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Regulation of adipose tissue mass: consequences for obesity and strategies for stabilising weight loss

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Regulation of Adipose Tissue Mass: Consequences for Obesity and Strategies for Stabilising Weight Loss

A thesis submitted in fulfilment of the requirements

for the award of the degree

DOCTOR OF PHILOSOPHY

from

University of Wollongong

by

Parisa Abolhasan B.Med.Chem. (Hons), Grad.Cert.Bus and Dip.Appl.Sci.

Department of Biomedical Science

2002

Declaration

I, Parisa Abolhasan, declare that this thesis, submitted in partial fulfilment of the requirement for the award of Doctor of Philosophy, in the Department of Biomedical Science, University of Wollongong, is wholly my own work and has not been published by others unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Parisa Abolhasan

ABSTRACT

This thesis describes the current obstacles that we are facing in the treatment of obesity. The aim of this study was to shed more light into the study of adipose tissue, which is hoped to provide more information about the difficulty of weight loss and more importantly, the difficulty of low weight maintenance.

The experiments performed are done on rats. Female rats were divided in two groups of sedentary and exercised animals. Exercised rats were placed under intense voluntary *exercise* regimen in running wheels. Total body weight changes and fat cell size changes of retroperitoneal fat pads were then compared in exercised and sedentary rats. The results presented indicated a substantial reduction in body weight and fat cell sizes in subcutaneous fat pad of exercised rats compared to sedentary rats.

A new group of female rats were again divided in sedentary and exercised groups. These rats were subsequently used to study *sympathetic innervation* of adipose tissue. Three-dimensional *whole mounts* of omental fat pads were prepared and stained using avidin-biotin peroxidase. The subsequent staining of the sympathetic innervation in these slides revealed a complex meshwork of nerves both in sedentary and exercised rats. Some qualitative differences between the innervation of exercised and sedentary animals were also observed. The exercised rats displayed a denser level of innervation compared to sedentary rats. Stained whole mount slides were further used to compare the sizes of fat cells in contact with nerve fibres and those with no nerve contact. The cells in contact with nerves were significantly smaller than those cells with no nerve contact both in sedentary and exercised animals.

This study further revealed the presence of *mast cells* mainly in the exercised rats. A close examination of these results revealed a high density of mast cells near nerve

fibres. The significance of this finding and its implications on the adipose tissue needs further research

The final part of this thesis explored the concept of *apoptosis* in mature isolated adipocytes. An apoptosis-inducing agent (DNA intercalator) was used to induce apoptosis in mature adipocytes. The isolated adipocytes incubated with this drug became apoptotic. The process of apoptosis was substantiated using qualitative analysis such as DNA gel electrophoresis, light and confocal microscopy.

Future study of the process of apoptosis in adipocytes is recommended using flow cytometry. This will provide valuable quantitative data looking at extent of apoptosis in these cells.

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Finally I would like to thank my wonderful family who have been so supportive of my decisions. I hope this thesis can be something they can be proud of. I also like to thank my partner Brad who has been so patient with me and given me joy and reason to carry on in times when it was so easy to give up. I hope I can give back all he has given me.

Abbreviations

A	Adrenaline
ANOVA	Analysis of variance
BAT	Brown adipose tissue
BMI	Body mass index
BMR	Basal metabolic rate
BSA	Bovine serum albumin
CT	Computer tomography
DEXA	Dual x-ray energy absorptimetry
DMEM	Dulbecoo's modification of eagle's medium
FBS	Fetal bovine serum
FFA	Free fatty acid
HSL	Hormone sensitive lipase
IOTF	The international obesity task force
LPL	Lipoprotein lipase
MRI	Magnetic resonance imaging
NA	Noradrenaline
NHS	Normal horse serum
NIDDM	Non-insulin dependent diabetes mellitus
PBS	Phosphate buffered saline

RME	receptor mediated endocytosis
SNS	Sympathetic nervous system
TEF	Thermic effect of food
TH	Tyrosine hydroxylase
VLCD	Very low calorie diet
WAT	White adipose tissue
WHO	World health organisation
WHR	Waist to hip ratio

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CHAPTER 1

Introduction

1.0) Synopsis

This thesis reports work that addresses the issue of *weight loss* and some of the changes that occur in adipose tissue as a result of weight loss. Weight loss is not a problem in many developing countries where under-nutrition remains an important health issue. In most affluent countries, however, obesity is reaching epidemic proportions¹. Reviews of the current treatments for obesity shows that new treatments may provide some hope for the obese. These treatments, however, do not provide a lasting solution and prevention of obesity may be the only effective long-term solution. This, however, will not help some 250 million (Bouchard *et al.*, 1999) people who already suffer from obesity and its related diseases. To help these already obese people a better understanding of how to achieve permanent weight loss is urgently needed.

The second half of the past century has been a time of radical change in many different aspects of society. Mechanisation of life through introduction of computers, and better transport among other factors, have resulted in our adoption of “easier” means of living. These changes have opened doors for a series of chronic diseases such as stroke, coronary heart disease (including arteriosclerosis, heart failure, and hypertension), type 2 diabetes, some cancers, osteoporosis, and sarcopenia (frailty in old age as a result of weak muscles) and obesity (Booth *et al.*, 2000). Obesity, among other chronic diseases² mentioned above, is slow in progression and is continuous and like others it is on a dramatic rise.

Even though there has been considerable research into the causes of obesity and its

¹ Over 65% of Australian men and 45% women are considered overweight (de Looper & Bhatia, 2001)

² Some healthcare professionals still do not consider obesity to be a disease (Banasiak & Murr, 2001)

treatments, there has been much less attention paid to the study of adipose tissue as an organ and the changes associated with weight loss. The process of weight loss is driven by at least two players, namely insulin and sympathetic output. Weight loss is associated with a reduction in insulin output (Henry *et al.*, 1985, Kelley *et al.*, 1993). This thesis however, concentrates on the role of sympathetic activity. A decrease in Sympathetic Nervous System (SNS) activity has been shown in animal and human models of obesity (Tataranni *et al.*, 1999, Vander Tuig *et al.*, 1982, Young & Landsberg, 1983). There are however, studies showing sympathetic over-activity in obesity (reviewed in Bjorntorp and Rosmond, 2000). It is clear that the sympathetic activity plays a major role in the process of weight change. Therefore, this topic needs more attention. Whereas the Physiology and pharmacology of this process has been well described, the anatomy is far from clear

The following thesis attempts to shed some light into the *neuroanatomy* of adipose tissue and the role of Sympathetic Nervous System (SNS) in adipose tissue, and its role in adipose tissue cellularity. An alternative approach into treatment of obesity is also proposed using the concept of *apoptosis* (cell suicide). It is hoped that this approach would result in controlled removal of excess adipocytes recruited during obesity.

Obesity at its fundamental level is defined as an excess accumulation of body fat, which results from positive energy balance. That is, when energy intake is larger than energy expenditure, the excess energy is stored. On top of this simple truth are overlaid multiple levels of subtlety which are reviewed briefly in this chapter.

1.1) Categorisation of Overweight and Obesity

The first step in the treatment of obesity is to recognise people who are over-weight or obese and categorise them accordingly. Two simple methods currently used to categorise obesity are measurements of body mass index (BMI) and waist to hip ratio (WHR). BMI is defined as the ratio of weight in kilogram ‘kg’ to height in meter ‘m²’. BMI≥30 kg/m² has been agreed as the international standard classification for obesity in adults (George *et al.*, 1999). Table 1.1 and 1.2 show the cut-off points for BMI in adult Europeans and Asia-Pacific nations according to the World Health Organisation (WHO, 1998). The BMI cut-off values have been based on mortality outcomes where the confounding influences of cigarette smoking and co-existing chronic diseases have been minimised (The Asia-Pacific Perspective, 2000).

Classification	BMI (kg/m²)
Underweight	< 18.5
Normal range	18.5-24.9
Overweight:	≥ 25
<i>Pre-obese</i>	25-29.9
<i>Obese I</i>	30-34.9
<i>Obese II</i>	35-39.9
<i>Obese III</i>	≥ 40

Table 1.1) *The BMI cut-off point in European population (WHO, 1998)*

Classification	BMI (kg/m²)
Underweight	< 18.5
Normal range	18.5-22.9
Overweight:	≥ 23
<i>At risk</i>	23-24.9
<i>Obese I</i>	25-29.9
<i>Obese II</i>	≥ 30

Table 1.2) *The BMI cut-off points for Asia-Pacific population (WHO, 1998)*

WHR is a simple measurement of waist to hip ratio, where a WHR>1 for men and >0.85 in women is another classification of subjects at increased risk of obesity related complications such as cardiovascular disease and diabetes mellitus (Seidel, 1999, Smith *et al.*, 2001). Another way to simplify this measurement is to measure waist circumference only. In fact it has been shown that WHR reduction does not predict changes in visceral adipose tissue reduction. Therefore waist circumference alone has been suggested to be used as an anthropometrical indicator of changes in abdominal obesity (Ross *et al.*, 1996a, Ross *et al.*, 1991, Stallone *et al.*, 1991). The WHO has used the waist circumference cut-off points of 102 cm for men and 88 cm for women to specify obesity (Després *et al.*, 2001).

More precise and technically sophisticated methods for determining body fat include underwater weighing, the assessment of total body water by dilution of tritiated water and measurement of body fat by dilution of an inert fat-soluble gas such as Xenon (The Asia-Pacific Perspective, 2000). Computed Tomography (CT) scan, ultrasound, Dual X-ray Energy Absorptimetry (DEXA) and Magnetic Resonance Imaging (MRI) have also been used to determine body fat (The Asia-Pacific Perspective, 2000).

These measures are all expensive, difficult to do, time-consuming and not applicable to doctors' office practice. Therefore BMI and WHR/waist circumference remain as the cheapest and most accessible methods used today by health professionals to categorise obesity in adult populations.

These methods, however, are not completely appropriate for all populations since it is evident that Asia-Pacific populations show some deviation from the international cut-off points (The Asia-Pacific Perspective, 2000). Age is another important factor that is overlooked by WHO recommendations, where the elderly may have more visceral deposits of fat even though they might have similar BMI measurements to their younger counterparts (Després *et al.*, 2001).

1.2) Impact of Obesity

The World Health Organisation has recently estimated that in the world there are 250 million obese people (BMI over 30) and 400 to 500 million overweight people (BMI over 25) (Bouchard *et al.*, 1999). The International Obesity Task Force (IOTF) has reported that obesity is increasing in all societies, not only in western ones, and that in many populations over 70% have a BMI over 25 (WHO, 1998).

They also predict that by the time we reach the mid decades of this century most of the US and Australian population will be at risk of serious illness because well over fifty percent of the population in these nations will be obese by 2030 (see figure 1.1).

In countries undergoing economic transitions, such as Asia and Latin America, a paradoxical situation of increasing under-nutrition and over-nutrition in different segments of the population is emerging. A shift from traditional life-style to those usually observed in affluent societies is responsible for the rise of obesity in these

societies. The abundance of energy dense foods and a reduction in energy expenditure through ‘mechanisation’, as the population becomes more industrialised, has occurred as cultures deviate from their more traditional forms of living (Droulers and Loughnan, 2001).

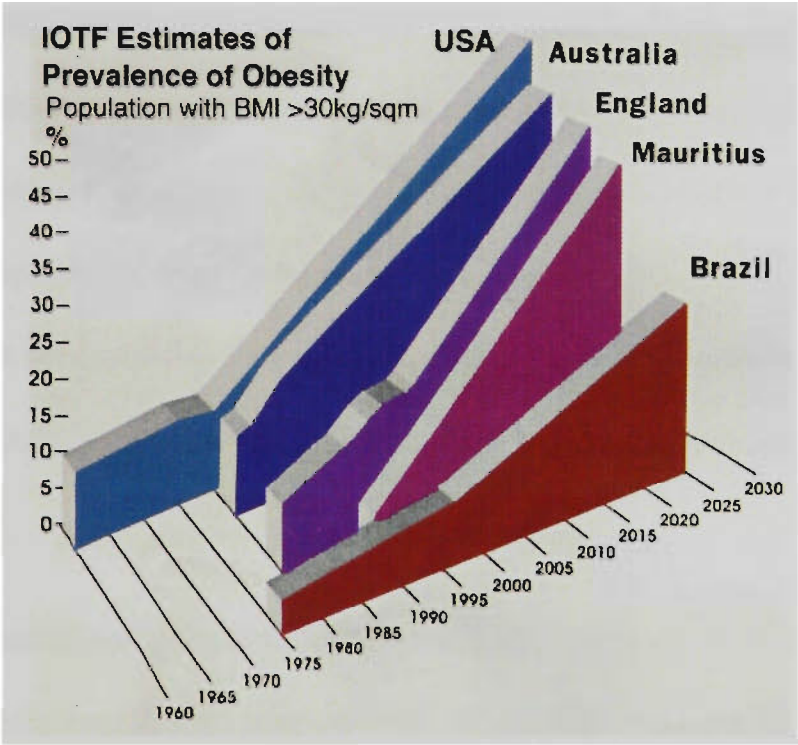


Figure 1.1) *The International Obesity Task Force (IOTF) prevalence and projected estimates of prevalence of obesity (BMI≥30).*

1.2.1) Socio-economic Impact

Obesity contributes to considerable loss of productivity, and to a wide variety of social effects ranging from stigmatisation, loss of self-esteem and frustration and anxiety (Hutton, 1994, Stunkard, 1996).

The direct economic impact of obesity is also large. The Nurses Health study estimated that 17% of all costs treating coronary heart disease (CHD) could be attributed to obesity (Bray & Macdiarmid, 1999). More recently, it has been

estimated that between 5.7 and 6.0% of all US health spending is directly attributed to obesity (BMI >30) (Wolf & Colditz, 1994). This equates to \$22 billion (Halpern, 1999, 1994 figures) or \$51.6 billion (James *et al.*, 1997, 1995 figures).

1.2.2) Medical Impact of Obesity

Obesity is no longer considered a cosmetic disorder but a major risk factor for chronic diseases. Obesity carries a heavy burden of risks including death, cardiovascular disease, hypertension, hypercholesterolaemia, insulin resistance, and type 2 diabetes, gall bladder disease and cancer of the breast, prostate and colon, liver disease, lower back pain, sleep apnoea, stroke, and urinary incontinence (NIH report, June 1998).

Obesity is now recognised as the second (to smoking) cause of preventable death at 300,000 deaths per year in the USA (Beebe, 2002, McGinnis & Foege, 1993, WHO, 1998).

The risk of medical complications is related to both the amount and location of excess body fat (Després *et al.*, 1989, Sparrow *et al.*, 1986). The more severe side effects of obesity are usually associated with increased central (visceral) adiposity (Carey, 1996, Després *et al.*, 2001, Ferrannini *et al.*, 1991, Seidell *et al.*, 1990). This has been suggested by some to be due to differential metabolic activity in different depots, with visceral adipose tissue having the highest lipid turn-over (Fried *et al.*, 1993, Martin *et al.*, 1992), which in turn may result in release of free fatty acids (FFA) into portal circulation with various negative consequences (Fujioka *et al.*, 1987, Hoffstedt *et al.*, 1997, Matsuzawa *et al.*, 1994). Fat accumulated around the viscera has been suggested to secrete less leptin than subcutaneous fat (Montague *et al.*, 1997a, Van Harmelen *et al.*, 1998). The low leptin signal from viscera has been suggested to result in lipid accumulation in this region (Montague *et al.*, 1997a). The exact nature

of the differences between depots is unclear at the moment and anatomical differences between depots have not been thoroughly investigated. Whether there are regional differences between different parts of the same depot is not discussed.

Historically, central adiposity has been associated with the male population having a higher visceral fat than women of equal fatness (Kvist *et al.*, 1989, Lemeiux *et al.*, 1993, Ross & Rissanene, 1994), whereas women have high subcutaneous adiposity, especially glutfemoral adiposity (Larsson *et al.*, 1984, Wing & Jeffery, 1995). This sex difference is, however, narrowed with increased severity of obesity. Women with high visceral obesity and menopausal women also show high risks of obesity related disorders (Bjorkelund *et al.*, 1996, Chang *et al.*, 2000, Colombel & Charbonnel *et al.*, 1997, Folsom *et al.*, 1990, Malacarta *et al.*, 1997, Ng *et al.*, 1997, Toth *et al.*, 2000).

It might be that men are genetically designed to burn fat quickly therefore they store the excess in the visceral region, whereas fat in women is needed only in times of pregnancy and lactation, in other words in times of great and continuous energy expenditure and secretion of fat in milk.

It is clear from the discussion above that there are important differences between different fat depots. The question however, remains as to why such differences exist. Historically, researchers have ignored adipose tissue. We propose that understanding the physiology and anatomy of adipose tissue could provide us with valuable knowledge towards answering above question. In order to understand the differences, we need to explore areas such as innervation and vascularisation of adipose tissue.

1.3) Finding Answers for Obesity

It is clear from all the data available that the prevalence of obesity is constantly rising. The question is why is obesity on the rise? How is it that some individuals remain fat despite restricted diets? Why is it so hard to lose significant amount of weight and nearly impossible to keep it off (Hensrud *et al.*, 1995, Weinsier *et al.*, 1995)? The traditional notion that obesity is simply the well-deserved consequence of overeating has led to unhelpful and sometimes incorrect answers to these questions.

In 1962 James V. Neel of the University of Michigan proposed the concept of a “*thrifty gene*” in our ancestors. According to this theory, thrifty genes boosted the ability to store fat from each feast in order to sustain people through the next famine. Since all human populations seem to share this genetic susceptibility, this genetic adaptation has become a liability (reviewed in Gibbs, 1996). Over 100 monogenic obesity genes have been recognized in rodents. These are being progressively isolated and completely characterized. Examples are, *obese*, *diabetes*, *tubby*, *fat*, *agouti yellow* (Kordik & Reitz, 1999). However, finding mutations of the above genes in humans have been somewhat unsuccessful and not adequate to explain obesity at a population level. Obesity is a complex disease, thus the combination of various gene mutations have been suggested to be involved, which makes pinning down a cause even harder.

It is naive to think that obesity can be completely explained by genetic factors since the likelihood of a significant change in our gene pool in the past 20 years is zero while obesity has increased by 30% and it is constantly on the rise (Filozof *et al.*, 2000a). This can also be concluded by looking at the dramatic rise in obesity in Asia-Pacific populations (The Asia-Pacific Perspective, 2000). It is estimated that genetic

factors are responsible for 40-70% of the variation in obesity-associated phenotypes in the general population (Kordik & Reitz, 1999). Therefore, this epidemic is most likely the result of the (inexorable) impact of environmental factors on a susceptible genotype. It seems that obesity occurs as soon as enough food and leisure are available to cause an imbalance between energy intake and energy expenditure.

Diet is the first environmental factor explored that could influence weight. It has been shown that weight loss in diet studies is dependent on macronutrient content. That is, weight loss is more strongly associated with change in percentage energy from fat than with change in total energy intake (Sheppard *et al.*, 1991, Toubro & Astrup, 1997). A high fat diet is, however, obesity promoting only when it produces positive energy balance. Some talk about over-consumption of food and weight gain in individuals consuming diets high in fat. This has been blamed on the low satiety properties and high caloric density of fat (Golay & Robbioni, 1997). However, reports looking at changes in diet have shown a reduction of fat and calorie intake over the last decade, suggesting a trend towards a physically inactive society (Heini & Weinsier, 1997, Poehlman *et al.*, 1995, Rissanen *et al.*, 1991, Samaras *et al.*, 1999).

This trend is also apparent in Australia, with a fall in consumption of fat from 54.5g/person/day in 1989-90 to 50.8g/person/day in 1998-99 (Figure 1.2). This is an average fall of 0.8% per year. This fall in fat consumption is in apparent conflict with the rise in overweight and obesity in that period. The 1999-2000 data shows that 2 in 3 adult males and almost 1 in 2 adult females were found overweight (Figure 1.3) (de Lopper & Bhatia, 2001).

Consumption of Fat in Australia

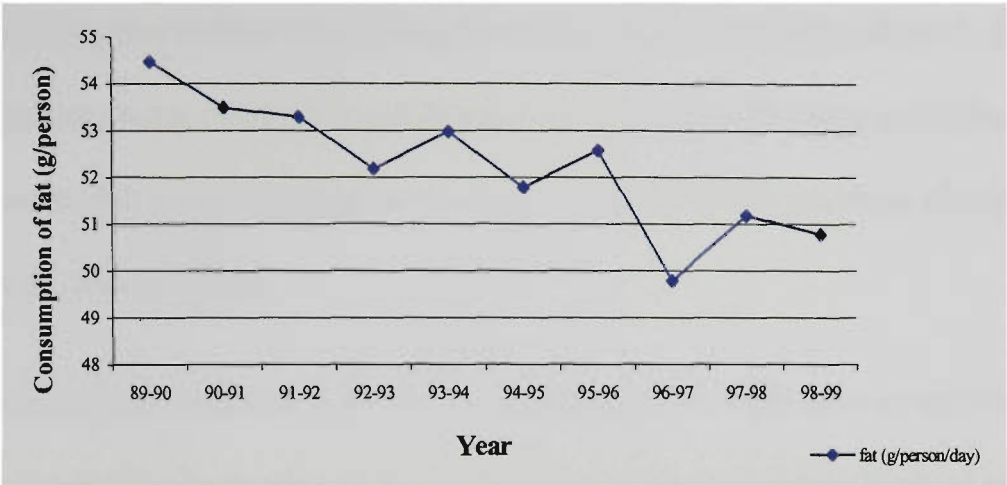


Figure 1.2) *The apparent consumption of fats in Australia from 1989 to 1999.*

There is a decrease from 54.5 g/person/day in 1998-90 to 50.8 g/person/day in 1998-99 an average drop of 0.8% per year.

Data taken from the Australian Health Trends 2001.

Percentage of Overweight Adults in Australia

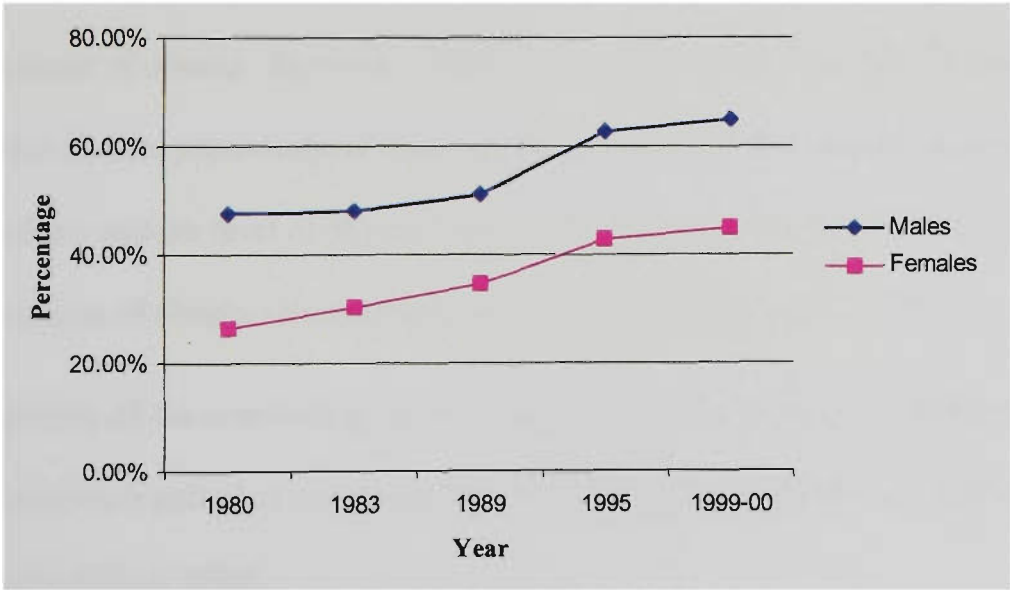


Figure 1.3) *The proportion of 25-64-year-olds who are considered overweight (BMI \geq 25).*

Notes, 1) persons were considered overweight if they had a BMI \geq 25, 2) Includes persons living in capital cities or urban areas, 3) The proportions were age-adjusted using the total Australian population as at 30 June 1991.

Data taken from the Australian Health Trends 2001.

It has been shown that high fat diet in combination with physical inactivity increase the chance of positive energy balance and therefore weight gain (Shepard *et al.*, 2001). Thus, under free-living conditions, combination of exercise and healthy eating has been recommended as an important non-surgical approach to the treatment of obesity (Tremblay & Doucet, 1999).

A lower thermogenic response to a meal, which remains low even after weight loss, has been suggested to be another cause of obesity in some individuals (Bessard *et al.*, 1983, Rippe & Hess, 1998). Studies of adipose tissue dynamics further reveal intrinsic characteristics of massively obese individuals fat cells. These studies show an abnormal expansion of fat cell number in these individuals, which results in greater fat cell mass in these individuals (reviewed in Hamilton, 1996).

It is clear that there are great inter-individual differences contributing to the development of obesity. However, in general, a combination of genetic factors, endocrine factors, psychological make up, socio-economic and cultural factors, diet composition and the level of physical activity have been suspected to be involved in manifestation of obesity (Houseknecht *et al.*, 1998, Sheppard *et al.*, 2001).

Putting aside all the controversy, all will agree that for overweight or obesity to occur there has to be a period of imbalance between energy intake and energy expenditure (McNeely & Goa, 1998).

1.3.1) Obesity & Basal Metabolic Rate

Energy expenditure is often described as a sum of basal metabolic rate (BMR), thermic effect of food (TEF), and physical activity. BMR is the energy required to maintain cellular function as well as body temperature (Shils *et al.*, 1994). TEF is the

heat lost through metabolism of intermediary substances as the meal is ingested (Peek *et al.*, 2001). Depending on the level of activity and fat-free mass, 55-70% of daily energy expenditure can be attributed to BMR (Leiter, 1985, reviewed in Prins & O'Rahilly, 1997). Physical activity includes the involuntary functions as well as the voluntary functions. Physical activity also includes non-exercise activity thermogenesis or simply referred to as fidgeting (Levine *et al.*, 1999). Regardless of particular BMR and TEF, one thing remains clear that the level of physical activity can be altered to readjust the amount of energy stored.

The process of weight gain and energy balance has been the subject of many years of research. As obesity is a result of imbalance between energy intake and energy expenditure, there have been many studies trying to determine whether a low metabolic rate is a key contributor to obesity. At least in small groups of the population high susceptibility to obesity can be explained by low BMR (Gannon, 2000, Sun, 2001, Weyer, 1999). On the whole, however, low BMR does not explain the cause of overweight/obesity in most of the population.

Weight loss studies have shown that upon weight loss, energy expenditure, as measured by BMR and spontaneous activity, is reduced and energy is conserved, which is said to retard the process of further weight loss and facilitate weight regain (Astrup, 1999, Connolly *et al.*, 1999, Even *et al.*, 1993, Geissler, 1987, Shetty, 1990, van Gemert, 2000, Pasman, 1999, Ravussin, 1985). Diet restriction studies show that obese subjects placed on a restricted diet, reduce their BMR (Apfelbaum, 1971, Klein, 1996, Levin *et al.*, 2000) and fat oxidation (Nicklas *et al.*, 1997, Larsson, 1995, Beumann, 1992). According to these studies there is an early drop in weight due to dietary restriction followed by a period where weight loss gradually plateaus (Gibbs,

1996, Nicklas *et al.*, 1997). Some studies suggest that by addition of an appropriate exercise routine to the diet restriction we can help inhibit the decline in energy expenditure and fat utilisation initiated by dietary restriction (Bryner, 1999, Hill, 1987, Niklas *et al.*, 1997, Thompson, 1998, van Aggel-Leijseen *et al.*, 2001, van Baak, 1999). Studies using different intensity exercise suggest that high intensity exercise favours negative energy and lipid balance and a lesser body fat deposition than low to moderate intensity exercise. This is thought to be related to an increase in post-exercise metabolism (Hunter *et al.*, 1998, Tremblay *et al.*, 1994).

A meta-analysis shows that post-obese individuals have a 3-5% lower BMR than never obese individuals (Astrup *et al.*, 1999), others show that post-obese have a BMR that is appropriate for their new body composition and the consequent drop in BMR seen in post-obese is due to loss in fat free mass (Filozof *et al.*, 2000a, Hainer *et al.*, 2000, Peuter, 1992, Weinsier, 2000, Wyatt, 1999). The authors of the meta-analysis explain the discrepancy as being the consequence of low sample numbers in the other studies. They suggest that a high sample number is a crucial factor when detecting only a 3-5% difference (Astrup *et al.*, 1999).

Putting aside the controversy about the appropriateness of BMR for a given body mass, one thing that the majority agree on is that obese and ex-obese individuals display impairment of skeletal muscle uptake and/or utilisation of fatty acids and therefore subsequent risk of weight re-gain (Astrup, 1994, Filzof *et al.*, 2000a, Hainer *et al.*, 2000, Nicklas *et al.*, 1997, Ranneries *et al.*, 1998, Zurlo *et al.*, 1990). This suggests a preferential storage of fat for individuals with a hereditary dysfunction of fat oxidation; therefore a diet low in fat has been the recommendation for weight maintenance (Cooling, 1998, Hainer *et al.*, 2000, Pasman *et al.*, 1999).

1.3.2) Role of Hypothalamus

Weight gain is a gradual process (Houseknecht *et al.*, 1998), which presumably allows the BMR to adjust to the new body weight, which thereafter protects and can resist weight change (Aronne *et al.*, 1995, Kalklin *et al.*, 1993, Kennedy, 1961, Wardlaw, 1996). Over the long-term energy intake, energy expenditure, and efficiency of storage are modified to maintain body adiposity. The *hypothalamus* is known as the centre for appetite and energy utilisation (Williams *et al.*, 2000). This region of the brain is under the influence of higher brain centres (reviewed in Snitker, 2000) that can influence food intake (Oro *et al.*, 1965). Lesions of the ventromedial nucleus of the hypothalamus induce obesity due to an increase in food intake (Bray & York, 1979, LeMagnier, 1983). The hypothalamus is also under the influence of a variety of hormonal substances that are secreted from peripheral tissues such as the gastro-intestinal tract, pancreas and adipocytes (reviewed in Kordik, 1999). Abnormalities in the secretion and synthesis of these hormones have been observed in many animal models of obesity (Gregoire *et al.*, 1998).

1.3.3) Leptin

The discovery of *leptin* (Zhang *et al.*, 1994) supports the idea of an endocrine feedback loop (reviewed in Prins & O'Rahilly, 1997). Leptin is secreted by adipocytes and it has been implicated in regulation of food intake and whole-body energy balance in animals (Campfield *et al.*, 1996, Eckel *et al.*, 1998). In mice it has been shown that leptin is a major afferent signal to the hypothalamus (Zhang *et al.*, 1994). Leptin plays an important role in lipostatic mechanism by which the brain of the animal senses body fat in order to keep body weight constant throughout life (reviewed in Guerre-Millo, 2002 and Janeckova, 2001). It seems that in animals the level of body fat sends

certain level of signals to the brain which signifies the state of body fatness at which point the brain sets the ground rules for further energy intake and energy expenditure to regulate body size and composition.

Circulating leptin concentrations are positively correlated with body fat or BMI (Considine *et al.*, 1996, Hickey *et al.*, 1996, Martin *et al.*, 1998). An increase in body fat results in a subsequent increase in leptin secretion, presumably as larger and more numerous adipocytes increase leptin release (Khort *et al.*, 1996, Hamilton, 1995, Shillabeer *et al.*, 1998). In animals, this increase in leptin in turn decreases food intake and increases energy expenditure thus lowering weight gain (Campfield *et al.*, 1995, Eckel *et al.*, 1998, Pellymounter *et al.*, 1995). Leptin also influences energy homeostasis via central regulation of thermogenesis (Collins *et al.*, 1996, Doring *et al.*, 1998).

In a group of leptin deficient mice (*ob/ob*) the level of fat is misread, they in turn reduce their BMR and increase their food intake accordingly (Forbes *et al.*, 2001).

The administration of exogenous leptin to *ob/ob* mice, results in reduced food intake, increased energy expenditure, increased physical activity, and normalisation of hyperglycaemia and hyperinsulinaemia (Considine *et al.*, 1996, Gregoire *et al.*, 1998, Hamilton *et al.*, 1995, Houseknecht *et al.*, 1996, Lonnqvist *et al.*, 1995). Leptin has also been suggested to act as a signal to “turn off” energy utilisation during periods of energy deficit (Ahima *et al.*, 1996).

Although leptin has been isolated from humans, its exact role in obesity is controversial but is under much investigation. In contrast to mice, there is no evidence of substantial leptin influence in energy expenditure in humans (Roberts *et*

al., 1997). Its direct involvement in obesity in humans is not as clear as in mice and only a few cases show direct involvement of a mutated gene in obesity (Clement, 1999, Montague *et al.*, 1997, reviewed in O'Rahilly, 2002). A rare case of human leptin deficiency has revealed excessive weight gain in early childhood leading to severe obesity. The leptin deficient individual unlike the *ob/ob* mice showed no evidence of BMR or total body expenditure impairment (Mantzoro *et al.*, 2000). Leptin supplementation of this individual showed positive results of weight loss mainly as a loss of fat mass mediated by reduction in food intake with no change in BMR and energy expenditure (Farooqi *et al.*, 2002). Generally in obese humans, the level of leptin in plasma is normal or even higher than normal weight individuals (Considine *et al.*, 1996, Geldszus *et al.*, 1996). There is also evidence of leptin reduction during weight loss (Williams *et al.*, 1999). Therefore a hallmark of human obesity does not seem to be the absence of leptin, but leptin resistance (Hamann & Matthaei, 1996) possibly due to a defect in receptor or in post-receptor processing. Other investigators have attributed a deficient uptake of leptin into the brain to be the reason for obesity (Caro *et al.*, 1996, Schwartz *et al.*, 1996).

More recently, leptin receptors have been detected in peripheral tissue, which has broadened the role of leptin into such processes as production of steroids in ovaries (Duggal *et al.*, 2000) and effects on adrenocortical steroidogenesis (reviewed in Smith *et al.*, 2002). Leptin also acts on reproductive physiology telling our brain if the body is ready to reproduce (Barb 1999, Chehab *et al.*, 1997, Trayhurn *et al.*, 1999, Wauters & Van Gaal, 1999) and is suggested to be involved in haematopoietic (Haluzik *et al.*, 2000) and immune system development (Gregoire *et al.*, 1998).

Conventional treatments for obesity result in a rapid and persistent decrease of leptin secretion in obese individuals (Considine, 1997, Filozof *et al.*, 2000b, Geldszus *et al.*, 1996, Rosenbaum *et al.*, 1997, Rosenbaum *et al.*, 2002) with a contemporaneous increase in appetite and decrease in energy expenditure despite the individual having achieved a normal weight (Geldszus *et al.*, 1996). This could be the reason why the original high weight is regained within 4 to 5 months by participants returning to inappropriate eating and exercise levels (Goodrick & Foreyt, 1991, Kral *et al.*, 1997). One solution to this problem is a permanent stimulation of leptin secretion to prevent relapse (Rosenbaum *et al.*, 2002).

Among other hormones secreted by adipose tissue are immune system related proteins. Some of these include *adipsin*, *acylation stimulation protein* (ASP), *adipocyte complement-related protein* (Acrp30/AdipoQ) now commonly referred to as adiponectin, and *tumour necrosis factor* (Gregoire *et al.*, 1998). Another addition to the proteins discovered secreted by adipocytes is called *resistin* (Steppan *et al.*, 2001). Studies in animals have shown that the concentration of this protein is said to be increased markedly in both genetic and diet induced-obesity. Administration of anti-diabetic drugs results in a reduction of resistin. These studies suggested resistin to be a hormone that could link obesity to diabetes (Steppan *et al.*, 2001). However, several studies since then have shown that insulin resistance and obesity are associated with a decreased expression of resistin in animals (reviewed in Ukkola, 2002). In human, resistin is secreted at very low levels with no apparent linkage between obesity and resistin expression (reviewed in Beltowski, 2003, Fain *et al.*, 2002, Nagaev and Smith, 2001, Savage *et al.*, 2001, Stumvoll and Haring, 2002). *Ghrelin* is another important hormone that is under intense investigation for its link to

energy balance. Ghrelin is a hormone released from the stomach that stimulates the release of growth hormones (reviewed in Dickson, 2002, Kojima *et al.*, 1999). It has been implicated in body weight and energy balance in rodents (Tschop *et al.*, 2000). The concentration of ghrelin has been shown to be lower in obese humans (Tschop *et al.*, 2001). Mutations of the ghrelin gene have been suggested to play a role in human obesity (Ukkola *et al.*, 2001).

1.4) Treatment of Obesity

Once it is established that a person is overweight or obese, treatments or life-style changes are recommended accordingly. Treatment of obesity might appear simple, since we tend to put on weight due to imbalance between energy intake and expenditure, we should theoretically lose weight by reversing the process, that is, by increasing our energy expenditure or decreasing intake. This, however, appears not to be the case and weight loss is not simply the reverse of weight gain. This is discussed in more detail in later chapters.

Medical practitioners of today try to emphasise the importance of the understanding of realistic weight loss goals in order to alleviate its medical complications (not cosmetic, physiological or orthopaedic purposes) since unrealistic ones often lead patients to disappointment and less successful outcomes. The unrealistic goals of weight loss of the 80's have changed to more realistic ones of today.

The primary goal of any current medical weight loss therapy is to achieve sufficient weight loss to improve health and function. This is usually achieved by a 5-10% reduction in body weight (Davidson *et al.*, 1994, Lean, 1997, Wadden *et al.*, 2001).

Short-term weight loss adequately corrects some of the associated complications such

as insulin resistance, diabetes mellitus, hypertension, hyperlipidaemia and blood coagulation abnormalities, as well as physical impairment (Blackburn 1995, Sjöström, 1995, Williamson 1999). A Swedish study (SOS; Swedish Obese Subjects) has examined the benefits of long-term weight loss. A 30 kg weight loss over 2 years showed a 60% reduction in plasma insulin, 25% reduction in glucose and triglycerides and a 10% reduction in blood pressure (Sjöström *et al.*, 1999).

Weight lost is usually not long lasting and obese patients who lose 10% of their body weight find themselves once more at risk by gaining the lost weight. Even though benefits have been established with this modest weight loss, majority of patients display dissatisfaction (Womble, 2001).

Pharmacotherapy is recommended to those individuals for whom other forms of therapy (usually diet and exercise) have failed. This is not to say that pharmacotherapy should be used as a single modality as clinicians are advised to use it in conjunction with diet, exercise, and behaviour modification to suit an obese individual (Moloney, 2000). The efficacy of pharmacological agents is improved when life-style modifications are added (Wadden *et al.*, 2001).

Surgery (intestinal bypass or restrictive gastric) seems to have the highest success rate (Kral *et al.*, 1997, Sjöström, 2000, The Asia-Pacific report, 2000), but it is reserved for patients with BMI of over 40 or sometimes lower, depending on morbidity conditions (Stunkard, 1996). However, surgery is not accessible to all obese individuals and is costly.

It is generally accepted that most dieters regain lost weight and we can maintain a reduced weight in the reduced-obese state only by permanent restriction of energy

intake (Peters *et al.*, 2002, Sarlio-Lahteenkorva, 1998). A survey on long-term weight maintenance of ex-obese individuals revealed that only 6% of patients losing 5-10% body weight would keep it off in the long term (Sarlio-Lahteenkorva, 2000).

The success of this small number of individuals is balanced by a high failure rate for a group who find themselves in the predicament of regaining more weight than they had lost (reviewed in Rippe, 1998). However, studies evaluating the influence of commercially available weight loss programs, suggest that those seeking and complying with such treatments tend to show a higher weight loss and more importantly weight maintenance than previously thought (Lowe *et al.*, 2001). They show that at 5-year follow-up, 42.6% of these individuals maintained a loss of 5% or more, 18.8% maintained a loss of 10% or more, and over 70% were below the initial weight (Lowe *et al.*, 2001).

The new pharmacological treatments provide positive results for reducing medical impacts of obesity (James *et al.*, 2000, Rossner *et al.*, 2000). They are, however, incapable of achieving a permanent weight loss (Halpern 1999, Hill *et al.*, 1999, James *et al.*, 1997). Despite many billions of dollars spent on weight reduction programs and in drug development and dieting, there is today no guarantee of maintenance of stable weight after weight loss. Hence with current available treatments, unrealistic dreams of reaching an ideal weight remain just that.

Today, an integrated weight management strategy is used to treat obesity, which consists of restricted diet, exercise, behaviour modification and pharmacotherapy (Astrup, 2000, 1993, Vansant *et al.*, 1999). It seems that life-style modification is the key to the success of long-term weight loss maintenance (McGuire *et al.*, 1999).

Whether obesity is caused by genetic or environmental factors or a combination of both, the outcome seems to be the same and the treatment rather disappointing. At our current environment, there is seemingly no lasting solution to mankind's tendency to gain weight and currently no strategy for stable and permanent weight loss and the increase in prevalence of obesity is occurring against a background of a steady reduction in energy expended for survival as well as an increase in energy intake (Bouchard *et al.*, 1999).

That this is so indicates that we have designed treatments for obesity that are aimed at the wrong targets. In view of the increase in the prevalence of obesity in adult population and the growing incidence of childhood obesity (reviewed in Must & Strauss, 1999), it is clear, now more than ever, that a more radical approach to this chronic disease is necessary. It is our contention that the biological consequences of lipid mobilisation at the tissue – organ level hold the key to understanding why weight loss is not stable. When we examine the literature on this process we find very little to tell. In order to treat obesity more effectively, we propose that a better understanding of weight loss at the tissue level is necessary to enable us to design interventions and therapies that are more effective in inducing permanent weight loss. In comparison to the wealth of information about weight gain, there is very little understanding of weight loss. The change in distribution of various components of adipose tissue during weight gain is well documented. However, the information available on weight loss concentrates on either the adipocyte (DiGirolamo, 1991) or the total fat mass (Ross *et al.*, 1997, Tremblay *et al.*, 1995) and does not examine the tissue and its components. For example, there is no description of changes to the vasculature and innervation after chronic weight loss and neither explanation for the

occurrence of mast cells in fat tissue nor for their preservation in weight loss. It appears that not only is weight loss more than the simple reverse of weight gain, but also the changes in fat tissue during weight gain and weight loss are more profound than a simple change in fat mass.

1.5) Adipose Tissue

Adipose tissue is now considered by some not to be a tissue but an organ (Cinti, 1999b). It has, however, rarely been studied as such. No more than eighty years ago it was believed that adipose tissue was another form of connective tissue with no apparent influence on energy metabolism with simple anatomy of fat cells, small number of capillaries and no innervation of fat cells (reviewed in Wirsén, 1965). The tissue that was considered to be simple and foam-like in structure with little or no influence over its fate or the function of the body is now regarded as highly organised and sophisticated with complex physiology and anatomy and the presence of at least two distinct types of this tissue (Pond, 2000).

The two types of adipose tissues present in mammals are white adipose tissue (WAT) and brown adipose tissue (BAT). BAT is multilocular in appearance and is present in infants and is the primary thermogenic tissue in infant metabolism (Figure 1.4). BAT has largely disappeared by adulthood because this role has been taken over by muscle tissue. BAT has a rich vascular supply along with noradrenergic and sensory innervation (Cinti, 1999, Haseberg, 1934). In contrast, WAT consists of unilocular adipocytes (Figure 1.5) with much less vascularisation and innervation than BAT (Cinti, 1999, Haseberg, 1934). WAT exists within abdomen and thorax, under the skin, in skeletal muscle, and in bone marrow (Scow *et al.*, 1970). Since obesity is an excess accumulation of WAT, the focus of this thesis will be on WAT and only when

specified otherwise, WAT is referred to simply as adipose tissue.

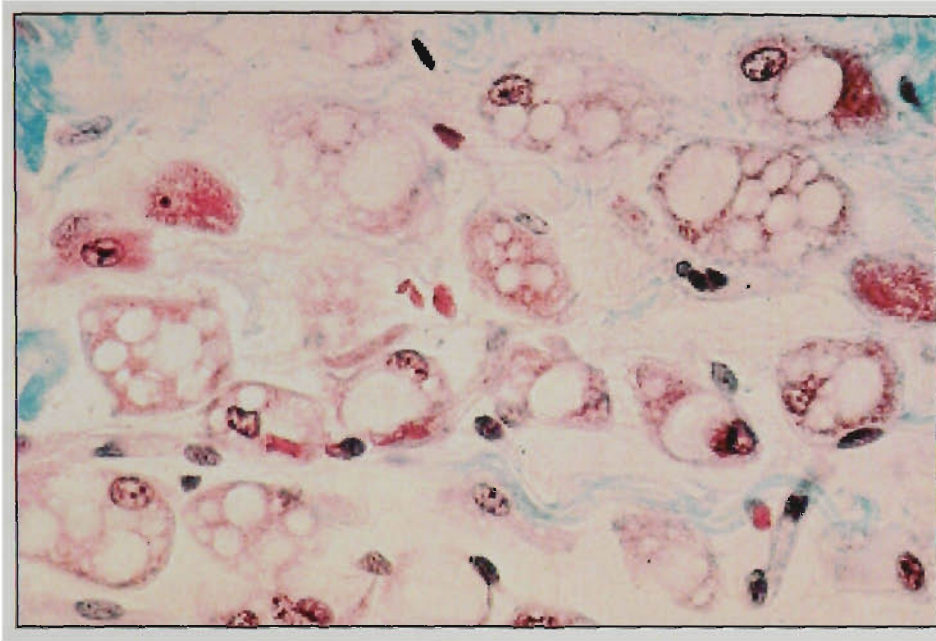


Figure 1.4) *A photograph of a section of Brown Adipose Tissue (BAT). The picture displays the presence of multilocular adipocytes*

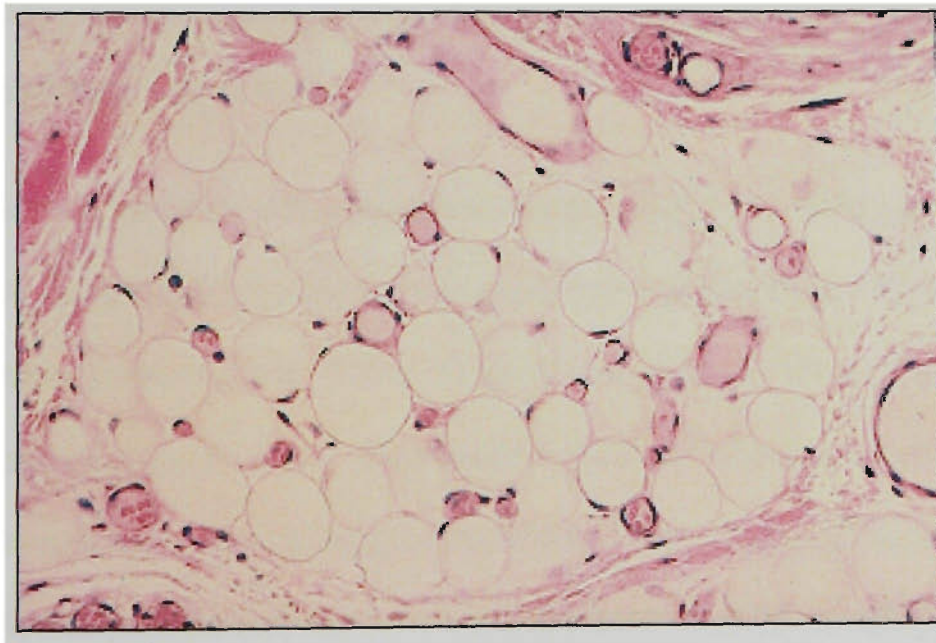


Figure1.5) *A photograph of a section of White Adipose Tissue (WAT). The picture displays the presence of unilocular adipocytes.*

The pictures taken from, www.udel.edu/Biology/Wags/histopage/colorpage/ca/ca.htm

Adipose tissue consists of adipocytes, pre-adipocytes undifferentiated stromal cells, vascular cells, nerve fibres, mast cells, and various interstitial cells among others. The changes in distribution of some of these components during weight gain are reasonably but not fully understood. Changes induced by weight loss have been studied at the level of the adipocyte, pre-adipocyte and stem cell (reviewed in Prins & O’Rahilly, 1997). Changes in nerve supply and vasculature have been mentioned in passing in the literature but apparently not subject to systematic study (reviewed in Bartness & Bamshad, 1998).

Adipocytes are by mass (possibly not number) the main cellular component of adipose tissue serving as storage units for lipid. The mass of adipose tissue is a function of the mass and number of its constituent adipocytes. A unique feature of adipose tissue is its enormous capability of size variation from a few to a few hundred-fold in extreme obesity (Björntorp *et al.*, 1982).

In mammals the major site of accumulation of triglyceride is the cytoplasm of adipocytes. Droplets of triglyceride coalesce to form a large globule, which occupies most of the cell volume (Stryer, 1995). Triglycerides are the most concentrated store of metabolic energy in the body. The yield from fatty acid oxidation is approximately 9 kcal/g in contrast to 4 kcal/g for glycogen (Stryer, 1995). Triglycerides are continually being synthesised and hydrolysed in adipocytes under the influence of various hormones. The mass of adipocytes depends on the rate of *lipolysis* (breakdown of fat) and *lipogenesis* (synthesis of fat) and the number of fat cells.

Despite popular historical misconception, it is now generally accepted by researchers that the number of adipocytes is not fixed. An increase in fat cell number is usually

seen in severe obesity and can persist in adulthood (Gregoire *et al.*, 1998, Sjostrom & Bjorntorp, 1974).

During weight gain, it appears that there is a phase of increasing adipocyte size followed by a period of increase in adipocyte number (Björntorp *et al.*, 1982, Björntorp 1991, Faust *et al.*, 1978, Prins *et al.*, 1997, Shillabeer *et al.*, 1994, Sjöström & Björntorp, 1974, Vasselli *et al.*, 1992). Therefore, while the volume of lipid an individual adipocyte can accumulate is finite, the capacity of adipose tissue to expand is virtually limitless. Number of studies have shown that there is no direct evidence that weight loss induced by energy restriction (Miller *et al.*, 1983, Taylor 1979) or diet restricting surgery such as stomach stapling (Kral *et al.*, 1977), exercise (Askew, 1984, Tremblay *et al.*, 1984) or pharmacotherapy (Qian *et al.*, 1998) induces loss of adipocyte numbers. The only practical means by which we can reduce fat cell number might be via lipectomy. This mode of cell removal is only performed on subcutaneous adipose tissue, which has the lowest adverse effects (Terry *et al.*, 1991). There are however, others who believe cell number is reduced upon weight loss. This is shown in studies done by Sjostrom and colleagues. They have shown in a group of ex-obese who have lost up to 30 kg (54%) of body fat using gastric bypass or gastroplasty, have lost a significant number of their fat cells (14%) (Naslund *et al.*, 1987, Sjostrom & Willim-olsson, 1981). The literatures presented here appear conflicting. Thus further studies are necessary to shed more light on this topic. However, the issue remains that "...the intractable component of severe obesity in man is a surfeit of fat cells...(Faust, 1980)", and individuals with high number of fat cells show a poor prognosis to weight loss and low weight maintenance (Bjorntorp *et al.*, 1975, Krothiewski, 1977).

An alternative and rather radical solution proposed in this thesis to overcome this problem is to eliminate extra adipocytes by inducing apoptosis (cell suicide). This is achieved by using DNA intercalators (drugs used to treat cancer) that attack cellular DNA and result in apoptosis. It is not a means to prevent obesity. It is a treatment for the obese condition that recognises the important distinction between gaining weight and losing weight. At the centre of the rationale for treatment is the hypothesis that weight loss is not the reverse of weight gain. We propose a therapy aimed at the restoration of homeostasis within adipose tissue stores. In this way many of the drives to the central nervous system may be normalised and the central defect ameliorated if not cured completely.

The following chapters look at adipose tissue more closely in the hope of achieving better understanding of the tissue particularly in regard to reduction in cell size and ultimately devising new methods by which weight loss can be maintained.

CHAPTER 2

Exercise Induced Weight Loss

2.0) Introduction

Maintaining energy balance is one of the most important functions for any living organism. The regulation of the energy balance is the work of many genes and gene products (for example, enzymes), receptors, and neurotransmitters, which govern energy expenditure, appetite, and metabolic signalling. However, for weight loss to occur there has to simply exist a period of negative energy balance, that is energy expenditure must exceed energy intake. A weight loss therapy is considered successful when there is a decrease in adipose tissue mass, a process that is caused by breakdown of stored fat.

Chemically fat is a range of triglycerides synthesised from fatty acid and glycerol (Kirtland, 1975), a process called *lipogenesis* under the influence of lipoprotein lipase (LPL). The endogenous and exogenous triglycerides are transported in plasma as lipoprotein complexes. LPL breaks down the lipoprotein complex and hydrolyses triglycerides to fatty acids and glycerol. The site of LPL is at the capillary endothelium. Fatty acids are then taken up by tissue for storage or oxidation (Nilsson-Ehle, 1980).

Triglycerides are anhydrous, which increases their energy content per unit mass, making them the most efficient form of energy storage. It has been estimated that the body stores of triglycerides are sufficient to provide adequate energy for 30-40 days of starvation for an individual of normal weight (Hales *et al.*, 1978). The majority of triglycerides are stored in adipose tissue. The remaining triglycerides are stored in other tissues such as liver, kidney, and muscle (Coppack *et al.*, 1999, Hales *et al.*, 1978). Stored triglycerides can undergo the process of *lipolysis*, which mobilises

stored fat. Lipolysis provides the body with the mobilised fatty acids as a vital source of energy in the post-absorptive state.

The process of fat mobilisation is under the influence of hormone sensitive lipase (HSL). HSL is the rate limiting enzyme in lipolysis in fat cells (Large *et al.*, 1998, Eden *et al.*, 1990) and catalyses the degradation of triglycerides into non-esterified fatty acids and glycerol (Barsh *et al.*, 1964). Activation of lipolytic activation associated with increasing the level of phosphorylation of HSL and deactivation of lipolysis is associated with decreasing the level of phosphorylation of HSL. A decrease in expression of HSL has been suggested to be the cause of the decrease in enzyme function and consequent impaired lipolytic activity of adipocytes in some cases of obesity (Large *et al.*, 1999).

Changes in fat cell size during weight gain and weight loss are a result of the orchestrated processes of lipogenesis and lipolysis that are controlled by the co-ordinated regulation of LPL and HSL. During fasting HSL is more active, whereas, after meals LPL becomes more active (Lafontan *et al.*, 1997). There are regional differences in expression of LPL, which could help explain differences in adipocytes seen in various fat depots (Lafontan *et al.*, 1997). A new pathway controlling human adipose tissue lipolysis via atria natriuretic peptides (ANP) is also under investigation, which operates via a cGMP-dependent pathway (Greenberg *et al.*, 1991, Sengenès *et al.*, 2000).

The liberated fatty acids are transported (bound to albumin) via the circulation from adipose tissue and are used as important oxidative fuel by liver, kidney, skeletal muscle, and the myocardium (Coppack *et al.*, 1994).

Usually the rate of lipolysis is higher than that of fat oxidation, which results in re-esterification of surplus circulating free fatty acids to form triglycerides (Elia *et al.*, 1987). The process of re-esterification usually occurs at two levels depending on the need for fat utilisation. The primary re-esterification is at the level of adipose tissue where adipose tissue immediately re-esterifies some of the excess fatty acids (Guan *et al.*, 2002). Secondary re-esterification is performed in liver, where the re-esterified fatty acids are stored, or packaged into lipoproteins and secreted into plasma (Hales *et al.*, 1978). The balance between triglyceride lipolysis and fatty acid esterification determines the size of fat stores, both the level of total fat stores and the transfer of fat from one fat depot to another. Energy balance sets the overall, long-term direction of the equilibrium.

Physical activity has also been shown to have a positive influence on the distribution of fat, with physically active people having smaller stores of intra-abdominal fat, which appears to bring the most pathological sequelae (reviewed in Rippe & Hess, 1998).

As discussed earlier, fat oxidation is impaired in obese and post-obese individuals (Filozof *et al.*, 2000a, Hainer *et al.*, 2000, Peuter, 1992, Weinsiet, 2000, Wyatt, 1999).

Some studies indicate a positive influence of exercise on fat oxidation in lean individuals (Calles-Escandon *et al.*, 1996, Hunter *et al.*, 1998, Larsson *et al.*, 1995, van Baak 1999). In contrast the effect of exercise on fat oxidation in obese and in post-obese individuals is less clear (van Baak, 1999). Whether exercise corrects fat oxidation in obese and post-obese or not, one thing is clear; that addition of an appropriate exercise program to a diet restriction results in a greater energy deficit

than diet alone, which presumably increases the use of fat stores as a source of fuel (Doucet *et al.*, 1999, Kempen *et al.*, 1995).

2.1) Size Fluctuation of Adipocytes in Adipose Tissue

It is generally believed that during weight gain, fat cells increase in size until they reach a critical mass (0.6µg in rats - 0.8 µg in human) (Björntorp *et al.*, 1982, Björntorp 1991, Faust *et al.*, 1978, Sjöström & Björntorp, 1974, Vasselli *et al.*, 1992) beyond which new adipocytes are recruited (Björntorp *et al.*, 1982, reviewed in Prins *et al.*, 1997, Shillabeer *et al.*, 1994). An increase in adipocyte number is caused by replication and differentiation of preadipocytes (reviewed in Prins *et al.*, 1997), with this process being continuous throughout the life of the individual (Kasubuchi *et al.*, 1979). Preadipocytes are precursor cells that remain dormant until needed, when they differentiate into mature adipocytes (Björntorp *et al.*, 1982, Prins *et al.*, 1997) capable of storing large amounts of lipid. This results in an increase in the number of adipocytes in obese humans and animals (Björntorp *et al.*, 1971a, Björntorp *et al.*, 1971b, Björntorp *et al.*, 1975, Hagger 1975, Hirsch & Knittle, 1970, Knittle, 1974, Sjöström & Björntorp, 1974). Excess energy (Faust *et al.*, 1978 Lemonier 1972, Vasselli *et al.*, 1992) along with genetic factors (Björntorp *et al.*, 1982, Faust *et al.*, 1978) has been blamed for an increase in adipocyte number during obesity.

On the other hand when we induce weight loss, lipid is mobilised from adipocytes reducing their size (Björntorp *et al.*, 1975, Dark *et al.*, 1989, Hirsch *et al.*, 1969, Hirsch & Knittle, 1970, Oscai *et al.*, 1972). Some studies show that once formed, fat cells do not reduce in number upon weight loss (Björntorp *et al.*, 1971a, Dark *et al.*, 1989, Hirsch & Han, 1969). This persistence in fat cells is evident after massive

weight loss due to exercise (Askew 1984, Tremblay *et al.*, 1984) or diet (Miller *et al.*, 1983, Taylor 1979). This has been convincingly shown in rats where even drastic starvation causes no significant reduction in cell number despite greatly reduced cell size (Miller *et al.*, 1983). In humans, a reduction in adipocyte size (but not number) can be induced by dieting, exercise or surgery (this does not include liposuction or lipectomy) and can be long lasting. Tremblay (1984) has shown that adipocyte cell diameter was reduced in individuals who had lost weight and taken up long distance running. Their adipocytes were half the volume of control, weight-matched, always-lean runners despite up to 10 years of running to achieve weight control (Tremblay *et al.*, 1984). Kral and colleagues (1977) showed that large losses of weight induced by gastric surgery were accompanied by large reductions in cell volume. In this study authors reported a small reduction in cell number. The authors were, however, unsure whether the cell loss was artefactual and caused by their inability to measure number or size of very small adipocytes (Kral *et al.*, 1977). There are however, others who hold a different view stating that cell number does reduce upon weight loss. Sjostrom and colleagues have shown that weight loss induced by gastric bypass and gastroplasty resulted in a significant reduction in cells number (Naslund *et al.*, 1988, Sjostrom & Willim-ollson, 1981). However, obese individuals still have an excess number of adipocytes after weight loss, which are now small and are presumably sending small satiety signals to the brain. This surfeit of adipocytes have been shown to result in individuals having poor prognosis in weight reduction and low weight maintenance (Bjorntorp *et al.*, 1975, Faust, 1980, Krothiewski, 1977).

The strategy of preserving adipocytes may explain the inability of the ex-obese to maintain weight loss. Preservation during weight loss of extra adipocytes recruited

during times of plenty results in a smaller adipocyte size for any given size of body fat because the lipid is distributed among a larger number of units. If, for example, leptin output were regulated at the level of the individual cell then such small adipocytes would produce subnormal amounts of leptin. This has been observed in women who have lost moderate amounts of weight. The secretion of leptin falls to subnormal levels even though the individuals were above normal weight (Considine *et al.* 1996, Geldszus *et al.*, 1996). These individuals would presumably be receiving a subnormal satiety signal and be more prone to eat even though their weight was above normal (but reduced). In evolutionary terms, this may not have been a disadvantage since such individuals would eat more readily, replace used fat and hence survive longer periods of starvation. It is only today when energy expenditure is rarely above energy intake that such a hormonal imbalance exposes a pathological consequence.

Weight loss and the changes that occur as a result of this lost weight need to be explored at the tissue level. For example, during weight loss do all cells and all fat depots contribute equally to weight loss? Results from previous research show that visceral adipose tissue is preferentially mobilised by diet and exercise (Askew, 1984, Ross *et al.*, 1996), which results in improvement in metabolic risk factors (Krempf & Farnier, 2001).

The study of anatomic changes in adipose tissue due to weight loss is a relatively unexplored area. One factor contributing to this is the difficulty in reducing fat stores by dietary restriction alone, particularly in the rodent. Rats preserve body weight remarkably well in the face of caloric restriction and significant depletion of lipid stores occurs only at a point of starvation that results in significant death (Miller *et al.*, 1983). This makes such studies an ethical dilemma. As an alternative we have

adopted a paradigm that results in significant depletion of adipose stores using the tendency of female rats to run extraordinary distances in running wheels.

The following study is not an attempt to show the benefit of weight loss using exercise. Instead exercise is used as a tool to reduce fat cell size and open the way for a range of different studies of adipose tissue. The first of those presented in this thesis is a study of the adipocyte size change as a result of exercise and the next chapter will examine these differences in adipocytes at a more detailed micro-regional level within the same depot.

2.2) Study Design

In this study female rats of the same age and same initial weight are divided into two groups. One group constitutes the sedentary (control) group and the other exercised group have unlimited access to running wheels.

Running data, body weight data, vaginal cytology and adipocyte cell sizes are investigated and correlations of various data are made in order to establish possible differences between the two groups of rats. The study concentrates on intra-abdominal fat depots because changes in size of these correlate with pathological consequences of excess fat storage.

2.3) Materials & Methods

Dulbecco's Modification of Eagle's Medium (DMEM) or Ham's F12 was obtained from ICN chemicals (Sydney). All other reagents and solvents were of analytical grade and obtained through standard suppliers.

A group of 10 female Wistar rats was obtained from Animal Resource Centre (ARC) in Perth. The average weight of these rats was 267.8 ± 9.5 g (mean \pm SD). The animals were divided into two groups of five and housed in temperature and humidity controlled environment, with lights on daily from 0600 hrs to 1800 hrs. The first group of animals was used as an exercise group and was housed separately in wire-bottomed cages equipped with a running wheel (34.5 cm diameter) (Figure 2.1) on which they could exercise voluntarily for 10 weeks.

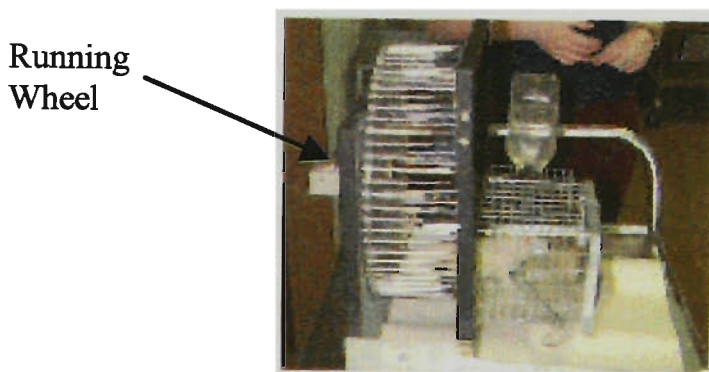


Figure 2.1) *An illustration of a running wheel cage used in this study.*

The running regimen was started for all rats on the same day. Magnetic latches enabled the total number of revolutions, the speed and the length of each bout of running to be logged continuously on a Macintosh computer using a MacLab (AD Instruments, Sydney). Exercised rats were weighed daily just before the beginning of dark period

The Wollongong University Animal Ethic's Committee requires that animals should not be housed separately unless stated as one of the objectives in the study design.

Therefore, the second group (control) of animals were housed in pairs in ‘metabolic’ cages where there was no facility for exercise for the entire 10 weeks. Water and rat chow were available *ad libitum* for both groups of rats. Sedentary rats were weighed weekly.

All the animal studies were approved by the University of Wollongong Animal Ethics Committee (Animal ethics number AE99/02).

2.3.1) Tissue Preparation

Rats were anaesthetized using intraperitoneal ketamine (80mg/kg) and intra-muscular rompun (12mg/kg) injections. The rats were then perfused through the left ventricle with oxygenated tissue culture medium – Dulbecco’s Modification of Eagle’s Medium (DMEM) or Ham’s F12 (100-200ml) followed by fixative containing a mixture of aldehydes (formaldehyde 1% w/v and glutaraldehyde 1% w/v) in 0.1M phosphate buffer, pH 7.4 at room temperature. For routine histology, retroperitoneal adipose tissue was post-fixed in the same fixative for 1 hour at room temperature and then overnight at 4°C and washed three times in 0.1M sodium phosphate buffer, pH 7.4, and stored in the same buffer at 4°C. The Anatomical Pathology Lab at the Wollongong Hospital performed paraffin tissue sectioning and routine Haematoxylin and Eosin staining (for specific details of the method see appendix I).

2.3.2) Analysis of Fat Cell Size

The sizes of approximately 100 fat cells were measured adopting a technique used by Sjöstrom (1971), viewing 4 µm thick slides under an Olympus BH2 light microscope equipped with an eyepiece micrometer at 40x magnification (Sjöstrom *et al.*, 1971). For adipocytes appearing as circular objects, one measurement of diameter was done.

For cells appearing as elliptical objects, a measurement of ‘dmax’ and a measurement of ‘dmin’ were performed (see figure 2.2).

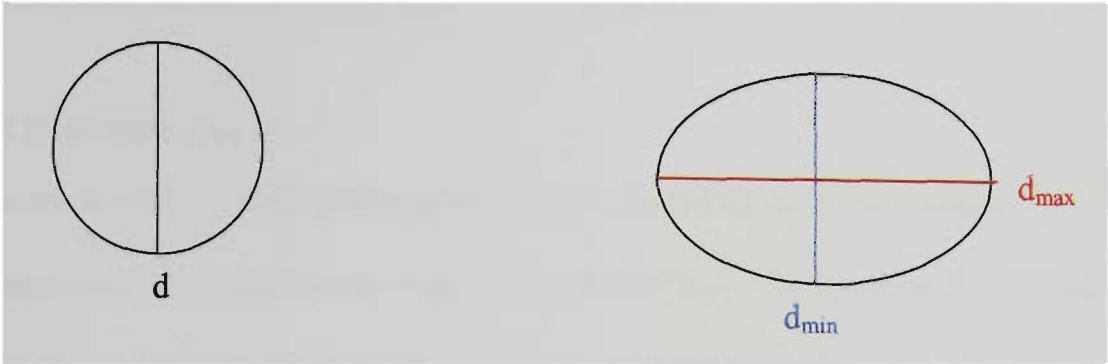


Figure 2.2) *The measurement of fat cell diameters based on their appearance under the microscope.*

The eyepiece micrometer was used to measure the distances at 40X magnification. a) circular cell b) elliptical cell. d_{max}) maximum diameter, d_{min}) minimum diameter.

2.3.3) Stereological Analysis

The diameter for each fat cell was corrected by the method of Weibel (1980),

$$D = 4/\pi d - t/2$$

d = measured diameter,
 D = corrected average diameter,
 t = section thickness (4μm).

This method corrects for the random sectioning of the tissue at any given tissue thickness (Weibel, 1980). The volume of each and every cell was calculated using the corrected diameter (D) (volume of sphere is $V=4/3 \pi r^3$, and volume of ellipsoid is $V=4/3 \pi (r_{max})(r_{min})^2$, where V = volume in Pico litres (pl), r = radius = ½ D in μm). The average volume for all fat cells was determined from these individually calculated volumes.

2.3.4) Statistical Analysis

Differences in sizes of adipocytes were analysed using the student's t-test. Analysis of Variance (ANOVA) was used to test the possible correlation of the data. Values of $p < 0.05$ were considered significant. All measurements are indicated as mean \pm SEM.

2.3.5) Estrus Cycle

The estrus cycle of the exercise-group was monitored by daily microscopic examination of vaginal smears taken at 1700-1800 hrs. The prepared slides were stained using routine *Papanicolaou* staining method and examined under a light microscope (for specific details see appendix II). Large, squamous-type epithelial cells without nuclei characterized the estrus cycle. Observation of vaginal cytology was continued every day until the animals of both groups were sacrificed.

2.4) Results

2.4.1) Control (sedentary) Rats:

Table 2.1 summarises the body weight and cell size (D=diameter in μm , Vol=volume in pl) data for 5 sedentary rats (S1-S5) from the start to the end of the 10-week study.

Rats	Start Wt. (g)	End Wt. (g)	Wt. Gain (g)	D \pm SEM (μm)	Vol. \pm SEM (pl)
S1	262	359	97	141.4 \pm 3.1	164.2 \pm 10.5
S2	270	390	120	132.9 \pm 3.2	137.9 \pm 10.2
S3	272	353	81	133.1 \pm 3.1	127.4 \pm 9.0
S4	270	346	76	146.3 \pm 2.9	189.0 \pm 10.3
S5	270	313	43	129.8 \pm 2.5	130.8 \pm 7.5
Average	268.8	352.2	83.4	137.8	156.4
SD	3.9	27.6	13.4	29.9	10.0
SEM	1.8	12.3	6.0	1.5	4.9

Table 2.1) *Data for sedentary rats after 10 weeks.*

S1-S5 represents the 5 sedentary rats used for the study. Start Wt.= body weight of the rats at the start of the study, End Wt.= body weight of the rats at the end of the study, Wt. Gain = the change in body weight over the period of the study. D= corrected average diameter in micrometers (μm), SD= standard deviation, SEM= standard error of the mean. Vol.= calculated average volume in picoliters (pl). Values are given as 'mean \pm SEM'.

Figure 2.3 shows a graph of the weight changes observed in sedentary rats during 10 weeks. There was a steady increase in weight of all rats during the 10 weeks. The weight increased from 268.8 ± 1.8 g to 352.2 ± 12.3 g, an increase of 83.4 ± 6.0 g (Figure 2.4). This change in weight was significant with $p < 0.01$ (t-test). The calculated average diameter of retroperitoneal adipocytes for these control rats was 137.8 ± 1.5 μm . This translates into a volume of 156.4 ± 4.9 pl.

*Body Weight Change of Sedentary Rats
Over 10 Weeks*

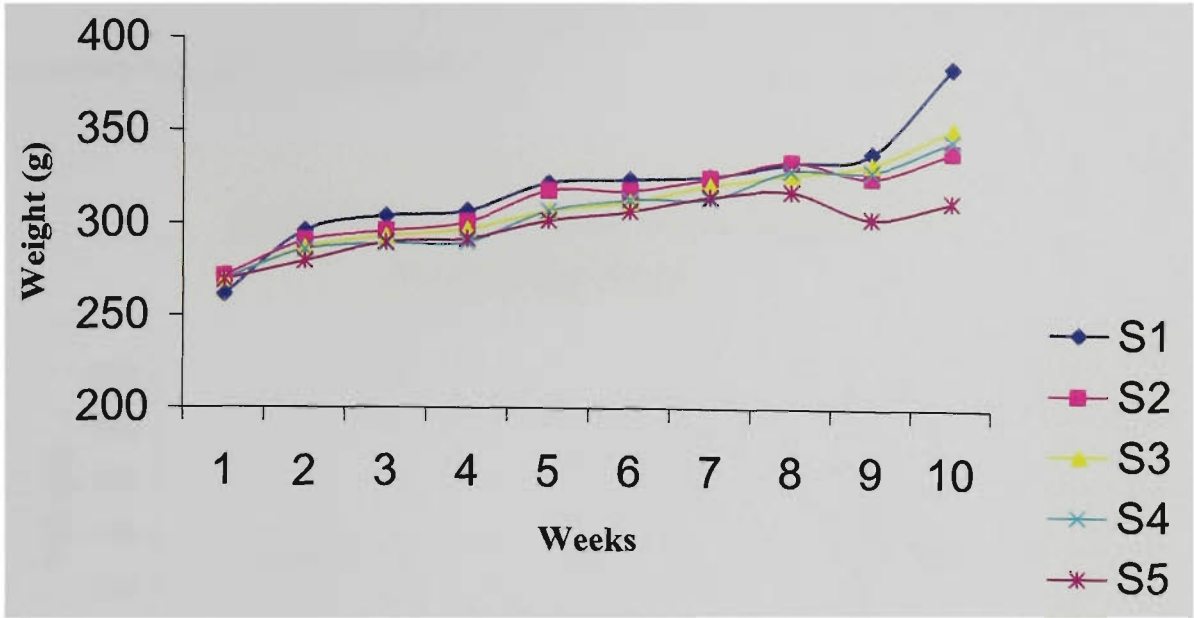


Figure 2.3) A graph of changes in rat weights during 10 weeks of sedentary life-style.

*Average Weight Change of
Sedentary Rats*

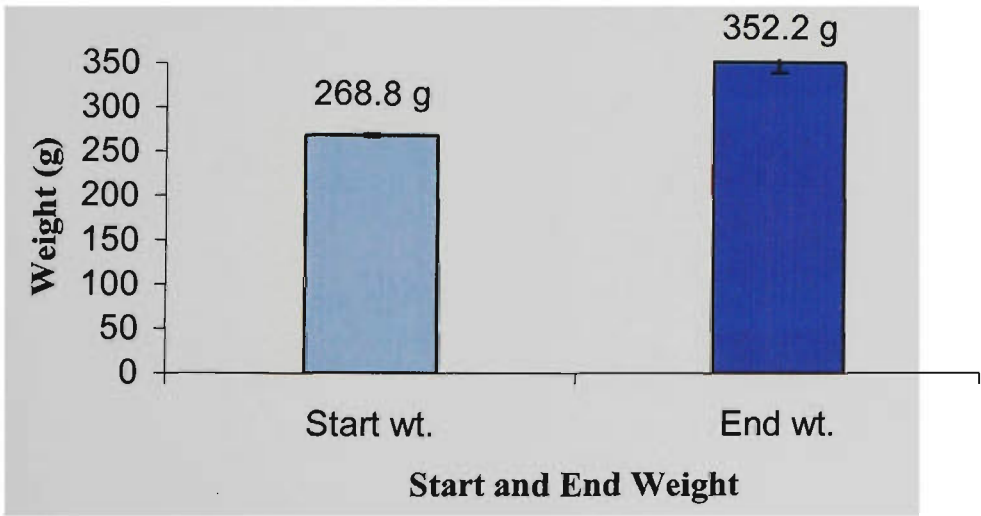


Figure 2.4) Comparison of starting weight ($268.8 \pm 1.8\text{g}$) with final weight ($352.2 \pm 12.3\text{g}$) for 5 sedentary rats after 10 weeks. The values are mean \pm SEM. $P < 0.01$ (t-test).

Figure 2.5 displays a scatter plot of cell volumes versus body weight for 5 sedentary rats. There was no correlation between final body weight and cell sizes in these 5 sedentary rats ($p>0.05$, ANOVA).

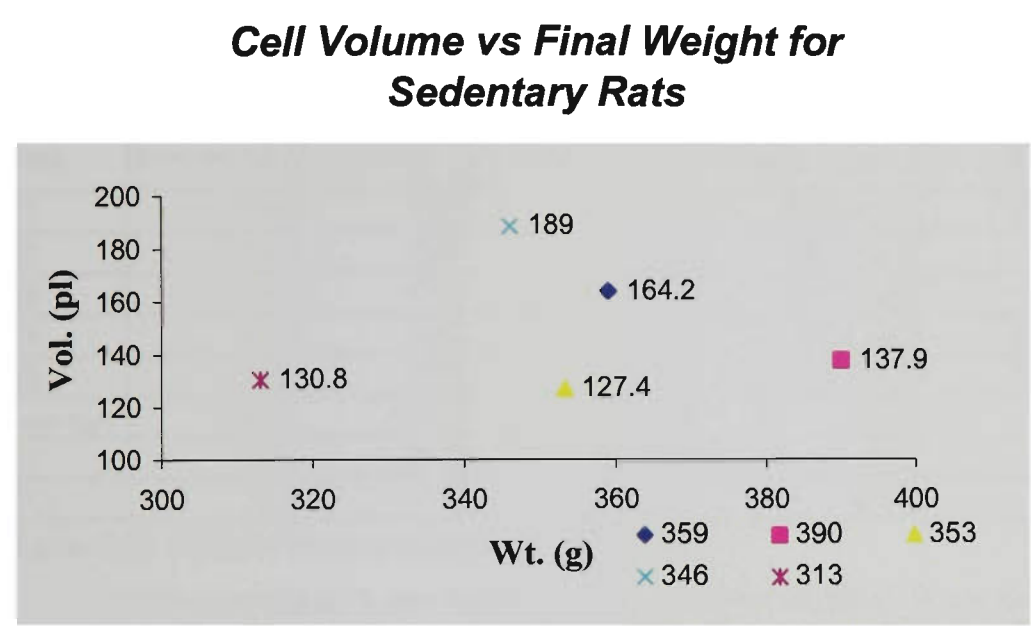


Figure 2.5) *A graphical comparison of body weight (g) and cellular volume (pl) for 5 sedentary rats.*

The calculated correlation is 0.0057 and $R^2=3\times10^{-5}$ ($p>0.05$, ANOVA).

2.4.2) Exercised Rats

Table 2.2 summarises the data for 5 exercised rats (E1-E5) after 10 weeks of study period. These rats had gained weight from 266.7 ± 5 g to 305.2 ± 11.3 g. The average cell volume of retroperitoneal fat pad was 35.3 ± 1.05 pl. The cell volumes of these rats varied from 22.6 ± 1.1 pl (E5) to 57.8 ± 3.5 pl (E1).

Rats	Start wt. (g)	End wt. (g)	Wt. Change (g)	D \pm SEM (μ m)	Vol. \pm SEM (pL)
E1	262	322	60	98.8 ± 2.0	57.8 ± 3.5
E2	271	320	49	78.2 ± 1.4	28 ± 1.6
E3	280	311	31	83.0 ± 1.5	34.1 ± 1.9
E4	261	302	41	84.4 ± 1.6	35.3 ± 1.7
E5	250	271	21	72.8 ± 1.3	22.6 ± 1.1
Average	266.7	305.2	40.4	83.2	35.3
SD	11.1	25.3	25.7	19.5	26.5
SEM	5.0	11.3	11.5	0.77	1.05

Table 2.2) Data for exercised rats after 10 weeks.

E1-E5 represents the 5 exercised rats. The corrected cell diameters ‘D’ are used to calculate the cell volumes for each rat. The values for ‘D’ and Vol. are given in ‘g’ and ‘pl’ respectively. Values are given as mean \pm SEM.

Table 2.3 shows the running result for these 5 exercised rats. The data is organised as the total distance run by each rat in “km” and the distance run on the day of sacrifice in “km”. The cumulative distance run by each rat varied from 420 km to 550 km. Rat 4 ran the greatest distance (550 km) during 10 weeks. This corresponds to approximately 7.9 km running per day. Rat 5 had the maximum distance run on the day of sacrifice (16.3 km).

Rats	Total Run (km)	Run at Sac. (km)
E1	528.1	1.8
E2	420.6	1.1
E3	427.0	1.8
E4	551.1	2.6
E5	430.1	16.3
Average	471.4	4.7

Table 2.3) *A summary of running data for five exercised rats.*

Total Run is the cumulative distance run by each rat over 10 weeks. Run at Sac. is the distance run by each rat on the day of sacrifice. All distances are given in kilometres (km).

Figure 2.6 shows the weight change versus cell volume data for the five exercised rats. According to these results there is no correlation between cell volume and weight change in these rats ($p>0.05$, ANOVA).

Cell Volume vs Weight Change for Exercised Rats

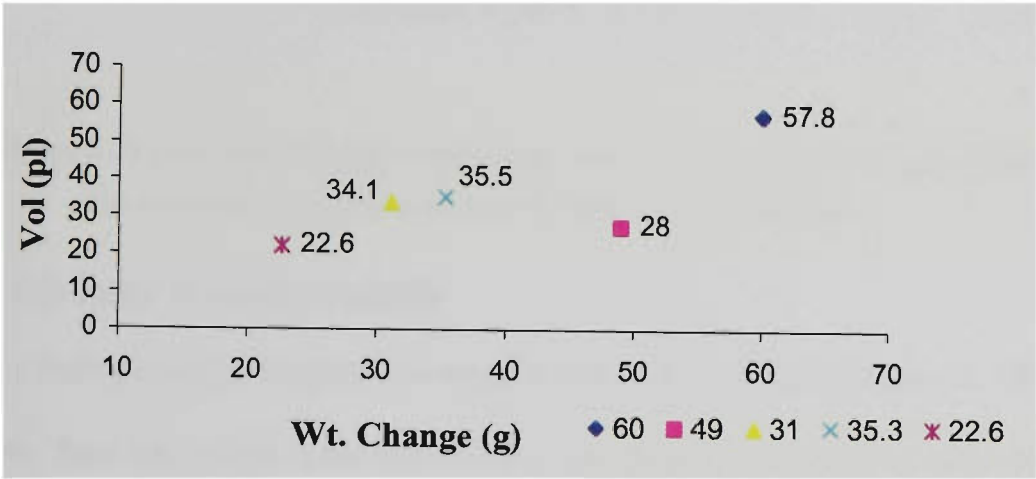


Figure 2.6) *Cell volumes (pl) versus weight change (g) for 5 exercised rats.*

The calculated correlation is 0.78 and $R^2=0.61$ ($p>0.05$, ANOVA).

Figure 2.7 shows the retroperitoneal cell volumes versus total cumulative distance run by rats at the end of 10 weeks. The results of this analysis showed no correlation between the total distance run and the cell volume for these rats ($P>0.05$, ANOVA).

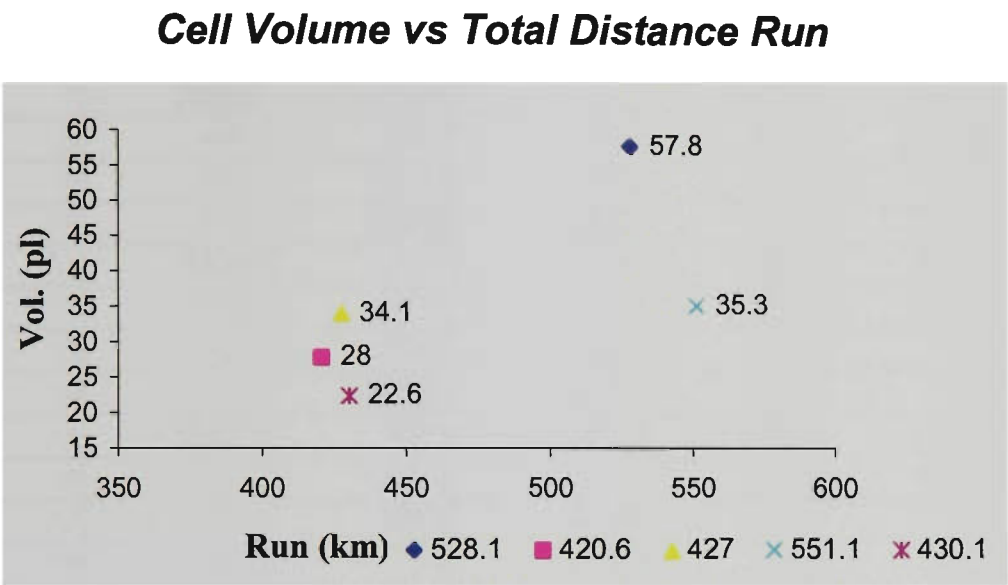


Figure 2.7) *Cell volumes (pl) versus total distance run (km) for 5 exercised rats.*
The calculated correlation is 0.64 and $R^2=0.43$ ($p>0.05$, ANOVA)

2.4.3) Daily Running Activity

The daily pattern of running was cyclical with a peak of activity every 4-5 days (Table 2.4). Rats ran mainly in the dark period. The peak running activity was 4-8 fold higher than the lowest running activity. Vaginal cytology showed that the rats had a 4-5 day estrus cycle. The maximum distance run was observed every 4-5 days corresponding to the day of estrus. Figure 2.8 is a simple graph of running pattern for the last 20 days until the day of sacrifice.

Cycle (days)	Run (m) R1	Run (m) R2	Run (m) R3	Run (m) R4	Run R5
1	0	0	0	0	0
2	12042	11304	12019	11993	4479
3	16242	1056	8223	15197	11664
4	7545	1126	5342	2844	14347
5	4761	3631	6462	8223	2383
6	12512	13513	16761	10903	3176
7	20649	1238	4544	19322	12826
8	4881	1931	7361	2512	16450
9	8280	10431	9765	11506	2419
10	9765	12206	15904	10737	6575
11	18281	4138	3489	15833	10557
12	6646	1301	4660	5098	19689
13	4211	6009	8496	4072	1246
14	12000	12920	16341	5464	2049
15	16501	1243	4938	17315	4042
16	5230	2915	4251	1429	12360
17	4362	9965	8756	7460	2411
18	3820	94	6366	2151	949
19	15157	1176	1546	13962	8577
20 (Sac.)	1781	1128	1846	2565	16257

Table 2.4) *The running pattern for 5 exercised rats for the last 20 days of the study.*

“Cycle (day) 1” corresponds to 20 days before sacrifice. The estrus cycle was estimated by microscopy examination of ‘pap stained’ vaginal smears. The shaded areas correspond to the day of estrus.

Pattern of Running for 5 Exercised Rats

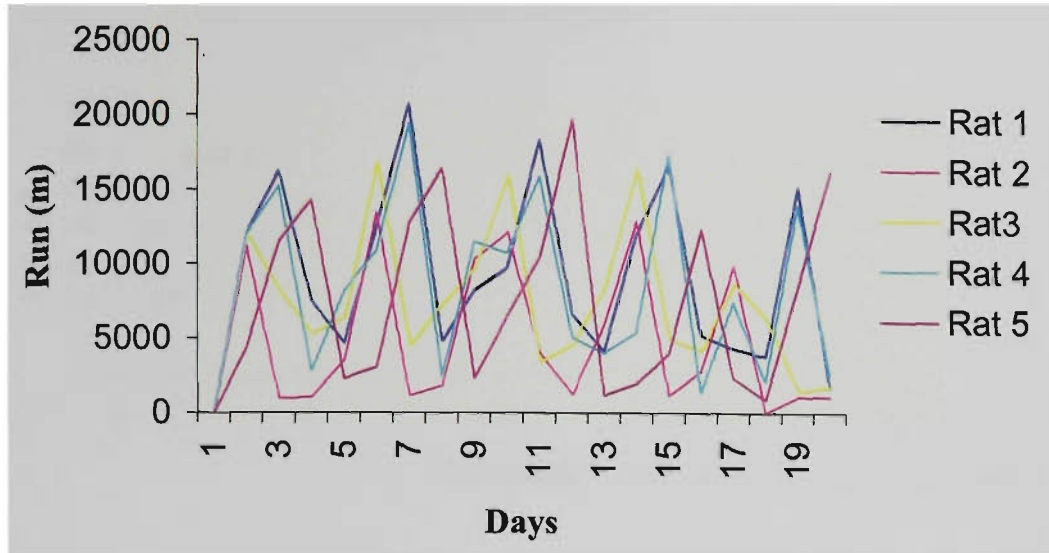


Figure 2.8) A graph representing the running pattern for the last 20 days of 5 exercised rats. There is a cyclical running pattern of every 4 to 5 days.

Examining these results shows that not all rats ran their maximum periodical run on the day of the sacrifice. Figure 2.9 shows a graph of the retroperitoneal cell volumes versus distance run by each rat on the day of sacrifice. There is no correlation between distances run on the last day and cell volumes ($p > 0.05$, ANOVA). The rat (E5) that ran the longest distance (16.3 km) had the smallest cells (22.6 pl) in the group.

Cell Volume vs Distance Run on the Day of Sacrifice

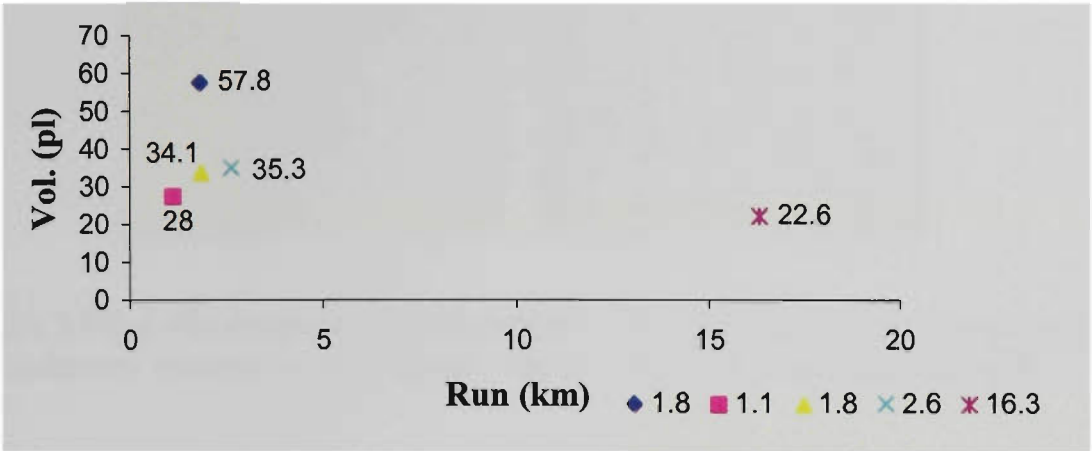


Figure 2.9) The distance run (km) on the day of sacrifice by each rat versus the cell volume (pl).
Correlation of -0.54 and $R^2=0.29$ ($p>0.05$, ANOVA). Only one rat (E5) ran the maximum distance on the day of sacrifice.

2.4.4) Exercised versus Sedentary:

Table 2.5 summarises the data for 5 sedentary and 5 exercised rats. The difference between the starting weight for sedentary and exercised rats is not significant ($P>0.05$, t-test). The final weight difference is, however, significant with $p<0.05$ (352.2 ± 12.3 g for sedentary versus 305.2 ± 11.3 g for exercised rats).

Rats	Start Wt.±SEM (g)	End Wt.±SEM (g)	Wt. Change±SEM (g)	D±SEM (µm)	Vol.±SEM (pl)
S	268.8 ± 1.8	352 ± 12.3	83.4 ± 6.0	137.8 ± 1.5	156.4 ± 4.9
E	266.7 ± 5.0	305.2 ± 11.3	40.4 ± 11.5	83.2 ± 0.77	35.3 ± 1.1
p value	0.5	0.02	0.004	4.403E-143	3.32404E-85

Table 2.5) A summary of weight changes and cell volumes for sedentary (S) and exercised (E) rats. The values are given as mean ± SEM.

Figure 2.10 is a photograph of a 4µm thick H&E paraffin section of retroperitoneal fat pad from a sedentary and an exercised animal. As it is evident from this picture, the cell sizes of the two rats look remarkably different.

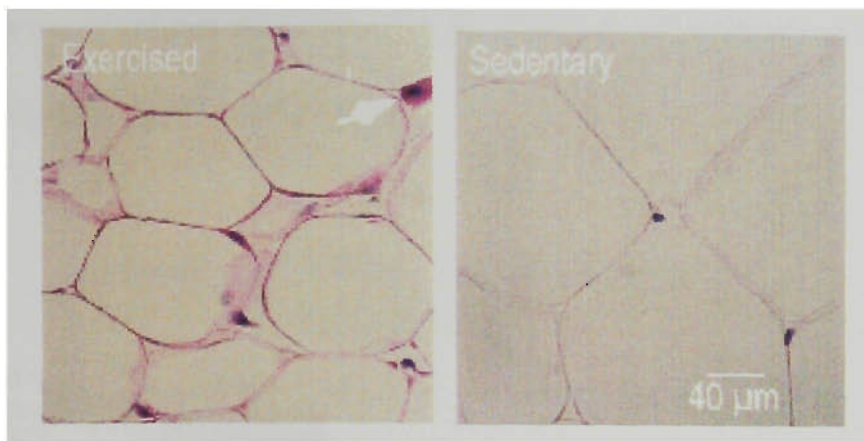


Figure 2.10) A 40x magnification photograph of two typical 4μm section of exercised and sedentary retroperitoneal fat pad. The arrow points to an interstitial cell.

Figure 2.11 shows the differences in weight changes for sedentary (S) and exercised (E) rats. According to these results, the weight change in sedentary animals was twice that of the exercised rats. This difference is statistically different with $p < 0.01$ (t-test).

Comparison of Body Weight Change between Sedentary and Exercise Rats

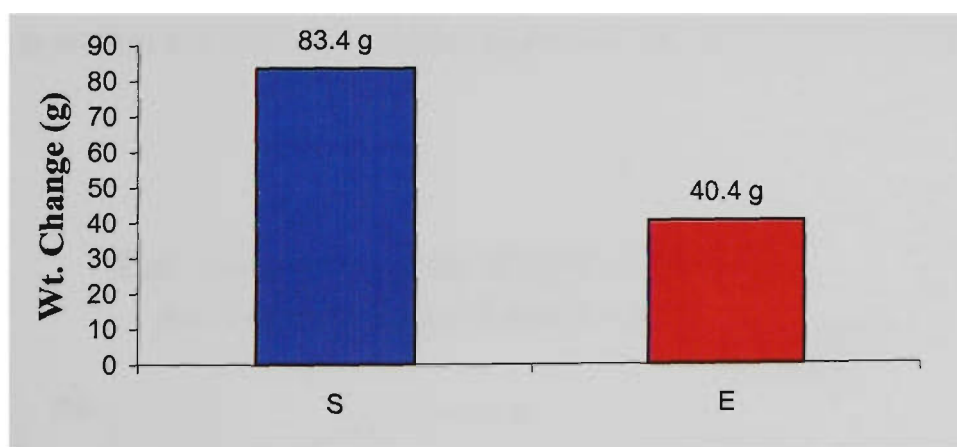


Figure 2.11) A graph comparing the body weight change in sedentary (S) and exercised (E) rats ($p < 0.01$, t-test).

Figure 2.12 shows the difference in cell volume in sedentary and exercised rats. Average cell volume for sedentary rats was 156.4 ± 4.9 pl, and the average cell

volume for exercised rats was 35.3 ± 1.1 pl. This 4.4 fold difference is highly significantly different ($p<0.001$, t-test).

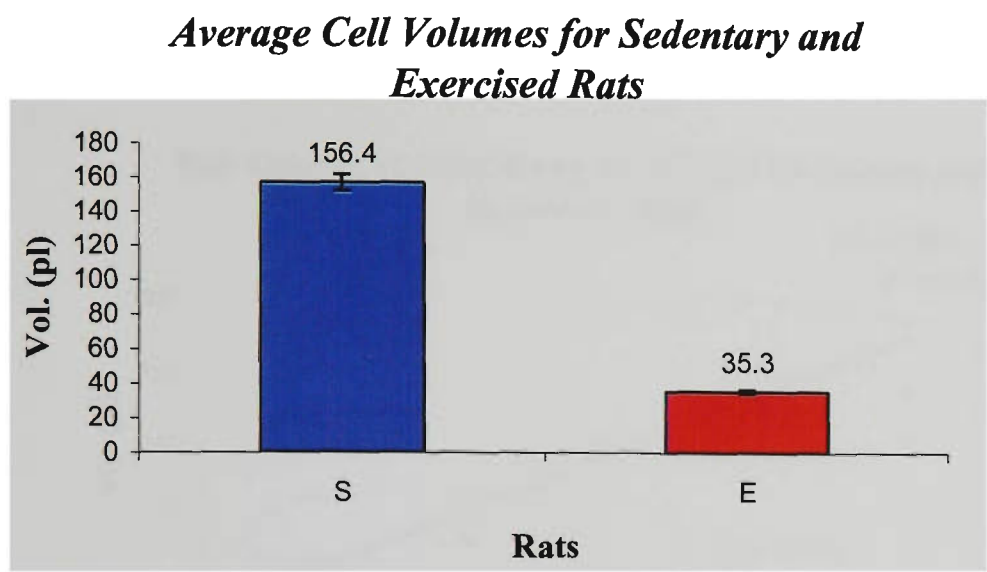


Figure 2.12) Comparison of average cell volumes of sedentary and exercised rats.

Sedentary rats display an average cell volume of 134.9 (SEM=11) versus exercised rats average cell volume of 30.4 (SEM=4.7), $p<0.001$ (t-test).

When cell volumes are plotted versus weight change for both groups of rats (figure 2.13), it is evident that there is a positive correlation ($R^2=0.55$, $P<0.05$, ANOVA).

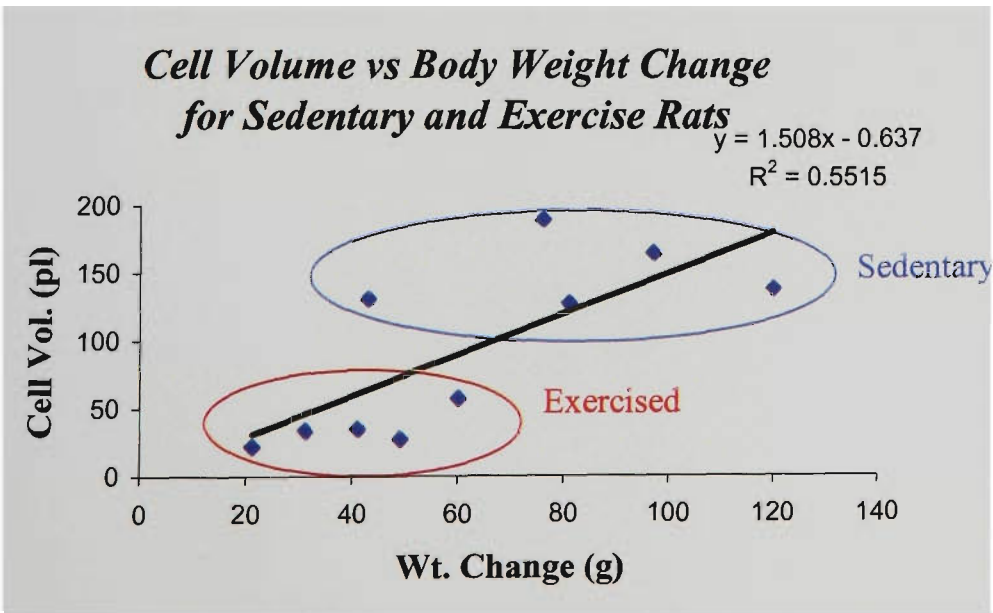


Figure 2.13) A graph of cell volume versus body weight change for sedentary and exercised rats ($p<0.05$, ANOVA).

There is a similar correlation when cell volume of all rats is plotted against their final body weight (figure 2.14). It is evident from this graph that there is a positive correlation between cell volumes and final body weight ($R^2=0.55$, $P<0.05$, ANOVA)

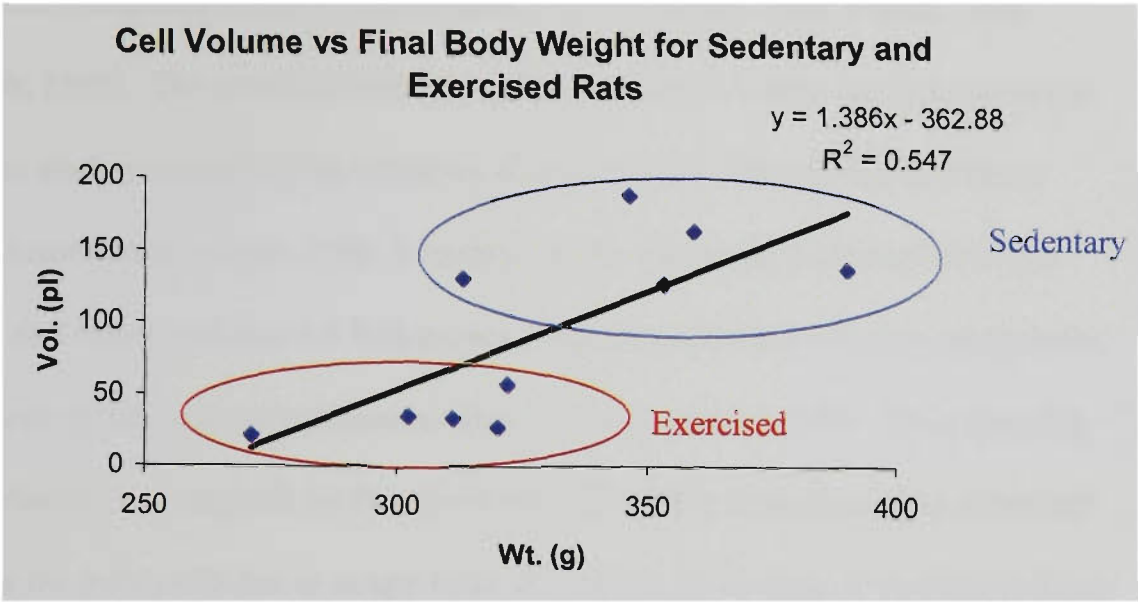


Figure 2.14) A graph of cell volume versus final body weight for sedentary and exercised rats ($p<0.05$, ANOVA).

2.5) Discussion

Voluntary wheel running has been studied in captive animals for almost a century (reviewed in Sherwin 1998). In this study female rats were used since previous studies done by others and in our own laboratory have shown the preference of female rats to run voluntarily (Anantharaman-Baar, 1989, Kennedy, 1964, Pierce, 1998, Sherwin, 1998). The periodical running pattern of every 4-5 days corresponds to the previous studies examining the voluntary exercise habits of female rats and estrus cycle (Anantharaman-Baar, 1989, Kennedy, 1964). Our study confirmed previous reports that female rats run 4-8 fold greater distances on the day of estrus compared to other days of the cycle (Anantharaman-Baar, 1989, Kennedy, 1964). There are other data collected, not analysed for this thesis that will enable someone else to determine whether the increase is due to longer bouts of running, more bouts of running or faster running.

The fat cell sizes in this study are obtained from thin paraffin sections of the retroperitoneal fat pad. When a 4 μ m thick section is cut through a cell it does not indicate what it may seem to show. This is because the fat cell diameter can be as large as 120 μ m which when cut randomly does not guarantee a slice through the middle and therefore the true cell diameter. Figure 2.15 shows that the sections cut through the cell will show a range of diameters from “a” to “d”. As it can be seen, the random cutting results in a collection of cell diameters progressively decreasing from the ‘true’ value.

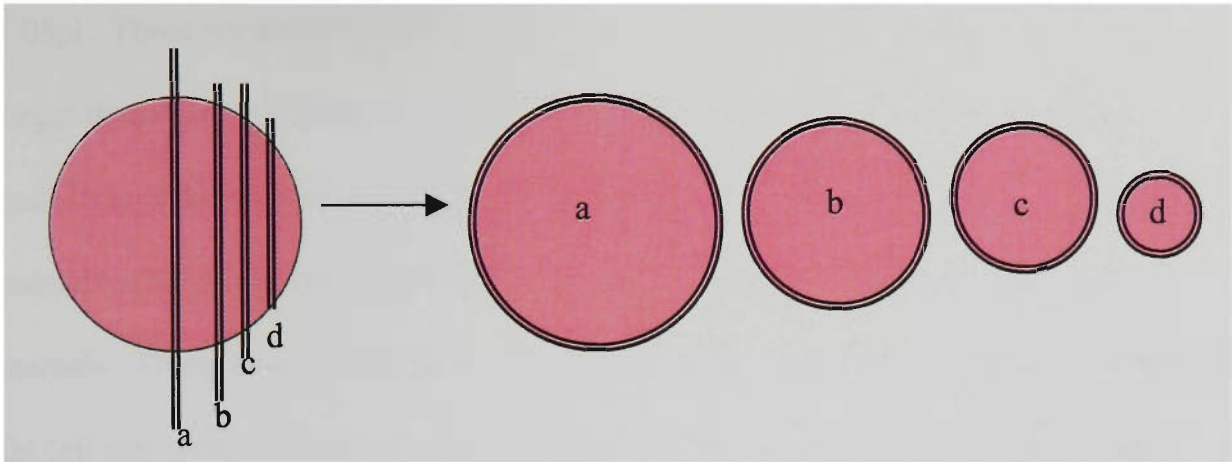


Figure 2.15) *Serial sections of a cell using routine sectioning techniques. The serial sectioning results in objects (a-d) with varying diameter.*

Since thin slices were taken as random samples, it was necessary to correct for the effect of tissue thickness. The measured cell diameters (d) of all cells were corrected by a method suggested by Weibel (1998), to give the corrected cell diameter (D). The volume of cells was calculated from ‘D’ and by assuming that fat cells are approximately spherical or ellipsoid, and then an average of these volumes was obtained.

2.5.1) Sedentary versus Exercised Rats

According to results produced in this study, there is a statistically significant positive correlation between gain in body weight and cell volumes when all rats are combined. The body weight of sedentary animals increased from an average of 268.8 ± 1.8 g (mean \pm SEM) to an average of 352.2 ± 12.3 g. This was an average of 83.4 ± 6.0 g increase in body weight. Whereas, the body weight of exercised animals increased from an average of 266.7 ± 5.0 g (mean \pm SEM) to an average of 305.2 ± 11.3 g. This was an average increase of 40.4 ± 11.5 g. The body weight change of sedentary animals was approximately twice that of exercised rats. When comparing the cell sizes we see a bigger difference, with sedentary rats having an average cell sizes of 156.4 ± 4.9 pl (mean \pm SEM) versus exercised animals displaying cells sizes of $35.3 \pm$

1.05pl. Therefore the cell sizes of sedentary animals are approximately 4.4 times larger than exercised animals. These results suggest that larger changes in weight gain seem to have resulted in larger cell volumes. The body weight change in these animals is subject to substantial muscle weight changes particularly in exercised animals. Therefore, the relatively smaller body weight differences compared to larger fat cell size differences could be due to lean body mass gained in exercised animals (Diaz-Herrera *et al.*, 2001). In order to be able to critically analyse the correlation between change in weight and change in cell volume, it is necessary to have the starting volume of the cells, which in this case was impossible. Addition of another group of aged matched rats that were sacrificed prior to being placed in running wheels or made sedentary would allow this determination (this experiment is currently being performed by another student in our laboratory). This experiment will allow us to discriminate between the two possible mechanisms of how the different cell sizes arose. One possibility (figure 2.16a) assumes that at the start of the experiment cell volumes were equal to or smaller than smallest cell size measured (22.6 pl); during the treatments cells in exercised rats **increase** minimally whereas those in sedentary rats increase several fold. The other possibility (figure 2.16b) assumes that at the start of the experiment cell volumes were bigger than the exercised cells but smaller than the sedentary ones; during the treatments cells in exercised rats **decreased** whereas those in sedentary rats increased but not as much as in the first assumption.

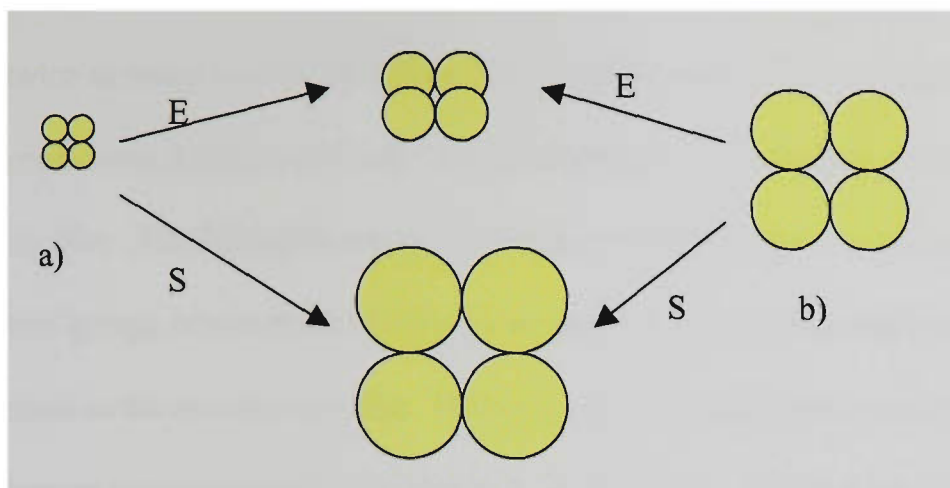


Figure 2.16) *Assumed process of size change in sedentary (S) and exercised (E) rats.*

a) Assumes that cell sizes are very small at the start following which the exercised animals only put on small amount of weight whereas the sedentary animals put on large amount of weight. b) Assumes that the cell sizes at the start were larger than cells of exercised animals but smaller than sedentary animals.

The above models assume that cell number in the two cases remains constant. From what we know about regulation of cell number, we might expect cells to be generated in sedentary rats as adipocytes reach their limit size (600-800 μg or approximately 650-875 pl) (Björntorp *et al.*, 1982, Björntorp 1991, Faust *et al.*, 1978, Sjöström & Björntorp, 1974, Vasselli *et al.*, 1992) and weight continues to be gained. The cell sizes measured here in sedentary rats were only a maximum of 189 pl, approximately four-fold lower than this theoretical limit. Thus it can be safely assumed that cell number is the same in both groups of rats. A similar logic argues that cells are not lost when rats exercise. Considering the ratio of body mass change and cell volume change further supports this. The ratio of difference in body mass change was 2 fold (83.4 ± 6.0 g sedentary rats versus 40.4 ± 11.5 g exercised rats). The corresponding ratio for volume was 4.4 fold (156.4 ± 4.9 pl sedentary rats versus 35.3 ± 1.1 for exercised rats). If the difference in body mass between the two groups of rats were entirely attributable to a change in fat mass, these two ratios should be the same if fat

cell number is constant. That they are two fold different argues that there should be twice as many smaller fat cells in the exercised rats. This is contrary to intuitive predictions where one would expect a decrease in cell number as fat stores become smaller. An alternative explanation is that the difference in body mass between the two groups of rats is attributable to an increase in lean body mass and a decrease in fat mass as the animals exercise. This is more likely and it has been observed in many human studies (reviewed in Tipton & Wolfe, 2001). The role of insulin is an important factor in the weight loss observed in these rats. As it was discussed earlier, weight loss is associated with a reduction in insulin output (Henry *et al.*, 1985, Kelley *et al.*, 1993), which would reduce its anti-lipolytic action. In exercised animals this could facilitate further weight loss and contribute to the substantial weight loss observed in results presented in this thesis.

We attempted to measure body composition using tritiated water as an indicator (Pace & Rathbun, 1945). Using this method exactly as reported gave body compositions with negative percentages of fat in exercised rats. Changing the coefficients in the equations of Pace & Rathbun enabled positive fat compositions to be calculated but the coefficient was chosen arbitrarily and changing the coefficient by very slight amounts could create any fat composition. Body composition can be reliably measured by chemical analysis. This method is incompatible with taking samples for other studies. Until a reliable method for fat content of small animals is developed, this question cannot be answered reliably in a longitudinal study of rats.

These results, however, suggest that voluntary exercise caused 4-fold reduction in fat cell sizes in the retroperitoneal fat pads of female rats compared to sedentary controls. This method of weight reduction is further used in the next chapter to study the

adipose tissue more closely. The reduction in fat cell size in adipose tissue is caused by lipolysis, which results in mobilisation of fat from adipose tissue. As discussed previously, changes in adipose tissue during weight loss results in a reduction in fat cell size. This implicates stimulating factors, which govern this fat mobilisation. The SNS is an important factor in fat mobilisation. Therefore, it is important to find out what happens to innervation of fat tissue upon weight reduction. It is also of interest to explore the possibility of heterogeneity of fat cell sizes near and far from the SNS innervation. This is done hoping to shed more light into the study of adipose tissue.

CHAPTER 3

Innervation of Adipose Tissue

3.0) Introduction

The understanding of the function of adipose tissue has changed greatly throughout history from its inconsequential beginning as a foam-like connective tissue to an organ playing a crucial role in the control of energy metabolism (reviewed in Pond, 2000). Adipose tissue plays an important role in stabilising fuel supply, holding the largest reserve of metabolic energy in the body in the form of lipid, and as a convenient source of fuel (Hauner, 1999). It accumulates fat during periods of overeating, and releases fuel during starvation. It synthesises fat, responds to hormones and nervous stimulation, and varies in its metabolic activity with the nutritional state of the animal. The presence of adipose tissue is necessary to maintain glucose homeostasis. Transgenic mice that contain no WAT show a number of metabolic abnormalities, including a fatty liver and elevated levels of plasma triglycerides (Moitra *et al.*, 1998). In conjunction with its major role in coordination of general energy metabolism, adipose tissue has other minor roles such as production of body heat (this function is however mainly restricted to BAT) (Sjöström, 1985), and to provide mechanical support to a few specific tissues, for example orbital fat (Wertheimer, 1965). Under some circumstances WAT can be converted to BAT such as exposure to cold and β_3 agonists (Guerra *et al.*, 1998). White fat has also recently been proposed to assist the immune system in fighting infection (Pond, 1999).

Adipose tissue is a heterogeneous metabolic organ with some depots showing less activity (lipid turn-over) than others. For example, the visceral depot, which includes omental and mesenteric fat, is more responsive to lipolytic action of catecholamines than other depots (reviewed in Arner, 1997). In contrast the subcutaneous region, which accounts for 80% of total body fat is more responsive to anti-lipolytic action of

insulin (reviewed in Arner, 1997). Since lipolysis is driven by either a decrease in circulating insulin or an increase in circulating catecholamines or by sympathetic nerve activity, an exploration of the neuroanatomy and vascularisation of different depots can help to explain the apparent differences seen in visceral and subcutaneous adipose tissues.

The study of SNS activity is important when looking at causes of obesity. A decrease in SNS activity has been shown in animal and human models of obesity (Bray, 1991, Tataranni *et al.*, 1999, Vander Tuig *et al.*, 1982, Young & Landsberg, 1983). There are other studies, which show an increase in SNS activity in obesity (reviewed in Bjorntorp and Rosmond, 2000). Thus a better understanding of regional SNS activity may have important clinical implications in understanding obesity (Coppack *et al.*, 1998, Fishman & Dark, 1987). Even though energy balance sets the overall long-term direction of equilibrium, over shorter time periods a range of physiological processes impacts on the relative rates of lipolysis and esterification. The lipolytic action of catecholamines on adipocytes can vary due to both physiological and environmental factors. Release of NA from sympathetic nerves is thought to stimulate lipolysis during strenuous exercise (Coppack *et al.*, 1994, Gilsezinski *et al.*, 1998, Hales *et al.*, 1978, Lafontan *et al.*, 1997, Songenes *et al.*, 2000), cold exposure (Arnett *et al.*, 1960, Garofalo *et al.*, 1996, Trayhurn *et al.*, 1998, Wilson *et al.*, 1970), stress (Coppack *et al.*, 1994, Lafontan *et al.*, 1997), or prolonged fasting (Migliorini *et al.*, 1997, Wolfe *et al.*, 1987). This and other evidence (Correll, 1963, Hales *et al.*, 1978) speaks of a clear physiological and biochemical link between sympathetic nerves and adipocytes (Correll, 1963, Hales *et al.*, 1978).

3.1) Regulation of Lipolysis

The metabolic processes in fat cells are regulated by neural, hormonal, and local factors. The process of lipolysis is influenced mainly by the action of two sets of hormones, *insulin* and the *catecholamines* (Gordon & Cherkas, 1958).

Insulin is a hormone that decreases the level of lipolysis and increases the rate of re-esterification, thus reducing the level of free fatty acids in plasma in humans (reviewed in Ferrannini *et al.*, 1999, Horowitz *et al.*, 1999). During conditions such as fasting, the level of insulin drops which in turn results in an increase in lipolysis (Horowitz *et al.*, 1999).

Catecholamines (*adrenaline* and *noradrenaline*) are neuro-hormones that originate from different sources. Adrenaline (A) and noradrenaline (NA) are released from the adrenal medulla and reach the tissue via the circulation, while noradrenaline is the neurotransmitter for postganglionic sympathetic neurons and is released locally in the tissue (Rang *et al.*, 1999).

Human adipocytes express four adrenoceptors subtypes which co-exist on the plasma membrane; three of which are stimulatory (β_1 , β_2 , β_3) and one which is inhibitory (α_2) (Lafontan *et al.*, 1995) and both share adrenaline and noradrenaline as their agonists (Bartness *et al.*, 1998). The β -adrenoceptors are coupled to excitatory GTP proteins and activate adenylyl cyclase (Bartness *et al.*, 1998). This in turn promotes the conversion of ATP to cyclic AMP (cAMP) increasing its concentration, resulting in the phosphorylation and activation of HSL, thus increasing the level of triglyceride degradation into fatty acids and glycerol (Barsh *et al.*, 1964).

In contrast, α_2 -adrenoceptors are linked to inhibitory G proteins, leading to an inhibition of lipolysis. The binding of catecholamines to the α_2 -adrenoceptor causes a decrease in concentration of cAMP, consequently dephosphorylating and deactivating HSL (Saulnier-Blanche, 1990).

Participation of each adrenoceptor in lipolysis differs according to fat pad, species, gender, age, and degree of obesity (Bartness *et al.*, 1998, Gilsezinski *et al.*, 1998, Goldrick & Foreyt, 1969, Harmelen *et al.*, 1997, Kasther *et al.*, 1977, Pond & Mattacks, 1991, Youngstrom & Bartness, 1995).

The concentration in the plasma membrane and the affinity for catecholamines differs between α and β -adrenoceptors. Generally most human adipocytes have a higher concentration of α_2 -adrenoceptors, which have high affinity for catecholamines (Lafontan *et al.*, 1995). Consequently, they are activated (bearing in mind that α_2 -adrenoceptors are inhibitory) at lower concentrations of catecholamines (Lafontan *et al.*, 1995). Thus the inhibitory action of α_2 -adrenoceptors modulates lipolysis at rest when the level of catecholamines is low, whereas the stimulatory action of β -adrenoceptor modulates lipolysis during exercise and trauma when the sympathetic nervous system is activated increasing the concentration of catecholamines (Arner *et al.*, 1990). In other words, for NA to stimulate lipolysis it has to overcome its first action (inhibition) as it arrives at the adipocyte - a paradoxical way of organising a regulatory system.

The success of weight-loss programs using very-low-calorie diets has been shown to depend on the sensitivity of α_2 -adrenoceptors to noradrenaline, where those with low sensitivity (lower anti-lipolytic activity) have lost more weight than others (Hellstrom

et al., 1997, Horowitz *et al.*, 1999). Therefore, the α_2 -adrenoceptor sensitivity to noradrenaline could be a metabolic predictor for weight loss during very low calorie diets. Lipolysis during fasting goes through early and late processes. Early fasting (>12h) does not result in an increase in the rate of lipolysis but an increase in release of fatty acids in circulation by decreasing the process of re-esterification as insulin levels fall. Late in fasting however, when the energy demand is high, the level of lipolysis is increased (Hausberger, 1935, Scow *et al.*, 1970). WAT is the only tissue that shows an increased stimulation of SNS during a fast, conversely SNS activity is decreased in heart, BAT, and pancreatic tissue (Migliorini *et al.*, 1997).

3.2) Neuroanatomy of Fat

It is widely assumed that sympathetic nerves directly regulate and mobilise lipid stores in WAT. However, despite extensive research, our knowledge of the anatomical aspects of adipose tissue in particular sympathetic innervation remains unclear. The spatial distribution of sympathetic innervation of white adipose tissue is unclear and the ideas regarding distribution are varied. Past studies have relied on indirect routes such as pharmacological evidence (Fredholm & Rosell, 1968) to study the role of SNS in lipolysis.

Some investigators, using direct observation, have denied a direct innervation (Wirsen, 1964), or described it only for multilocular (presumably brown adipose tissue) within white fat pads (Wirsen, 1965). Morrison (1999) hypothesised a separate subtype of sympathetic innervation regulating “metabolism” as opposed to vasculature (Morrison, 1999). Ballard (1974) described an intense but regionally variable innervation of dog adipocytes (Ballard *et al.*, 1974). Earlier work has described a one-on-one innervation of adipocytes with nerves forming peri-cellular

baskets around individual adipocytes (Bartness, 2002, Diculescu & Stoica, 1970, Dogiel, 1898).

At the ultrastructural level the relationship between nerves and adipocytes has been described as sufficiently intimate that varicose, presumably sympathetic, fibres pass, within a distance of 40 to 100 nm from adipocytes (Slavin & Ballard, 1978) and that they are accepted as having functional contact as is the case in other tissues. This contact appears much easier to demonstrate in fasted animals and is not evident in the fed state (Slavin *et al.*, 1978). Whether the latter is a difference created by sampling or represents a functional change in innervation caused by fasting is unknown but obviously an important issue. The strongest statement about a direct innervation of white adipocytes and the concurrent vasculature was achieved using a histofluorescence technique combined with confocal microscopy. This study reported that catecholaminergic nerve fibres were seen in direct contact with adipocytes (Rebuffe-Scrive *et al.*, 1991). This data was, however, presented in a preliminary form within a review and did not include micrographs and quantifications and has not been reported in a peer-reviewed publication. Youngstrom and Bartness demonstrated the anatomical evidence of continuity of the SNS directly innervating white adipose tissue in 1995 (Youngstrom & Bartness, 1995). They showed that when fluorescent anterograde tract tracers are injected into sympathetic chain ganglia, a fluorescent ring envelops individual adipocytes. However, one limitation of this study was the inability to determine the type of neurotransmitter present in the nerve terminals.

Despite this uncertainty about innervation of adipocytes, it is uniformly recognised that the majority of nerve fibres are associated with the vasculature (Hauseberger

1934, Scow *et al.*, 1970). It seems to be generally assumed that the bulk of noradrenaline released and active in inducing lipolysis in white adipose tissue is initially released from perivascular nerves. Presumably this noradrenaline, at high concentration within the vasculature, exerts a vasoconstrictor role at α receptors on vascular smooth muscle before diffusing to vasodilator β receptors and eventually into the parenchyma. The magnitude of the resultant concentration gradient of neurotransmitter and its geometric relationship with the adipocytes is never discussed.

It was curious to us that biomedicine had apparently described possibly five different adrenergic receptors on adipocytes but could not identify with any certainty the source of the agonist that acted on them. The strategic interaction of noradrenaline in control of release of fat from adipocytes combined with the storage of so much fat in so many overweight individuals added impetus for us to clarify the nature of the distribution of the noradrenaline in adipose tissue.

Recently the notion of 'regional heterogeneity in fat pads' has been proposed to describe a broad range of regional differences in adipose tissue located at different sites. Metabolic activity (lipid turn-over) is highest at visceral depots (Hoffstedt *et al.*, 1997), less in abdominal subcutaneous fat and lower still in gluteo-femoral fat (Arner, 1997). This high metabolic activity in visceral fat has been suggested to be due to the high expression of β -adrenoceptors (in particular β_3) and a lower expression (and function) of α_2 -adrenoceptors (Van Harmelen *et al.*, 1997).

In addition to lipolytic specificity between different depots, differences within the same depot have been reported (Pond, 1999). The heterogeneity within a single site may provide answers regarding the source of fuel supply during starvation. During

starvation, supplying triglycerides to the body could take range of pathways between the following two possible extremes. First, all adipocytes (some 10^{10} in humans, 10^7 - 10^8 in laboratory rodents (Pond, 1999) would contribute equally, each liberating a small quantity of fatty acids. Second, a minority of adipocytes could contribute large amounts while the rest contribute little or nothing (Pond, 1999). This would imply differences in cell size, catecholamine sensitivity, intrinsic activity, and responsiveness within a single depot.

In the following study, neuroanatomy of fat in sedentary rats is compared to weight-reduced rats of the same age. Here heterogeneity within a single site is described which adds an anatomical perspective to the debate about the source of fuel supply during starvation. Also a new technique is described that displays the entirety of the sympathetic innervation in a single fat pad using avidin-biotin-peroxidase immunohistochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis.

3.3) Materials and Methods

Mouse monoclonal anti-TH antibody was purchased from DiaSorin, Dade Diagnostics (Australia). The secondary antibody, Biotin-SP-conjugated, affinity-purified, donkey anti-mouse IgG, was purchased from Jackson ImmunoResearch Laboratories Inc (Australia). Normal Horse Serum (NHS), ExtrAvidin, merthiolate, diaminobenzidine, chromic potassium sulphate and glucose oxidase were all purchased from Sigma (Sydney). Female rats were purchased from Gore Hill (Sydney). DPX was purchased from Fronine Laboratories (Sydney).

Rats were divided into two groups of exercised and sedentary (control) rats with 4 rats in each group. Runners and sedentary rats were housed and fed as before (see section 2.3).

3.3.1) Tissue Preparation

At the completion of the 8-week study, rats were anaesthetised as before (see section 2.3.1). A piece of omental lobule was removed immediately for whole mount immunohistochemistry. The omental fat pad was chosen for immunohistochemistry since it is thin and elastic in nature and therefore easier to stretch flat in order to maximise staining for whole mount analysis. The rest of the rat was perfused and a small piece of retroperitoneal fat pad was removed for routine histology and cell size analysis as per section 2.3.1 and 2.3.2.

3.3.2) Avidine-biotin-peroxidase Immunohistochemistry for TH

The piece of omental fat pad was stretched and pinned on balsa wood. The tissue was then oxygenated in culture medium for 1 hour. The tissue was fixed in a mixture of aldehydes (formaldehyde 1% w/v and glutaraldehyde 1% w/v) in 0.1M sodium

phosphate buffer, pH 7.4, for 1 hour then washed in 0.1M sodium phosphate buffer, pH 7.4 at room temperature and stored in the same buffer at 4°C for later immunohistochemistry analysis.

The concentrations of primary and secondary antibody as well as incubation times and temperatures and tissue treatments prior to application of antibody were varied in order to obtain optimal conditions for positive staining (methods adapted from protocols used by Ida Llewellyn-Smith, Flinders University, Australia, to stain whole mounts of intestine). The final procedure adopted for staining of sympathetic neurons in the fat pad was as follows:

Day 1. The tissue was placed in 3 changes of immunobuffer (IB) (Tris-PBS [10mM Trizma base, 10mM sodium phosphate buffer, 0.9% NaCl, 0.05% Merthiolate, pH 7.4]) then with 50% ethanol in water or IB containing 0.3% Triton X100 for 30 min each to increase the permeability, non-specific staining then blocked by 10%-NHS-IB for 20 minutes. Anti-TH antibody (1:10,000) in 10% NHS-IB was applied to the section and incubated at room temperature for 4 days with shaking. The primary antibody also contained 0.05% sodium azide as a preservative. A control antibody (raised against the protein, α -elonase) was also utilized to control antibody specificity.

Day 5. The tissue was washed in 4 changes of Tris-PBS for 40 minutes each. Biotinylated donkey anti-mouse IgG antibody (1:500)- the secondary antibody- in 1%NHS-IB was applied and samples left for 2 days at room temperature with shaking. The tissue was washed again in Tris-PBS as described above.

Day 7. The sections were incubated with ExtrAvidin (1:1500) in IB and left overnight at room temperature with shaking.

Day 8. The sections were washed again in Tris-PBS as described above.

(NB. All steps were done on a shaking water bath)

A Nickel-intensified diaminobenzidine-glucose oxidase (Ni-DAB-GOD) reaction was performed on the tissue (no longer than 15 minutes) and the sections placed in distilled water (method adopted from Llewellyn-Smith *et al.*, 1993). The tissue was mounted on glass slides that had been made adhesive in 1.5% gelatine and 0.05% chromium potassium sulphate and air dried overnight.

Day 9. Finally the sections were dehydrated by immersion overnight in each of 50%, 90%, 95%, 99% and twice in 100% ethyl alcohol and cleared twice with xylene.

Day 17. The sections were cover-slipped using DPX (a mountant for microscopy).

3.3.3) Mast Cell Staining

Unstained 4µm paraffin sections of retroperitoneal fat pad were mounted on ordinary microscope slides without a coverslip. The sections were cleared and rehydrated and stained in toluidine blue solution (1% toluidine blue in 50% isopropanol) for 30 minutes then placed in absolute isopropanol for 1 minute. Sections were dehydrated by immersion for 2 minutes in each 50%, 70%, 90%, 95%, and twice in 100% ethyl alcohol and cleared twice with xylene for 5 minutes. The sections were then cover-slipped using DPX ³.

³ The staining of mast cells was done with the assistance of a few undergraduate students at Wollongong University, as part of a BMS 302 course.

3.3.4) Cell Size Analysis of 4µm Thick Sections

Tissue sections of 4µm thick were prepared and cell sizes analysed as per section

2.3.2.

3.3.5) Cell Size and Sympathetic Nerves Analysis in Whole Mounts

Full thickness lobes of omental fat pad up to 5 cm² and approximately 1mm to 2mm thick from both sedentary and exercised rats were examined by light microscopy. The three dimensional distribution and structure of the sympathetic nerves were analysed and photographed with a conventional film or a digital camera.

The size of adipocytes was estimated for approximately 100 cells from each of innervated and non-innervated area for each rat. This was done using Camera Lucida drawings measuring the profile area at the point where the diameter was maximum (calliper diameter). These drawings of contiguous cells were then retraced as separate outlines. These drawings were scanned, digitised and then processed in Adobe Photoshop (version 5.5). Scanned drawings of individual adipocytes were filled with black using the paint bucket after sealing the circumference. Images were exported to NIH image and the area of the profiles measured using the “analyse particles” routine. Sizes were measured against images of a calibrated graticule ruled in 10µm lines (see section 3.4.9).

3.4) Results

3.4.1) Whole Mount

The omental fat pad was used for a whole mount study. Figure 3.1 is a photograph of a piece of omental fat pad. The fat pad was stretched (flattened) and stained prior to mounting on a conventional microscope slide. The fat pad in this figure is over 2cm long and 1cm wide and 2mm thick.

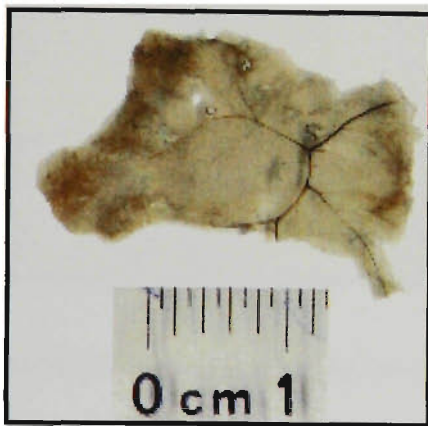


Figure 3.1) *A photograph of a piece of omental fat pad mounted on a conventional microscope slide.*

3.4.2) Success of Staining

The staining of innervation shown in the following figures are the best staining achieved by adjusting the protocol as discussed earlier in the methods. This protocol gave no staining of tissue with control antibody indicating specific staining. Figure 3.2 shows a low power whole mount photograph of a piece of omental fat pad stained for tyrosine hydroxylase. The black lines represent the stained sympathetic nerve fibres. The figure shows the nerve trunks following the major blood vessels and the separation of the nerve fibres into parenchyma. The staining of the nerve fibres is much more evident near and around blood vessels than the parenchyma with some parts of the adipocytes in the tissue receiving no visible innervation. This picture allows a view of the entire whole mount innervation. The extent of the staining is

explored by following the three-dimensional penetration of the nerve fibres in the tissue. By following the nerve up and down the tissue, it was revealed that the nerves penetrated the whole depth of the tissue. This also revealed that the staining penetrated into the middle of the tissue.

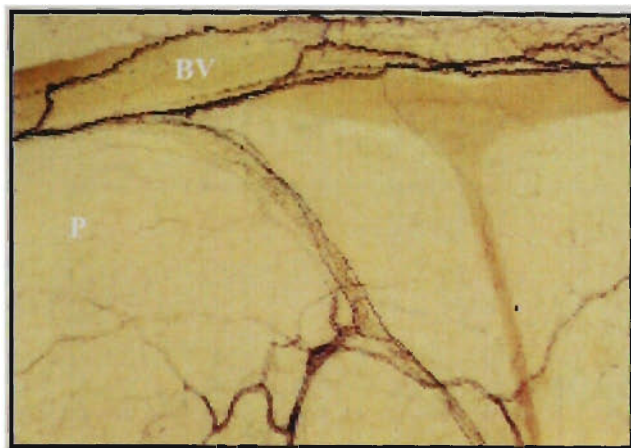


Figure 3.2) *A 10x magnification photograph of an omental fat pad stained for tyrosine hydroxylase.*

The black lines seen are the stained sympathetic nerve fibres. The whole mounts were viewed under a conventional microscope. The whole depth of the tissue was scanned to follow the 3-D structure of the fibres and blood vessels. BV=Blood vessel, P=Parenchyma.

The staining of the fibres occasionally revealed a break in the fibre. This indicates the risk of breaking of the nerve that could happen during stretching of the tissue (Figure 3.3). This phenomenon is, however, not common.

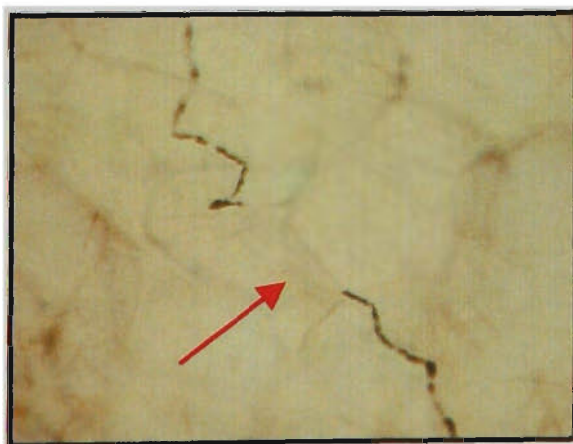


Figure 3.3) *A 40x magnification photograph showing a break in the middle of the nerve fibre. The arrow points to a break in the nerve.*

3.4.3) The Origin of Nerve Fibres

The complexity of the innervation is again evident in figure 3.4. The nerve trunks in this picture run across the tissue supplying nerve fibres to the tissue. Some of the nerve trunks do not run along-side blood vessels and appear to be independent of the blood vessel. This is again seen in figure 3.5. This figure shows a nerve trunk in the tissue without showing any apparent connection to the blood vessels.

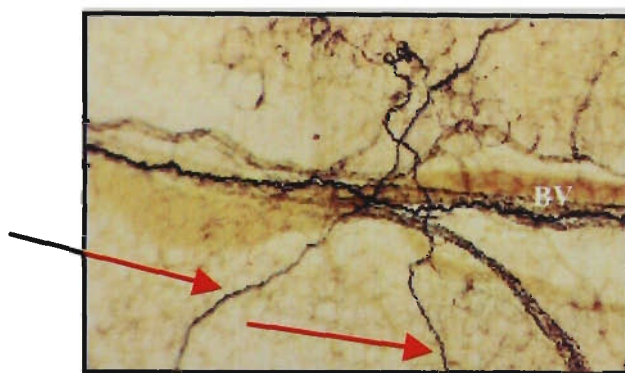


Figure 3.4) *A 10x magnification photograph showing the sympathetic innervation in a piece of omental fat pad.*

The complexity of the innervation is shown in this figure. Some nerve trunks run perpendicular to the blood vessels suggesting their lack of involvement with blood vessels. The arrows point to two nerve fibres, which appear to have no involvement with blood vessels. (BV= blood vessel).

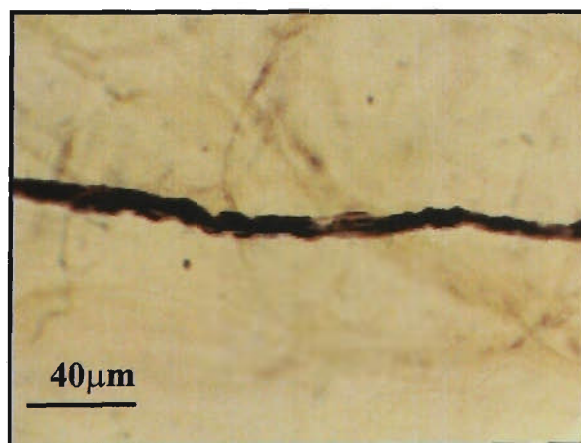


Figure 3.5) *A 100x magnification photograph of a nerve trunk in the middle of parenchyma separate from a blood vessel.*

3.4.4) Innervation of Blood Vessels

Figure 3.6 shows a photograph of a whole mount at higher magnification displaying the sympathetic innervation of an artery and a vein. The sympathetic nerve wraps itself around the vein and the artery. The 3-D structure of the nerve is followed revealing the extent of the penetration of staining to include the entire nerve trunk and fibres.

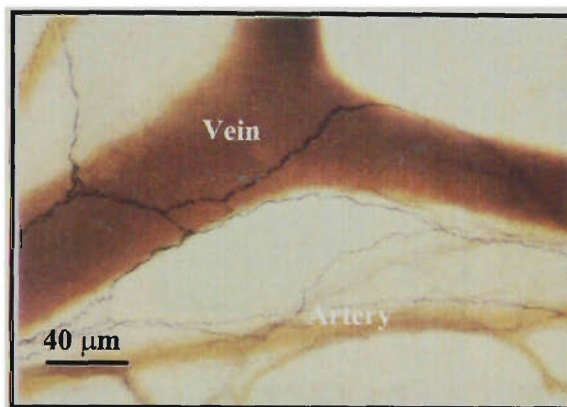


Figure 3.6) *A 40x magnification photograph showing the innervation of major blood vessels.*

The innervation of a blood vessel is again displayed in figure 3.7. This figure shows the complexity of innervation at the point of arterial branching. The nerve fibre has formed a meshwork of tightly packed nerve fibres surrounding the arterial branching.

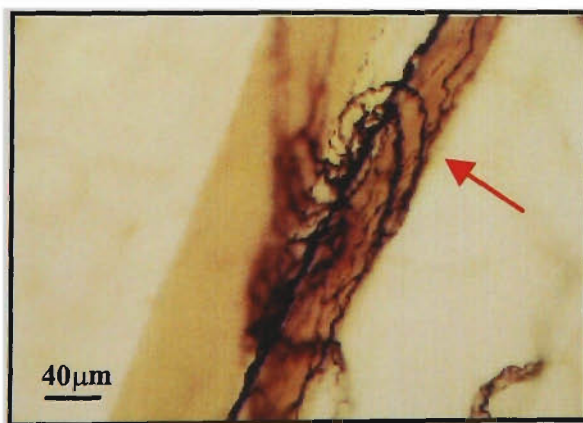


Figure 3.7) *A 100x magnification photograph showing a closer look at the innervation of an artery. The arrow point to the artery.*

The nature of innervation of blood vessels is explored in figure 3.8. This figure shows that the majority of innervation is around the artery with the vein receiving less innervation. Where the artery and vein run next to each other, again the artery receives the majority of the innervation. The nerve fibres appear to cross from the artery to the vein that is they seem to share the same innervation (see also figure 3.6).

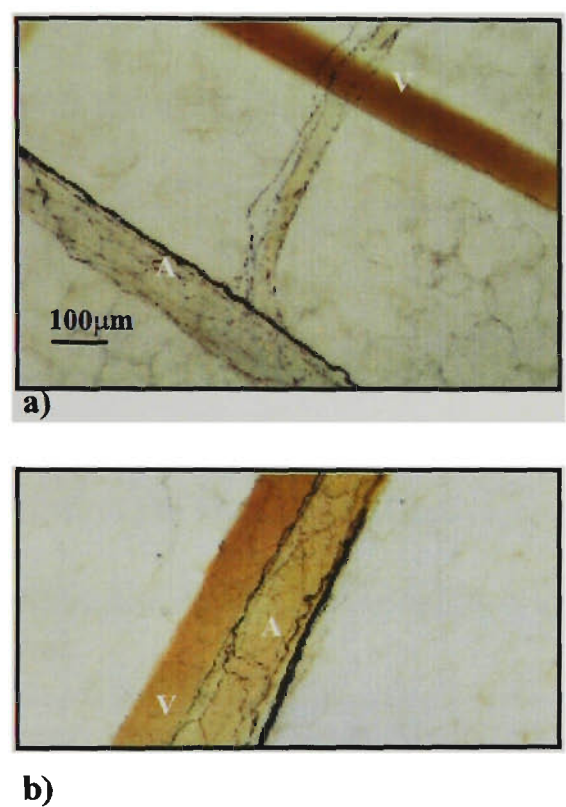


Figure 3.8) 25xPhotographs of omental fat pad showing the innervation of artery (A) and vein (V).
(a) Artery and vein are apart from each other. (b) Artery and vein run next to each other.

3.4.5) Innervation of Adipocytes

It is of interest to see where the nerves supplying the parenchyma originate. Figure 3.9 shows that the origin of some nerve fibres supplying the tissue can be traced back to the nerve trunks running along side the blood vessels. In other words the nerve trunks supplying the parenchyma do not start at a separate route. This figure also shows that the majority of parenchyma seems to lack any innervation.

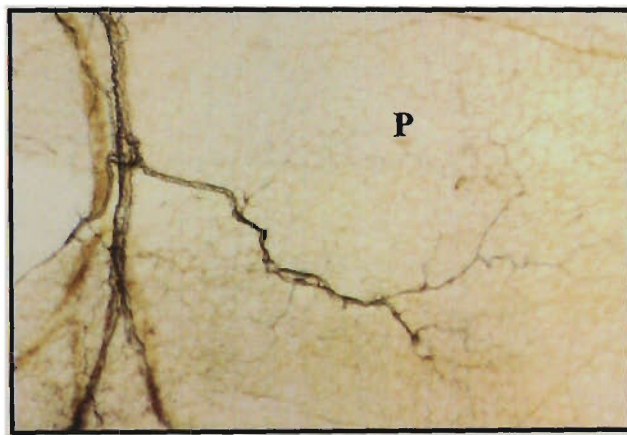
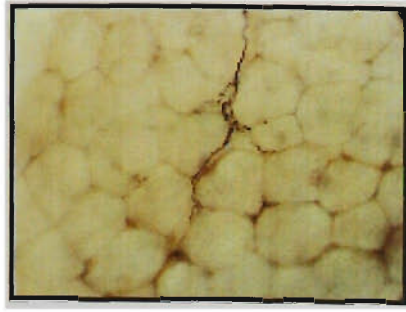


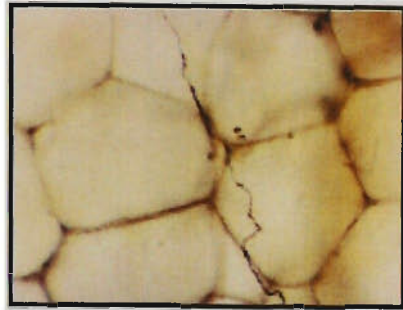
Figure 3.9) *10x magnification photograph showing the entirety of innervation in a piece of omental fat pad.*

The low power magnification allows a view of the entire fat pad. The nerve fibres in this picture can be traced back to reveal their point of origin. P=parenchyma

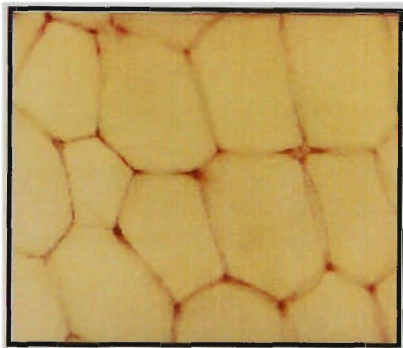
Figure 3.10 compares an innervated region of adipose tissue being supplied with one single fibre with an area that lacks any visible innervation. This figure reveals the limited number of adipocytes that are in contact with nerve fibres and it displays the lack of direct innervation to the majority of cells.



a)



b)



c)

Figure 3.10) *Photographs of the tissue at various magnifications, showing the extent of innervation of single adipocytes.*

(a) A 40x magnification photo showing small number of cells that are in contact with the nerve fibre. (b) A closer examination (100x magnification) of cells in contact with the nerve, and (c) a photo showing 100x magnification of cells, which lack any innervation.

The innervation of adipocytes is further analysed by closer examination of nerve fibres supplying individual adipocytes. Figure 3.11 shows a picture of a nerve fibre with varicosities along the fibre. This suggests that all cells in contact with the fibre can potentially become activated simultaneously.

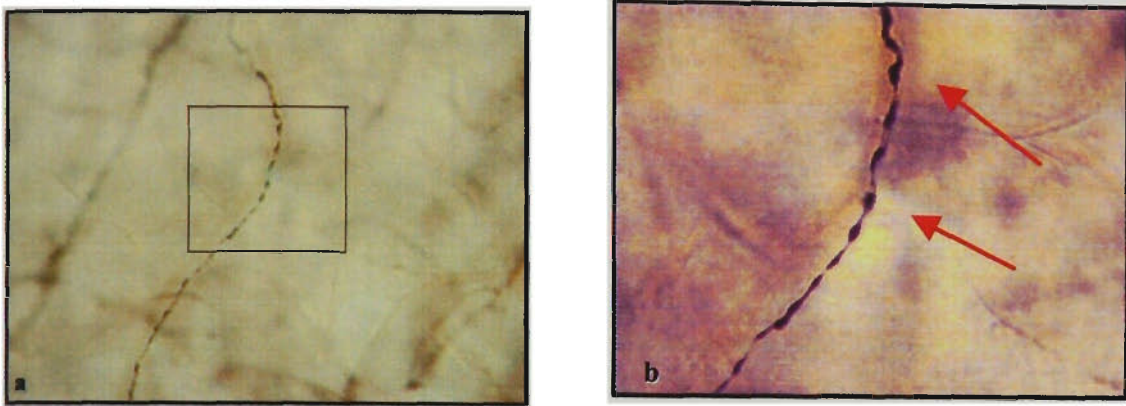


Figure 3.11) *A close examination of a nerve fibre in the parenchyma.*

(a) A 40x magnification photograph of a nerve fibre with varicosities, in the parenchyma of the omental fat pad. (b) A closer examination (100x magnification) of the same nerve showing the varicosities along the nerve fibre. The arrows point to the varicosities.

It is of interest to investigate the nature of nerve endings in the tissue. How the nerve fibres terminate on particular adipocytes(s) is shown in figure 3.12. This figure shows how a nerve terminal forms a bouton at the point of its termination.



Figure 3.12) *A 40x magnification photograph of a nerve fibre showing the nature of nerve ending.*

The point of termination is where no further tracing of the nerve fibre can be done. The arrow points to the end of the nerve, which in this case is a terminal bouton.

Whereas majority of the nerves terminate in terminal boutons, occasionally some nerve fibres wrap themselves around adipocytes forming peri-cellular baskets. Figure 3.13 shows such a basket surrounding an entire fat cell. Such baskets are seen in the middle of the parenchyma with no blood vessels visible.

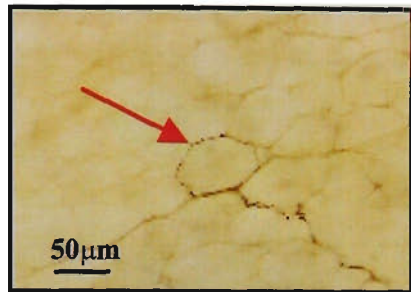


Figure 3.13) *A 25x magnification photograph of a nerve ending forming a terminal peri-cellular basket. The arrow points to a peri-cellular basket.*

The presence of such baskets is more evident in exercised animals. Figure 3.14 shows a picture of two baskets in close proximity to each other in an exercised rat.

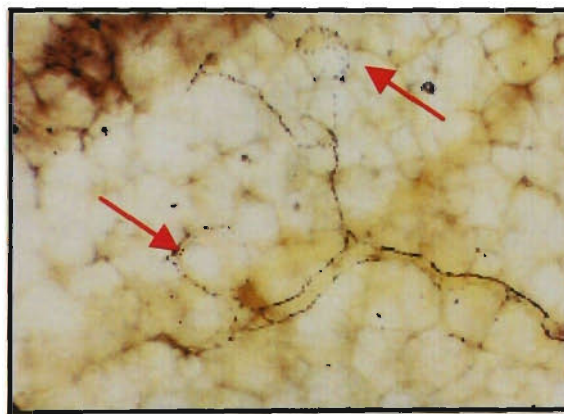


Figure 3.14) *A 25x magnification photograph showing the presence of multiple baskets in an exercised rat. The arrows point to the terminal peri-cellular baskets.*

Whereas some baskets engulf one adipocyte others might engulf more than one adipocyte. This phenomenon is shown in figure 3.15 where the terminal peri-cellular basket has formed a complex basket around more than one adipocyte.



a)



b)

Figure 3.15) *25x magnification photographs of two baskets in two exercised rats.*

A closer examination of the basket shown in figure 3.15 (b) reveals the presence of multiple boutons on cells engulfed by this basket (figure 3.16).

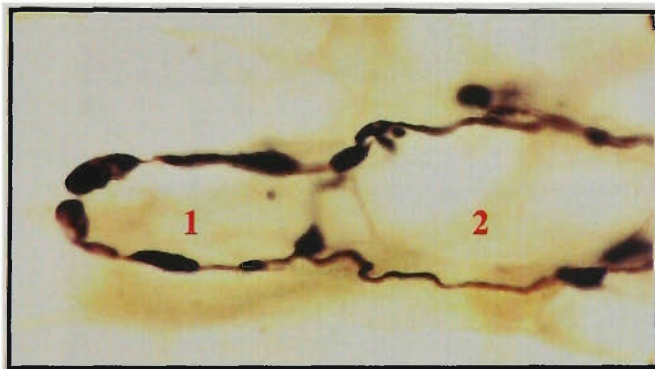


Figure 3.16) *A closer look (100x magnification) at the terminal basket from figure 3.15b. Numbers 1 & 2 indicates two adipocytes that are engulfed by this basket.*

3.4.6) Neuroanatomy of Sedentary versus Exercised Rat

The anatomical difference between the innervation of sedentary and exercised animals is explored in figure 3.17. This figure shows how exercised rat appears to be more densely innervated compared to the sedentary rat. The nerve fibre in the exercised rat looks tangled, whereas the sedentary rat displays a straight nerve fibre. This difference is commonly seen between sedentary and exercised rats.



a)



b)

Figure 3.17) *A comparison between the nature of innervation in a sedentary and an exercised rat.*

(a) A 40x magnification photograph of a nerve fibre in a sedentary rat. (b) A 40x magnification photograph of a nerve fibre in an exercised rat. The exercised rat displays tangling / twisting of the fibre compared to the sedentary rat which lacks any nerve tangling.

3.4.7) Mast Cell Analysis

The advantage of whole mount examination of entire fat pad innervation is evident in all figures shown above. Closer examination of such whole mounts can reveal other characteristics of the tissue. An important feature, which is seen commonly in these whole mount pictures, is the presence of ‘mast cell-like’ structures in exercised animals. Figure 3.18 shows the abundance of these ‘cells’ in the tissue taken from one exercised animal, the presence of which is somewhat less obvious in sedentary animals.

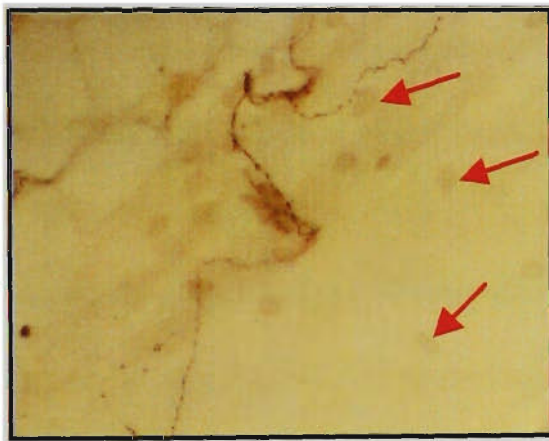


Figure 3.18) *A 40x magnification photograph of a piece of omental fat pad taken from an exercised animal.*

The arrows point to some of the commonly seen structures near nerve fibres of exercised rats. These structures are like mast cell in appearance.

The presence of these cells is again explored more closely in figure 3.19. This figure reveals the presence of these cells along side a nerve fibre.

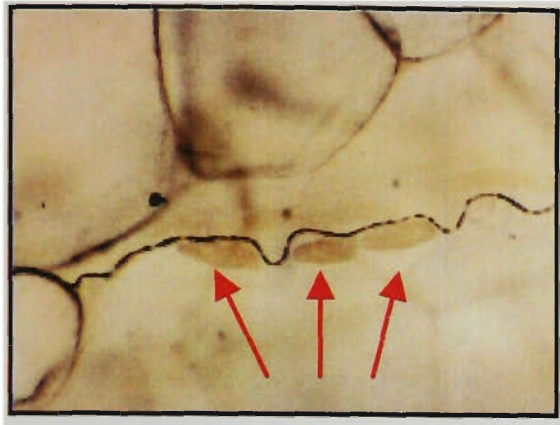


Figure 3.19) *A 100x magnification photograph showing the presence of mast cell-like structures in contact with a nerve fibre in an exercised rat.*

The presence of mast cells like structures is also evident in H&E stained 4µm thick paraffin sections of retroperitoneal fat pads. Figure 3.20 shows the presence of a number of these mast cells in a H&E stained section of retroperitoneal fat pad taken from an exercised animal. Figure 3.21 takes a closer look at these ‘mast cells’.



Figure 3.20) *A 40x magnification photograph of an H&E stained section of retroperitoneal fat pad taken from an exercised animal. The arrows point to two ‘mast cells’.*



Figure 3.21) *A 100x magnification of H&E stained section previously seen in figure 3.20*

r) Red blood cells, m) mast cells previously seen in figure 3.20

Occasionally some ‘mast cells’ are observed in sections of sedentary animals. Figure 3.22 is a H&E stained section of retroperitoneal fat pad taken from a sedentary animal.

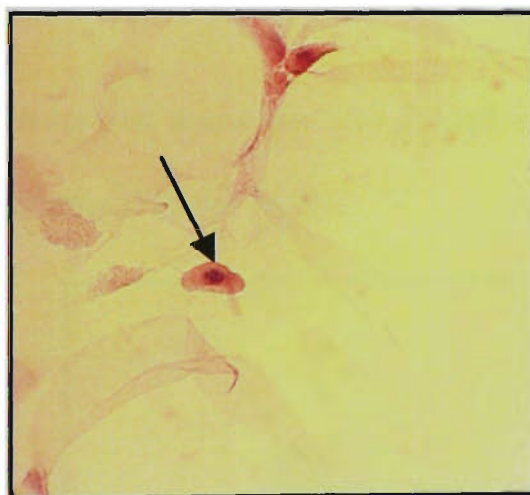


Figure 3.22) *A 40x magnification photograph of a H&E stained section of 4 μ m thick showing one mast cell in retroperitoneal fat pad taken from a sedentary animal. The arrow points to the mast cell.*

Mast cells are not readily identified in H&E stained sections (the granules are refractile and do not stain). Toluidine Blue staining is further used to confirm the presence of mast cells in these sections. Figure 3.23 shows a photograph of a 4 μ m thick paraffin section of retroperitoneal fat taken from an exercised animal. This figure reveals the abundant presence of these cells in adipose tissue. This staining

demonstrates the strongly sulphated acid mucopolysaccharide content of mast cell granules revealed by the Toluidine Blue stain.

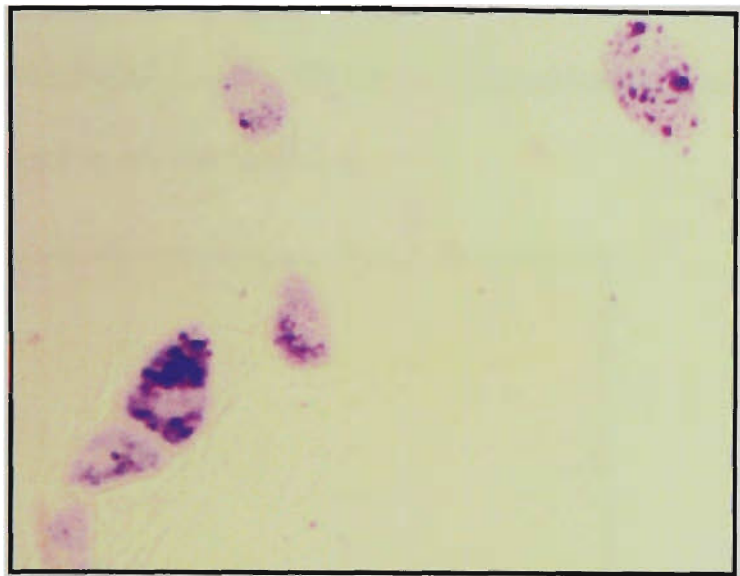


Figure 3.23) *A 40x magnification photograph of a 4µm thick paraffin section stained with Toluidine Blue. This section was taken from retroperitoneal fat pad of an exercise animal.*

Figure 3.24 takes a closer look at one mast cell stained with Toluidine Blue. The granularity is strongly stained in this figure.

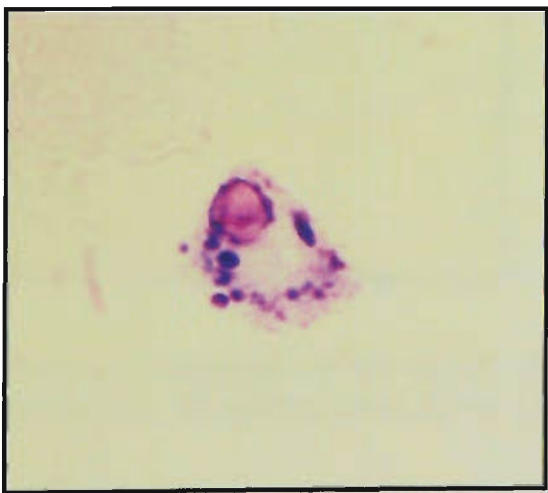


Figure 3.24) *A 100x magnification photograph of one mast cell stained with Toluidine Blue.*

3.4.8) 4μm Thick Sections

Figure 3.25 looks at the size differences of a typical 4μm thick section of retroperitoneal fat taken from a sedentary and an exercised rat. Slides like these were used to measure the cell diameters with the aid of a conventional microscope and an eyepiece micrometer as per section 2.3.2.

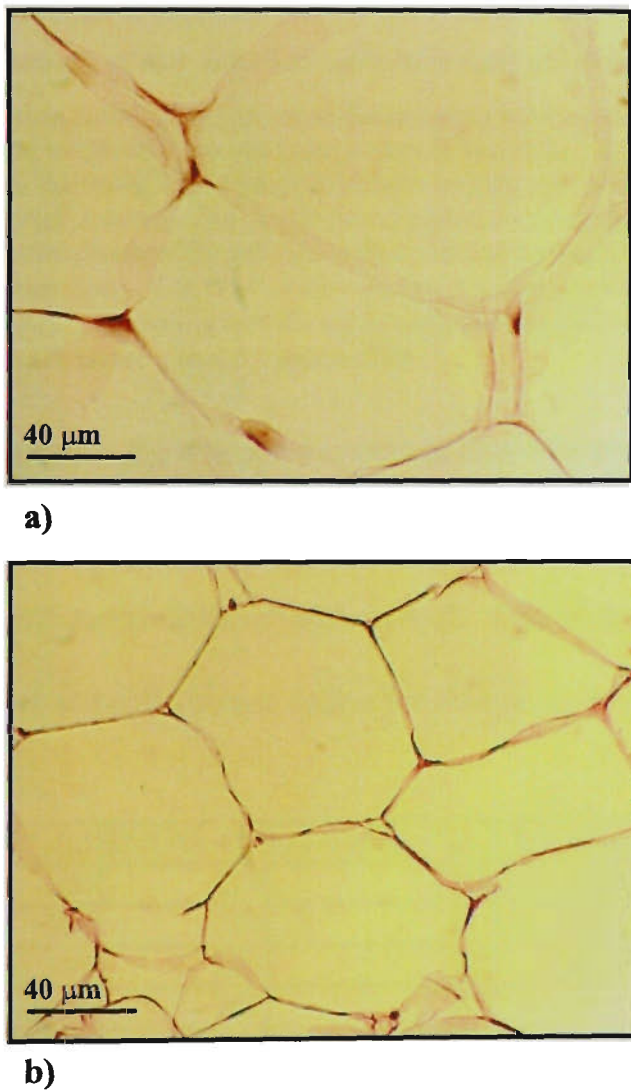


Figure 3.25) 40x magnification photographs of two typical 4μm paraffin sections taken from retroperitoneal fat pad. a) sedentary rat, b) exercised rat.

Table 3.1 shows the results for 4 sedentary female rats (S1-S4) after 8 weeks. A piece of retroperitoneal fat pad was used for the measurements of cell volumes. The weight of these rats increased from an average of 225.3 ± 1.4 g to 284 ± 12.3 g, an increase of 58.8 ± 6.0 g. This weight change is significant with $P < 0.01$ (t-test). This table also

shows the corrected cell diameter and cell volume data for these rats.

Rats	Start Wt. (g)	End Wt. (g)	Wt. Change (g)	D ± SEM (µm)	Vol. ± SEM (pl)
S1	225	287	62	98.5 ±2.8	57.4 ± 4.7
S2	229	302	73	110.8 ± 2.8	81.3 ± 5.5
S3	222	274	52	139.2 ± 2.7	159.7 ±9.1
S4	225	273	48	149.4 ± 2.6	191.1 ±9.2
Average	225.3	284	58.8	129.1	134.6
SD	2.9	13.6	11.2	32.8	96.1
SEM	1.4	12.3	6.0	1.7	1.2

Table 3.1) Summary of the data for sedentary rats (S1-S4).

Each column in this table represents the summary of the data for four sedentary rats. ‘Start Wt.’ is the weight of these rats prior to start of the study. ‘End Wt.’ is the weight of the rats at the end of the study. The change in weight from the start to the end of the study is given in the ‘Wt. Change’ column. The weight data are given in grams (g). The values shown for ‘D’ is the corrected diameter for cells taken from *retroperitoneal* fat pad measured for each rat in micrometers (µm). The ‘Vol’ is the calculated volume from ‘D’ of these cells for each rat in pico litres (pl), (assuming fat cells are spherical or ellipsoids in shape). The values of D and Vol. for each rat are given as mean ± SEM.

Table 3.2 summarises the data for 4 exercised rats after 8 weeks of running. The rat weight incresed from an average of 224.3 ± 1.5g to 274.8 ± 12.3g. This change in weight was significantly different with p<0.01. This table also shows a summary of the cell diameter and cell volume values for exercised rats.

Rats	Start Wt. (g)	End Wt. (g)	Wt. Change (g)	D ± SEM (µm)	Vol. ± SEM (pl)
E1	225	273	48	83.2 +/- 2.3	34.3 +/- 2.5
E2	228	279	51	76.6 +/- 1.6	26.1 +/- 1.6
E3	223	284	61	80.3 +/- 2.2	31.1 +/- 2.8
E4	221	263	42	63.7 +/- 2.0	15.9 +/- 1.7
Average	224.3	274.8	50.5	76.4	27.3
SD	3.0	9.0	7.9	18	19.6
SEM	1.5	12.3	6.0	1.1	1.2

Table 3.2) Summary of the data for 4 exercised rats (E1-E4).

Starting weight, end weight and weight change is shown for exercised rats. The corrected diameter ‘D’ for cells of *retroperitoneal* fat pad, and the calculated volume ‘Vol’ of these cells is given as before in ‘µm’ and ‘pl’ respectively. The individual values of ‘D’ and Vol. are given as mean ± SEM.

Table 3.3 further shows the total distance run by exercised rats. These rats had run an average of 126 km with rat 3 running the most distance of over 205 km and rat 1 the least distance of over 73 km.

Rats	Total Run (km)
E1	73.3
E2	145.3
E3	205.7
E4	79.9
Average	126.1

Table 3.3) *A summary of the running data for 4 exercised rats.*

The total distance run for each exercised rat is given in kilometers (km).

Figure 3.26 compares the cell volumes versus total distance run by exercised rat. The result shows no correlation between the total distance run and the cell volumes of these rats ($P>0.05$, ANOVA).

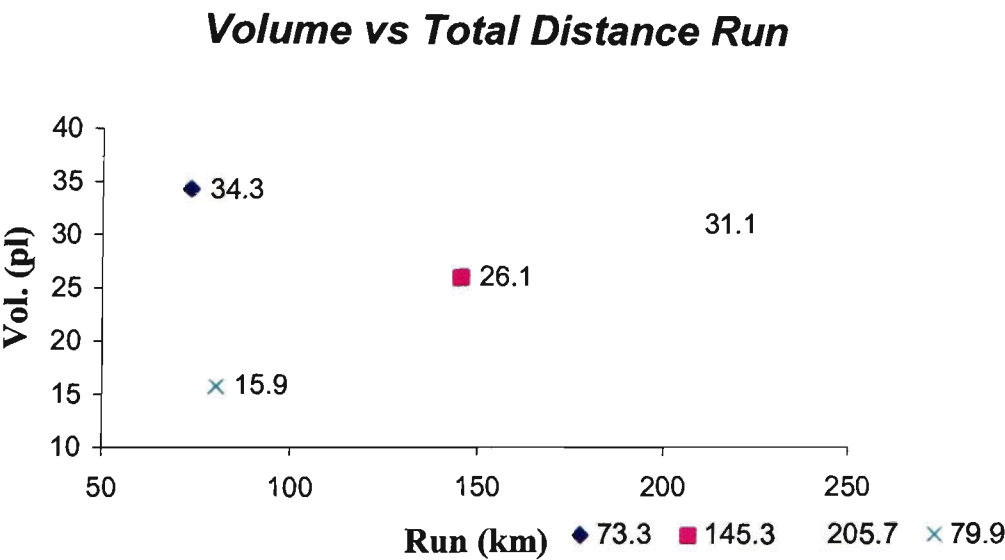


Figure 3.26) *Cell Volume versus total distance run for exercised rats.*

The calculated correlation= 0.29, $R^2 = 0.084$, $p>0.05$ (ANOVA).

The graph shown in figure 3.27 compares the average cell volumes of sedentary and exercised rats. The average cell volume of sedentary rats was approximately 5 times larger than exercised rats. This is significantly different with $p<0.001$ (t-test).

Average Cell Volume for Exercised and Sedentary Rats

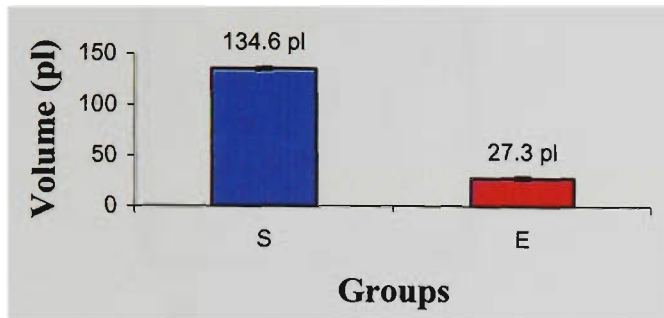


Figure 3.27) *The average cell volumes for sedentary (S) and exercised (E) rats.*

The average cell volumes of cells of retroperitoneal fat pad taken from sedentary (134.6 ± 1.2 pl) and exercised (27.3 ± 1.2 pl) rats differ significantly, with $p < 0.001$ (t-test). The values are given as 'mean \pm SEM'.

3.4.9) Fat Cells Size Analysis of Whole Mount

Figure 3.28 is an example of a Camera Lucida tracing of a whole mount. The fat cells are in contact with each other. Figure 3.29 is the secondary tracing from Camera Lucida drawings, which was then altered by Photoshop and NIH Image to analyse traced areas (Figure 3.30).

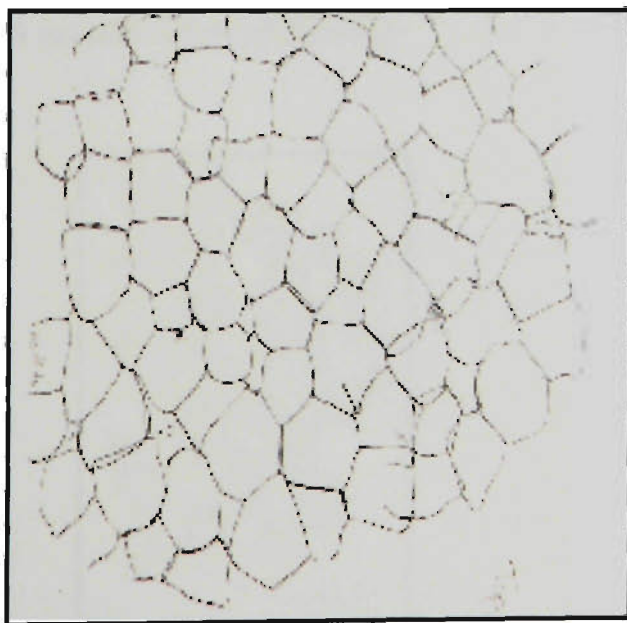
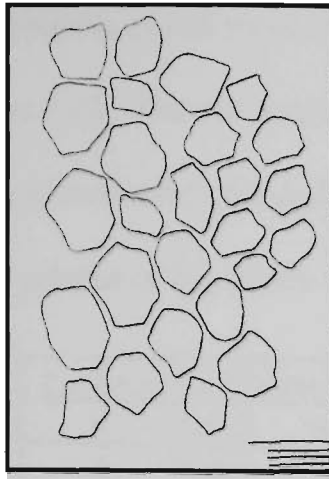
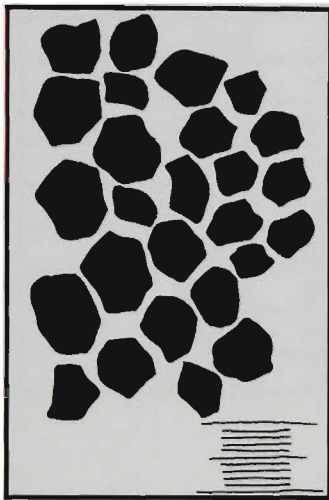


Figure 3.28) *An example of a Camera Lucida drawing.*



a)



b)

Figure 3.29) *An example of the tracing from cells traced through Camera Lucida and scanned in Photoshop (a) and filled with black paint (b). The scale bar is traced and scanned for each magnification of drawing.*

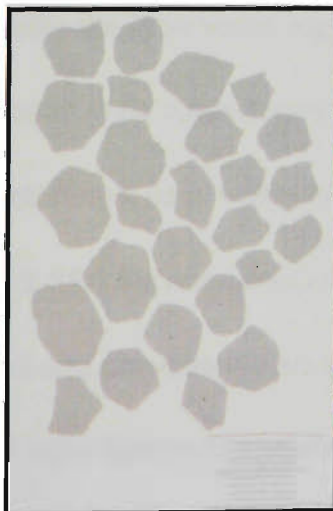


Figure 3.30) *The NIHImage picture of cells shown in figure 3.29*

This step is done in order to measure the area for each cell drawn.

Table 3.5 displays the results of cell areas obtained using NIH Image evaluation of the tracing shown in figure 3.30. The cell area data are further used to obtain cell diameters from which volumes for each cells are calculated. Cell volumes were calculated to give the volume of the sphere of equivalent cross sectional area.

Cells	Area (µm ²)	Cells	Area (µm ²)
1	4696.3	14	2905.4
2	5556.2	15	3923.4
3	6286.9	16	3361.1
4	2817.7	17	5548.5
5	2807	18	8837.3
6	8080.7	19	2342.2
7	4503.2	20	4939.5
8	3912.2	21	9395.8
9	7597.4	22	5746
10	3216.4	24	6189.4
11	4526.1	25	5541.3
12	3686.9	26	3866.6
13	8902.8	27	4600.3

Table 3.5) *Display of the cell areas achieved through NIHImage for the cells in figure 3.30.*

Table 3.6 shows the summary of the results of cell volumes of whole mounts for 4 sedentary animals. Cells, which are in direct contact with one or more nerve varicosities, characterize the innervated regions and cells that are two or more cells away from the innervated cells characterize non-innervated regions. The innervated regions in sedentary rats have cells with volumes 25.0 ± 15.3 pl (mean \pm SD) versus 29.8 ± 17.9 pl (mean \pm SD) for non-innervated areas. The difference between the cell volumes of innervated and non-innervated areas is significant with $p<0.05$ (Two Way ANOVA). Rat 3 had cells that were remarkably smaller than other rats both in the innervated and non-innervated region.

Rats	Volume Inn. \pm SD (pl)	Volume Non-inn. \pm SD (pl)
S1	29.8 \pm 11.2	32.4 \pm 9.5
S2	26.6 \pm 9.4	32.5 \pm 9.9
S3	7.2 \pm 3.5	7.3 \pm 3.9
S4	43.0 \pm 17.5	56.1 \pm 17.23
Average	25.0 \pm 15.3	29.8 \pm 17.9

Table 3.6) *A summary of the size of omental adipocytes of sedentary rats.*

The volume of innervated and non-innervated areas for each sedentary rat is shown in ‘pl’. The average cell volume is achieved by combining all innervated and all non-innervated cell volumes. The difference between innervated and non-innervated cells is significant (p<0.05, 2-Way ANOVA). The individual cell volumes are given as ‘mean \pm SD’.

Table 3.7 shows a summary of the data for fat cell sizes of whole mounts of exercised rats. The innervated and non-innervated regions are characterized as before. The average volumes of innervated cells were 17.8 \pm 10.3 pl (mean \pm SD) versus 22.4 \pm 13.5 pl (mean \pm SD) for non-innervated cells. The difference between cell volumes of the innervated and non-innervated areas was significant with p<0.05 (2-way ANOVA).

Rats	Innervated \pm SD (pl)	Non-innervated \pm SD (pl)
E1	27.0 \pm 11.8	32.6 \pm 12.7
E2	7.8 \pm 2.6	8.7 \pm 3.2
E3	16.5 \pm 5.1	19.5 \pm 7.3
E4	19.8 \pm 9.6	28.6 \pm 13.6
Average	17.8 \pm 10.3	22.4 \pm 13.5

Table 3.7) *A summary of the size of omental adipocytes of exercised rats.*

The individual values for each rat is given as ‘mean \pm SD’.

3.5) Discussion

An obvious method to examine distribution of innervation across the whole fat pad is to produce serial sections, however, this would be a tedious and time-consuming process. A typical paraffin section is 4 μm thick. Depending on the region selected for sectioning the tissue could have zero to heavy innervation. Several hundred to several hundred thousand sections would have to be examined to build an image of the entire innervation of a full thickness piece of a fat pad, equivalent to the ones shown here.

In this study sympathetic nerves were revealed in whole mounts of white adipose tissue using avidin-biotin-peroxidase immunohistochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis and a marker for sympathetic nerves. The innervation patterns were then examined. The origin and termination of the fibres is explored at various depths. Here for the first time we explore the 3-D innervation of a whole piece of fat pad in rats, revealing substantial information. The technique described analyses large sections of the fat pad up to 5 cm^2 and the full thickness of the fat pad as a whole-mount.

3.5.1) Staining Methodology

Previous attempts to perform immunohistochemistry have been hampered by poor penetration of reagents into the tissue. This study overcame these problems by adapting techniques developed by Llewellyn-Smith and colleagues at the Flinders University, Australia, to stain whole mounts of intestine. The success of this method depends on three key factors. First, penetration enhancers are used to create more rapid diffusion pathways through the tissue. Two were used in this study, soaking in ethanol (50%) or addition of the detergent TritonX100 (0.3%) to all incubations.

Ethanol was used in order to enable future electron microscopic examination of the tissues. Triton treated tissue is only suitable for slides prepared for light microscopy.

Second, incubations were performed for long times (days not hours) and at higher temperatures (room temperatures not 4°C). Third, in order to perform long incubations at room temperature, high concentration of biocides (sodium azide or merthiolate) were added to prevent growth of bacteria and consequent degradation of reagents. In this study concentrations of all reagents were optimised by performing chequerboard titration of each reagent to investigate optimal staining by trialling different serial dilutions of each reagents for different times.

Parameters were investigated to ensure the staining of nerves throughout the piece of tissue with the minimum of background staining (for example the adipocytes themselves). Thus incubation times and reagent concentrations were lengthened to improve penetration and reagent concentrations were reduced to limit background staining. The final working method described in section 3.3.2 is the result of this series of experiments. This method of staining is also suitable for tissues fixed in a variety of fixatives such as, formaldehyde / glutaraldehyde (1%/1%), 4% formalin and Zamboni.

The fact that nerve fibres can be followed in the whole depth of the tissue without interruption, assures that the reagents penetrated into the tissue adequately.

3.5.2) Innervation of Adipocytes and Vasculature in Adipose Tissue

Mapping in this way confirms the relatively sporadic nature of direct innervation of adipocytes in contrast to the rich innervation of the vasculature. Our results show that the previous descriptions of the innervation of adipocytes that appear to contradict

each other, do not contradict but reflect descriptions of different parts of the tissue that have been sampled unrepresentatively. The innervation of major blood vessels is clearly seen in results presented here. The results describing a heavy plexus of nerves around blood vessels confirm previous studies (Hauseberger 1934, Scow *et al.*, 1970) and add some important new information. We confirm that most of the arterial tree is richly innervated. Where veins and arteries run parallel to each other, fibres sometimes criss-cross from artery to vein. This aspect requires further investigation at both anatomical and physiological level. Nerves fire in an all or none fashion that is, if one individual fibre is depolarised all endings along its length release neurotransmitter in concert. If individual fibres pass from artery to vein then depolarisation of such fibres will have consequences on both vessels and supply and return blood flow and distribution in both elements of the vasculature will be altered.

The presence of innervation around capillaries requires further research and may require electron microscopic investigation. Such studies are possible with material collected in this thesis as the fixation conditions and penetration enhancements used for some tissues are compatible with electron microscopy. The vascularisation of adipose tissue is currently being explored in our laboratories. A combination of blood vessel staining and innervation staining can shed more light into the extent of innervation around capillaries. The new discovery that branches in the arterial tree are more heavily innervated indicates that regulation of blood flow may be quite complex.

The origin and dividing regions of vascular and parenchymal nerves were closely examined in this study to determine whether there were two distinct systems, one supplying only vascular elements and the other only parenchyma as suggested by Morrison (1999). Detecting bifurcations where a nerve branched and supplied

varicose fibres that innervated a blood vessel as well as innervating adipocytes would rule out such a possibility.

The majority of nerve trunks run along the blood vessels. There are some nerve trunks, which at first appear to not have any relationship with the blood vessels.

These nerve trunks, however, eventually join blood vessels. Some of the nerve trunks were cut off, thus no prediction can be made about their origin or destination. Here we describe the source of parenchymal innervation as being continuous with the vascular innervation. Our results do not rule out the existence of an entirely separate innervation for some intra-parenchymal nerves. Even though the origins of the majority of nerve trunks were traced back to the blood vessels, we could not detect whether the same nerve fibre that forms varicosities with the blood vessels formed varicosities with fat cells. This is due to the limitation of the visualisation of varicosities on nerve fibres that form nerve trunks at the light microscopic level.

If separate, sympathetic stimulation would influence either parenchymal adipocytes or the vasculature, but not both. If they were an integrated system, sympathetic stimulation would cause all nerves to release NA, thus activating adipocytes and the vasculature simultaneously. Since vascular cells also express adrenergic receptors, the elevation of catecholamines results in multiple effects. Initially, nerve stimulation causes α -receptor-mediated vasoconstriction. This is followed by β -receptor-mediated vasodilatation, in response to circulating catecholamines (Crandall *et al.*, 1997). Physiologically this allows the concurrent control of both lipolysis and blood flow. At low concentrations of NA, activation of α -receptors causes the inhibition of lipolysis and vasoconstriction, thus producing a rapid reduction in blood flow and decreased outflow of fatty acid into blood and decreased uptake of fatty acids and

triglycerides from blood. When sympathetic drive increases, subsequent activation of β -receptors promotes lipolysis and vasodilation, thereby transporting the fatty acids away from the adipose tissue (Reviewed in Crandall *et al.*, 1997). Whether this is actually the way blood flow and lipolysis is coordinated *in-vivo* is conjectural at this stage. Studies in several laboratories around the world (for example, Frayn KN and colleagues at Oxford, UK) where studies of blood flow and lipolysis are being performed simultaneously will clarify this issue.

3.5.3) Regional Heterogeneity in Lipolysis

Our results indicate that the cells responding to sympathetic nerve stimulation would do so on a regional basis in proportion to the level of innervation. We show that different portions of a fat pad may have absolutely no innervation of adipocytes, others may have sparse innervation. Small pockets of the tissue may have areas with adipocytes having direct innervation. In any of these innervated regions there may be tight pericellular baskets where a single nerve fibre wraps several times around a single adipocyte whilst nearby adipocytes receive no contact.

The discovery that some adipocytes have no direct innervation begs the question what drives lipolysis. Several possibilities can be considered. One possibility is that such cells are far from nerves and do not normally come under lipolytic influence. These cells represent lipolytically quiescent cells. Their role may be to act as long-term fat stores. These cells remain large until directly innervated cells are reduced substantially in size by chronic energy imbalance (for example, starvation) thus reducing the distance between large fat cells and nerves. At this time, NA from nerve terminals several cells away is sufficiently concentrated to activate receptors. Another possibility is that neurotransmitter from fibres innervating vessels is able to diffuse

into the parenchyma and reach a sufficiently high concentration to stimulate receptors. Given that the density of innervation around vessels contributes by far the majority of the innervation this is an attractive possibility. A third possibility is that cells distant from nerves have a higher receptor density than directly innervated cells in a process analogous to denervation super sensitivity, whereby the number of receptors increases after denervation (Rang *et al.*, 1999). A final possibility is that these cells respond only to circulating catecholamines. In this instance the stimulation of lipolysis will depend on the interaction of plasma catecholamines and blood flow to adipose tissue. It might be predicted that in some circumstances the increase in plasma catecholamines might occur when blood flow to fat is severely reduced. For example, during the flight or fight response, blood catecholamines rise but blood flow is preferentially directed to muscle and away from the viscera and skin. In these circumstances it is hard to see how efflux of fatty acids from major fat depots would be stimulated.

Regions where cells have contact with terminals are presumably capable of greater response than regions with zero innervation. The latter cells will receive larger and more frequent stimuli than the former and presumably lipolysis will be more active in these cells. Since cell size is determined by the balance between uptake of fatty acids and release after lipolysis, it was of interest to investigate cell size differences between innervated and non-innervated regions. The results presented here showed that innervated cells were significantly smaller than neighbouring but non-innervated cells. Use of neighbouring cells controlled for other effects that may affect adipocyte size such as proximity to blood vessels. This sympathetic distribution supports the

hypothesis that a few ‘pacemaker’ adipocytes within any given depot are involved in rapid release (and possibly therefore re-uptake) of triglycerides (Huking *et al.*, 2003).

We propose that sympathetic drive is discretely organized in a way that has not been appreciated in the past. We propose that at low levels of sympathetic drive, lipolysis is driven by NA released from nerves to peri-cellular baskets and multiply innervated adipocytes. We propose that high concentrations of neurotransmitter occur only in the vicinity of the adipocytes within the basket. High levels of NA activate β receptors. The NA is either taken back into the nerve terminal or diffuses away decreasing in concentration of NA so receptors are less stimulated and therefore there is less lipolysis and only a small proportion of lipid flows into the circulation. The affinity of the adrenoceptors for NA is $\alpha_2 > \beta_1 > \beta_2 > \beta_3$ (Lafontan *et al.*, 1997). Indeed the lower concentration of NA will stimulate only α_2 receptors that are much more sensitive to NA thus causing cells to switch off lipolysis. This will restrict the zone of lipolytically active cells, by de-activating cells that neighbour the basket. This is illustrated schematically in figure 3.32. Upon initiation of strenuous exercise or during a prolonged fast, noradrenaline released from nerves that are in contact with adipocytes would stimulate stimulatory β receptors in these cells only (figure 3.31a) resulting in lipolysis and release of fatty acids, whereas only α_2 receptors in neighbouring cells become stimulated (due to low the concentration of NA) resulting in inhibition of lipolysis in these cells (figure 3.31b). Close proximity to blood vessels would enable the swift removal of released fatty acids. The loss of triglycerides would result in a smaller volume and of these cells. The refilling would be presumably performed in the resting period, during which all adipocytes including

quiescent adipocytes would release fatty acids as basal lipolysis (figure 3.21c), which are then taken up by neighbouring adipocytes and re-esterified (figure 3.31d).

In this study, it was shown that adipocytes in areas with higher density of innervation are significantly smaller than cells in areas clear of innervation suggesting that they (the smaller cells) have been under the influence of stronger lipolytic stimulation.

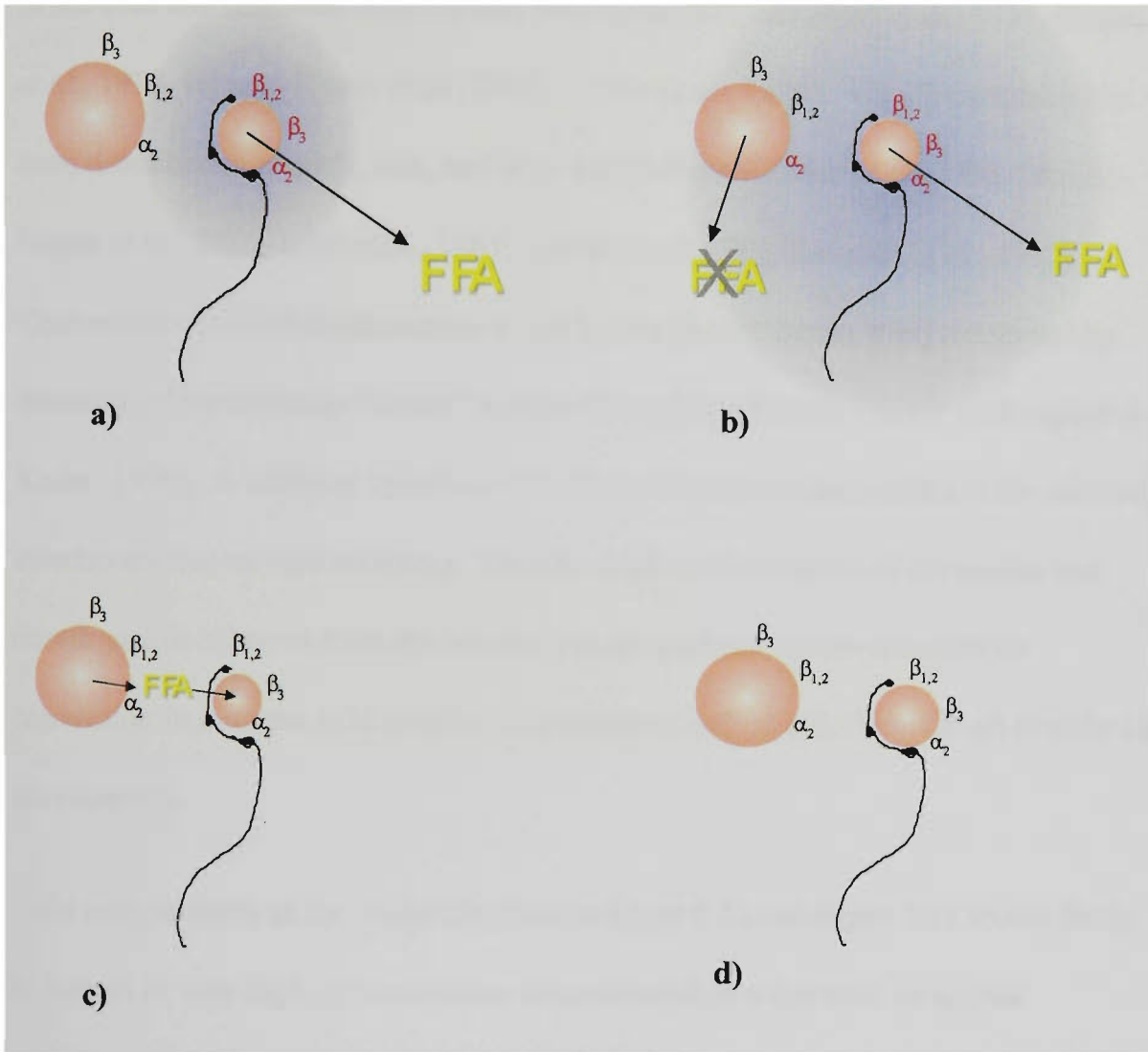


Figure 3.31) *The possible pattern of NA release from nerve terminals affecting active (pacemaker) and quiescent cells.*

Fat cell in contact with nerve (the pacemaker cell) becomes stimulated releasing FFA (a), and fat cell lacking any contact with nerve is not stimulated (b), during resting period, basal lipolysis releases free fatty acids from all fat cells and uptake of such fatty acids will replenish small adipocytes (d).

The presence of tight pericellular baskets around adipocytes especially in exercised animals raises interesting possibilities in relation to the conversion of white fat to brown fat. A number of interventions are known to cause the appearance of clusters of “brown” adipocytes within traditionally “white” fat. These include placing animals in the cold and treatment with various beta agonists (Champigny *et al.*, 1991, Guerra *et al.*, 1998, Himms-Hagen *et al.*, 2000, Lončar *et al.*, 1989). The phenomenon has been described in rodents, cats, and dogs and humans (Cousin *et al.*, 1992, Himms-Hagen *et al.*, 2000, Lean *et al.*, 1986, Lončar *et al.*, 1986, Ricquier *et al.*, 1982). Conversion of the white phenotype to the brown phenotype has been confirmed by detection of the uniquely “brown” marker Uncoupling Protein 1 (UCP1) (Ricquier & Kader, 1976). A unifying hypothesis for the mechanism of conversion is the action of catecholamines on beta receptors. Thus the high concentrations of adrenaline and noradrenaline released from the adrenal and sympathetic nerves stimulate the conversion in extreme cold whereas beta agonists such as (CL-316243) act directly on the receptors.

Cells such as those in the basket depicted in figure 3.13 and figure 3.15 would likely be bathed in very high concentrations of noradrenaline when such an animal experienced a cold environment. It is thus possible that the small adipocytes at the centre of pre-cellular baskets are the cells that initiate the process of conversion from the white phenotype to the brown phenotype (that is; *ucp1* expression).

Preliminary experiments conducted in our laboratory have shown that when rats are placed into the cold (4°C) for 16 days that adipocytes are significantly reduced in size (Kubbinga, 2001). Furthermore baskets of sympathetic fibres can be seen around some white adipocytes. These adipocytes are smaller than non-innervated adipocytes

(Hogson *et al.*, 2002).

Experiments are in progress to determine whether more extensive exposure to cold causes conversion of phenotype of the cells in the centre of the baskets. This will be done by performing double immunostaining for UCP1 and TH using the whole mount technique developed here.

3.5.4) Mast Cells

This study revealed the presence of cells, which appeared to be mast cells in whole mounts and paraffin sections. The Toluidine Blue staining of 4µm thick sections of the tissue confirmed the existence of these mast cells in the fat tissue.

Mast cells release number of substances with potential activity relevant to adipose tissue including, histamine, 5HT, heparin and TNFα. Release of these substances has different effects on the tissue, for example histamine increases the permeability of small blood vessels and heparin translocates LPL off the endothelium. It is of interest to investigate the role of these substances in fat tissue and the role of mast cells in supply of TNFα in adipose tissue, which has never been considered.

3.5.5) Sedentary versus Exercised Rats

The results presented in this study show substantial size differences (5-fold) between the cells obtained from retroperitoneal fat pad of exercised and sedentary animals (27.3 ± 1.2 pl for exercised rats versus 134.6 ± 1.2 pl for sedentary rats). The size differences seen in omental fat pad of this group of rats, however, was not as substantial as those seen in the retroperitoneal depot (22.4 ± 13.5 pl for exercised rats versus 29.8 ± 17.9 pl for sedentary rats). Size analysis from whole mounts showed

that sedentary rats had cells which were approximately 1.5 times larger than exercised rats. The omental fat cells were also much smaller in both sedentary and exercised rats compared with cells in retroperitoneal fat pad. This confirms that there is significant difference between the two fat pads and thus each needs to be studied separately (Pond, 1999).

The level of innervation between active and sedentary rats was also compared in this study. It is well established that exercise causes an increase in sympathetic activity (Coppack *et al.*, 1994). The results in this study display innervation variation between exercised and sedentary rats. The exercised rats appear to have denser innervation compared to sedentary rats. The higher number of peri-cellular baskets in exercised rats also points to the notion of innervation variability in these two groups of animals. It is, however, unclear whether this difference is just a passive response to removal of lipid from the cells, making the tissue collapse, or a response to reorganization of the nerve around cells. It is also of interest to find the physical relationship between nerve fibres and cells in contact with them. That is, is there evidence of a functional relationship and do the varicosities seen at light microscopic level contain synaptic vesicles? This will be possible by electron microscopic evaluation of tissue prepared in this study.

Another important phenomenon observed in this study is the presence of large number of mast cells in adipose tissue of exercised rats compared to sedentary rats and they are seen in close proximity to the nerve fibres. The question is whether the presence and proximity of these mast cells to innervation is a random event. Is mast cell number in exercised rats real or due to collapse of cell size making density of these cells higher? What is the function of mast cells in adipose tissue? Are they important

in adjusting blood vessel permeability at the time of exercise? These and many other questions need to be considered in future research of adipose tissue.

Results presented here indicate that the sympathetic innervation of adipose tissue is an important factor in the regulation of fat cell sizes. Further research is necessary in this topic in order to investigate other possible impacts that SNS might have on adipose tissue. Most of the information about receptor types and responsiveness in adipose tissue has been derived from homogenates, from isolated adipocytes or from small prisms isolated at random that have either taken no account of or have homogenised such microheterogeneity. We may be able to make faster progress in understanding obesity and its associated pathology problems of weight loss if we re-evaluate phenomena such as the distribution of adrenergic receptors, production of leptin and other cytokines, rates of lipolysis and esterification with the knowledge that these factors may not act uniformly throughout the tissue. We predict that the sympathetic nervous system will influence the expression of some genes expressed in adipocytes and given the newly discovered regional heterogeneity that this expression will be correspondingly regionally heterogeneous. In this light the influence of sympathetic nerves on leptin and IL-6 expression requires urgent re-evaluation at a micro regional level. Some of the dysfunction that causes obesity or particularly that prevents weight loss will be caused by imbalance in the system regulating the different micro regions of adipose tissue. Direct experimentation is necessary to investigate this matter.

CHAPTER 4

Apoptosis of Adipocytes

4.0) Introduction

The majority of the studies on adipocytes cellularity indicate that, adipocytes do not disappear during weight loss. There are circumstances when cell size is reduced massively. In these cases adipocytes simply reduced in size, with the number of adipocytes remaining the same. There are however, other studies, which clearly show a reduction in cell number in weight-reduced individuals. Considering the discrepancy observed in the literature, further research is valuable.

It was stated earlier that the high number of fat cells in obese individuals could result in poor prognosis observed in weight loss and low weight maintenance. The following chapter explores the topic of cell death in adipocytes and proposes the idea of eradication of extra number of adipocytes in order to improve the prognosis of weight maintenance.

The study of apoptosis in adipose tissue is a new area of research. The following study explores the available data on apoptosis in this tissue. It also introduces a new way by which apoptosis is induced in “isolated adipocytes” a previously unexplored area.

Cell death can be accidental or programmed in a multi-cellular organism. Evidence supports the proposition that there is a ‘suicide program’ inherent in vertebrate cells that can be activated when the cell’s death is desirable for the good of the rest of the community (Cohen, 1993). Some cells in the body, such as blood cells and lymphocytes are continuously renewed whereas, other cells such as neurones, have a very limited capacity for renewal and often survive for the life of the organism (Thompson, 1995). The failure to undergo apoptosis appears to be involved in the

pathogenesis of a variety of human diseases, including cancers, autoimmune disease, and viral infections (Thompson, 1995). In contrast, an accelerated rate of physiologic cell death has been implicated in disorders such as neurodegenerative disorders, AIDS and osteoporosis (Thompson, 1995).

4.1) Mechanisms of Cell Death

Generally, cell death occurs via two distinct mechanisms, *necrosis* and *apoptosis* (Kerr *et al.*, 1972). Figure 4.1 is an illustration of the morphological features of necrosis and apoptosis. Necrosis is a degenerative phenomenon and refers to the morphology most often seen when cells die from severe and sudden injury, such as ischaemia, sustained hyperthermia, physical or chemical trauma (Cohen *et al.*, 1993). This process is sometimes referred to as accidental cell death (Cohen *et al.*, 1993). In necrosis, the plasma membrane may be the major site of damage. The membrane might lose its ability to regulate osmotic pressure, leading to the cell (and their organelles like mitochondria) swelling and rupture (Figure 4.1a). This process results in spillage of cell contents into the surrounding tissue and provokes an inflammatory response (Boehringer Mannheim, 1998)

Apoptosis on the other hand is a mechanism of genetically programmed cell death. It is a normal component of the development and health of multicellular organisms, which operates during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the neuronal system and endocrine dependent tissue atrophy (Boehringer Mannheim, 1998). Each day countless millions of cells such as blood cells, skin and intestinal cells are removed without causing any inflammatory or unwanted effects.

In contrast to necrosis where the cells have no control on their death, apoptotic cells play an active role in their own death. Therefore this process is often regarded as an active suicidal response to various physiological and pathological stimuli (Kamesaki *et al.*, 1993).

Apoptosis is characterised by a series of biochemical and morphological changes during cell death that are clearly different from those seen in necrosis (Figure 4.1b). One of the first morphological changes of apoptotic cells is the ruffling and blebbing of the plasma membrane. Following this process the cell breaks into small apoptotic bodies. Unlike necrosis, the broken pieces of cell remain sealed and retain their osmotic gradient. The final fate of apoptotic bodies is the *in-vivo* engulfment (phagocytosis) by macrophages “professional” phagocytes implicated in clearing loads of apoptotic cells in the liver, spleen, bone marrow, lymph node, and inflamed tissue) or adjacent epithelial cells (Savill *et al.*, 1990). The phagocytes break down the cell fragments and ‘recycle’ the components for other cells to use. Due to the efficient mechanism by which the apoptotic bodies are removed, no inflammatory response is triggered (Boehringer Mannheim, 1998).

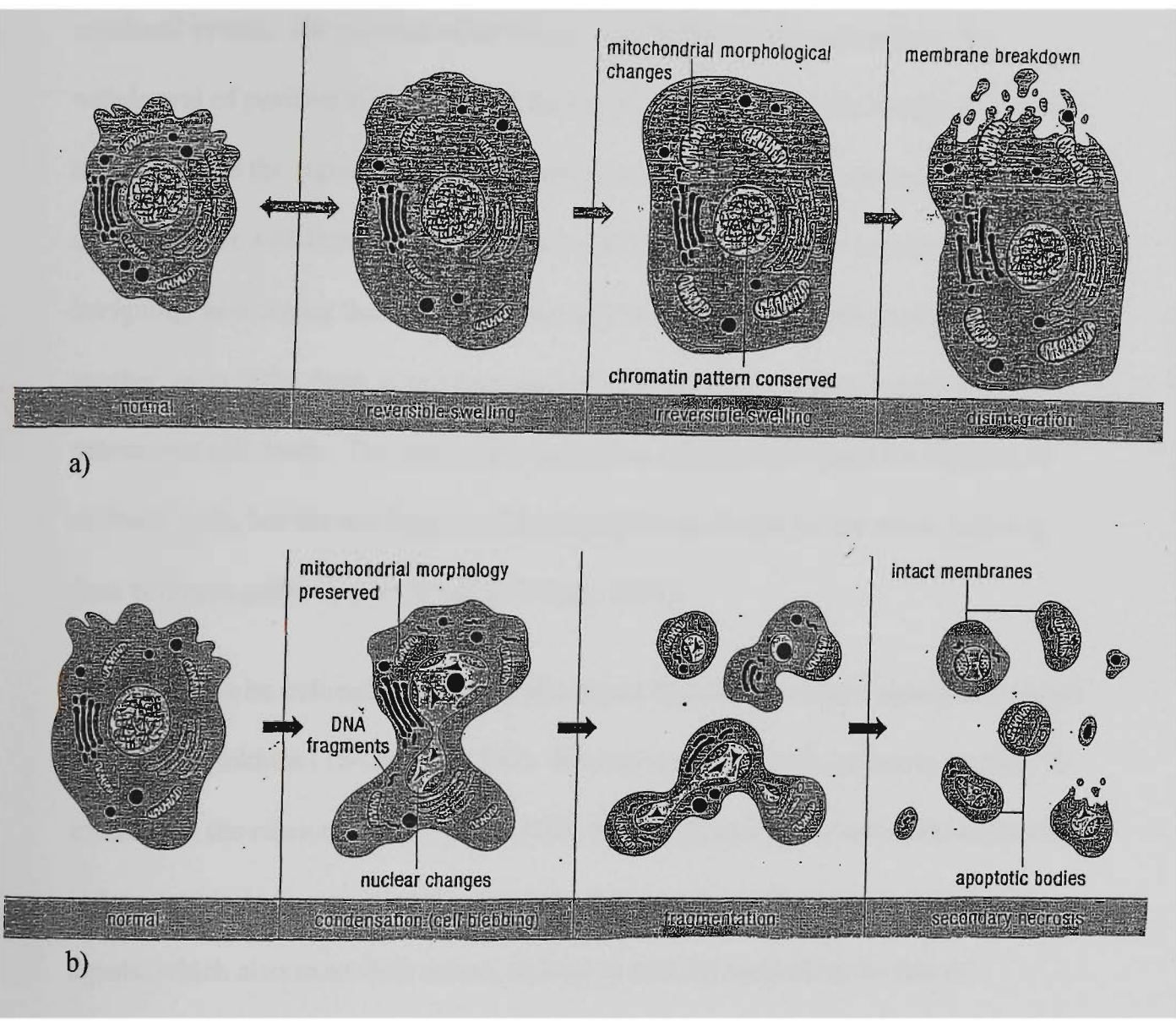


Figure 4.1) *Graphical representation of mechanisms of necrosis and apoptosis.*

a) a necrotic cell during different stage of the process. This cells shows swelling followed by final disintegration and rupture, which results in spillage of contents in surrounding environment. b) an apoptotic cell during different stage of the process. The first sign of apoptosis is shown as the condensation followed by cell fragmentation and blebbing. The final stage of this process is the formation of apoptotic bodies.
This figure is taken from Boehringer Mannheim, 1998.

4.1.1) Apoptosis Inducing Agents

There are various stimuli that lead to cell death and not all of these stimuli are unnatural events. The process of apoptosis is a response to two processes: the withdrawal of positive signals needed for continued survival or the receipt of negative signals. Often the signal to die comes from the environment. For example, an exposure to or withdrawal of a hormone or growth factor. This is conceptually intriguing, as it means that the fate of one cell is dependent upon the activity of another, even if the death is programmed, it is the events occurring outside that determines cell death. The pathway to activation of apoptosis might be different in different cells, but the mechanism of death itself may always be the same, that is, a final common pathway (Reviewed in Cohen, 1993).

Apoptosis can be induced by extracellular signal delivery of various agents and ‘death inducing’ cytokines (Thompson, 1995). Several agents including glucocorticoids, X-irradiation, the calcium ionophore A23187, tributyltin oxide, and anti-CD3 antibodies induce apoptosis in immature thymocytes. Anticancer agents are among the other agents, which also exert their action, at least in part, by increasing the rate of apoptosis (Sun *et al.*, 1994).

4.1.2) Genes Involved in Apoptosis

In several well-studied models of apoptosis, there appear to be several genes, which are associated with apoptosis. These genes are required for both morphological changes and death to occur. These observations lead to the extraordinary conclusion that cells are not “murdered” but “commit suicide” in response to various signals (Cohen, 1993). Individual genes have been associated with apoptosis in two ways:

either they are expressed in cells undergoing apoptosis, or their modulation affects the process. Among the latter, the *c-myc* proto-oncogene may play a part in regulating the choice between proliferation and apoptosis. The anti-oncogene *p53* has also been associated with apoptosis. The product of this gene arrests cell proliferation, and may, instead, switch the cell to the differentiation mode. In many cell lineages, differentiation equals death, and *p53* will cause apoptosis when expressed in myeloid or epithelial cell lines (Cohen 1993).

Fas is a gene whose product is a membrane-spanning protein homologous to both tumour necrosis factor (TNF) and nerve growth factor receptor. In a cell that expresses *fas*, either naturally or by transfection, cross-linking by antibody to *fas* induces apoptosis.

The putative oncogene *bcl-2* has the properties suggestive of an ‘anti-apoptotic’ gene. It does not, however, prevent apoptosis in all circumstances; for example, it will protect cells from cytotoxic T cells but it does not seem to work in every cell lineage (Cohen 1993). There are some genes whose expression increases in apoptotic cells, although their role in the process, if any, has yet to be determined. TRPM-2, whose protein product is known by many names including clusterin and SGP-2, is expressed during apoptosis in a number of tissues, primarily of the urogenital tract (Cohen 1993).

Several enzymes have been implicated in the induction of apoptosis. Relevant to the following study are the **topoisomerases** (enzymes that resolve the topological and conformational changes in DNA) and their role in DNA repair. The orderly progression of cellular processes such as DNA replication, transcription, and the

separation of daughter chromosomes at cell division, is dependent upon the action of nuclear topoisomerase. Inhibition of topoisomerases can disrupt cell cycle progression and generate intracellular signals that lead to cell death by apoptosis or prolonged cytostasis. Several classes of cytotoxic drugs are now recognised as potent topoisomerase inhibitors (Smith, 1994). There are generally two types of topoisomerase inhibitors; they include DNA intercalators and non-intercalators. For the purpose of this study, only DNA intercalators are explored.

4.1.3) DNA Intercalators as Apoptotic Agents

The DNA intercalators are divided into four groups; the **anthracyclines**, **ellipticines**, **aminoacridines**, and **anthracenediones**. Intercalative agents have to be planar and most often aromatic ring systems, which are held between the flat purine and pyrimidine rings by van der Waal's forces and charge transfer complex formation. The intercalation of a topoisomerase poison in the vicinity of the breakage-reunion site of the DNA may misalign the two broken DNA ends by 10-30 degrees (depending on the unwinding angle of the intercalator). This misaligned state of the enzyme-drug-DNA complex may in turn prolong the disjoined state of DNA strands. In this project, an acridine DNA intercalator was studied to explore apoptosis in adipocytes (Figure 4.2).

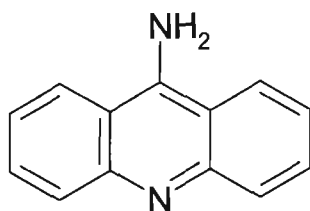


Figure 4.2) *The structure of an acridine-based compound, which intercalates between the base pairs of DNA.*

4.2) Apoptosis in Adipocytes

As discussed earlier, there is an increase in fat cell number in obese individuals (reviewed in chapter 2). Weight loss results in a large number of endocrinologically starving adipocytes which now presumably send an abnormally low level of leptin (and possibly other hormones) to the brain thus increasing hunger and telling the individual to eat more. This could help explain the ease in which ex-obese tend to put on their lost weight.

If we ask why adipocytes are preserved *in-vivo* during periods of energy deficit what explanations can we offer? The answer might be an evolutionary one. A strategy of reducing cell size but not cell number would confer an evolutionary selective advantage to individuals at times of inconsistent food availability. These individuals would have had the ability to store as much as fat as they could in times of plenty to be used when food was scarce. In contrast elimination of lipid-depleted adipocytes after prolonged periods of starvation would place the individual at a disadvantage when food becomes abundant. Such individuals would have a limited storage capacity for excess energy and additionally they would have to endure periods of time with toxic or pathogenic amounts of blood borne lipid while new adipocytes were generated and differentiated. Presumably, a strategy of maintaining a stable number of small, lipid-depleted adipocytes would allow facile storage of freshly ingested lipid. In contrast to the studies discussed above, others believe that fat cell number is reduced upon weight loss. This possibility is also evolutionary valid since preservation of small and extra fat cells is a disadvantage since these cells won't be performing their fundamental function of energy storage.

It was discussed earlier that obese individuals have excess of fat cells, which are not fully eliminated upon weight loss. Examining the limited literature suggests that adipocytes do not undergo apoptosis under 'normal' conditions *in-vivo*. Resistance of mature fat cells to undergo apoptosis has been shown by Magun and colleagues (1998). This study shows when 3T3-L1 preadipocytes differentiate into adipocytes; they acquire the ability to resist the apoptosis-inducing stimulus of growth factor deprivation (Magun *et al.*, 1998).

The evidence for a lack of elimination of excess adipocytes *in-vivo* is in apparent conflict with studies claiming that apoptosis can be induced in animal and human adipocytes *in-vitro*. Prins and colleagues first showed that human adipocytes undergo apoptosis *in-vitro* when deprived of growth factor or due to mild heat injury (Prins *et al.*, 1994a). Several other studies, have confirmed that a variety of pharmacological treatments or pathological situations can induce adipocyte apoptosis *in-vitro* (reviewed Sorisky *et al.*, 2000). The list of pro-apoptotic agents includes tissue necrosis factor (Prins & O'Rahilly, 1997, Sorisky *et al.*, 2000), cachexia and leptin administered into the brain (Qian *et al.*, 1998) (ICV) (Qian *et al.*, 1998). Adipose tissue explants from cancer patients also display evidence of apoptosis (Prins *et al.*, 1994b). Whether the conclusions drawn from *in-vitro* and *in-vivo* studies are contradictory or reflect the removal of influences protective in the whole animal awaits further studies.

One study shows a massive loss of lipid after leptin injection or via an adenovirus vector. This loss however, does not display a loss in cell number (Reviewed in Sorisky *et al.*, 2000). This is consistent with an absence of apoptosis *in-vivo*.

One study shows *in-vivo* loss of adipocytes detected in whole adipose tissue. The mechanism of induction was pathological relying on induction of diabetes in rats by streptozotocin (Geloan *et al.*, 1989). In this experiment it is not clear whether adipocytes were lost by apoptosis or necrosis. It is also not clear whether the effect was direct and induced by the streptozotocin that killed the pancreatic beta cells or secondary to the loss of one of the body's major anabolic, growth-promoting and apoptosis preventing hormones, insulin.

Therefore, under normal energy balance, apoptosis in adipocytes seems only to occur in extreme circumstances. However, more quantitative studies are necessary to evaluate the level of apoptosis in this tissue.

However limited they might be, the above studies “do” provide the evidence for apoptosis in adipose tissue. Therefore, adipocytes ‘must’ contain the necessary signalling pathway for apoptosis. It is the intention of this study to introduce a hypothesis by which the body harnesses the orderly reprocessing system of apoptosis to redistribute triglycerides from a large number of small adipocytes to form a normal number of normal sized adipocytes (Figure 4.3). During obesity there is an increase in fat cell size and eventually fat cell number (refer to section 2.1). The recruitment of new adipocytes ensures that new space is ready for excess lipid accumulated. Weight loss results in reduction of fat cell size and the phenomenon of endocrinologically starving fat cells.

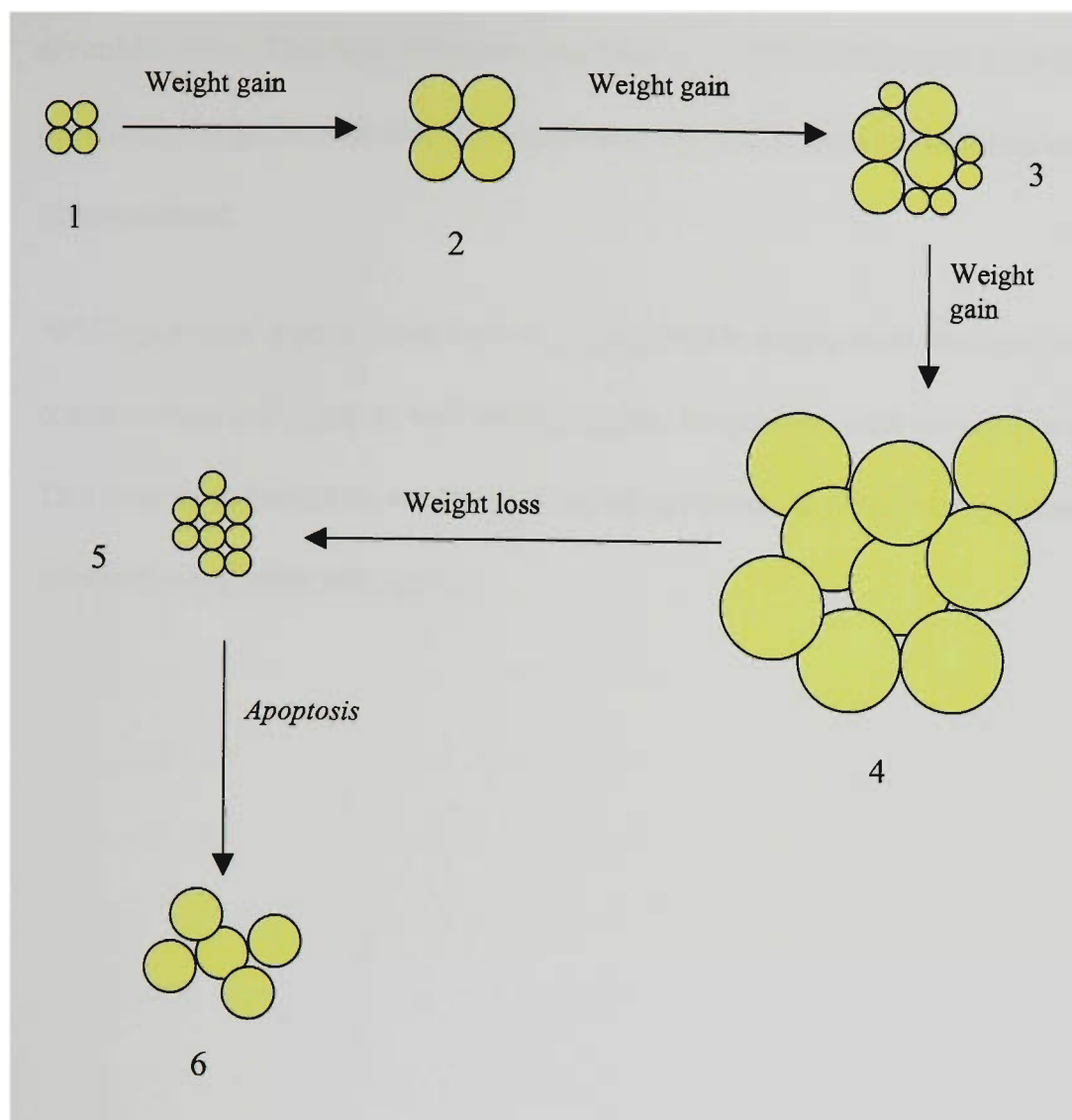


Figure 4.3) *The process of weight gain and weight loss and the action of the proposed drug on this process.*

The cells start at a certain number (1) from which weight gain results in accumulation of fat in these cells until they reach a certain limit (2) upon which new cells are recruited (3). The recruited cells help in the process of further fat accumulation (4). Weight loss using current treatments results in reduction in cell sizes and the problem of excess number of very small adipocytes (5). The proposed treatment aims at reducing the number of these small cells and the re-accumulation of the spilled lipid (6).

It is hoped that by harnessing apoptosis we can eliminate excess number of shrunken (via dietary intervention, exercise or other) adipocytes. The remaining adipocytes would then take up the triglycerides released from elimination of previous cells. This may result in adipocytes, which now send normal satiety signals (including but not limited to leptin) to higher feeding centres in the central nervous system. It is necessary to emphasise that the core of this treatment concentrates on elimination of

shrunk cells. This is to eliminate any chance of excess release of lipid from previously large fat cells into the circulation which otherwise could have negative consequences.

While previous studies have looked at apoptosis in explants of fat tissue, which contain many cell types as well as adipocytes, isolated fat cells are used in this study. This is done to ensure an exclusive study of apoptosis in adipocytes without the interference of other cell types.

4.3) Materials and Methods

9-Aminoacridine (a DNA intercalator) was supplied by Acros Chemicals (Sydney). Collagenase (I.U.B.3.4.24.3) type II, was supplied by Worthington Biochemicals (Australia). Tissue culture media was obtained from ICN Chemicals (Sydney). All other reagents and solvents were of analytical grade and obtained through standard suppliers. Water was purified to $>18\text{m}\Omega$ using an Elgastat purification system.

4.3.1) Isolation of Adipocytes

Adipocytes were isolated by a modification of the method of Rodbell (1964). Rats were sacrificed using CO_2 followed by exsanguination through the heart. The abdominal fat pads were removed and rinsed in PBS pH 7.4. Thin portions from each pad were cut into small pieces (0.5 cm). For every gram of fat tissue 10 mg of type II collagenase was dissolved in 3 ml of PBS/0.15% bovine serum albumin (BSA). The tissue was incubated in collagenase for 1 hour at 37°C in a shaking water bath. The suspension was also manually shaken every 10 minutes.

After 1 hour of shaking, the cell suspension was left for 5 minutes on the bench to allow the isolated adipocytes to float to the top. The cells were then aspirated from the solution and re-suspended in PBS, pH 7.4 at 37°C . The resuspension of supernatant was repeated 2-3 times in order to minimise cell breakage.

4.3.2) Apoptosis of Adipocytes

Isolated adipocytes from 2 gram of fat tissue were divided equally and cultured in 2 sterile flasks containing 25 ml sterile culture medium, a mixture of DMEM and Ham's F12 medium, 50:50, supplemented with Fetal Bovine Serum (FBS) 10% (v/v). The cells were then incubated for 24 hours at 37°C in an atmosphere of CO_2 (5%) in

humidified air. Flask 1 containing the control (no drug) and the other containing cells treated with 10 μ M concentration of 9-aminoacridine. The culture media of both groups of cells contained penicillin, streptomycin, kanamycin, and amphotericin to decrease the chance of bacterial and fungal growth. This was because the cells were collected from animals under non-sterile conditions as they were often sourced from other studies to reduce the number of animals needed to comply with ethical guidelines.

Cells were recovered after 24 hours by aspiration of the top layer of the medium and washed with 37°C PBS and each suspension were divided into two aliquots. Aliquot 1 (control & treatment) was used for confocal microscopic analysis and aliquot 2 (control & treatment) was used for analysis by DNA gel electrophoresis (Figure 4.4).

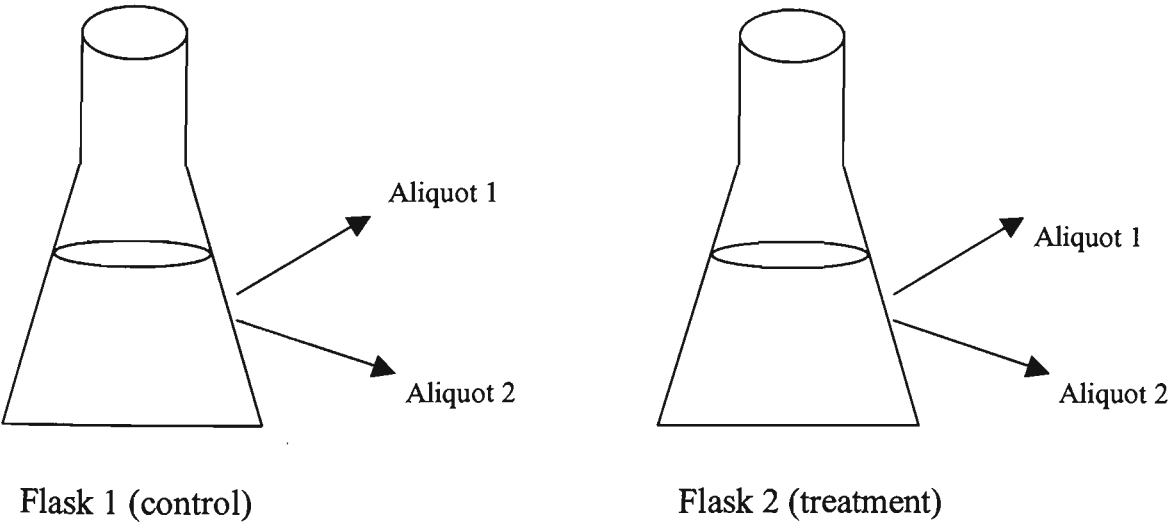


Figure 4.4) *Schematic diagram of the process of cell recovery for further analysis.*

Flask 1 contained the control cells and flask 2 contained the treatment cells. Both flasks were divided into two portions, aliquot 1 & 2, which was further used for either DNA gel electrophoresis or confocal microscopy.

4.3.3) DNA Extraction and Gel Electrophoresis

Aliquot 2 samples from each flask were treated with 0.5% (v/v) Triton X100 detergent and centrifuged for 20 minutes at 1500 rpm to lyse the cell membrane and allow temperature isolation of nuclei. The recovered pellet was then washed with PBS and centrifuged a second time. The final pellet containing nuclei was used for DNA gel electrophoresis analysis.

Cellular DNA was isolated using Proteinase K / SDS extraction buffer solution (0.3 mg/ml Proteinase K in SDS extraction buffer). This method was adapted from Kaufman (1989). Isolated nuclei were incubated with the extraction buffer overnight. The DNA was purified using phenol:chloroform extraction. The isolated DNA was stained with DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% w/v sucrose in buffer) and allowed to run on a 1.8% agarose gel with TAE (0.04 M Tris-acetate, 0.001M EDTA) as the running buffer. The gel was run for approximately 70 minutes at 64mA current. The gel was stained in ethidium bromide for 20 minutes and examined with a UV camera (for full details see appendix III).

4.3.4) Light Microscopy

Prior to cell culture procedure, a preliminary experiment was done to evaluate the state of the isolated adipocytes using light microscopy. To enable processing of the cells by routine histology, the cells needed to be handled as an aggregated pellet. Aggregation of cells was done using a fibrin clot technique. A sample of 200-500µl of isolated adipocytes was placed in a small tube, to this 200µl of citrated human plasma and 100µl of thrombin was added. The sample was then shaken gently and placed in a 37°C water bath for a few seconds to allow clotting to form. The fibrinogen in the plasma reacts with the thrombin to form a disc of fibrin that is jelly-

like in consistency. The jelly like clot contained embedded adipocytes that then could be handled in further processing. The embedded adipocytes were fixed using formaldehyde 4% (w/v) in 0.1M sodium phosphate buffer, pH 7.4.

The Anatomical Pathology Laboratory at Wollongong Hospital performed the paraffin tissue sectioning of above sample. The cells embedded in small prisms of paraffin blocks were cut in serial sections of 4 μ m and mounted on microscope slides (for details see appendix I). The slides were observed under an Olympus BH2 microscope and photographed or images were captured with a digital camera.

4.3.5) Confocal Microscopy

Confocal microscopy was used to provide evidence of apoptosis on cultured cells.

The morphology of the nucleus upon 24-hour incubation with or without the drug was evaluated using confocal microscopy. Approximately 1 ml of the cells from aliquot 1 were incubated with 1 μ M acridine orange (a fluorescent marker of the cellular nucleus) for few minutes, and examined using Leica confocal microscope at 488 nm excitation and 520-600 nm emission.

4.4) Results

4.4.1) *Light Microscopy of Isolated Cells*

Figure 4.5 shows the light microscopy result of 4µm thick section of adipocytes prior to collagenase treatment and after 60 minutes of collagenase treatment. Initially the cells are all connected to each other and to other cell types such as vascular cells, erythrocytes and interstitial cells. The adipocytes appear as hollow cells, the empty void representing the space occupied by the lipid droplet that was removed during the histological procedure. Thin ribbons of cytoplasm surround the lipid body. Not all cells have a nucleus because the plane of section is only 4µm thick. Thus in any section each adipocyte (diameter up to 120µm) will not necessarily be sectioned through its nucleus (diameter 5-7 µm). Figure 4.6 shows the success with which adipocytes were separated from other cell types after 60 minutes incubation in collagenase. After 60 minutes the cells were clearly intact and separated from each other and other cell types surrounded by fibrin. Nuclei and ribbons of cytoplasm could be seen to be intact. Of the 100 cells observed all (100%) had features that indicated that they were adipocytes, namely they had a large (>20µm) lipid body.

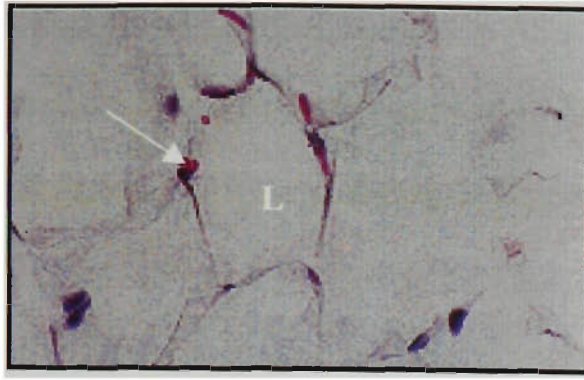


Figure 4.5) *A photograph of a 4 μ m paraffin section of intact rat adipose tissue before cellular isolation.*

This figure shows adipocytes, red blood cells and interstitial cells. The hollow core (L) is where the lipid body did occupy the cell before it was removed during the histological procedures.

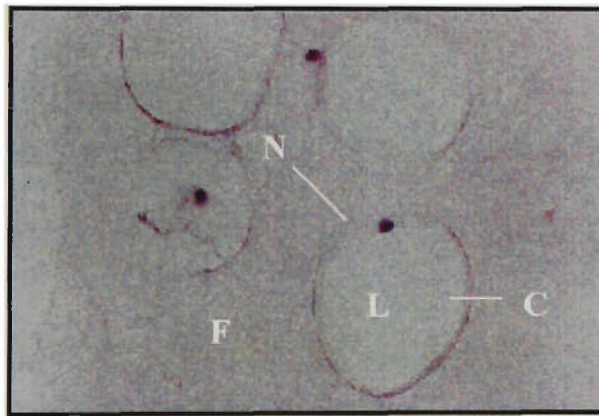
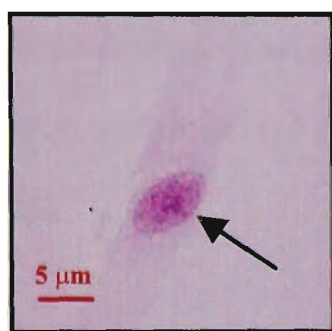


Figure 4.6) *A paraffin section of isolated adipocytes 60 minutes after collagenase treatment.*

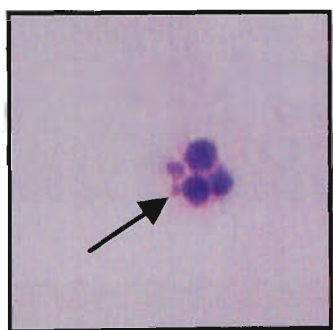
Fibrin (F) surrounds the isolated adipocytes that have intact nuclei (N) and cytoplasm (C) surround the empty void where lipid (L) was situated.

4.4.2) Light Microscopy of Control and Drug Treated Cells

Figure 4.7 shows the light microscopic photograph of the nucleus harvested from a normal and a treated fat cell. The normal nucleus is larger and less condensed than the treated nucleus. The treated nucleus has been broken down into pieces.



a)



b)

Figure 4.7) *A 100x magnification light microscopic photograph of the nucleus harvested from a normal (a) and 9-aminoacridine treated (b) fat cell.*

The control cell has an intact and larger nucleus than the treated cell. The arrows point to the nucleus of each cell.

4.4.3) Confocal Microscopy

Figure 4.8 shows the confocal microscopic photograph of adipocytes following 24 hours of incubation with or without drug treatment. The cells are stained using acridine orange as a fluorescent dye to aid confocal examination. Figure 4.8a is an example of a typical control cell, which was not exposed to 9-aminoacridine. Figure 4.8b is an example of a cell exposed to 10 μ M concentration of 9-aminoacridine for 24 hours. This cell has a much more condensed nucleus in volume and is much less intensely fluorescent compared to control cell and the nucleus in this cell again seems to have broken down into pieces.

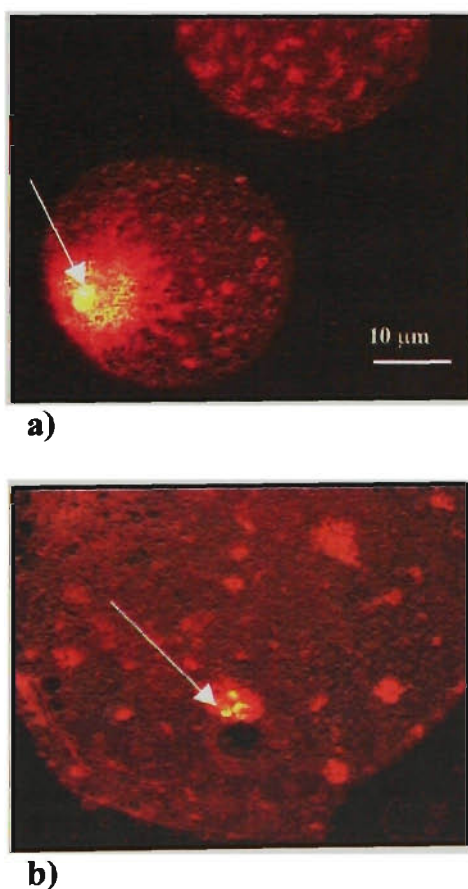


Figure 4.8) *A 100x magnification confocal microscopic picture of a control (a) and a treated cell (b) stained with acridine orange.*

The control cell (a) has an intact and brighter nucleus whereas treated cell (b) has a condensed and broken nucleus. The arrow points to the nucleus.

The isolated cells were further treated with detergent (Triton X100) prior to DNA gel electrophoresis. Figure 4.9 shows the confocal microscopic result of isolated nuclei. This method has cleaned the cell of its cytoplasm and lipid. The nuclei are further used for DNA electrophoresis.

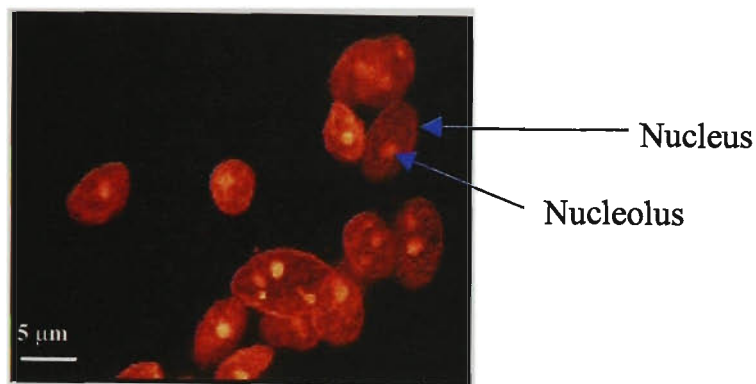


Figure 4.9) *A 100x magnification confocal microscopic picture showing isolated nuclei of cells after treatment with the TritonX100 and stained with acridine orange.*

4.4.4) DNA Gel Electrophoresis

Figure 4.10 shows the DNA gel electrophoresis result of control and treated adipocytes (the nuclei of these cells used for DNA electrophoresis). Lane 1 contains DNA from nuclei of cells that have been incubated without any drug. This lane does not show any evidence of laddering. Lane 2 contains DNA of cells incubated for 24 hours with 10 μM concentration of 9-aminoacridine. The DNA in this lane has broken down which is seen as ladder formation. As it can be seen from this figure, the treated cells show degradation of DNA into ladder, one of the characteristics of apoptotic cells.



Figure 4.10) *The DNA gel electrophoresis of DNA extracted from control (lane 1) and treated (lane 2) cells.*

The control DNA displays no evidence of DNA laddering, whereas the treated DNA shows a classical DNA laddering evidence of apoptosis.

4.5) Discussion

To show that adipocytes undergo apoptosis requires one of the tests for apoptosis to be applied to isolated cells. The nature of adipocytes raised several methodological problems. First, adipocytes are large (up to 120 μ m in diameter); they thus will not pass through the orifice of the flow cytometer (the average cell sizes which can be evaluated by flow cytometer is 30-40 μ m). Second, adipocytes have low density, they thus float in aqueous medium, conventional cell separation procedures and cell culture procedures must therefore be modified. Third, since fat cells contain a large amount of lipid, a new method had to be devised to isolate cellular nuclei prior to DNA extraction. This ensured a clean DNA gel electrophoresis result without the interference from the excess lipid which otherwise can cause streaking on the gel. Furthermore, because adipocytes were too large for flow cytometry study, apoptosis in these cells was studied using other diagnostic methods such as gel electrophoresis and confocal microscopy.

A new method was developed to isolate nuclei of adipocytes in order to improve the quality of the DNA gel electrophoresis by minimising the level of lipid present therefore prevent streaking. It should be noted that the concentration of detergent (TritonX100) used to isolate nuclei might vary in cells of different sizes. Therefore future experiments might require a preliminary experiment to find the necessary TritonX100 concentration for adipocytes of different sizes.

Apoptosis is a complex phenomenon; several factors should be taken into account when inducing apoptosis in cells. They include: cell type, exposure time, chemical feature and concentration. Indeed compounds, which cause apoptosis at low concentrations, can cause cell necrosis at higher concentrations or at longer times of

exposure. Therefore, precise protocols have been developed to classify chemicals according to the mechanism by which they cause cell death (Zanetti *et al.*, 1996).

9-Aminoacridine is a chemically simple drug that has features of other DNA intercalators, such as a flat polymeric ring structure, which can intercalate into DNA base pairs. To date, there are no studies showing that 9-aminoacridine induces apoptosis in any cell type. The results of this study show that 9-aminoacridine is capable of inducing apoptosis. More importantly, however, this study confirms the previous notion that adipose tissue can undergo apoptosis. This is shown in confocal microscopic evaluation of incubated cells. Cells that were incubated over-night without the drug do not show any evidence of apoptosis. The nuclei of these cells appear intact and larger than treated cells. However, cells that were incubated with 9-aminoacridine display distinct signs of apoptosis such as condensation of nucleus and presence of apoptotic bodies.

Additional confirmation of this apoptosis comes from the DNA gel electrophoresis performed. Past studies have analysed apoptosis in whole adipose tissue which contains several other cells as well as adipocytes. However, this study examined apoptosis induced exclusively in adipocytes. This notion becomes more important when examining the qualitative results of a gel electrophoresis since the presence of other cell types can contribute to the final DNA ladder evidence seen on a gel electrophoresis. The DNA ladder seen on cells exposed to the drug compared to the normal results seen from the control cells reveals more convincingly that these cells are capable to undergo apoptosis upon exposure to 9-aminoacridine. Therefore this study adds a new agent (DNA intercalator) to the growing list of pro-apoptotic agents of adipocytes.

A time course experiment conducted in our laboratories have shown that, the time required for apoptosis to be induced in adipocytes is much longer (24-hours) than other types of the cells (HL-60) exposed (6-hours) to the same concentration of the drug. These results provide evidence of adipocytes resistance to apoptosis. This phenomenon could also be due to drug delivery problem. Therefore, this process needs further research.

4.5.1) Future Work

A more quantitative result of apoptosis is necessary in adipocytes using flow cytometry. However, as mentioned earlier, the size of adipocytes creates technical difficulties. Overcoming the size issue with adipocytes might be solved by the isolation of nuclei from incubated adipocytes. As it was shown in this study, it is possible to isolate nuclei and perform gel electrophoresis. Similarly we can perform flow cytometry on isolated nuclei, which now are of appropriate size. By using appropriate nuclear markers then we might be able to quantify apoptosis in these cell types.

The second and perhaps the pivotal part of the proposed treatment strategy is to selectively target adipocytes. To selectively target fat cells for death we propose a novel cell targeting system that uses a combination of ligands, which work together as addressing moieties. The drug-addressing moieties are delivered to adipocytes by the action of receptor-mediated endocytosis (RME). The process of RME is an important and relatively unexplored process in adipocytes and is a vital part of the cell killing concept. Figure 4.11 represent a sketch diagram of the proposed cell-targeting drug. The presence of specific ligands which act as addressing moieties ensure accurate and safe delivery of otherwise toxic drug to the required cells and final release of the

active drug inside the cells which then causes apoptosis in intended cells (adipocytes). This concept needs vast array of synthetic chemistry and biological testing, which was outside the scope of our study. However, it is hoped that the above results will pave the way for the ultimate goal, which is to provide a radically new and lasting solution for obesity.

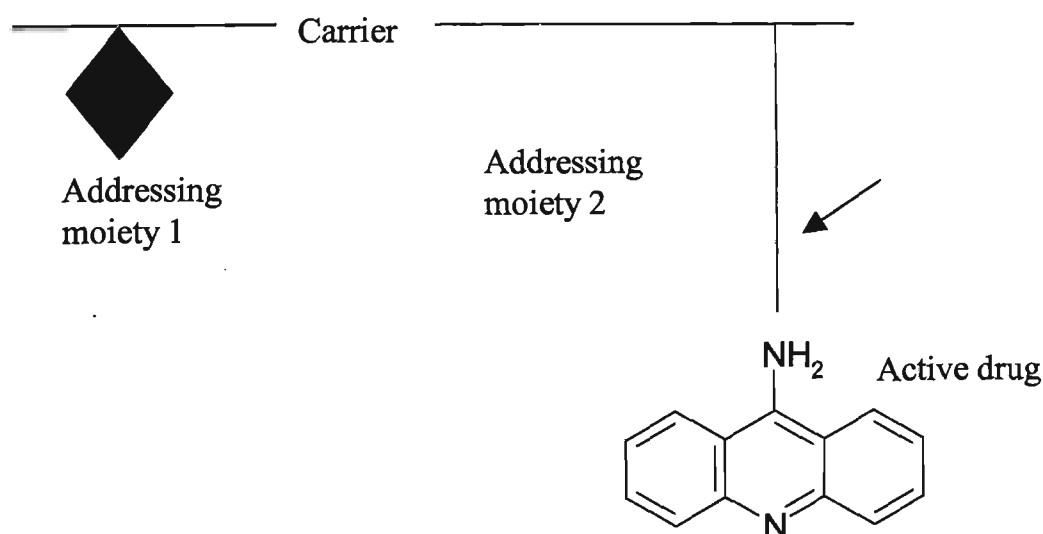


Figure 4.11) *Schematic diagram of the proposed “stealth drug”.*

The proposed active drug is cytotoxic in nature, which necessitates the presence of addressing moieties to ensure a safe delivery of the active drug to the intended site (adipocytes). The carrier has to be biologically inert and capable of carrying as many addressing moieties as necessary to ensure a safe delivery of the drug. Once inside the intended cells, the carrier and the moieties are detached from the active drug. The arrow points to a side sequence that is degraded once inside the intended cells.

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Appendix I

H & E staining of 4 μ m thick tissue slides

Slides with mounted paraffin sections were placed in an oven (50°C) for 20 minutes to dry. They were then placed in the following solutions:

- 1) Histolene for 4 minutes
- 2) Histolene for 1 minute
- 3) Alcohol for 1 minute
- 4) Alcohol for 1 minute
- 5) Alcohol for 1 minute
- 6) Wash in running water for 1 minute
- 7) Harris haematoxylin for 3 minutes
- 8) Wash in running water to clear
- 9) Place in acid alcohol for 30 seconds
- 10) Wash in running water for 1.5 minutes
- 11) Place in Scotts Blue stain (10% lithium carbonate in water) for 1 minute
- 12) Wash in running water for 1 minute
- 13) Place in eosin 1% (alcoholic) for 1 minute
- 14) Place in absolute alcohol for 1 minute
- 15) Place in absolute alcohol for 1 minute
- 16) Place in absolute alcohol for 1 minute
- 17) Histolene 2 minutes
- 18) Histolene 2 minutes

Appendix II

Pap Staining

The vaginal smears were air-dried on slides then placed in the following reagents:

- 1) Fix slides in 95% alcohol for 20 minutes
- 2) Rinse in water
- 3) Place in Harris haematoxylin for 40 seconds
- 4) Rinse in water (until runs clear)
- 5) Place in 1% acid alcohol for 20 seconds (dipping)
- 6) Rinse in water
- 7) Place in Scotts Blue (10% lithium carbonate in water) reagent for 20 seconds
- 8) Rinse in water for 2-5 minutes
- 9) Rinse in 95% alcohol for 40 seconds
- 10) Place in OG6 for 40 seconds
- 11) Dip in 95% alcohol for 40 seconds (x2)
- 12) Place in EA50 for 1 minute
- 13) Dip in 95% alcohol for 30 seconds
- 14) Dip in 100% alcohol for 30 seconds
- 15) Place in Histolene for 2 minutes (x2)

Appendix III

DNA Isolation

The recovered nuclei from adipocytes of control and treated cells were used for DNA gel electrophoresis. The apoptosis in these cells was evaluated as follows:

- The recovered (clean) nuclei were re-suspended in 400 μ l of pre-heated (50°C) lysis buffer to lyse the outer and inner nuclear membranes. The lysis buffer contained, Tris 0.5M, EDTA 2mM, NaCl 10mM, SDS 2% to which 0.3 mg/ml Proteinase K was added to destroy DNA proteases. These proteins result in degradation of DNA. The re-suspending in lysis was done quickly with several pipetting actions up and down the tube to disperse the cells.
- The suspension was incubated overnight at 50°C.
- The DNA of the incubated suspension was isolated using 3 successive phenol:chloroform extraction followed by one chloroform extraction.
- 1/10 volume of sodium acetate 3M, 1/100 volume MgCl₂ and 2.5 volume of 100% ice-cold ethanol was added quickly to the solution.
- The mixture was stored overnight in a freezer at -20°C.
- The recovered solution was centrifuged for 20 minutes at 4°C to pellet DNA.
- The supernatant was carefully discarded, while the pellet was dried at 40°C.
- The dried DNA was dissolved in 50 μ l of distilled water.
- The amount of DNA in the sample was measured by reading absorbance of

1:200 dilution of the sample at 260nm and 280nm.

- 2µl RNase (RNA digestive enzyme) cocktail was added to each sample and incubated for 20 minutes. The presence of RNA would also be noticed in the gel; therefore it is necessary to remove it.
- 1µl of DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% w/v sucrose in buffer) was added for every 10µl of the sample.
- The gel was prepared by dissolving 0.48g of agarose in 30ml of Tris Acetic Acid / EDTA (TAE) running buffer.
- The samples were loaded and let to run for 70 minutes at 64mA current.
- The recovered gel was stained with ethidium bromide for 20 minutes to visualise the gel under ultraviolet (UV) illumination. Ethidium bromide is a DNA stain that fluoresces under UV.