph nodes present among the fat. For females, take care not to confuse the fat pad and the mammary gland, which is involuted.
4. If sampling is done to study gene expression, depending on the size of the pad, it may be preferable to reduce the pad to powder, in order to use only the amount required for the study.

3.4.2   Sampling Gonadal AT in Mice or Rats (see Fig. 2.2)

1. Open the abdominal wall.
2. Extract the genitals (ovaries or testes, according to the sex) from the abdominal cavity.
3. Remove carefully, by dissecting the fat tissue or handling the gonadal tract with one hand, and separate fat from other tissues by gently pulling them with the other hand.
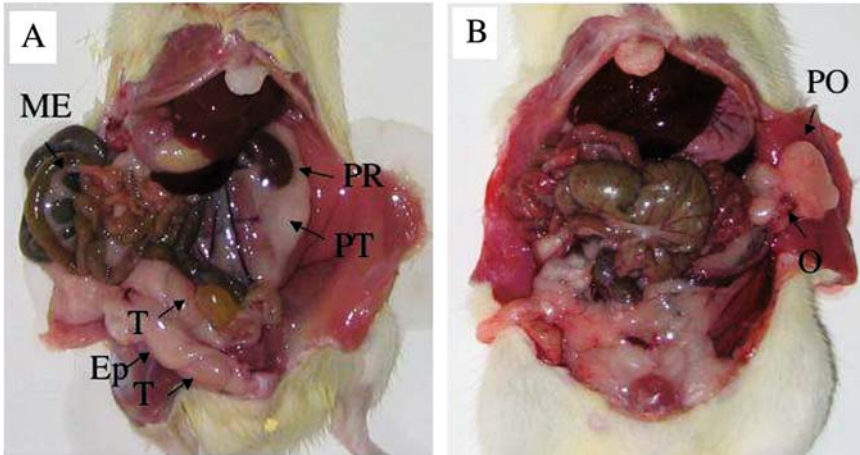
Fig. 2.2 Internal AT in male or female older rats. **(A)** The abdominal cavity of a 200-g weight male rat is opened. The left part corresponds to the natural appearance of abdominal cavity. In the right part, the testis (T) and the associated epididymal adipose pad (E) have been put in a prominent position. **(B)** Appearance of abdominal cavity of an older rat (body weight: 400 g) **(C)** Intestines are put on the right side and the left ovary (O) and periovarian pad (PO) placed on the opened abdominal wall. **(D)** The genital part was removed, and it is then possible to better distinguish the retroperitoneal (RP) and the perirenal fat (PR)
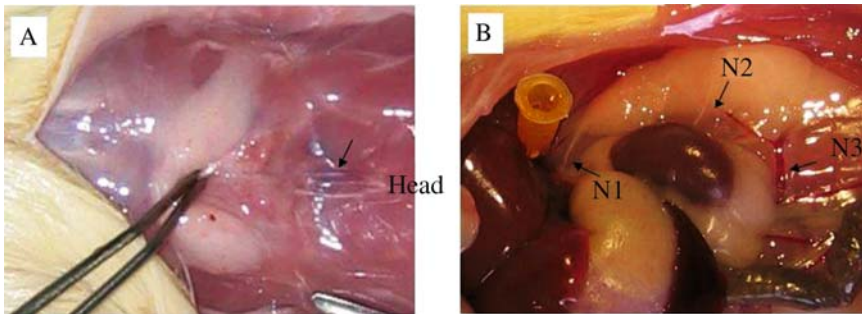

Fig. 2.3 Innervations of fat tissues. **(A)** Upper panel: The IBAT is lifted by fingers and the nervous fibers can be easily identified (arrow). **(B)** Lower panel: a, b, and c correspond to the three nervous fibers innervating the retroperitoneal pad

3.5 Denervation Studies

3.5.1 IBAT *(see Fig. 2.3)*

1. Proceed as in **Subheading 3.3.** to reach the BAT.
2. Carefully separate AT from muscle above the shoulders.

3. Carefully start to raise the IBAT; nerve fibers can now be seen arising from under each shoulder muscle.
4. Cut them at two points and remove the fragment to block regeneration.
5. Suture skin.

3.5.2 Retroperitoneal AT

1. The retroperitoneal fat pad is innervated by three nerve fibers, and the contralateral pad can be used as control. In this case, use the left or right one at random.
2. Proceed as in **Subheading 3.6.**, but the opening must be as small as possible. The goal is to maintain the animal alive after surgery.
3. Cut the three nerves at two points and remove the fragments.
4. Close the abdominal wall, then the skin.

3.6 Investigations via Vascular System

Almost all fat pads are individually vascularized, and it is possible to use such a feature to investigate some parameters (blood flow, arteriovenous differences, etc.) via the vascularization system or to inject particles (e.g., virus) with good preservation of the anatomy and the cellular interactions of the pad.

3.6.1 IBAT (see Fig. 2.4)

1. To easily obtain cannula with different diameters, use yellow tips for pipettes. Bring the center of the tip, which you hold at each end, near a flame. When the tip begins to melt and becomes translucent, rapidly stretch it to obtain catheters. The diameter depends on length of stretch. Cut one end obliquely.
2. Lay the anesthetised animal on one side, and carefully dissect the arterial vessel
3. Put in position the surgical silk, before doing a small incision in the artery.
4. After introducing the catheter into the artery, one can inject any solution (drugs, viral particles, etc.).

3.6.2 White Pad

A very good description of surgical and technical procedure was made by Scow *(72)* for the periovarian pad. Similar experiments can be performed with most fat pads.
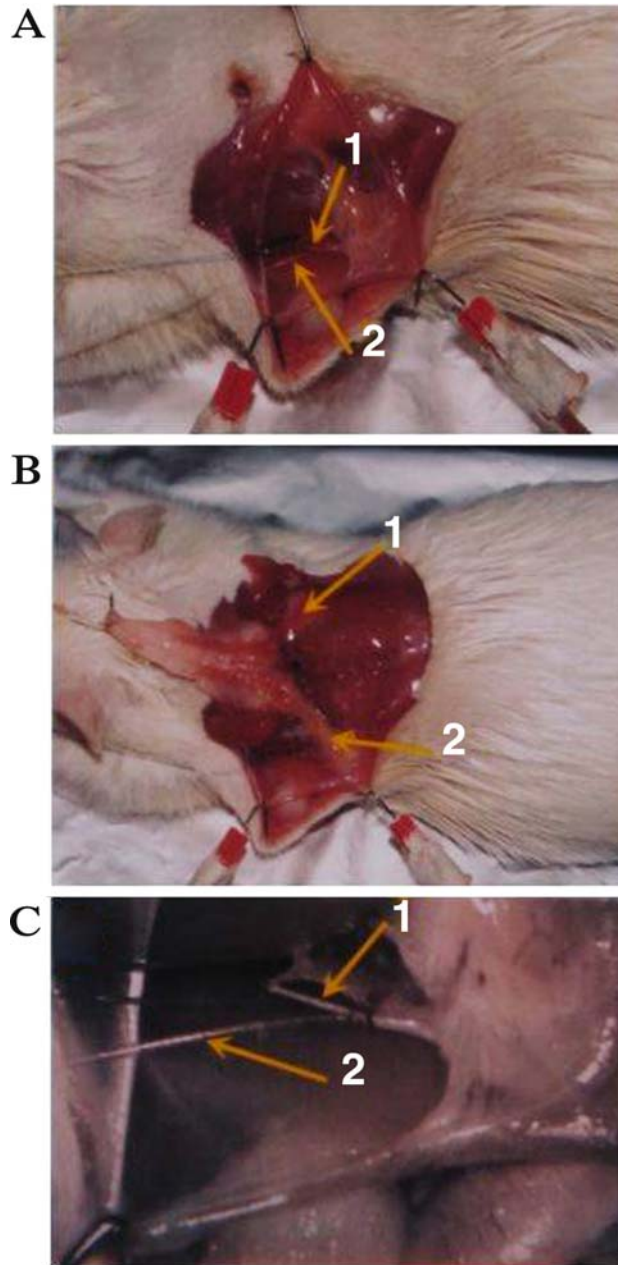
Fig. 2.4 Catheterization of arterial vessel of IBAT. **(A)** The rat is opened on the side and , after careful dissection, the arterial vessel of IBAT can be visualized (arrow 1). A catheter (arrow 2) is introduced into the vessel, and a surgical silk positioned to secure it in place. **(B)** After catheterization, the IBAT is washed with physiological buffer, and the IBAT is dissected and exposed. The washed part of the pad (arrow 2) appears clearer than the other part (arrow 1). **(C)** Enlargement of the catheterization. 1, arterial vessel; 2, catheter

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