THE EFFECT OF STEROID HORMONES ON THE SIZE OF MYOMETRIAL CELLS: A MORPHOMETRIC STUDY.

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Thesis submitted in fulfillment of the requirements for the Master's Degree in Biomedical Technology (M Tech) in the School of Life Sciences at the Cape Technikon.

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I declare that this thesis is my own work. It is being submitted for the Master's Degree in Technology (M Tech), to the Cape Technikon, Cape Town. It has not been submitted before for any diploma, degree or examination at any other Technikon or tertiary institution. The work was carried out in the Department of Anatomical Pathology, University of Cape Town/Groote Schuur Hospital, Cape Town. The opinions and conclusions drawn here are not necessarily those of the Cape Technikon.

B Seymour

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February 1997

Date

Summary

The aims of this study were to measure:

1. Myometrial cells of menopausal uteri to establish whether they atrophy after the menopause.
2. Myometrial cells at different phases of the menstrual cycle to investigate the influences of oestrogen and progesterone during the cycle.
3. Myometrial cells in the fundus and lower uterine segment to establish whether they differ in size.
4. Myometrial cells of pregnant uteri to investigate the effect of the hormonal status of pregnant women on the size of myometrial cells.
5. Neoplastic cells of leiomyomas of the uterus to investigate whether these benign tumours behave in the same manner as myometrium or, because they are neoplastic, they react differently.

A preliminary investigation was undertaken to establish the optimal methodology for this study to measure myometrial and leiomyoma nuclei in the uterus. The aims of this preliminary investigation were:

1. To test the reproducibility of measurements of myometrial and leiomyoma nuclei in transverse and cross section.
2. To test five histological staining methods to ascertain the best method for a morphometric study on uterine cells.
3. To find the minimum sample size of nuclei per section of myometrium or leiomyoma in order to yield statistically significant results.

This preliminary study found that the Haematoxylin and Eosin stain gave the most statistically reproducible measurements. Subjective assessment of the five staining methods also found Haematoxylin and Eosin to be optimal.

It was also found during the preliminary study that measuring the myometrial nuclei in cross rather than transverse section gave the most statistically reproducible measurements. It was also found that it was best to use an axial ratio criterion of 0,9 when measuring cross-sectioned myometrial nuclei. The optimum sample size per section was also investigated and it was found that measuring 100 nuclei was optimal.

It was found that in the uteri used in this study there was no statistically significant decrease in nuclear size after the menopause. It was also found that there was no statistically significant difference in nuclear size during the different phases of the menstrual cycle. There was also no notable difference in nuclear size between nuclei in the fundus and lower segment of the uteri in this study. It was found that there was a significant increase in the size of nuclei in leiomyomas compared to the normal myometrial nuclei from the same patient.

The myometrial nuclei from pregnant uteri were also significantly larger than those from non-gravid uteri.

Opsomming

Doelstellings van die studie

The doelstellings van hierdie studie was om die volgende te meet:

1. Miometriale selle van menopousale uterusse om vas te stel of hulle na die menopouse atrofeer.
2. Miometriale selle tydens verskillende fases van die menstruele siklus om die invloede van estrogeen en progesteroon gedurende die siklus te ondersoek.
3. Miometriale selle in die fundus en laer segment van die uterus om vas te stel of hulle in grootte verskil.
4. Miometriale selle van uterusse tydens swangerskap om die effek van die hormonale status van swanger vrouens op die grote van miometriale selle te ondersoek.
5. Neoplastiese selle van leiomyomas van die uterusse om vas te stel of hierdie nie-kwaardaardige gewasse op dieselfde manier reageer as die miometrium of, omdat hulle neoplasties is, verskillend reageer.

Materiaal en Metodes

'n Voorlopige ondersoek was onderneem om die optimale metodiek vir die meting van miometriale en leiomyoma kerne in die uterus vas te stel. Die doelstellings van hierdie voolopige was:

1. Om die reproduseerbaarhied van metings van miometriale en leiomyoma kerne in transversale en dwarsvlakke te toets.
2. Om vyf histologiese kleurmetodes te ondersoek om sodoende die beste metode vir 'n morfometriese studie van uterusselle vas te stel.

3. Om die minimum monstergrootte van kerne per snit miometrium of leiomyoma te vind om sodoende statisties beduidende resultate te lewer.

Hierdie voorlopige studie het bevind dat Haematoksilien en Eosien kleuring die mees statisties reproduseerbare metings lewer. Subjektiewe evaluasie van die vyf kleurmetodes het ook bevind dat Haematoksilien en Eosien optimaal is.

Tydens die voorlopige studie was ook bevind dat meting van die miometriale kerne in dwars- eerder as in transversale vlak die mees statisties reproduseerbare metings lewer. Dit was ook bevind dat 'n aksiale ratio van 0.9 die beste is vir die meting van miometriale kerne in dwarsvlak.

Die optimum monstergrootte per snit was ook ondersoek en daar is gevind dat die meting van 100 kerne optimaal is.

Resultate

Daar is bevind dat in die uterusse wat in hierdie studie gebruik is, daar geen statisties beduidende vermindering in kerngrootte na die menopouse was nie.

Daar is bevind dat daar geen statisties beduidende verskille in kerngrootte gedurende die verskillende fases van die menstruele siklus was nie. Daar was ook geen opmerklike verskille in kerngroottes van die fundus en laer segment van die uterusse nie.

Daar is bevind dat daar 'n beduidende vergroting van kerne in leiomyomas was vergelykend tot die normale miometriale kerne van dieselfde pasiënt.

Die miometriale kerne van uterusse tydens swangerskap was beduidend groter as dié van nulligravida uterusse.

For Mom, Dad, Colleen and Anthony who have encouraged and cherished every endeavour.

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Introduction.

This study will use morphometry to obtain quantitative data in order to answer questions about the behaviour of myometrial cells at different stages of reproductive life in women. The puposes of this study are to measure:

1. Myometrial cells of menopausal uteri to establish whether they atrophy after the menopause.
2. Myometrial cells at different phases of the menstrual cycle to investigate the influences of oestrogen and progesterone during the cycle.
3. Myometrial cells in the fundus and lower uterine segment to establish whether they differ in size.
4. Myometrial cells of pregnant uteri to investigate the effect of the hormonal status of pregnant women on the size of myometrial cells.
5. Neoplastic cells of leiomyomas of the uterus to investigate whether these benign tumours behave in the same manner as myometrium or, because they are neoplastic, they react differently.

Routine microscopic observation suggests that the muscle cells and their nuclei of post-menopausal uteri are smaller than those seen in pre-menopausal women and that those of pregnant uteri are larger than both of these.

Uterine muscle cells are approximately five to ten microns wide and 50 to 800 microns long (Norris *et al*, 1973) and are markedly altered by hormonal and physical factors. Oestrogen causes pronounced cellular hyperplasia as well as hypertrophy

while progesterone acting on a uterus previously stimulated by oestrogen causes further hyperplasia and hypertrophy of the myometrium (Norris *et al*, 1973).

Leiomyomas are benign tumours of smooth muscle origin and are present in the general population in about one in four women in active reproductive life (Robbins *et al*, 1984). These neoplasms usually present during the reproductive years and stabilize or regress after the menopause (Gompel and Silverberg, 1985). They have not been reported before the menarche (Kistner, 1979). Little is known about the epidemiology or aetiology of these tumours despite their prevelance (Ross *et al*, 1986). There is much debate over the role of hyperoestrinism in the growth of these tumours although it is known that they shrink and become fibrosed and possibly calcified post-menopausally (Robbins *et al*, 1984). During pregnancy their rapid increase in size is accompanied by cellular proliferation which increases the possibility that these tumours may be caused by excessive oestrogen stimulation (Robbins *et al*, 1984).

Experimental proof is still lacking and there is no evidence that oestrogen initiates their formation or does more than maintain their size (Robbins *et al*, 1984). It was thought that since uterine leiomyomas were known to be oestrogen-dependent tumours they constituted a contraindication to oral contraceptive use (Bonnar, 1990). In 1986 Ross *et al* found the first evidence that the risk of fibroids is reduced by the use of oral contraceptives. This study also found that term pregnancies actually reduce the risk of fibroids and that this risk is further reduced with each additional term pregnancy (Ross *et al*, 1986).

The present study will attempt to correlate information on hormonal status with measurements of leiomyoma and normal uterine myometrial cells to investigate the influence of steroid hormones on these cells. No studies have been found in the literature to quantify changes in size of these cells and to relate them to the hormonal milieu. It is hoped that these investigations will add to current knowlege of leiomyomas and ultimately lead to their successful treatment.

Fresh uteri from patients who had undergone hysterectomy for various benign uterine conditions with special emphasis on patients with leiomyomata were collected. On receipt of the fresh specimens the uteri were cut in the sagittal plane. One 2mm slice from the fundus and one 2mm slice from the lower segment was taken through the thickness of the uterine wall to include endometrium and myometrium.

Cases were selected from departmental records to include pregnant patients.

Two to four micron paraffin sections were cut on the tissue collected prospectively and retrospectively. Fixing and processing methods were standardized to reduce standard error resulting from the distortion of the tissue during these procedures.

The efficacy of measuring transversely or cross-sectioned nuclei was evaluated. Axial ratio parameters to determine "roundness" or "longness" of the nuclei was tested and set to produce the smallest standard deviation.

An investigation to determine the staining method which gives optimal clarity of nuclear and cytoplasmic membranes was carried out. This is important as the image loses resolution when it is projected from the microscope to the monitor of the VIDS

III system. This makes nuclear and cell boundaries indistinct unless the section is very thin and the stain yields enough contrast.

The optimum number of nuclei per slide to be measured was estimated using an average summation graph. This is produced by calcluating a mean value after each set of observations. After the first few sets of observations the mean oscillates considerably, but after a number of sets it attains a steady state. When this is achieved there is no virtue in continuing observations as they will not alter the mean significantly (Aherne & Dunnill, 1982).

The myometrial nuclei were measured using a computer programme called VIDS III which is a high resolution, semi-automatic, image analysis system. The equipment includes a monitor which displays the magnified image generated by the microscope and a graphics tablet with a mouse which were used to outline the nuclei.

The area of nuclei will be measured using the general drawing mode where the boundaries of the nuclei are traced and which provides 16 different measurements for each nucleus.

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Chapter 1

Literature Review

1.1 Introduction.

The first part of this literature review will be an overview of the literature on the subject of morphometry while the second part will be concerned with the literature devoted to the morphology and physiology of the uterus and leiomyomas.

Morphometry may be defined as a group of methods used to obtain quantitative information about microscopic and macroscopic anatomical structure (Aherne and Dunnill, 1982). The term morphometry embodies planimetry, stereology and the counting of elements (Baak and Oort, 1983). Planimetry is the term applied to the measurement of features in a two dimensional plane, although these features may be three dimensional (Baak and Oort, 1983). Stereology is the study of mathematical relationships between three dimensional structures and their two dimensional representative sections (Loud and Anversa, 1984). Loud and Anversa (1984) explain the relationship between stereology and morphometry by stating that morphometry is the measurement of structures using stereologic principles. An example of the counting of elements or enumeration is the counting of blood cells suspended in a known volume in order to determine the number of cells per unit volume of blood (Aherne and Dunnill, 1982).

Morphometry can thus be utilized as a powerful tool that may be used to extract valuable information from representative sections of tissue (Loud and Anversa, 1984) The "powerful elegance" of morphometry lies in the fact that by using fundamentally simple techniques to quantify biological

structures a third dimension may be added to microscopical investigation (Aherne and Dunnill, 1982). This dimension brings numerical precision and a statistical basis to the field of morphology (Loud and Anversa, 1984).

1.2 Basis and biological applications of morphometry.

Morphometry is a set of methods designed to obtain quantitative information about anatomical structure and thus embodies measurement, probability and statistics. It is based on theories and methods that stem from geometrical probability (Aherne and Dunnill, 1982).

The quantitative description of histological sections has long been subservient to the qualitative description (Baak and Oort, 1983). Histopathologists use pattern recognition to make judgements based on impressions subconsciously tested against a mental image of the normal pattern rather than quantitative appraisal. Quantitative measurement in histology has fallen behind the other disciplines of medical technology, the main reason being that simple linear or areal measurements of the microscope image are seldom sufficient to extract information about a three dimensional structure (Gore, 1979). Geologists and metallurgists, faced with similar problems of quantitation, have developed methods which have been applied to histology (Weibel *et al,* 1966). The purpose of this section is to describe the development of geometrical probability and statistics in geology and metallurgy and their incorporation into morphometric methods used in histopathology.

1.2.1 The mathematical basis.

The origins of the theory of probability are rooted in Italy. A commentary on Dante's "Divina Commedia" printed in Venice in 1477 contains the earliest mention of probability (Todhunter, 1949). The emergence of probability as a new branch of mathematics was finally realised by Blaise Pascal (1623 - 1662) and Pierre de Fermat (1601 - 1665) who are acknowledged as the founders of the Theory of Probability (Aherne and Dunnill, 1982). From their different approaches to a gambling problem posed by Chevalier de Mere it is possible to derive the supposition that "the probability of an event is a number attached to a realized subset of some universal set of possible outcomes."(Aherne and Dunnill, 1982).

Pascal then developed his Arithmetical Triangle to consider similar problems (Rouse and Ball, 1912). The Arithmetical Triangle is formed when each number is the sum of that immediately above it and that immediately to the left of it (Todhunter, 1949). This triangle is essentially a method of finding the coefficients of a binomial expansion (Figure 1.1)

1	1	1	1	1	1	1	1	1	1
1	2	3	4	5	6	7	8	9	
1	3	6	10	15	21	28	36		
1	4	10	20	35	56	84			
1	5	15	35	70	126				
1	6	21	56	126					
1	7	28	84						
1	8	36							
1	9								
1									

Figure 1.1: Pascal's Arithmetical Triangle.

The Ars conjectandi (1713) written by Jacques Bernoulli was the first book devoted entirely to the theory of probability (Smith, 1925). This book arose from Pascal's combinatorial theory based on his arithmetic "triangle" (Aherne and Dunnill, 1982). This was the first written exposition of the theory of probability in which the properties of the binomial distribution were worked out. Binomial variables e.g.: area and volume fractions, which are important factors in morphometry, are still known as Bernoulli variables (Aherne and Dunnill, 1982).

Calculus was invented at the beginning of the eighteenth century and was applied to the problems of both continuous and discrete probability. Via Calculus, Buffon in 1777, brought geometry and probability together when he challenged colleagues of the French Academy with his famous Needle Problem, Figure 1.2 (Todhunter, 1949). The importance of this problem is that the probability of success can be calculated exactly and can also be estimated experimentally. Buffon asked what the probability would be of a

rod falling across a line if the rod was thrown across a plane ruled with equidistant parallel straight lines and the rod is shorter than the distance between the parallel lines (Todhunter, 1949). The problem involves a compound probability as the centre of the rod may fall at any point within one of the figures and the rod may take all possible positions by turning around its centre. The Needle Problem laid the foundations for a new division of mathematics based on the theory of geometrical probability. This branch of stereological mathematics is the basis of morphometry.

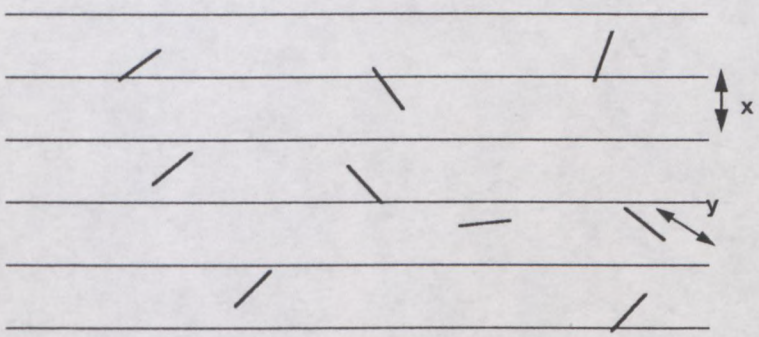

Figure 1.2: Buffon's needle problem. The parallel lines are x units apart and the "needle" is y < x units long. The diagram shows ten resting positions of a randomly thrown needle.

In 1812 Pierre-Simon Laplace (1749-1827) wrote the "Theorie analytique des Probabilties" (Aherne and Dunnill, 1982). The significance of his work for morphometry was that he moved the Buffon Needle Problem into a second dimension by calculating the probability of the rod falling across a line when it is thrown on to a grid consisting of two sets of uniformly spaced parallel lines with spacings a and b (Laplace, 1812).

After Laplace the interest in probability theory dissipated until 1868 when British analyst Morgan Crofton delivered a paper, " on the theory of local probability, applied to straight lines drawn at random in a plane," to the Royal Society (Aherne and Dunnill, 1982).

1.2.2 The influence of geology on the development of morphometry.

In conjunction with mathematics the work in the field of geology has been vital to the development of morphometry (Aherne and Dunnill, 1982). In the nineteenth century geology had become a quantitative science. In order to quantify the different chemical components of rocks difficult separation techniques and chemical analyses had to be employed. In 1842 Delesse, a French geologist, made public his method of estimating area (Delesse, 1848). This method was used to determine the volume of components of rock since Delesse had realized that the volume fractions occupied by constituent minerals could be estimated from their areal profiles on polished rock surfaces. Delesse's theorum introduced a new concept that mean area is an unbiased estimator of volume which has become an integral factor in quantitative microscopy (Aherne and Dunnill, 1982). This concept enables conclusions about three dimensional objects to be drawn from measurements of their two dimensional sections.

Rosiwal in 1898 (Rosiwal, 1898) introduced his ideas on linear analysis when he found that volumes could be determined from a study of one dimensional measurements. Rosiwal's method was extremely tedious as it involved measuring individual linear intercepts over the various components of homogenous rock with a simple micrometer. It is at this point in the

history of morphometry that the value of instrumentation was realised with the advent of a recording micrometer stage (Aherne and Dunnill, 1982).

This instrument, introduced by Shand in 1916, could measure linear intercepts rapidly and efficiently and perform the necessary calculations (Shand, 1916). S. J. Shand was from Victoria College, which later became the University of Stellenbosch, and the first recording micrometer was made from his drawings by Mr T.A. Linton from the South African College, Cape Town (Shand, 1916). This new tool allowed for the full realization of Rosiwal's linear analysis although it could only measure two components of rock at one time; the constituent under investigation and the remainder of the rock (Wentworth, 1923). Linear analysis was given further impetus when Shand's design was modified by C.K. Wentworth (Wentworth, 1923) and W.F. Hunt (Hunt, 1924) to enable more than three components of the rock specimen to be measured during one crossing.

In the 1930s a Russian petrographer, Glagolev, introduced a method of point counting using an integrating microscope stage linked to a recording meter (Glagolev, 1934). This application revived the principle of Delesse as the original method had been very tedious (Aherne and Dunnill, 1982). Delesse had used the paper weight method of tracing, cutting and weighing the objects of interest, working on the assumption that the paper or cellophane has a uniform thickness (Shand, 1916). Glagolev used a fixed point under which the image of the sample was moved automatically in a stepwise motion. Each time a "hit" on any one of six component minerals was made this was noted on the meter. He used the binomial Bernoulli distribution to discuss its sampling error owing to the probabilistic nature of this method. Glagolev calculated that the probable error for a component

that constituted 50 % of the specimen and had accumulated a thousand points would be about one percent. His apparatus could determine the composition of six features simultaneously and could be modified to determine more than six if necessary.

Glagolev's method was taken a step further when F.Chayes, an American petrographer, invented a simple modification of an ordinary microscope stage in 1949 (Chayes, 1949). This made the procedure more accessible and consolidated Glagolev's idea. An American physiologist, H.W. Chalkley, brought further change to the methodology in 1943 by keeping the sample stationary while a number of points were projected onto it and counted (Aherne and Dunnill, 1982). Chalkley's method was based on the fact that if points are made randomly on a block of tissue, the percentage of points lying in any morphological structure will, as more points are accumulated, approach the percentage of the tissue block made up of that structure. In 1949 Chalkley *et al* extended this method to obtain estimates of the volume-surface ratio of morphologic structures such as cells or nuclei.

These developments led to the introduction of a series of point-counting eyepiece graticules. In 1955 Haug proposed a graticule with 121 points while Hennig and Zeiss produced a graticule which had twenty-five points (Aherne and Dunnill, 1982). These were arranged in an hexagonal pattern to enable the whole circular field of view to be examined. Weibel, Kistler and Scherle designed a multipurpose graticule in 1966 and in 1968 Merz produced a curvlinear grid for the study of anisotropic structures (Aherne and Dunnill, 1982).

In the 1930s and 1940s the pace of advancement in the field of morphometry quickened as the speed, efficiency and controllable precision which characterize the methods of probability became apparent. As had previously been the case in the history of the development of the field, the new advances were made by geologists and metallurgists and were only incorporated into histology later (Aherne and Dunnill, 1982).

In 1945 another Russian petrographer, Saltykov, discovered that the surface area of a metallic inclusion is directly proportional to the number of intersections which its two dimensional profile makes with a grid which is laid on the plane of polish (Aherne and Dunnill, 1982).

Serge Tomkeieff's work in 1945 provided the historical link to Buffon's Needle Problem by enlarging on Crofton's theorems. Tomkeieff enumerated the following equations:

" 1. In a plane convex figure (polygon, circle) the average length of projection is equal to the perimeter divided by Pi and the average linear intercept (*L*) is equal to the area divided by the average length of projection:

$$L = \frac{\text{Pi x area}}{\text{perimeter}}$$

2. In a three dimensional convex figure (polyhedron, sphere) the average area of projection is equal to the surface area divided by four, and the average linear intercept is equal to the volume divided by the average area of projection.

$$L = \frac{4 \text{ x volume}}{\text{surface area}}$$ "

These equations are characteristic of geometrical probability; they reduce three dimensional concepts to two dimensions and two dimensional to one dimension. Even though Tomkeieff thought that these formulae were only of academic interest they were seen as important by fellow geologists as well as biologists (Aherne and Dunnill, 1982).

1.2.3 The role of morphometry in cellular biology.

By the early 1960s stereological methods had been developed to a point where they could be applied to problems of cell biology (Weibel, 1981). In 1962 the principle of linear analysis was used to obtain the first quantitative measurements of the area of endoplasmic reticulum surface per unit volume of cytoplasm in the liver cell (Loud, 1962). In this study Loud refers to the work of Chalkley *et al* whose determination of the volume-surface ratio of morphologic components was based on the analogous method of linear analysis used in metallurgical studies. Loud also conducted a morphometric study in 1965 (Loud *et al*, 1965) to compare the effects of different fixing and embedding media on the cytoplasmic structures of rat liver cells. In 1968 Loud found that quantitative observations of cytoplasmic morphology often contradicted interpretations derived from non-quantitative studies. The data from his electron micrograph study corrected the belief that centrilobular cells have fewer microbodies (peroxisomes) than peripheral cells and that rough-surfaced endoplasmic reticulum is less abundant in peripheral cells.

Morphometry has an important role in the study of the relationship between the structure and function of tissues since the quantitative estimation of the volume fraction of the cellular compartment is relative to the tissue's functional capacity (Loud and Anversa, 1984). In 1969 Weibel *et al*

conducted a study to correlate morphometric data and biochemical studies on liver cells. Previous studies had shown that biochemical data were usually quantitative while morphological information had been restricted to semiquantitative descriptions based on subjective judgements. This investigation found that morphometric methods permit efficient and reliable measurement of structures which allows quantitative data, obtained on histological sections, to be correlated with information from subcellular fractions.

Loud and Anversa (1984) described the measurement of the microvasculature in neonatal rats to determine the early adaptive growth of the myocardium. They found that the measurement of certain properties of the microvascular compartment at a specific time can be used to characterize a tissue at a particular stage and that measurements at different times can reveal normal or pathological changes.

Morphometry at a cellular level can be used to determine the size, number and shape of cells and their components allowing the quantitative analysis of cellular hypertrophy or atrophy, hyperplasia or cell loss as well as the maintenance of cell configuration. A morphometric study by Korecky and Rakusan in 1978 was conducted to determine the normal and hypertrophic growth in the rat heart. It concluded that normal cardiac growth in the rat from weaning to adulthood is almost entirely due to the growth of existing cells i.e. cellular hypertrophy.

Morphometry has been used extensively in cancer research. The purpose of a study by Baak *et al* (1981b) was to discriminate between benign, borderline and malignant mucinous ovarian tumours by measuring the mean

area, mean perimeter and mean of the short axis of the nucleus as well as the volume percentage of the epithelium and mitotic activity. They found that by using quantitative microscopy a reproducible, objective assessment of nuclear and histological features could be obtained and that this was diagnostically useful. Ooms *et al* (1981) have shown that morphometric parameters of T1 bladder tumours can have prognostic as well as therapeutic significance. In 1985 Baak *et al* found that morphometry of the primary tumour in breast carcinoma is a more accurate indicator of the prognosis than axillary lymph node status and that the combination of morphometry and conventional prognostic indicators gives a significant improvement of the prognosis prediction .

In 1965 Chayes observed that the improvement in the quality of imaging in electron microscopy had enabled cytologists to embark on quantitative analysis and advised electron microscopists to begin their work at the level of contemporary geologic methodology. Morphometric technique has since been successfully applied to electron microscopy since cytoplasmic composition expresses the functional state of a cell, for example, the concentration of mitochondria relates to ATP synthesis and the presence of myofibrils indicates the work capacity of muscles (Loud and Anversa, 1984).

Thus it can be seen that the groundwork for morphometry has been laid as a result of a merging of three sciences as diverse as geology, metallurgy and histopathology.

1.3 Tissue preparation for morphometry.

In order to investigate the internal structure of organs it is necessary to section fixed and embedded tissue. By sectioning, the integrity of the tissue is preserved in two dimensions while the third dimension is sacrificed for the advantage of resolution (Weibel *et al,* 1966).

1.3.1 Processing of tissue

In a histological preparation a fixed cell can only bear a resemblance to the living cell and the method of processing and staining determines how close that resemblance will be (Culling, 1974). The basis of good tissue preparation is adequate and complete fixation since faults in fixation cannot be remedied at any later stage (Culling, 1974).The fixative used is important since it is essential to ensure that cellular and tissue architecture are not radically altered by the effects of osmosis (Aherne and Dunnill, 1982). It is essential to add salts to slowly penetrating fixatives to provide an osmotic pressure similar to that of cells in order to avoid distortion of the tissue due to the swelling and bursting of cells (Young, 1935).

The fixative most widely used in morphometric studies was 10% buffered formalin, pH7, (Baak *et al*, 1981b, Tosi *et al*, 1986, Ooms *et al*, 1981, Crocker *et al*, 1983). Aherne and Dunnill (1982) also recommend a phosphate-buffered formol saline solution for light microscopic work. Yoshikawa *et al* (1981) describe a double fixation method for their morphometric study on glomeruli from renal biopsies. In this study Tru-Cut® needle biopsies were fixed immediately after removal in Bouin's alcoholic solution for five to six hours followed by fixation in 10% buffered formalin for

twenty-four hours (Yoshikawa *et al*, 1980). Bouin's fixative gives very good trichrome staining (Culling, 1974) which was necessary in this study as the sections were subsequently stained with periodic acid-Schiff and Masson's trichrome stain (Yoshikawa *et al*, 1980).

Before tissues are processed i.e. treated to facilitate their impregnation with a solid medium in order to produce sections for microscopy, it is important to ensure that fixation is complete (Bancroft and Stevens, 1982). Dehydration, which is the first step in the processing of fixed tissues may cause a high degree of shrinkage if the tissue is immersed in a high concentration of alcohol following an aqueous fixative (Culling, 1974). Culling (1974) recommends a 70% ethanol and water mixture as the first in a series of graded alcohols to prevent distortion of the tissue. This view is supported by Aherne and Dunnill (1982) since lower concentrations of ethanol cause swelling of fixed cells.

After being dehydrated the tissue is "cleared" in a solution to remove the dehydrating agent and to prepare the tissue for impregnation with the embedding agent (Bancroft and Stevens, 1982). Xylene is a commonly used clearing agent and since it causes minimal distortion (Bancroft and Stevens, 1982) it is highly suitable for the preparation of tissue for morphometry.

Since tissue is impregnated with molten paraffin wax which has a melting point of between 40 and 70 ^{0}C it is subjected to high temperatures which may adversely affect certain tissues (Bancroft and Stevens, 1982). Compression takes place when a tissue section is cut and the degree of compression is inversely proportional to the thickness of the section. This

problem can be overcome if care is taken when the sections are floated out before being placed on slides (Aherne and Dunnill, 1982).

1.3.2 Section Thickness.

The ideal tissue sample for morphometric analysis is an infinitely thin section since the methods depend, in theory, on measurements made on the surface of sections taken through tissue (Aherne and Dunnill, 1982). Since thick sections exaggerate any departure from randomness they reduce the credibility of measurements derived from them (Philp and Buchanan, 1971). If the section is very thin relative to the size of the structures under observation then section thickness can be discounted and the section may be regarded as a two dimensional image of the internal tissue structure (Weibel *et al*, 1966). This view is shared by Philp and Buchanan (1971) who agree that a fairly accurate estimate can be obtained from measurements of a section which is thin in relation to the object whose relative volume is being determined. Exceptionally thin sections are necessary when measuring components of individual cells e.g. nuclei (Aherne and Dunnill, 1982).

The characteristics of an infinitely thin section are as follows:

1. Every tissue component is cut at random by a non-oriented section.
2. Each structure loses one dimension; bodies are observed as areas, surfaces as lines and lines as points.
3. The quantitative occurrences of images on sections may be determined by the quantitative presence of structures in the tissue (Weibel *et al*, 1966)

This principle when applied to infinitely thin sections of tissue makes it possible to use sections for quantitative morphological studies.

In many cases the finite thickness of a section cannot be ignored since this would introduce systematic error (Weibel *et al*, 1966). The Holmes' effect, first described by Holmes in 1921 (Holmes, 1921) is the effect that section thickness has in making a component appear larger than it is to the eye placed above the section (Aherne and Dunnill, 1982) - Figure 1.3. Finite section thickness will introduce a systematic error due to Holmes' effect which is dependent on the ratio of section thickness to the mean diameter of the structures under investigation. It is generally accepted that the Holmes' effect can be disregarded if this ratio is much less than one (Weibel *et al*, 1966). According to Aherne and Dunnill (1982), it is necessary to make some allowance for the Holmes' effect if the mean diameter of the structures to be measured is less than twelve times the section thickness.

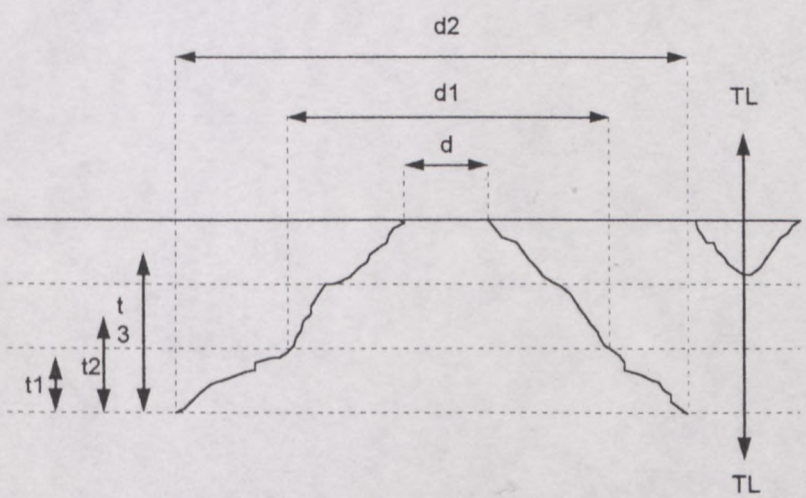

Figure 1.3: Cross sectional view of a section to show the Holmes effect and the "optically lost cap." d is the diameter of the cell segment on the left of an infinitely thin section. Depending on the thickness of the section (t) the observer will actually see d1 or d2. The smaller segment on the right may not be seen because there is not enough stained substance to absorb any transmitted light (TL)

Tissue processing and sectioning distorts and shrinks cells which makes it impossible to equate the measurements on tissue sections with those *in vivo*, however it may be acceptable to compare similar tissue which has been treated in the same way (Philp and Buchanan, 1971). Aherne and Dunnill (1982) confirm this by stating that for comparative morphometry, measurement of alterations in tissue structure caused by fixation, processing and embedding is irrelevant provided that all material is treated in an identical manner.

1.4 Sampling.

For morphometric studies, the aim of a sampling strategy is to obtain the maximum amount of quantitative structural information for a given total cost or effort (Gundersen and Osterby, 1981). It is a characteristic of probabilistic morphometry that exhaustive counts or measurements are not done; rather, only the necessary amount of observations are made (Aherne and Dunnill, 1982). Morphometric methods are dependent on statistical analysis (Aherne and Dunnill, 1982) and sampling methods are central to statistics (Kirkwood, 1988).

As it is not possible to investigate an entire organ morphometrically it is necessary to analyse a small sample of tissue which possesses the same quantitative characteristics as the entire organ (Aherne and Dunnill, 1982). An organ is isotropic if structures occupy the same space and are randomly distributed throughout the whole organ while an anisotropic organ shows marked variation of the geometrical properties of its components (Aherne and Dunnill, 1982). These properties of organs and tissues may result in large variation among a set of random samples due to the lack of

homogeneity in a structure (Aherne and Dunnill, 1982). A morphometric result only has meaning if the method of sampling has ensured that the subject under observation has been adequately represented (Loud and Anversa, 1984). Blocks should be taken uniformly throughout the tissue volume and microscopic fields should be evenly distributed over tissue sections to ensure a systematic sample (Loud and Anversa, 1984). Systematic sampling is superior to random sampling because it yields a smaller standard error (Aherne and Dunnill, 1982). This principle is endorsed by Weibel *et al* (1966) who state that an even distribution of the sample over the tissue is an important factor in reducing error. In this present study sections from both the fundus and the lower segment of the uterus will be measured in an attempt to achieve a systematic sample and therefore reduce the standard error.

1.5 Sample size.

Ultimately , the practical question in morphometric sampling is the number of measurements to be collected (Loud and Anversa, 1984). Morphometric analysis provides estimates of actual measurements within certain confidence intervals which depend on the size of the sample (Weibel *et al*, 1966).

The criteria necessary to ensure that a sample is adequate are:

1. that the variation between sample sites is statistically insignificant and
2. that the mean value of the measurement derived from a sample at any one site should have a standard error which is acceptably small (Aherne and Dunnill, 1982).

The standard error of the sample mean measures how precisely the population mean is estimated by the sample mean (Kirkwood, 1988). The size of the standard error depends on how much variation there is in the population and on the size of the sample, therefore, the larger the sample, the smaller the standard error (Kirkwood, 1988). The relationship between the number of observations and the precision of a mean is represented by the formula for the standard error: S.E. = $\frac{s}{n}$

where: s = standard deviation of the sample

n = sample size.

Since the standard error of the mean is inversely proportional to the square root of n (sample size), to halve the standard error, the sample size must be quadrupled (Ipsen and Feigl, 1970). Weibel *et al* (1966) agree that the quality of the result can be improved by increasing the sample size, however this improvement is proportional to the square root of the sample size.

In order to estimate a population mean it is not difficult to determine the sample size necessary to achieve a specified precision, provided that estimates of the population mean and standard deviation are available (Ipsen and Feigl, 1970). A pilot sample may be drawn from a population and the sample mean and standard deviation may be used as estimates of the population mean and standard deviation (Daniel, 1974).

Statistical accuracy is dependent on the size of the sample which can be equated with the number of specimens in a biological experiment (Loud and Anversa, 1984). Since there is natural variation within each specimen it has been shown that by increasing sample size one gains more overall precision than by increasing the number of observations within each unit (Nicholson,

1978). Mathieu *et al* (1980) also state that it is more efficient to measure less precisely more fields from a large number of sections than to achieve high precision on a few subsampled fields. Gundersen and Osterby (1981) summarize this principle by stating that it is better to look at more individuals rather than measure them more precisely i.e. " Do more less well!".

Since this study will investigate nuclear dimensions as an indicator of change in cell size particular note was made of references in the literature to nuclear morphometry. In the literature reviewed there is great variation in the size of the samples used in the studies which measured nuclear dimensions. The studies of Ooms *et al* (1981), Abbot *et al* (1982), Jagoe *et al* (1982) and Crocker *et al* (1982) all employed a sample size of less than twenty-five patients and each study measured varying numbers of nuclei. Ooms *et al* (1981) and Jagoe *et al* (1982) measured twenty and thirty nuclei respectively per case while Abbot *et al* (1982) and Crocker *et al* (1982) each measured 200 to 400 and 500 nuclei respectively on each case.

Five of the papers reviewed studied nuclei from between forty and one hundred patients. Baak *et al* (1981b) examined forty-two cases of ovarian tumours and measured twenty-five nuclei from each. Crocker *et al* (1983) reported on forty lymph nodes and measured five hundred nuclei from each case. Only two of the papers examined had samples larger than one hundred cases. Baak *et al* (1981c) investigated one hundred and ten cases of endometrial hyperplasia and carcinoma and Baak *et al* (1985) examined 271 patients with breast cancer measuring twenty-five nuclei from each. There seems to be great differences in sample size in the literature and the concept of "Do more less well" (Gundersen and Osterby, 1981) is adhered to by only a few of the researchers.

1.6 Statistical Methods.

This study employed the Student's T test and the one-way analysis of variance (ANOVA). One-way ANOVA analyses were predominantly used to assess the reproducibility of measurements when ascertaining optimal measurement and staining techniques. Student's T test was used to test for significant differences between sets of myometrial nuclear measurements.

The investigations of Tosi *et al* (1986), Yoshikawa *et al* (1981) and Cagle *et al* (1992) have all applied Student's T test to their results. The t-distribution is used to calculate confidence limits for the population mean when the sample standard deviation is known but not the population standard deviation (Hall, 1988). The t-distribution is used to determine confidence intervals for a smaller sample size, for a normally distributed population (Kirkwood, 1988). The t-distribution was discovered by W.S. Gosset in 1908 and improved by R.A. Fisher in 1926 (Snedecor and Cochran, 1967). This distribution is used to deal with the statistics of small samples (Snedecor and Cochran, 1967) and is composed of many different distributions which are differentiated by their degrees of freedom (d.f.) and like the normal distribution, the t-distribution is symmetrical, unimodal and bell-shaped (Daly *et al*, 1991). Since this distribution is more spread out it allows for the increased variability introduced into the determining of confidence levels when only the standard deviation of the sample is known (Daly *et al*, 1991).

Many of the papers reviewed used non-parametric methods to analyse their results. Baak *et al* (1981a, 1981b, 1981c), Van der Valk *et al* (1983), and Oom *et al* (1982) all used a form of Wilcoxon's test. Partin *et al* (1989) used

the Mann-Whitney and Wilcoxon's tests. Non-parametric methods are statistical techniques used to analyse numerical data without making assumptions about the normal distribution of the data (Kirkwood, 1988). In addition they test a null hypothesis by relating to median values rather than mean values (Daly *et al*, 1991). Non-parametric methods are very useful for the analysis of two-sample quantitative data (Daly *et al,* 1991). In general parametric tests are more powerful in detecting differences between populations of normal distributions. Non-parametric tests are not useful for estimation purposes while confidence levels for estimates are usually difficult to calculate (Daly *et al*, 1991). The hypothesis to be tested by non-parametric methods usually relates to the nature of the distribution as a whole rather than to the values assumed by its parameters (Armitage and Berry, 1987). The criteria for using non-parametric tests include:

1. Examining a population with an obvious abnormality,
2. Investigating a population too small to establish a possible abnormality,
3. The necessity for a rapid statistical technique.
4. When it is necessary to account for a rank order of observations e.g. for degrees of clinical improvement (Armitage and Berry, 1987).

Methods of multivariate analysis have been used by Baak *et al* (1981b, 1981c, 1985) and Mitmaker *et al* (1992). Multivariate analysis is a collection of methods used when the random variation in several variables has to be investigated simultaneously (Armitage and Berry, 1987). Multiple regression is a powerful method of studying the simultaneous effect on a random variable of various predictor variables with no special conditions of balance being imposed on their values (Armitage and Berry, 1987).

1.7 Instrumentation for morphometry

The development of computer-assisted instrumentation for morphometry has enabled the rapid expansion of the systematic study of histological material (Barry and Sharkey, 1985). The instrumentation used for image analysis may be divided into automatic and semi-automatic systems. Crocker and Curran (1979) used the Zeiss Microvideomat which is an automatic image analysing system to measure the nuclear diameters from tonsil and lymph node imprints. A grey scale is used as a reference of light intensity and is adjusted to a level of sensitivity which corresponds to a particular feature. The light density of the object examined under the microscope is converted to a black and white monitor picture and in this way it is possible to measure the percentage of a selected field occupied by a feature discriminated in the grey scale (Crocker and Curran, 1979). Since the scanning occurs in the microscope image, neither the light source nor the microscope stage moves during the scanning (Aherne and Dunnill, 1982). When an automatic system is used to quantify individual objects e.g.: nuclei, it is necessary to be able to discriminate the nuclei visually and electronically in terms of grey scale intensity and the nuclei must be presented to the machine as separate entities (Crocker and Curran, 1979).

Where identification of features cannot be made by grey-level density and the high level of discrimination achieved by human eye and brain is essential, semi-automatic systems offer a realistic solution (Gore, 1979). These systems use a graphic or digitizing tablet on which the features are outlined by a cursor whose position and movements are recorded in a microprocessor (Aherne and Dunnill, 1982). The position, area, perimeter

and shape of these features are analysed and reproduced as either a data printout or a visual display (Aherne and Dunnill, 1982).

The limitations of automatic image analysers can be overcome with the use of a semi-automatic system as the user can draw around selected features so that adjacent or overlapping nuclei in histological sections can be sized with ease and a good degree of accuracy (Crocker *et al*, 1982). The semi-automatic systems also have the advantage of being less expensive than the fully automatic systems (Baak and Oort, 1983). The present study will utilize a computer programme called VIDS III which is a high resolution, semi-automatic, image analysis system.

Mathieu *et al* (1981) compared the efficiency of three methods for planar analysis namely, point-counting, semi-automatic computer image analysis with MOP (Manual Optic Picture Analyser) AMO3, Kontron AG and automatic image analysis with Quantimet. This study found that Quantimet could not be used to analyse the electron micrographs of a biological sample as it could not recognize the relevant features (Mathieu *et al*, 1981). It was also found that compared to point-counting the use of the MOP considerably increased the accuracy of individual measurements although this took longer (Mathieu *et al*, 1981). Abbot *et al* (1982) used a television-based image analyser, the Quantimet 720 and a semi-automatic electronic planimeter, the Kontron MOP/AMO3, for morphometric analysis of tissue sections from a series of non-Hodgkin's lymphoma. In this study it was found that even when thin plastic sections with a high contrast nuclear stain were used, boundary features were still not well defined by the Quantimet 720 (Abbot *et al*, 1982). When the image analysis system relies on the

operator's manual definition of the feature under observation as in semi-automatic systems, morphometric data are more readily and reliably obtained (Abbot *et al*, 1982).

1.8 The Uterus.

1.8.1 Normal myometrium.

Part of this study is concerned with the measurement of myometrial cells pre-menopausally, post-menopausally, during pregnancy and during the menstrual cycle. It was therefore deemed necessary to review the literature pertaining to the normal structure of the musculature of the uterus.

Uterine muscle is only one constituent in the complex tissue which forms the uterine wall (Norris *et al.*, 1973). The other constituents include connective tissue, nerve cells and secretory elements which all influence the behaviour of the myometrial cells (Norris *et al.*, 1973). Myometrial cells are smooth muscle cells which are small, spindle-shaped and embedded in an abundant connective tissue network. They are about 5 to 10 microns wide and 50 to 800 microns long but these dimensions can be altered by hormonal and physical factors (Norris *et al.*, 1973). These alterations in size will be used in this study as indicators of the effects of menopause, pregnancy and menstrual cycle on these cells.

Schwalm and Dubrauszky (1966) investigated the percentage muscle content of sexually mature (aged 25 to 45 years), menopausal and pregnant uteri. In the sexually mature group they found that most of the musculature was in the wall of the upper part of the corpus below the fundus and that the percentage of muscle decreased caudally with very low figures in the cervix. They also found that the menopausal uterus contains much less muscle than the uterus in the period of sexual maturity and that the reduction in the percentage of musculature from the corpus toward the cervix is perceptible

but no longer definite. This study did not record an accompanying reduction in size of individual myometrial cells. In the pregnant uterus at 7 months it was found that there is a sharp increase in the proportion of muscle in the corpus and isthmus while the cervix contains approximately the same amount of muscle as in the non-pregnant state. Schwalm and Dubrauszky (1966) also noted that the passive enlargement of the corpus uteri which occurs during pregnancy does not alter the composition of its wall i.e. the ratio between muscle and connective tissue. They found that the increase in muscle percentage occurs from the third to the fourth month and that subsequently no further increase occurs. As with the menopausal uteri no conclusion was made in this study concerning the increase in size of individual cells. Norris *et al.* (1973) state that during pregnancy the myometrial cells increase in size rather than in number. A myometrial cell may increase from a diameter of 5 microns and a length of 90 microns at the beginning of pregnancy to 10 microns by 800 microns at term (Norris *et al.*, 1973).

1.8.2 Uterine endocrinology.

Part of this study includes the investigation of the size of myometrial cells at various stages of the endometrial cycle. In order to correlate hormonal status obtained from serum analysis with the morphology of the myometrium at different stages it is necessary to understand the effects of the endocrine system on the uterus.

The uterus is an organ markedly influenced by reproductive steroid hormones, the most important hormonally induced changes being those caused by oestrogens and progesterones (Wynne, 1977). Endocrine

physiology in the female which controls sex determination, conception, foetal development, birth, growth, puberty, reproduction and menopause illustrates the complexity and responsivity of this highly differentiated endocrine system (Norman and Litwack, 1987). Its integrated operation is dependent on the interaction of hormonal and neural signals between the central nervous system, the pituitary and the ovary (Norman and Litwack, 1987).

1.8.3 The ovarian cycle.

The very existence of the ovary and the reproductive axis as a whole is designed to serve the single purpose of generating a mature fertilizable ovum. The ovaries are paired organs situated in the abdominal cavity and contain primordial follicles derived from gut mesentery which are laid down in foetal life and persist scattered throughout the adult ovary (Hillier, 1990). At birth there are one to two million follicles in the ovary (Norman and Litwack, 1987). Atresia and cell death leads to a decrease in number so that by puberty there are only 100 000 to 300 000 oogonia to support the monthly ovulatory cycles over the next 35 to 40 years (Norman and Litwack, 1987).

Ovaries are unique among glands of internal secretion since their primary function is to produce gametes (oocytes) and all their endocrine activities subserve this function (Hillier, 1990). Reproductive life in the female is directly controlled by the gonadal hormones elaborated by the ovary i.e.: oestrogens, progesterones, androgens and relaxin (Botella-Llusia, 1973). These hormones stimulate target organs e.g. uterus, Fallopian tubes, breast as well as pituitary and hypothalamus (Hillier, 1990). The ovarian cycle is

designed to ensure that mature female gametes are produced in good condition in the appropriate number at ovulation to enable fertilization by sperm (Hillier, 1990). It is a means of restoring potential fertility following a sterile mating, an event which is infrequent in most mammals with the exception of humans since the widespread use of contraceptives leads to repeated ovarian and menstruation cycles (Hillier, 1990).

1.8.4 Gonadotrophins.

Gonadal function and reproduction are controlled primarily by pituitary hormones which bind to specific receptors in the ovary to regulate steroidogenesis and gametogenesis (Yen and Jaffe, 1991). These actions are exerted predominantly through the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are secreted by the same cell in the anterior pituitary in response to gonadotrophin-releasing hormone (GnRH) (Hillier, 1990). FSH, LH and GnRH act specifically on the pituitary and ovaries, respectively, while the steroid hormones, especially oestrogen and progesterone, have a wide range of actions in many tissues (Norman and Litwack, 1987).

There is increasing evidence that in addition to gonadal steroids FSH secretion is also regulated by a protein hormone termed inhibin (Norman and Litwack, 1987). Yen and Jaffe (1991) confirm that inhibin has long been proposed to preferentially inhibit FSH secretion. Inhibin is a protein formed in the ovaries and acts on pituitary gonadotropes to suppress the formation and release of FSH while its production is stimulated by FSH thus forming a typical closed-loop negative feedback mechanism to regulate gonadotrophin secretion (Yen and Jaffe, 1991). It has also been proposed that decreased

levels of inhibin is the ovarian signal for the initiation of the next round of FSH-mediated follicle recruitment (Roseff *et al*, 1989).

1.8.5 The corpus luteum.

When the mature follicle ruptures and releases the ovum at ovulation, the cells comprising the follicle, under the influence of LH, enlarge and differentiate into lutein cells which are the principal sites of production of progesterone and oestrogen after ovulation (Norman and Litwack, 1987). Subsequently capillaries grow around these cells, giving rise to the corpus luteum which is a typical endocrine organ (Norman and Litwack, 1987). The corpus luteum increases in size for 10 to 12 days if the released oocyte is not fertilized within one to two days (Norman and Litwack, 1987). This is followed by regression of the gland to produce a small white ovarian scar called the corpus albicans and concomitant cessation of progesterone and oestrogen secretion (Norman and Litwack, 1987). All available evidence suggests that the human corpus luteum is dependent throughout its lifespan on support by pituitary LH (Hillier, 1990).

1.8.6 The feedback system.

Ovarian cyclicity is controlled by a feedback system involving the hypothalamus, anterior pituitary and the ovaries (Hillier, 1990). The hormones produced by the corpus luteum have profound effects on the secretion of gonadotrophins by the anterior pituitary (Hillier, 1990). Over 90% of the ovarian hormones during the luteal phase is secreted by the corpus luteum and hence the secretory products of this structure dominate the feedback system (Baird & Fraser , 1975).During the luteal phase under

the combined effects of oestradiol and progesterone the concentration of LH is suppressed to the lowest level observed throughout the cycle (Hillier, 1990).

1.8.7 The menstrual cycle.

Part of this study includes the measurement of the nuclei of myometrial cells at different stages of the menstrual cycle to investigate whether the cyclical changes of progesterone and oestrogen have any influence. Blood taken at the same time as hysterectomy will be analysed to determine the steroid serum levels. Since this information will be an indication of the stage of the menstrual cycle it is important to understand the endocrinology of the cycle.

The menstrual cycle is a repetitive expression of the operation between the hypothalamus, pituitary and ovaries which results in associated structural and functional changes in the target tissues i.e. uterus, oviducts, endometrium and vagina (Yen and Jaffe, 1991). The average length for a cycle is 28 days although this varies among women and is usually more irregular near puberty and menopause (Crouch, 1972).

Each cycle culminates in menstrual bleeding, the first day of which is accepted as a reference point marking the beginning of a menstrual cycle (Yen and Jaffe, 1991). During the menstrual cycle, the endometrium, myometrium and cervix show cyclic changes related to changes in the steroid secretory pattern of the ovary (Norris, 1973). Menstruation occurs approximately between days one to five when progesterone secretion declines as the corpus luteum begins involution (Crouch, 1972), after which

there is an increase in the rate of synthesis of oestrogens as the ovarian follicles develop (Norris, 1973).

The first half of the cycle which lasts until about day 14 is referred to as the follicular phase and is characterized by a progressive increase in circulating levels of oestradiol by the developing Graafian follicle (Yen and Jaffe, 1991). The length of the follicular phase is determined by the time taken for a small antral follicle to reach maturity when it secretes sufficient oestrogen to provoke a preovulatory surge of LH (Hillier, 1990). In humans this process takes at least 14 days because the largest healthy antral follicle, containing approximately one million granulosa cells, is only two to four millimeters (Hillier, 1990). After menstruation the myometrium is relatively firm (Wynne, 1977). As the synthesis of oestrogen increases the muscle cells of the myometrium increase in size, especially in length and spontaneous contractions are apparent by about day 9 of the cycle (Wynne, 1977). No evidence has been found in the literature about the quantitative analysis of this increase in cell size.

Ovulation occurs at about Day 14 and marks the beginning of the ovulatory phase during which levels of oestrogen drop as progesterone secretion by the corpus luteum increases (Crouch, 1972). The contractions of the myometrial cells reaches a peak at ovulation and for a few days later (Wynne, 1977). After ovulation the increased blood levels of progesterone inhibit the contractions of the myometrium (Wynne, 1977).

The luteal phase follows and is characterized by a shift from the oestrogen-dominated follicular phase to progesterone dominance (Yen and Jaffe, 1991). The peak concentrations of progesterone and oestradiol reached at

the midluteal phase constitute the three day window in which the secretory endometrium is conducive to implantation (Yen and Jaffe, 1991). Unless implantation occurs the corpus luteum begins to degenerate (Crouch, 1972) leading to a decline in circulating progesterone, oestradiol and inhibin levels during the last four to five days of the functional life of the corpus luteum (Yen and Jaffe, 1991). Since the corpus luteum is the source of oestradiol and inhibin during the luteal phase and their secretion is controlled by LH and not FSH, there is no negative feedback loop to control the secretion of FSH below the threshold necessary to sustain the growth of medium sized antral follicles (Hillier, 1990). Thus during the luteal phase of the menstrual cycle all antral follicles more than 5 mm in diameter are atretic and make an insignificant contribution to the secretion of ovarian hormones (McNatty *et al.*, 1983). During this stage of the cycle follicular development is suppressed and only resumes when the concentration of FSH and LH rise during regression of the corpus luteum (Baird *et al.*, 1984).

The follicular growth of the ensuing cycle is dependent on the regression of the antecedent corpus luteum. The key event is the inverse relationship between the fall of inhibin levels and the rise in FSH levels that occurs two days before the onset of menses, thereby initiating follicular recruitment for the following cycle (Mais *et al.*, 1987). This luteal-follicular transition represents a sequence of dynamic changes involving the termination of luteal function and the reactivation of the GnRH-gonadotrophin system (Yen and Jaffe, 1991). These changes are a result of the withdrawal of the inhibitory effects of the corpus luteum steroids and inhibin (Filicori *et al.*, 1984)

The secretory activity of the corpus luteum and its functional life span are dependent on appropriate LH support (McLachlan *et al.*, 1989). Interruption of LH activity by a GnRH antagonist during various stages of the luteal phase induces a rapid decline in the levels of progesterone, oestradiol and inhibin followed by luteolysis and the onset of menses (McLachlan *et al.*, 1989). FSH levels are suppressed during the luteal phase to reach the lowest levels of the cycle since FSH is not required for the maintenance of the corpus luteum (Yen and Jaffe, 1991). The combination of inhibin with oestrogen and progesterone synergistically suppresses FSH secretion and thus prevents the initiation of folliculogenesis (Yen and Jaffe, 1991).

1.8.8 Oestrogen and progesterone

A principal objective of this study is to investigate the influences of progesterone and oestrogen on the myometrium of the uterus.

To accomplish their functions steroid hormones must bind and activate receptors which are a group of specific gene-regulatory molecules (Yen & Jaffe, 1991). Receptors are proteins present in cells in low amounts that bind steroid hormones specifically and very tightly (Yen & Jaffe, 1991).

The regulatory actions of oestrogens in granulosa cells are thought to be mediated by the binding of the steroid to receptors which regulate rates of transcription from oestrogen responsive genes (Hillier, 1990). Receptors bind hormone agonists or antagonists with affinities which generally correlate with their biological potency (Baulieu & Kelly, 1990). The oestrogen receptor (ER) binds oestradiol, a potent oestrogen, more strongly than oestrone or oestriol, which are weak oestrogens (Baulieu & Kelly, 1990).

Yen and Jaffe (1991) describe the biochemical pathway of steroid hormone action in cells as follows:

1. The hormones are secreted into the bloodstream, 95% of these are bound to plasma transport proteins, and provide a reservoir for steroid supply to cells.
2. The free steroids then diffuse into cells and combine with specific receptors in the target cells in which they will exert their functions.
3. After binding tightly to their specific receptors, the steroids cause a conformational change in the receptor's structure which converts the receptors from an inactive to an active form.
4. The receptors are then able to bind to the regulatory elements of genes and activate or suppress their function.

Oestradiol is the principal oestrogen secreted by the ovary in pre-menopausal women while oestrone is the principal oestrogen in post-menopausal women (Mishell *et al*, 1991). Oestrogens may be classified as either natural or synthetic according to their occurrence and by their chemical structure as either steroidal or nonsteroidal (Yen & Jaffe, 1991). The specific, high affinity binding of oestradiol in a tissue determines its capacity to retain the hormone and indicates the degree to which the target tissue is sensitive to the hormone's action (Farber *et al.*, 1972).

Oestrogen is an intrafollicular autocrine regulator since it acts on the cell which produces it (Hillier, 1990). The principal action of oestrogens on target tissues of the reproductive tract is tissue growth following binding of the steroid hormone to its receptor (Wilson *et al.*, 1980). This response in the uterus involves increased synthesis of RNA, proteins and certain enzymes

(Norris *et al*, 1973). Oestrogens affect the metabolic properties of both endometrium and myometrium (Norris *et al.*, 1973). Oestradiol has varied and extensive effects on the uterus including increasing the blood supply, increased capillary permeability, and increased uptake of water electrolytes and amino acids by myometrial cells (Norris *et al*, 1973). Wynne (1977) states that when oestradiol is administered to a rat the effects on the uterus include a rapid increase in the uptake of water, glucose, and amino acids as well as increased glucose metabolism, increased formation of glycogen, increased lipid synthesis, accumulation of lactate, increased rate of RNA and protein synthesis, increased cell division and increase in uterine weight. It is not stated whether the size of the myometrial cells increase which is an important question raised in this study.

Progesterone is the only natural progestogen which has biological significance and is secreted by the ovary and adrenal and, in pregnancy, by the placental trophoblast (Mishell *et al*, 1991). As with oestrogens, progesterones may also be classified as natural or synthetic (Yen & Jaffe, 1991).

Progesterone has long been considered an antagonist of oestrogen action and the delicate balance and interactions between these ovarian hormones are essential for many reproductive functions (Hsueh *et al.*, 1975). It is thought that progesterone could be antagonistic to oestrogen by suppressing the quantity of cytoplasmic oestrogen receptor (Hseuh *et al.*, 1975). The antagonistic effects of progesterone on uterine weight and protein content probably reflect the ability of progesterone to redirect the manner in which certain uterine cell types will respond to oestrogen (Hseuh *et al.*, 1975). Oestrogens and progestins bind to separate cytoplasmic

receptor molecules and therefore receptor-steroid complexes do not compete for the same nuclear sites (Hseuh *et al.*, 1975).

Progesterone produces cellular differentiation in the uterus (Baulieu & Kelly, 1990). It exerts a relaxing effect on the myometrium and the disappearance of this "progesterone block" is an important factor leading to myometrial contractions during parturition or abortion (Baulieu & Kelly, 1990). The myometrium contains progesterone receptors and since progesterone decreases uterine sensitivity to oxytocin stimulation it works synergistically with relaxin to reduce uterine motility and inhibit propagation of uterine contractions (Yen & Jaffe, 1991).

Progestins are steroids which mimic the effects of progesterone, which ensures the undisturbed development of pregnancy (Yen & Jaffe, 1991). They may be defined as agents that induce secretory changes in the proliferative endometrium (Yen & Jaffe, 1991).

1.8.9 Pregnancy.

During this study the size of uterine cells during pregnancy will also be analysed. No indication of quantitative studies on myometrial cells in the pregnant uterus has to date been found in the literature.

The series of morphologic and endocrinologic events which result in successful implantation of the blastocyte involves both systemic and local mechanisms (Yen and Jaffe, 1991). The human oocyte-corona-cumulus complex secretes high levels of progesterone, oestradiol and prostaglandins (Yen and Jaffe, 1991). In humans preimplantaion embryos are able to

secrete human chorionic gonadotrophin (hCG) which stimulates increased secretion of progesterone by the corpus luteum (Fishel *et al.*, 1984). If pregnancy is to be successfully established the maintenance of the corpus luteum and continued secretion of progesterone is essential (Hillier, 1990).

The corpus luteum of a non-fertile menstrual cycle has a finite lifespan of fourteen to sixteen days (Hillier, 1990). In the event of pregnancy its secretory activity is prolonged due to the secretion of HCG by trophoblastic cells of the placenta (Hillier, 1990). HCG and LH are structurally similar glycoprotein hormones which appear to interact with the same cell-surface receptor on primate luteal cells to stimulate adenyl cyclase (Cameron and Stouffer, 1982). It may be assumed, therefore, that prolonged gonadotrophic stimulation of luteal cells by HCG is the mechanism responsible for the rescue of the corpus luteum (Hillier, 1990). HCG secreted by the trophoblast appears in the maternal circulation soon after implantation and approximately nine days after ovulation (Yen and Jaffe, 1991). This HCG rescues the corpus luteum which would otherwise regress and a rise in progesterone, 17 alpha-hydroxyprogesterone, oestradiol and oestrone levels reflects the continued and augmented corpus luteum activity in response to HCG stimulation (Yen and Jaffe, 1991).

The functional capacity of the corpus luteum diminishes after the seventh week of gestation although it may continue to produce small amounts of progesterone for the duration of pregnancy (Tulchinsky and Hobel, 1973). The luteal-placental shift occurs after the seventh week and the placental trophoblast and decidua assume the role of progesterone production and continue to be the principal source until parturition (Yen and Jaffe, 1991).

The myometrium contains progesterone receptors and progesterone is known to decrease uterine sensitivity to oxytocin stimulation (Yen and Jaffe, 1991). Thus it works synergistically with relaxin to reduce uterine motility and inhibit propagation of uterine contractions (Yen and Jaffe, 1991). In more than 90% of lactating women menses resumes by three months after delivery and by six months in more than 90% of women who breast feed (Gold & Josimovich, 1987).

1.8.10 Menopause

A further aim of this study is to test the hypothesis, by morphometric means, that the nuclei of myometrial cells atrophy after the menopause. A literature search has not revealed any quantitative information to support or disprove this hypothesis. It is necessary to understand the menopausal changes in hormonal status to adequately study this question.

Throughout life there is a progressive loss of the fixed stock of oocytes in the ovaries and in women between the ages of 40 and 50 the depletion is such that very few oocytes remain and ovarian cyclicity ceases (Hillier, 1990). The latter stages of reproductive life where ovarian activity gradually declines and menstruation eventually stops is known as the climacteric (Chakravarti *et al.*, 1976). The menopause is the last menstrual period and is characterized by ovarian failure with raised levels of gonadotrophins (Hillier, 1990). As women approach menopause, the first detectable endocrine change is a gradual increase in plasma FSH (Soules *et al*, 1976). Some time after the rise in FSH, the production of oestradiol decreases slightly and LH serum levels rise (Gold & Josimovich, 1987). The increased resistance to gonadotrophins of the remaining follicles may explain the

decreased oestrogen production while the progressive decline in the number of ovarian follicles may be responsible for decreased production of ovarian inhibin which accounts for the increased levels of FSH (Gold & Josimovich, 1987). As oestradiol secretion falls to very low levels, both FSH and LH concentrations rise to post menopausal levels and remain elevated for many years (Chakravarti *et al.*, 1976).

During menopause oestrogen production is decreased, cyclicity is lost and oestrone, not oestradiol, is the major circulating oestrogen (Gold & Josimovich, 1987). Oestradiol secretion is reduced during the climacteric and after the menopause the contribution from the ovaries is negligible (Chakravarti *et al.*, 1976). Before the cyclicity of oestradiol secretion is lost, during the perimenopausal years, there is a slight decrease in oestradiol production, reflecting the waning steroidogenic capability of the aging follicles (Reyes *et al.*, 1977). After menopause the levels of oestradiol and oestrone drop (oestrone to a lesser extent), reflecting the absence of maturing ovarian follicles (Gold & Josimovich, 1987). The circulating concentration of oestrone is higher than that of oestradiol after the menopause (Yen & Jaffe, 1991). Chakravarti *et al.* (1976) found that concentrations of androstenedione, oestrone, and oestradiol all fall to about 20% of their premenopausal values within a year after menopause.

In postmenopausal women the average blood concentration of FSH and LH stays elevated for the remainder of life and although the postmenopausal ovary is not totally unable to secrete steroids the production level is markedly reduced (Norman and Litwack, 1987).

1.8.11 Hormonal contraceptives.

It is assumed that many women in this study will be using steroid contraceptives. There are three major types of oral steroid contraceptives (OC) formulations: fixed dose combination, combination phasic, and daily progestin (Mishell *et al*, 1991). The combination formulations which are the most effective and the most widely used are tablets which contain an oestrogen and a progestin given continuously for three weeks (Mishell *et al*, 1991). Combined OC's contain synthetic forms of the naturally occurring female steroid hormones, oestrogen and progesterone (Baulieu & Kelly, 1990).

The effectiveness of the combined OC's is a result of their interference with the reproductive process on several levels (Yen & Jaffe, 1991). They consistently inhibit the midcycle gonadotrophin surge by inhibiting hypothalamus and anterior pituitary stimulation of the ovaries (Mishell *et al*, 1991). They also prevent the changes that normally occur in the cervix which causes the mucus to remain thick, viscid and scanty which in turn retards sperm penetration (Yen & Jaffe, 1991). OC's also alter the motility of the uterus and oviduct, thus impairing transport of both ova and sperm as well as changing the endometrium to reduce its glandular production of glycogen and thus less energy is available for the blastocyte to survive in the uterine cavity (Mishell *et al*, 1991). They may also alter ovarian responsiveness to gonadotrophin stimulation (Mishell *et al*, 1991).

The daily progestin-only preparations do not consistently inhibit ovulation, but they do have the same effects on the reproductive system as the combined formulations i.e. they exert their action on the cervical mucus, motility of the uterus and Fallopian tubes and the endometrium (Mishell *et al*, 1991). As a result of their inconsistent ovulation inhibition, their

effectiveness is significantly less than that of the combination types of OC's (Mishell *et al*, 1991).

1.9 Leiomyomas.

This study will incorporate an investigation into the size of cells of uterine leiomyomas to determine whether these tumours behave in the same manner as myometrium or, because they are neoplastic, they react differently.

Uterine leiomyomas are benign neoplasms of smooth muscle. They are usually multifocal and their aetiology is unknown (Meloni *et al*, 1992). Leiomyomas are the most common benign neoplasm of women and occur in approximately 25% of women of childbearing age (Norris *et al*, 1973). They are also referred to as myomas, fibroids or fibromyomas (Robbins et al, 1989). These tumours occur subserosally, intramurally, or directly beneath the endometrium (Ackerman & Rosai, 1974). Microscopically, leiomyomas are formed by interlacing bundles of smooth muscle cells separated by varying amounts of connective tissue and are usually well vascularized (Ackerman & Rosai, 1974).

The symptoms associated with these tumours are dependent on their size, number and location (Soules & McCarty, 1982). The clinical effects of leiomyomas include impaired fertility, pain and pressure on the urinary bladder and abnormal, irregular vaginal bleeding (Robbins *et al*, 1989). Leiomyomas in pregnant women increase the frequency of spontaneous abortion, foetal mal presentation, uterine inertia, and postpartum haemorrhage (Robbins *et al*, 1989). The severity of these symptoms

underline the importance of examining the possible influences on the growth of these tumours in this study.

1.9.1 The influence of steroid hormones on leiomyomas.

It has long been recognized that there is a relationship between ovarian function and leiomyomas. These tumours thrive during the years of greatest ovarian activity and their involution follows ovarian regression (Miller *et al.*, 1955).

There is much debate over the role of hyperestrinism in the production of these tumours since it is known that they shrink and become fibrosed and even calcified postmenopausally (Robbins *et al*, 1989). Leiomyomas may be considered as endocrine-dependent lesions whose growth or size is dependent on oestrogens (Robbins *et al*, 1989). They rarely arise following the menopause and castration makes them atrophy premenopausally. An important observation has been that during pregnancy leiomyomas frequently undergo a rapid increase in size as a result of considerable cellular hypertrophy and cellular proliferation accompanied by some variability in nuclear and cell size as well as some mitotic figures (Robbins *et al*, 1989). This evidence is a basis for the hypothesis that leiomyomas are caused by excessive oestrogenic stimulation. However, Robbins *et al* (1989), state that experimental proof in animals is lacking and that there is no evidence that oestrogen initiates their formation or does more than maintain their size.

1.9.2 Steroid hormone receptors.

Wilson *et al* (1980) state that although the growth of leiomyomas is usually autonomous, its response to the steroid milieu suggests that these neoplasms have oestrogen receptors similar to normal tissues. Farber *et al* (1972) found that leiomyomas bind approximately 20% more oestradiol per milligram of cytoplasmic protein than does normal myometrium of the same organ which supports the observation that certain leiomyomas are oestrogen-sensitive neoplasms. Soules and McCarty (1982) found that myometrium and leiomyomas from the same patient each appeared to undergo similar changes throughout the menstrual cycle, both in quantity and pattern for oestrogen and progesterone steroid receptors. This study also found that the highest levels of these receptors occurred at midcycle, the exception being progesterone receptor in myometrium. The implications of these findings to this present study include the expectation that the myometrial and leiomyoma cells measured should be larger during midcycle as a result of the influence that oestrogen has on the growth of these cells. Soules and McCarty (1982) concluded that leiomyomas behave as normal uterine tissue in regard to steroid receptors.

A study by Chrapusta *et al* (1990) opposes the hypothesis that leiomyomas have oestrogen levels similar to normal uterine myometrium. This investigation takes the view that differences between the pattern of changes of oestrogen receptor and progestin receptor levels in leiomyomas and their parental myometria may result from a tumour-related modification of the local hormonal environment, since leiomyomas can synthesize oestrogens by the aromatization of circulating androgens. This study found that aromatase activity was much higher in leiomyomas than in their parental myometria in both follicular and luteal phases of the menstrual cycle (Chrapusta *et al.*, 1990). The purpose of this study was to compare the

pattern of changes of the oestrogen receptor and progestin receptor levels in leiomyomas of different histological types and in their parental myometria. The authors conclude that histological heterogeneity of leiomyomas may contribute to the discrepancies between earlier reports on the oestrogen receptor and progestin receptor regulation in the tumours and to the non-uniformity of the results of hormonal treatment of the tumours.

Further evidence supporting the link between steroid hormones and leiomyomas is given by Ross *et al* (1986) who investigated risk factors for leiomyomas and established that risk was consistently decreased with increased number of term pregnancies and also decreased with increased duration of oral contraceptive use. This study also confirmed that menopause was associated with an appreciably reduced risk of leiomyomas. Ross *et al* (1986) conclude that these have previously been identified as risk factors for endometrial carcinoma which strongly suggests that the underlying risk factor is unopposed oestrogen.

1.9.3 The role of progesterone in the growth of leiomyomas.

Although it is generally accepted that leiomyomas grow under the influence of oestrogen, a study on mitotic activity in these tumours during the menstrual cycle by Kawaguchi *et al* (1989) found that increased mitotic activity under the hormonal milieu of the secretory phase suggests that growth may also be affected by progesterone. Another study on mitotic activity (Tiltman, 1985) found that patients using a progestin only oral contraceptive preparation have significantly higher mitotic activity in leiomyomas than patients using either a combined oestrogen/progesterone oral contraceptive or patients who had never used any exogenous hormone.

Contrary to these findings a review article by Buttram and Reiter (1981) reports that several studies have suggested that progesterone may inhibit growth of leiomyomas. Pollow *et al* (1978) reported that the concentration of progesterone receptors in leiomyomas was significantly lower than in the surrounding myometrium.

1.9.4 The growth potential of leiomyomas.

The growth potential of leiomyomas is extremely variable even when multiple within a single organ. The biological basis for these variables has not been established (Cramer *et al*, 1985). This study observed that heterogeneity in the host hormonal milieu and in the ability of uterine leiomyomas to respond to various hormones may be important factors contributing to the wide variation in their growth potential. They were unable to confirm whether uterine and fibroid enlargement could be ascribed to hypertrophy or hyperplasia; a question which will be addressed by this present study. Cramer *et al* (1985) concluded, after repeated demonstration, that since human myometrial cells proliferate in culture they may also do so *in vivo*.

Additional factors have been implicated in the production and growth potential of leiomyomas; Buttram and Reiter (1981) reported that factors responsible for the initial neoplastic transformation are unknown but that two factors, growth hormone and progesterone, in addition to oestrogen may influence the rate of growth of these tumours. Their review article stated that Pollow *et al* (1978) demonstrated a significantly lower conversion of oestradiol into oestrone in leiomyomas than in myometrium which suggests that increases in local oestradiol concentrations may play a role in the

pathogenesis of leiomyomas. Grattarola and Li (1959) demonstrated that growth hormone is synergistic with oestradiol in the induction of increases in uterine weight. This leads to speculation that the high incidence of growth of leiomyomas during pregnancy may be caused by the synergistic effect of oestradiol and hPL rather than by oestradiol alone (Buttram and Reiter, 1981).

1.9.5 The treatment of leiomyomas.

In the face of the evidence that leiomyomas are oestrogen and/or progesterone sensitive, steroid depletion has been used as a method to treat these tumours. This is accomplished by using agonistic analogues of gonadotrophin-releasing hormone (GnRH) which, after a transient stimulation of steroid production, are capable of lowering oestrogen levels to the post-menopausal range (Andreyko *et al.*, 1988).

Regression of uterine leiomyomas has been observed after treatment with progestins (Goldzieher *et al.*, 1966), antiprogestin (Coutinho *et al.*, 1986) and GnRH antagonists (Andreyko *et al.*, 1988). In 1992 Coddington *et al.*, conducted a study to determine if a two month course of leuprolide acetate would be sufficient to decrease the size of leiomyomas as an adjunct to surgical treatment. Previous studies had shown that gonadotrophin releasing hormone analogues (GnRHa) caused a reduction in size of these tumours after three to six months of therapy. Traditional therapy had been limited to medical therapy with progestins as well as surgery. More recently GnRHa's have been used to induce hypoestrogenic states and reduce leiomyomas (Friedman *et al.*, 1991). The size of leiomyomas was noted to decrease by 50% of the volume during a six month course of therapy, presumed to be a result of the hypoestrogenic state. Although it was hoped that the reduction in size would be permanent, this did not occur as the

tumours increased in size shortly after discontinuing the medication. Coddington *et al.* (1992) also measured the changes in size of the uterus using ultrasound during treatment with GnRHa and found that the volume decreased significantly by 35% after one month and by 44% after completion of therapy. This study also noted changes in gonadotrophin levels; LH levels were suppressed after one month of therapy and remained suppressed during the pre-operative period while FSH levels were only minimally affected. These effects of GnRHa on the size of the uterus and leiomyomas underlines the importance of correlating serum hormone levels with morphometry data in this present study. RU 486, a synthetic steroid with antiprogesterone and antiglucocorticoid activities, has also been found to inhibit the growth of uterine leiomyomas (Murphy *et al.*, 1993) and 80% of patients in the study had a 25% or greater reduction in leiomyomata volume after three months of treatment.

Chapter 2

Materials and Methods

2.1 Introduction

A preliminary investigation was undertaken to establish the optimal methodology for a morphometric study on myometrial and leiomyoma nuclei in the uterus. Once this had been established the main study was undertaken using this methodology.

2.2 Preliminary study

2.2.1 Introduction to preliminary study

The aims of this initial investigation were:

1. To test five histological staining methods to ascertain the best method for a morphometric study on uterine cells.
2. To test the reproducibility of measurements of myometrial and leiomyoma nuclei in transverse and cross section.
3. To find the optimal axial ratio criterion for measuring cross-sectioned nuclei.
4. To find the minumum sample size of nuclei per section of myometrium or leiomyoma in order to yield statistically significant results.

2.2.2 Methodology for preliminary study

Six uteri were collected from hysterectomy procedures. These uteri were all from pre-menopausal women in the proliferative phase of the menstrual cycle with at least one accompanying leiomyoma. The fresh specimens were

dissected in the sagittal plane and two blocks of tissue were taken, one from the myometrium in the fundus and one from a leiomyoma in each uterus (Table 2.1).

Case	Age	Endometrium	Leiomyomas	Reproductive history
1	30	weakly proliferative	multiple	G0 P0
2	35	weakly proliferative	multiple	G1 P1
3	35	proliferative	multiple	G2 P2
4	37	proliferative	single	G2 P2
5	38	weakly proliferative	multiple	G2 P2
6	39	proliferative	multiple	G0 P0

Table 2.1: Patients for preliminary investigation

Note: e.g. G2 P1 - gravid 2, para 1

The blocks were all fixed in a 10% formal saline solution and processed in a vacuum impregnation processor. They were embedded in paraffin wax and 2 to 4µm sections were cut.

Nuclei in cross and transverse section were measured on sections stained with each of the five stains under investigation. A transversely sectioned nucleus was defined as having a shortest to longest axial ratio of less than 0,3 and a cross sectioned nucleus as having a ratio of more than 0,6.

A progressive mean graph as well as frequency histograms were drawn to ascertain the smallest sample size of nuclei necessary to obtain statistically significant results.

2.2.3 Staining techniques for preliminary study

All of the sections were stained with each of the five stains under investigation.

The stains tested were Haematoxylin and Eosin, Van Gieson, Giemsa, Masson's Trichrome and Mallory's Phosphotungstic Acid Haematoxylin. These stains were assessed according to the following criteria:

1. clarity of nuclear boundaries
2. nuclear/cytoplasmic contrast
3. reproducibility of measurements
4. reproducibility of staining results

The following staining methods were used:

2.2.3.1 HAEMATOXYLIN AND EOSIN (Bancroft & Cook, 1984) -Univerisity of Cape Town/Groote Schuur Hospital modification.

a. Method.

The slides were stained using an automatic staining machine.

I. The fixed sections were hydrated through graded alcohols to water,
II. stood three minutes in Mayer's haematoxylin (reagent i),
III. rinsed in water,
IV. stood 25 seconds in Scott's tap water solution (reagent iii),
V. rinsed in water,
VI. stood 50 seconds in the eosin/phloxine mixture (reagent ii) and finally
VII. dehydrated through graded alcohols, cleared in xylol and mounted.

b. Reagents

i. Mayer's Haematoxylin solution.

Haematoxylin	1g
Distilled water	1000ml
Potassium alum	50g
Sodium iodate	0,2g
Citric acid	1g
Chloral hydrate	50g

The haematoxylin, alum and sodium iodate were disolved in distilled water and left to stand overnight at room temperature. The chloral hydrate and citric acid were then added to the mixture which was boiled for five minutes, cooled and filtered.

ii. Eosin/Phloxine solution.

Two parts 1% eosin (yellowish) in distilled water.

One part 1% phloxine in distilled water.

The equal parts of the above were left to stand overnight.

iii. Scott's tap water solution.

Sodium bicarbonate	3,5g
Magnesium sulphate	20g
tap water	1000ml

The salts were dissolved separately and then mixed.

2.2.3.2 VAN GIESON (Bancroft & Stevens, 1982).

I. **Method**.

II. The fixed sections were taken to water.

III. They were stained with Weigert's iron haematoxylin (reagent i) for 20 minutes,

IV. rinsed in water,

V. stained in Van Gieson's stain (reagent ii) for 3 minutes,

VI. blotted and rapidly dehydrated in absolute alcohol, and

VII. cleared in xylol and mounted.

b. Reagents.

i.Weigert's Iron Haematoxylin (Weigert, 1904).

Solution A:

Haematoxylin	1g
Absolute alcohol	100ml

This was dissolved with gentle heat.

Solution B:

30% aqueous ferric chloride	4ml
Concentrated hydrochloric acid	1ml
Distilled water	100ml

Equal volumes of solutions A and B were mixed together just before use.

ii. Van Gieson's stain.

Saturated aqueous picric acid

1% aqueous acid fuchsin

Equal volumes were boiled for three minutes, cooled and filtered.

2.2.3.3 GIEMSA (Bancroft & Cook, 1984).

a. Method.

I. Fixed sections were taken to distilled water, and

II. stained with Giemsa (Merck) diluted 2 in 3 with distilled water.

III. Differentiation in 0,2% acetic acid was checked microscopically before

IV. Dehydrating rapidly through graded alcohols, clearing in xylol and mounting.

2.2.3.4 MASSON'S TRICHROME (Bancroft & Stevens, 1982).

a. Method.

I. i Fixed sections were taken to water.

II. The nuclei were stained with Wiegert's iron haematoxylin (2.2.2, reagent i) for 15 minutes,

III. blued in running water for 10 minutes,

IV. stained with Ponceau's Acid Fuchsin (equal volumes of 0,5% Ponceau 2R in 1% acetic acid and 0,5% acid fuchsin in 1% acetic acid),

V. rinsed in running water,

VI. mordanted and differentiated in 1% aqueous phosphomolybdic acid until collagen was decolourized and muscle, red blood cells and fibrin remained red. This was checked microscopically.

VII. The slides were drained and counterstained with 2% light green in 2% acetic acid for 8 minutes,

VIII. rinsed, dehydrated through graded alcohols, cleared in xylol and mounted.

2.2.3.5 PHOSPHOTUNGSTIC ACID HAEMATOXYLIN (PTAH) (Culling et al., 1985): University of Cape Town/Groote Schuur Hospital modification.

a. Method.

I. Fixed sections were taken to water,

II. placed in 4% iron alum for 30 minutes,

III. rinsed in distilled water,

IV. placed in PTAH solution (reagent i) overnight - 12 to 16 hours, and

V. dehydrated rapidly through graded alcohols, cleared in xylol and mounted.

b. Reagents.

i. PTAH solution.

Haematoxylin	0,1g
Phosphotungstic acid	2g
Distilled water	100ml
1.79% potassium permanganate	1ml

The haematein was dissolved in half of the distilled water and the phosphotungstic acid in the other half and mixed. The mixture was allowed to "ripen" for 24 hours before use.

2.2.4 Instrumentation

The nuclear areas of the myometrial and leiomyoma nuclei were measured using the VIDS III morphometry computer system in both the preliminary and main investigations. This system comprises a monitor and graphics tablet which allows the structure under investigation to be traced and which yields

16 measurement parameters per nucleus (Analytical Measuring Systems, VIDS III manual). Of these the following were used for this study: longest and shortest dimensions, axial ratio, and area measurement. VIDS III is a high resolution semi-automatic image analysis system utilising a high performance television camera to provide direct imaging of microscopic sections. The system comprises an IBM compatible computer, a colour monitor, a digitising tablet with four button cursor, video camera, printer and VIDS III software disk.

Figure 2.1: VIDS III semi-automatic morphometry system.

2.2.5 Results of preliminary study

A combination of statistical analyses and subjective assessments of staining methods was used to determine the best methodology for the study in the preliminary investigation.

2.2.5.1 Statistical analyses of staining methods for preliminary study

Statistical tests were used in the preliminary study to answer the following questions:

1. Which histological staining method gave the most reproducible measurements?
2. Was it better to measure myometrial nuclei in cross or transverse section? i.e. which method gave the most reproducible results.
3. Which axial ratio was best for area measurements of cross-sectioned nuclei?
4. What was the minimum sample size of nuclei per section of normal myometrium or leiomyoma necessary to yield statistically significant results?

2.2.5.2 Reproducibility of measurements: staining methods

Analyses of measurements of nuclei from the same uterus, stained by the five stains under investigation demonstrated no significant variation (p value > 0,05) between four sets of repeated measurements between measurements on Masson's Trichrome and Phosphotungstic Acid Haematoxylin on transversely sectioned nuclei in leiomyoma sections. Reproducibility was also demonstrated between measurements performed on Van Gieson stained sections of longitudinally sectioned myometrial nuclei. (Table 2.2)

	LONGITUDINAL NUCLEI (p values)	
Stain	**Normal myometrium**	**Leiomyoma**
H+E	0.0088	0.0429
Giemsa	0.0029	0.2776
Masson's Trichrome	0.0000	0.9820
PTAH	0.0000	0.0502
Van Gieson	0.0000	0.0000
	TRANSVERSE NUCLEI (p values)	
H+E	0.0000	0.0000
Giemsa	0.0170	0.3325
Masson's Trichrome	0.0000	0.0199
PTAH	0.0000	0.0087
Van Gieson	0.1828	0.0000

Table 2.2: One way ANOVA analyses: Significance values. A *p* value of more than 0.05 was significant at a 95% confidence level.

2.2.5.3 Reproducibility of measurements: cross vs transversely sectioned nuclei.

The measurements of transversely sectioned nuclei from normal myometrium were found to be reproducible across all stains (Figure 2.2). Measurements of longitudinally sectioned nuclei from leiomyomas were not reproducible with Giemsa, Masson's Trichrome and Phosphotungstic Acid Haematoxlylin stains. Only measurements on H+E stains demonstrated reproducibility (Table 2.2).

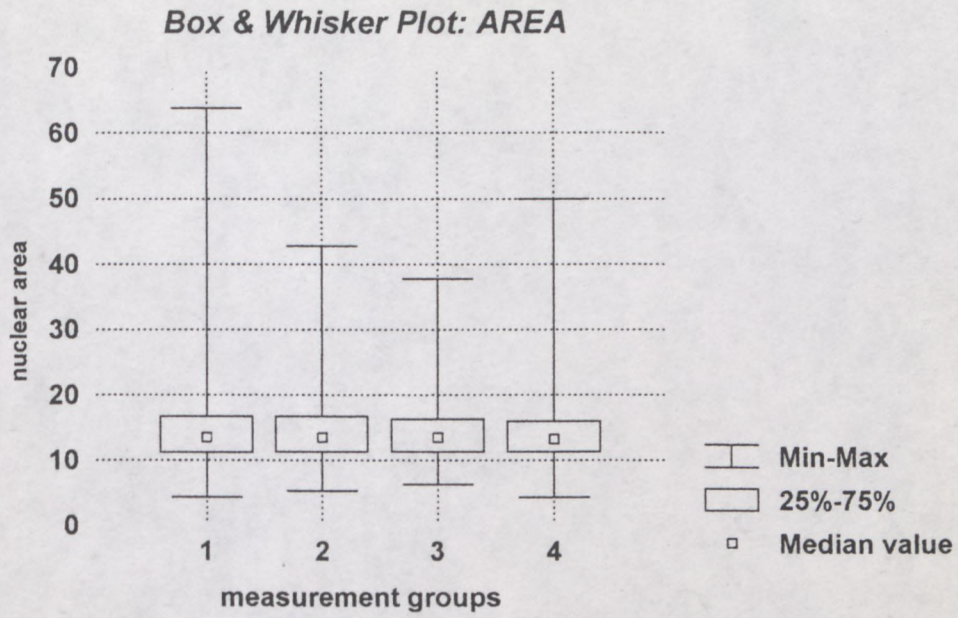

Figure 2.2: Box and Whisker plot of areas of transversely sectioned nuclei measured on an H+E section. The measurement groups represent four different sets of measurements of transversely sectioned nuclei. 100 nuclei were measured in each group.

2.2.5.4 Axial ratio:

A frequency histogram of all cross-sectioned nuclei in the preliminary study was drawn to include all nuclei with an axial ratio greater than 0,6 (Figure 2.3). This histogram demonstrated a normal distribution.

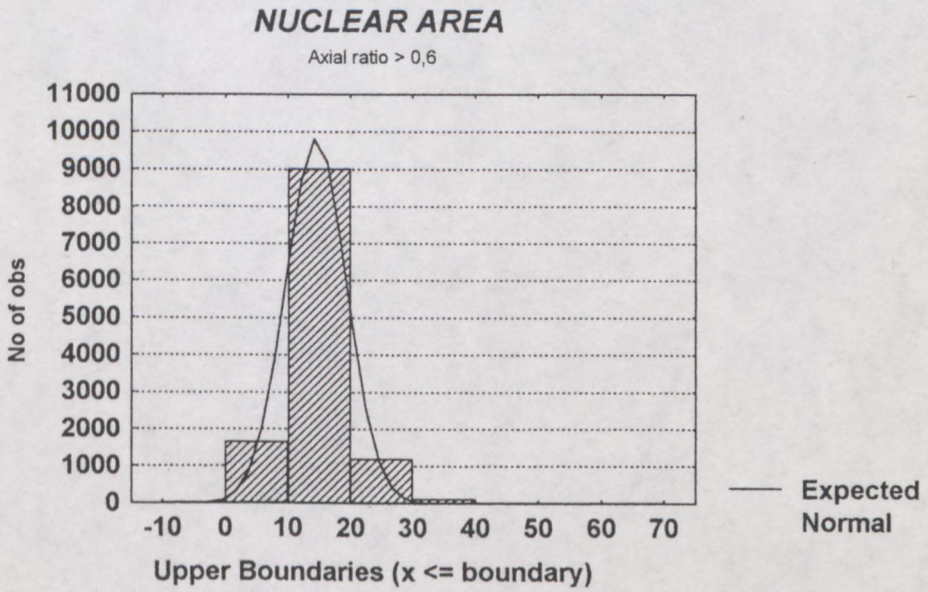

Figure 2.3: Frequency histogram of cross-sectioned myometrial nuclei: Axial ratio > 0,6

It was found that on changing the axial ratio criterion of the transversely sectioned nuclei to exclude all nuclei with an axial ratio of less than 0,9, the standard deviation and co-efficient of variation became considerably less (Table 2.3). The frequency histogram of this data illustrates a normal distribution (Figure 2.4).

	Axial Ratio		
	0,6	**0,9**	**0,95**
Mean	15,44	15,57	15,6
SD	5,53	5,06	5,05
CV	35,8	32,52	32,8

Table 2.3: Cross-sectional nuclei: Axial ratio parameters

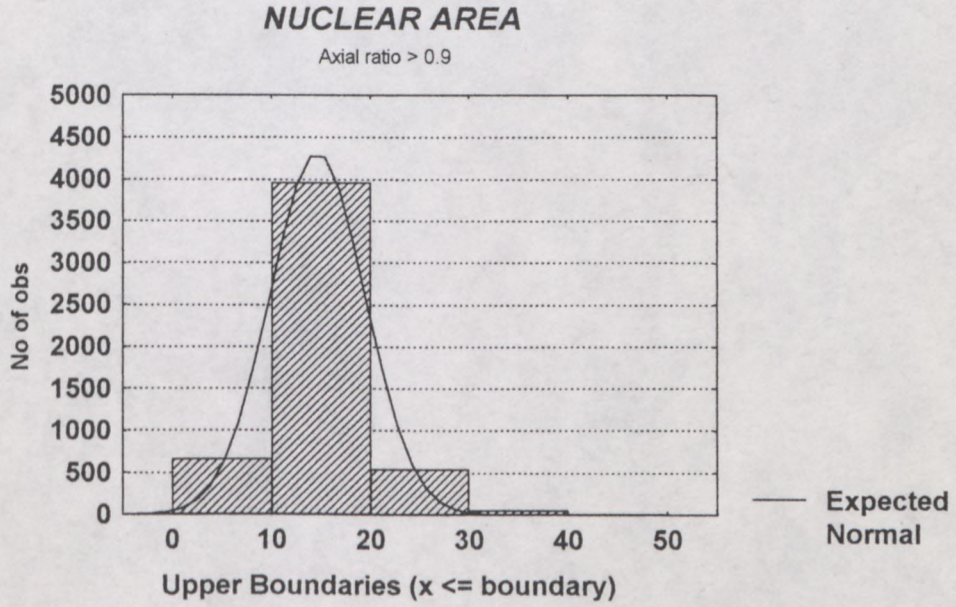

Figure 2.4: Frequency histogram of cross-sectioned myometrial nuclei: Axial ratio >0,9

When all nuclei with an axial ratio of less than 0,95 were excluded there was no significant change in these two parameters. A frequency histogram of this data was also drawn (Figure 2.5).

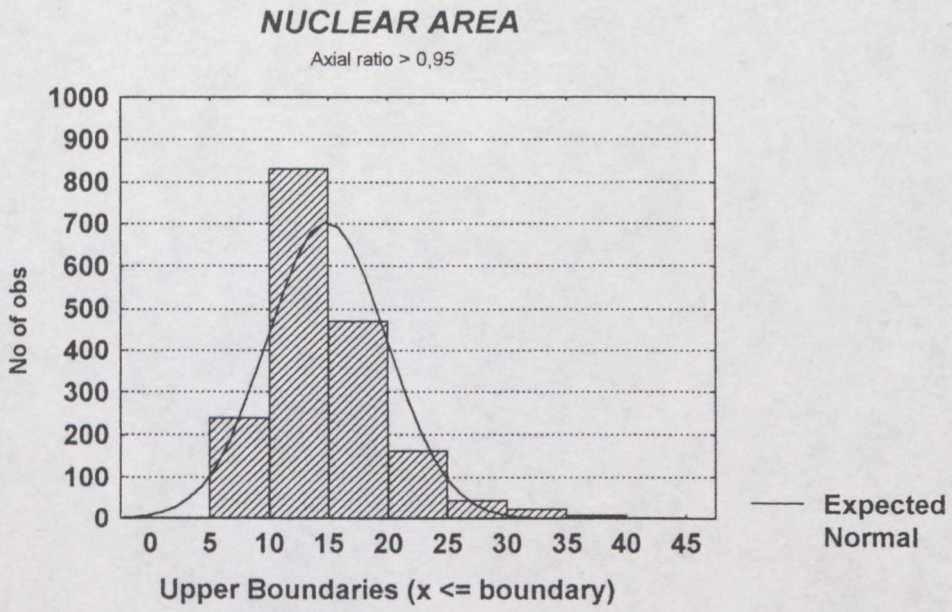

Figure 2.5: Frequency histogram of cross-sectioned myometrial nuclei: Axial ratio > 0,95.

2.2.5.5 Sample size

A progressive mean graph provides a graphical running measure of the precision of the mean value during a count (Aherne & Dunnill, 1982) and was used to assess optimal sample size. It was constructed by calculating the cumulative mean value after every 10 measurements of nuclear area. It was found that over the first 50 measurements the mean oscillated considerably but after a number of observations it became confined to narrow limits. This steady state was achieved at approximately 100 measurements and it was found that continued measurements did not alter the mean significantly. (Figures 2.6 and 2.7).

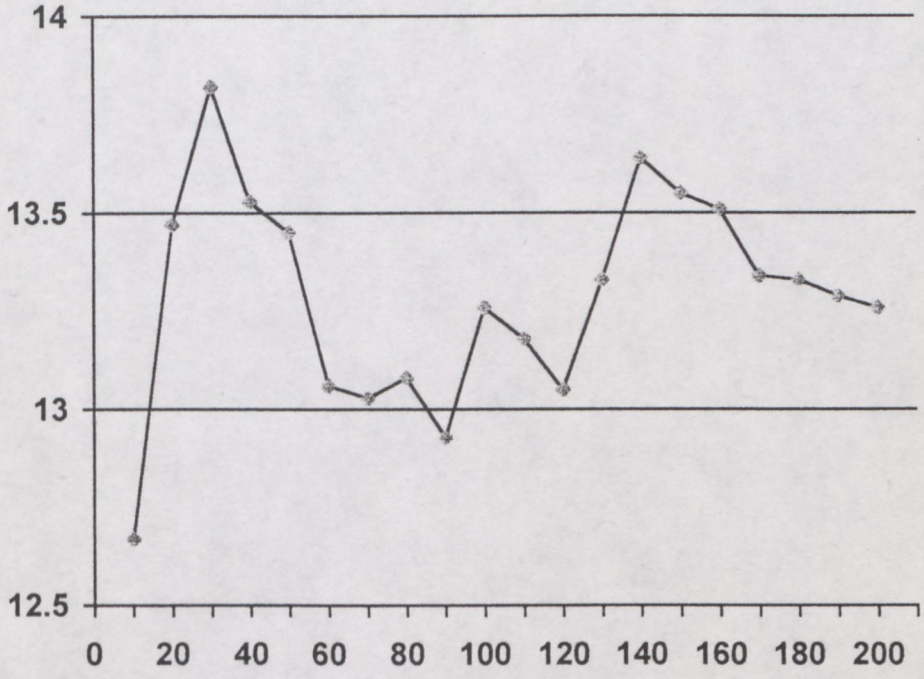

Figure 2.6: Progressive mean graph: cross-sectioned myometrial nuclei

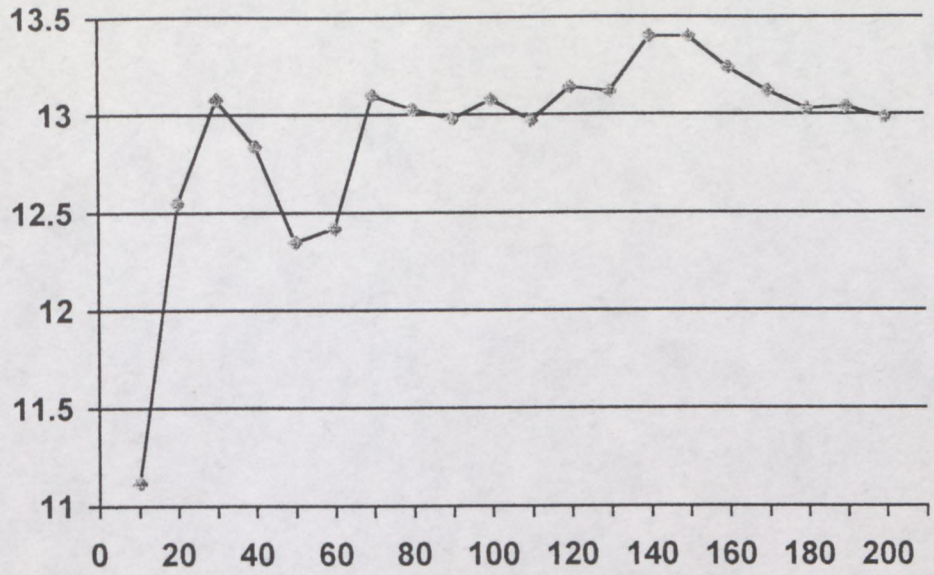

Figure 2.7: Progressive mean graph: cross-sectioned leiomyoma nuclei.

Analysis of the standard error of the different groups of sample size showed that the standard error decreased considerably from 50 to 100 measurements per section and thereafter the decrease was much smaller (Table 2.4)

Sample size	Standard error
50	0,51
100	0,36
150	0,3
200	0,25

Table 2.4: Relationship between sample size and standard error of cross-sectioned nuclei in normal myometrium.

2.2.5.6 Staining: Subjective assessment

A subjective assessments of the stains was made on the image of the section translated to the monitor used for measuring the nuclei. The stains were assessed using a 100x oil immersion objective. Each group of sections was stained with the same solutions to ensure reproducibility of staining results.

Subjective assessments were made of:

1. the clarity of nuclear boundaries
2. nuclear/cytoplasmic contrast
3. reproducibility of staining results,

2.2.5.6.1 Haematoxylin and Eosin

The Haematoxylin and Eosin stained myometrium image on the VIDS III monitor demonstrated clear nuclear membranes. The blue colour of the nuclei contrasted well with the pink-staining cytoplasm of the cells. These sections were stained on an automatic staining machine and thus resulted in uniform staining across all the sections (Figures 2.8 and 2.9).

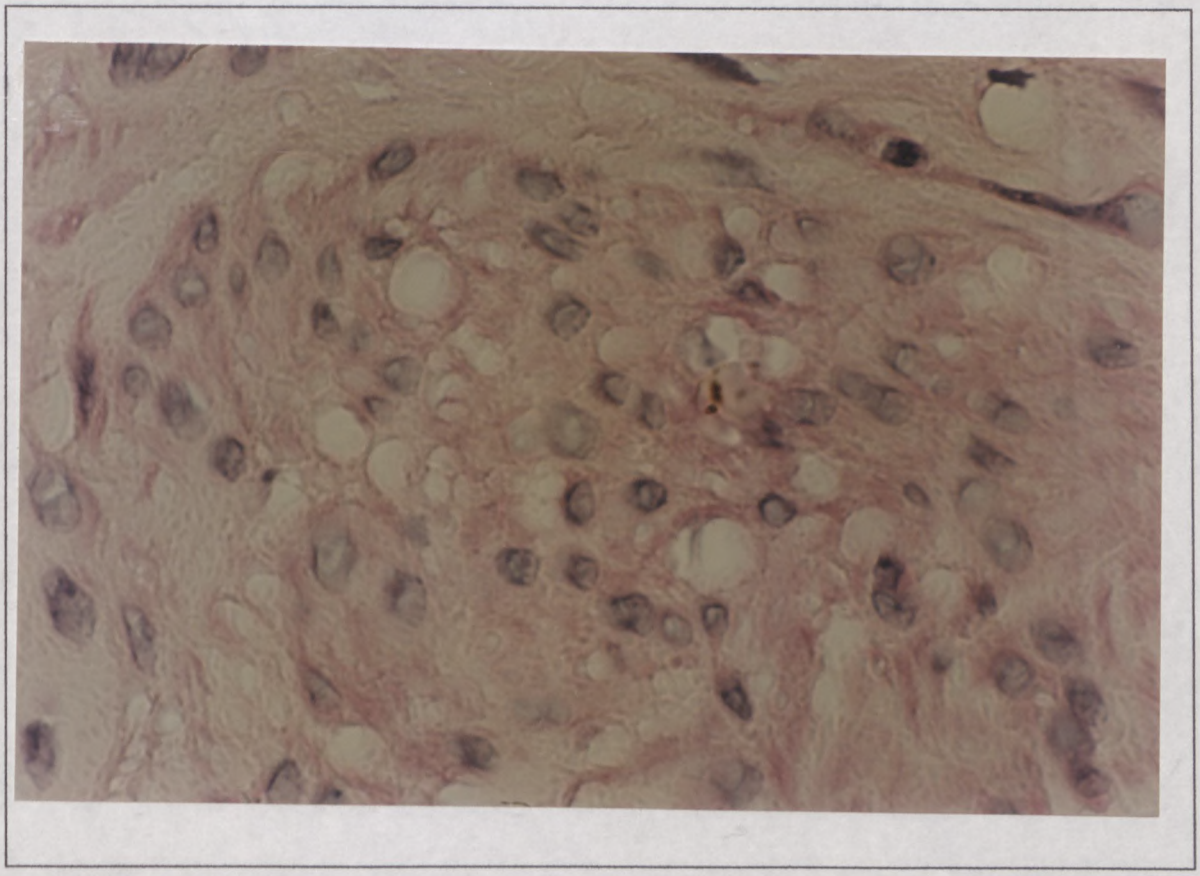

Figure 2.8: Haematoxylin and Eosin: Cross-sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

Figure 2.9: Haematoxylin and Eosin: Transversely sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

2.2.5.6.2 Van Gieson.

The iron haematoxylin used in the Van Gieson stain demonstrated the nuclear boundaries very well. Although they appeared pale under the microscope lens the image transmitted to the monitor was clear and demonstrated well demarcated nuclear boundaries. The yellow/brown cytoplasmic staining of the myocytes was pale enough to produce contrast. Reproducibility of the quality of staining was good across all sections (Figures 2.10 and 2.11).

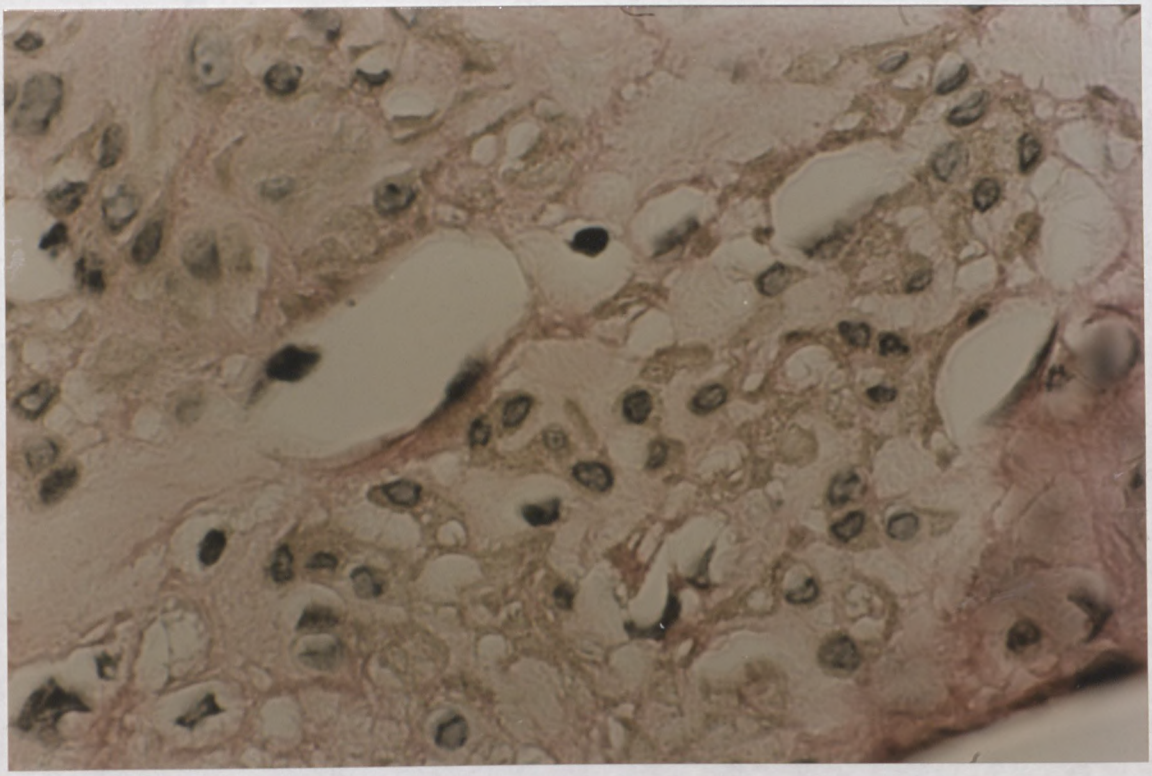

Figure 2.10: Van Gieson: Cross-sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

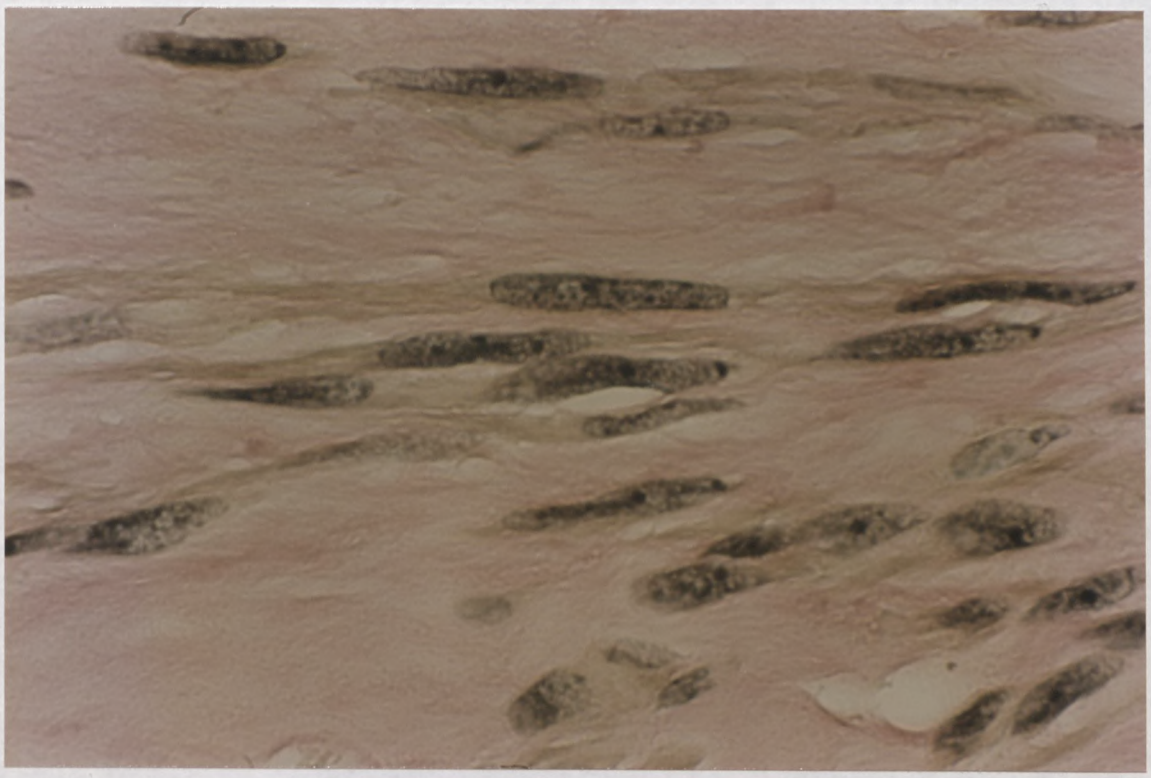

Figure 2.11: Van Gieson: Transversely myometrial nuclei. Magnification: 100x oil immersion objective.

2.2.5.6.3 Giemsa

Nuclear boundaries were well demonstrated with this stain and the pale pink staining cytoplasm of the myometrial cells provided excellent contrast to the dark blue/purple nuclei. It was difficult to produce uniform staining across all sections because each slide had to be differentiated separately (Figures 2.12 and 2.13).

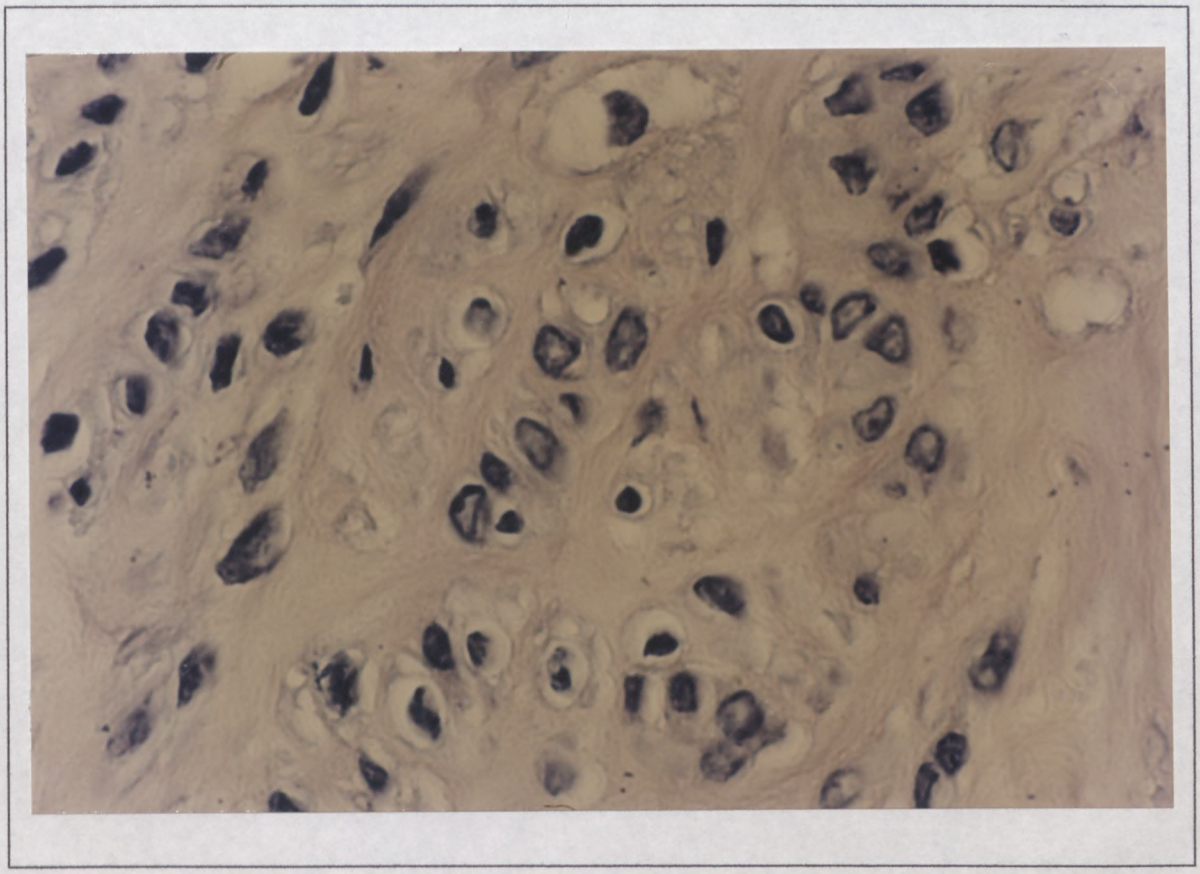

Figure 2.12: Giemsa: Cross-sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

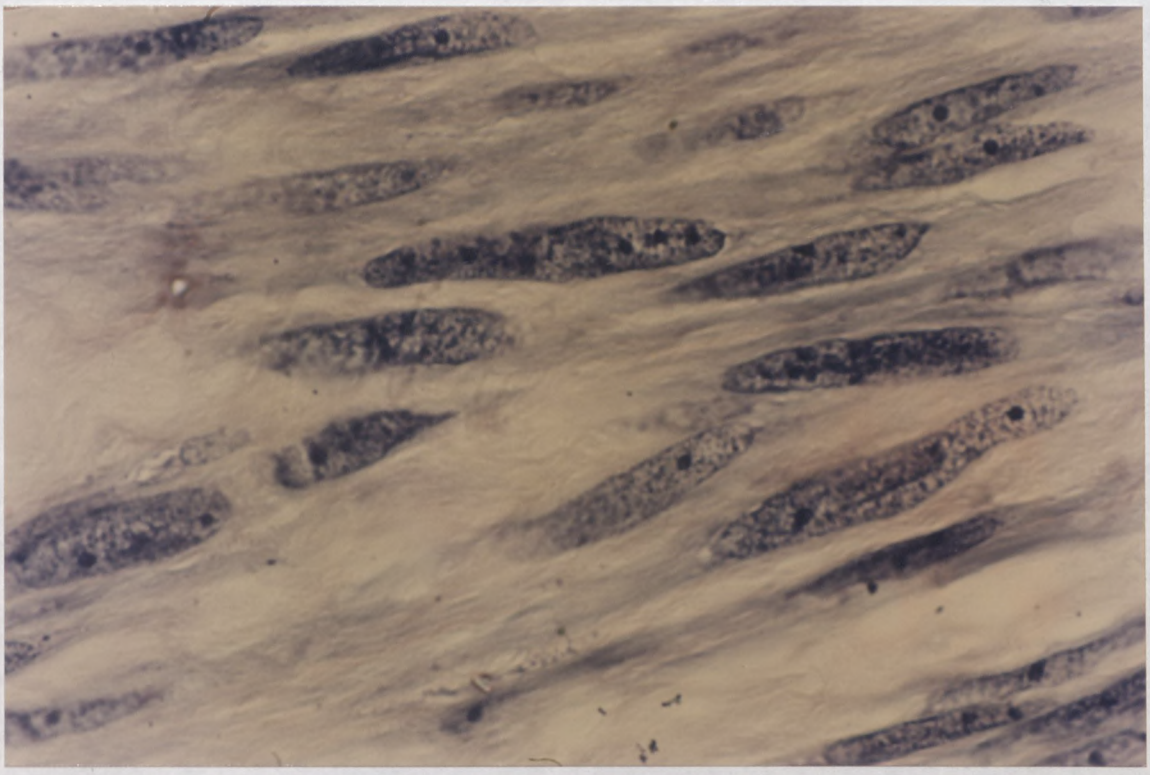

Figure 2.13: Giemsa: Transversely sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

2.2.5.6.4 Masson's Trichrome

The Weigert's iron haematoxylin used for this staining method produced very clear nuclear boundaries, an open chromatin pattern and clearly seen nucleoli. The contrast between the blue/black nuclei and the red cytoplasm of the myocytes was not good and it was difficult to distinguish nuclear borders against this background. The reproducibility of this stain was good across all sections (Figures 2.14 and 2.15).

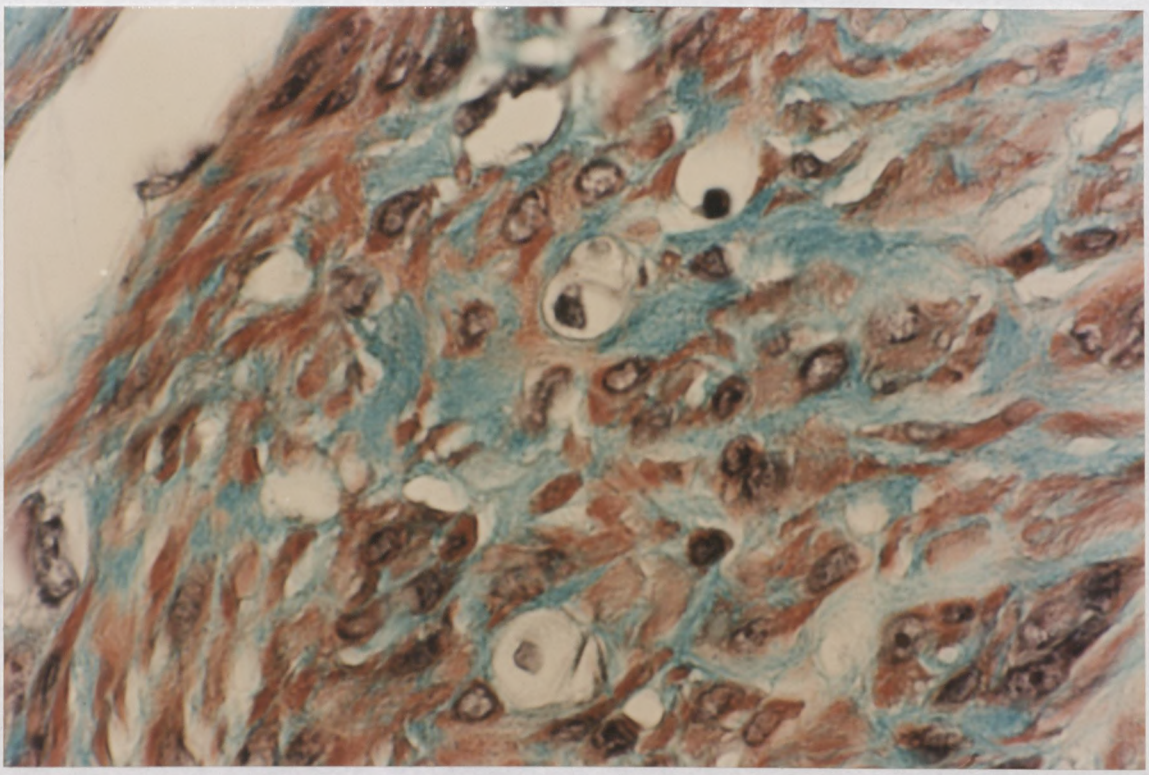

Figure 2.14: Masson's Trichrome: Cross-sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

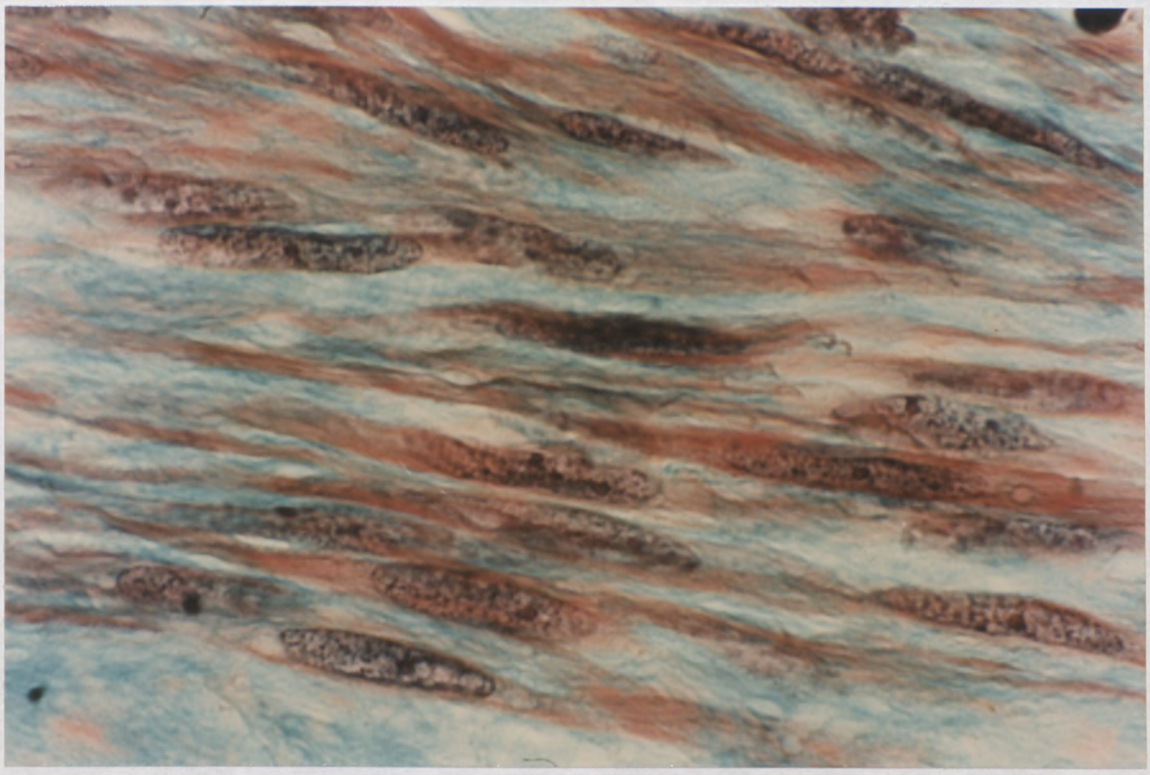

Figure 2.15: Masson's Trichrome: Transversely sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

2.2.5.6.5 Phosphotungstic Acid Haematoxylin

An open chromatin pattern and nucleoli were well demonstrated in most of the nuclei stained with this method. Nuclear boundaries however were difficult to discern owing to the stain's affinity for myofilaments in the cytoplasm of the myometrial cells. This gave many of the nuclei the appearance of being attenuated at the ends *i.e.* tapering off into fine filaments and making it extremely difficult to distinguish a boundary to measure around. Nuclear/cytoplasmic contrast was not good owing to this problem. Although the red/orange staining cytoplasm afforded good contrast with the purple nuclei the myofilamentous material surrounding the nuclei

severely impaired the contrast. The staining of these sections was well reproduced (Figures 2.16 and 2.17).

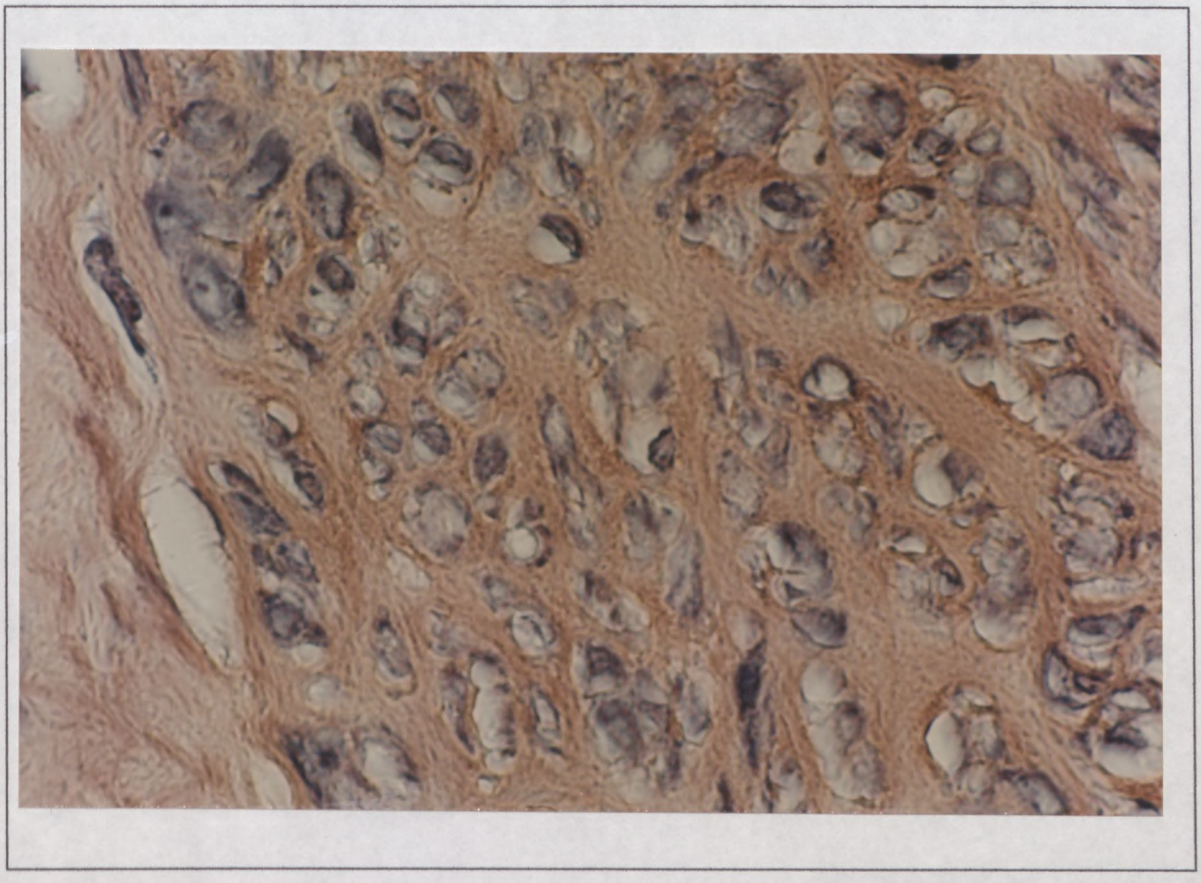

Figure 2.16: PTAH: Cross-sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

Figure 2.17: PTAH: Transversely sectioned myometrial nuclei. Magnification: 100x oil immersion objective.

2.2.6 Discussion: Preliminary study

When conducting a study of the area measurements of myometrial and leiomyoma nuclei to determine whether their size is affected by steroid hormones, should the area measured be the transverse or longitudinal cross section of these nuclei?

2.2.6.1 The three dimensional shape of myometrial nuclei

When measuring tissue sections the problems of dimension reduction must be taken into account as observations about three dimensional changes in tissues are interpretations made from two dimensional sections (Baak &

Oort, 1983). The general problem of size determination has theoretical and practical difficulties since it is necessary to deduce size from a study of transections which may be subject to ambiguities. The difficulties may be mitigated by making simplifying assumptions about the shape of the object at the cost of introducing bias.

In order to understand the measurements of uterine myometrial and leiomyoma cells it is necessary to consider their three dimensional shape. Most of our knowledge of these cells is gained from two dimensional sections.

From these it is possible to say that they are elongated spindles with sharply pointed ends. The thickest part of each fibre lies about the middle of its length and is occupied by a single elongated nucleus (Bourne, 1960). Ultrastructurally, using transmission electron microscopy the fibres are seen as bundles of spindle-shaped cells with fusiform, blunt-ended nuclei. On cross section they appear as large polygonal cells with irregular cellular outlines. The neoplastic smooth muscle cells of leiomyomas are identical in architecture and organization to normal myometrial cells (Ferenczy & Richart, 1974).

2.2.6.2 Reproducibility of nuclear measurement

Reproducible measurements may be obtained from cross-sectioned nuclei since the closer they are to round the more chance there is that they were absolutely transversely cut. However, even if sectioned at exactly right angles, polyhedral nuclei (Ferenczy & Richart, 1974) might not appear round. But the strict axial ratio criterion should ensure that most of the nuclei

measured have been sectioned almost at right angles. It was, in fact, observed that at 1000x magnification many nuclei appear to be round on cross section. (Those which were not may either be cross-sectioned polyhedral nuclei, or nuclei sectioned tangentially).

If the transverse sections are measured there is no way of knowing how their angle of sectioning has deviated from 90^0 and therefore no meaningful conclusions can be made from such measurements.

2.2.7 Conclusions: Preliminary study

2.2.7.1 Staining methods

In order to assess the optimum staining method for the study statistical tests were done to ascertain reproducibility of measurement results of each stain under investigation. In addition a subjective assessment was made based on the clarity of nuclear boundaries, nuclear/cytoplasmic contrast and reproducibility of staining results.

The measurements of transversely sectioned nuclei from normal myometrium and leiomyomas were found to be reproducible on H+E, and Van Gieson stains. The measurements of cross-sectioned nuclei from normal myometrium and leiomyomas were reproducible on H+E, Masson's Trichrome and PTAH (Table 2.5). From these statistical

results it was concluded that the H+E stain yielded the most reproducible measurements.

Stain	Normal myometrium *p* value	Reproducible ?	Leiomyoma *p* value	Reproducible ?
H+E	0.0000	yes	0.0000	yes
Giemsa	0.0170	yes	0.3325	no
Masson's Trichrome	0.0000	yes	0.0199	yes
PTAH	0.0000	yes	0.0087	yes
Van Gieson	0.1828	no	0.0000	yes
All Stains	0.0000	yes	0.1169	no

Table 2.5: One way ANOVA analyses of cross-sectioned nuclei. A *p* value of more than 0,05 was significant at a 95% confidence level.

The subjective assessment concluded that although Van Gieson and Giemsa stains proved to facilitate nuclear measurement, the H+E stain yielded comparable results with the additional advantage that the staining results were consistently reproduced due to the utilisation of an automatic staining machine.

2.2.7.2 Cross vs transversely sectioned nuclei

Repeated measurements of nuclei demonstrated that across all the stains tested the measurements of transversely sectioned nuclei from normal

myometrium were not reproducible. The measurements of cross-sectioned nuclei however were found to be reproducible over all the individual stains except for Van Gieson as were the results of all the staining techniques on normal myometrium sections (Table 2.5). All the measurements of cross-sectioned nuclei from leiomyomas were reproducible except for those stained with Giemsa while transversely sectioned nuclear measurements from leiomyoma tissue were only reproducible on tissue stained with H+E and Van Gieson.

It was therefore concluded that for this study it was better to measure nuclei that had been cut in cross-section.

2.2.7.3 Axial ratio

It was decided at the beginning of the study to use the axial ratio of the nuclei as the criterion for "roundness" i.e. whether the nuclei had been cut in cross section. A frequency histogram drawn of all nuclei in the study with axial ratios of 0,6, 0,9 and 0,95 respectively demonstrated little difference in the normal distribution between the three populations. Analysis of the standard deviation and coefficient of variations however demonstrated that these two parameters were considerably less when nuclei with and axial ratio of less than 0,9 were excluded from statistical analyses. It was concluded that it is best to use an axial ratio of over 0,9 to determine whether or not a myometrial nucleus had been cut in cross-section.

2.2.7.4 Sample size

Progressive mean graphs of the measurements of nuclei from normal myometrium and leiomyomas demonstrated that at approximately 100 measurements per section the mean of these observations achieved a "steady state" i.e. became confined to narrow limits. It was also found that the standard error was markedly decreased from 50 to 100 measurements but thereafter the decrease was not significant enough to warrant further measurements. It was therefore decided to measure 100 nuclei per section for this study.

The standard deviation, standard error and coefficient of variation were used to analyse sample size. The standard deviation is a measure of the spread of a population (Ipsen & Feigl, 1970). The coefficient of variation is the standard deviation expressed as a percentage of the mean and so was used to compare the relative amount of variation between the populations. The standard error of the mean was also used since the precision of a mean is quantified by the standard error which depends on the standard deviation and sample size (Ipsen & Feigl, 1970). The standard error increases with increasing variability in the original population and decreases with sample size (Daly *et al.*, 1991).

In order to assess optimal sample size groups of 50, 100, 150 and 200 cross sectioned nuclei were measured on the same section. The following parameters were investigated to assess this:

1. Progressive mean graph (Aherne & Dunnill, 1982).
2. Standard error.

3. Normal distribution.

2.3 Measurement of myometrial nuclei for main study

Fields of nuclei were selected under a 40x objective. Nuclei were measured using a 100x objective under oil immersion. This microscopic image was viewed on the VIDS III monitor and the nuclei were individually outlined using the tablet and cursor. The nuclear areas were measured in micrometers, squared, and recorded as such for subsequent statistical analysis. The fields were chosen to ensure wide representation across the section, as were the nuclei, and care was taken not to choose the same field more than once.

The axial ratio (or form factor) (Crocker *et al.* 1983) of each nucleus was used to decide whether it had been cut in either transverse or longitudinal section. The axial ratio is defined as the shortest dimension over the longest dimension as seen in cross-section. The axial ratio of a perfect circle is one while a shape tending towards oval will be less than one. For this study nuclei with an axial ratio of more than 0,6 were considered to be cut in cross section while those with an axial ratio of less than 0,3 were considered to be cut in transverse section.

2.4 Statistical Analyses

The one way analysis of variance method (ANOVA) was used to test for variance between the measurements of samples of nuclei to establish whether the area measurements of either transverse or longitudinal nuclei were reproducible and which special stain yielded the most reproducible

measurements. One way ANOVA is used to test the null hypothesis that all of several population means are equal (Moore & McCabe, 1993). Large variations between measurements indicate that there is no reproducibility while no statistically significant difference demonstrates that the results are reproducible (Altman, 1991). The Statgraphics VI software was used to perform the one way ANOVA analysis and significance levels of less than 0,05 meant that the response variable differed significantly between the different groups of nuclei.

Chapter 3

Results

3.1 Introduction.

Once the preliminary study was completed the main part of the study incorporated 49 sets of nuclei from the uteri of pre-menopausal women, 20 sets from post-menopausal women and 10 sets from pregnant women for measurement. Of the pre-menopausal and post-menopausal women, 28 sets were from proliferative, 16 secretory, 6 menstrual and 19 from uteri with inactive endometriums.

In addition, repeated measurements from the preliminary study were included giving a total of 100 sets of measurements from pre-menopausal women in addition to the 20 sets of post-menopausal measurements. Of these 79 sets were from proliferative, 16 from secretory, 6 from menstrual and 19 from uteri with inactive endometriums. This resulted in the areal measurements of 13000 nuclei being included in this study.

Not all the cases measured for the main study had all four sites i.e. fundus, lower segment, small leiomyoma, large leiomyoma (larger than 3cm in greatest dimension) sampled; either the uterus had no leiomyomas or only small or only large ones. The measurements from 7 pre-menopausal and 3 post-menopausal uteri with all four sites sampled were included in the study. These measurements were included with all other measurements for statistical purposes and in addition were recorded on a separate spreadsheet to enable dependent statistical tests to be performed.

After each set of nuclei was measured using VIDS III the measurements were saved in a .DAT file. These files were then imported into Quattro Pro and saved as .WK1 files i.e. in Lotus format.

The area and axial ratio of each nucleus was preserved in Quattro Pro spreadsheets and "tagged" with the following information; case, site, endomet., meno., count, stain. It is necessary to prepare data in this way before analyzing it in a statistical package to allow the statistical package to group data appropriately for the required statistical test. "Case" referred to the case number given to uteri in the study given in consecutive order as the uteri arrived from surgery. "Site" referred to whether the nuclei were from the fundus (A), lower segment (B), small leiomyoma (FS) or large leiomyoma i.e. larger than 3cm in greatest dimension (FL). Measurements from pregnant uteri were labelled 'pg' in the site category. "Endomet." referred to the endometrial status of the uterus i.e. proliferative (prolif), secretory (sec), menstrual (mens) or inactive (inactive). "Meno." referred to the menopausal state of the patient at surgery either pre (designated 'pre') or post-menopausal (designated 'post').

This manipulation of the data in Quattro Pro resulted in two files of 8000 and 5000 rows respectively each with 8 columns of variables. It was necessary to split the data since the version of Quattro Pro used (version 4.0) only allowed 8100 rows per file. The data was then imported into Microsoft Excel (version 4) and merged. This meant that the data containing measurements and information for 13000 nuclei could be run through a statistical package using only one file. It was also advantageous to use Excel to facilitate interchange with the statistical package, Statistica, which is also a Microsoft product.

3.2 Statistical analyses of nuclear measurements.

3.2.1 Normal distributions

In order to statistically analyse the nuclear measurements for the main part of the study, it was first established whether the measurements obtained from H+E stained sections all had normal distributions. The normal distribution represents one of the empirically verified elementary "truths about the general nature of reality, " and its status can be compared to the one of fundamental laws of natural sciences. The exact shape of the normal distribution (the characteristic "bell curve") is defined by a function which has only two parameters: **mean and standard deviation.**

A characteristic property of the normal distribution is that 68% of all its observations fall within a range of ± 1 standard deviation from the mean and a range of ± 2 standard deviations includes 95% of the scores. (On line help Statistica 5). For this study it was necessary to establish that the measurements fell within a normal distribution curve to ensure that the measurements accurately represented a normal population.

All measurements of area were plotted and found to have a normal distribution (Figure 3.1). This was done by using the data file containing all the nuclear measurements and having 8 variables.

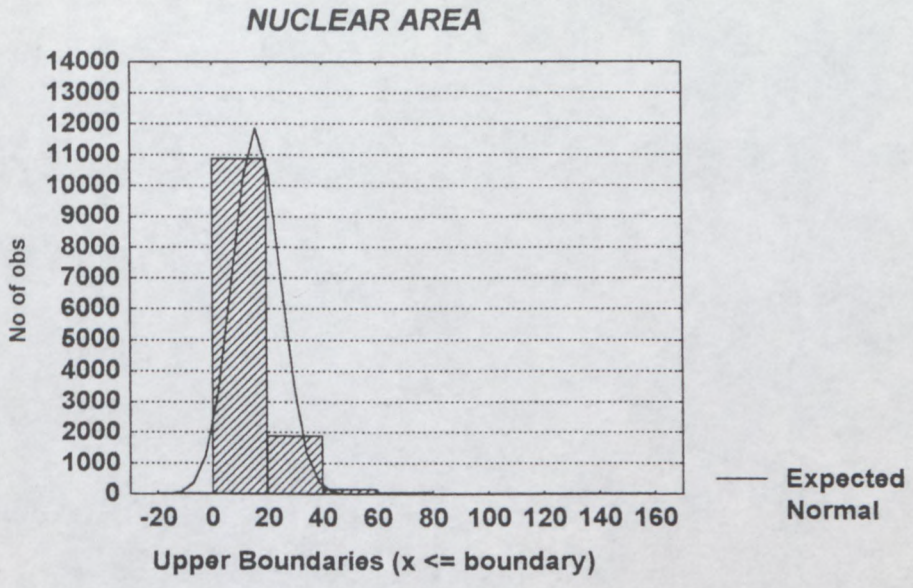

Figure 3.1: Frequency histogram to demonstrate the normal distribution of all the measurements of cross-sectioned nuclei.

Measurements of nuclear area from uteri with inactive, proliferative, secretory and menstrual endometriums were also plotted separately and found to have normal distributions (Figures 3.2 to 3.5).

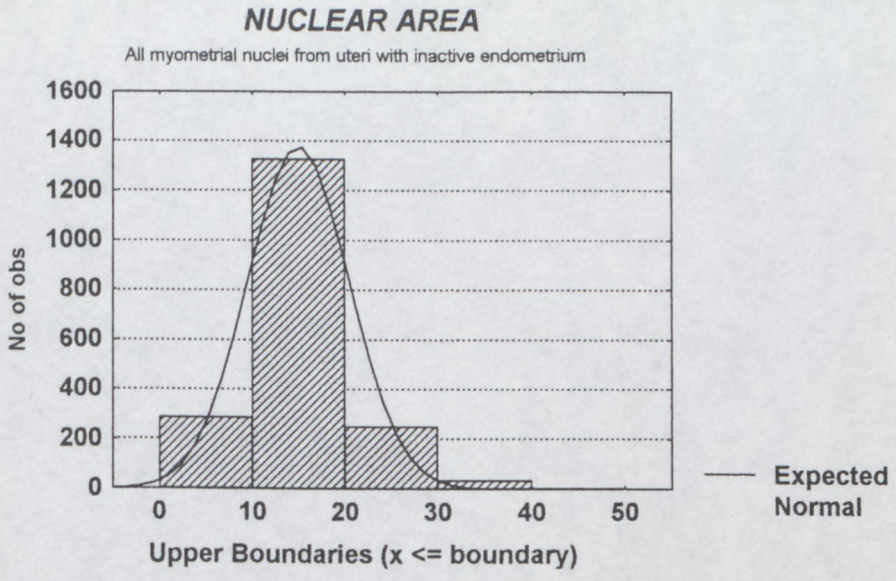

Figure 3.2: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from uteri with an inactive endometrium.

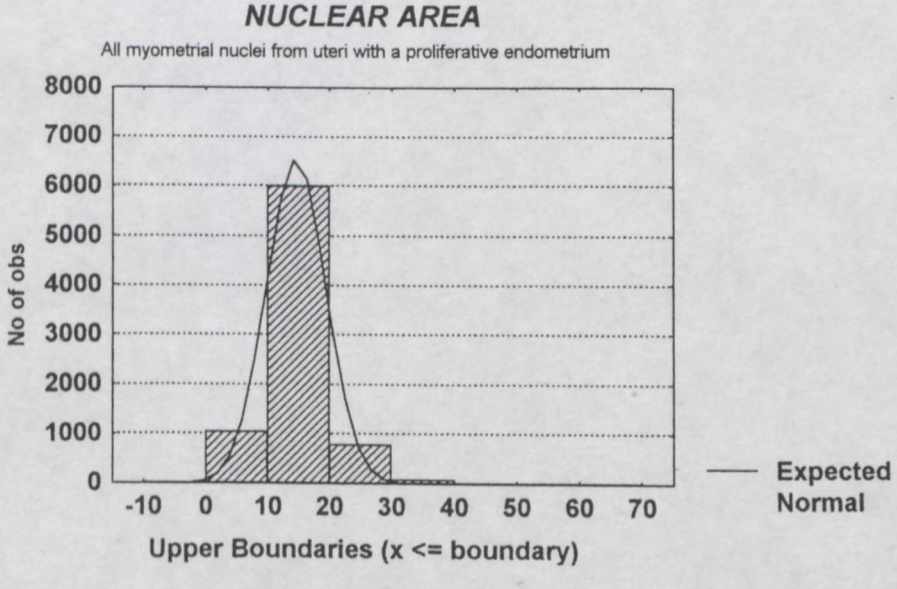

Figure 3.3: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from uteri with a proliferative endometrium.

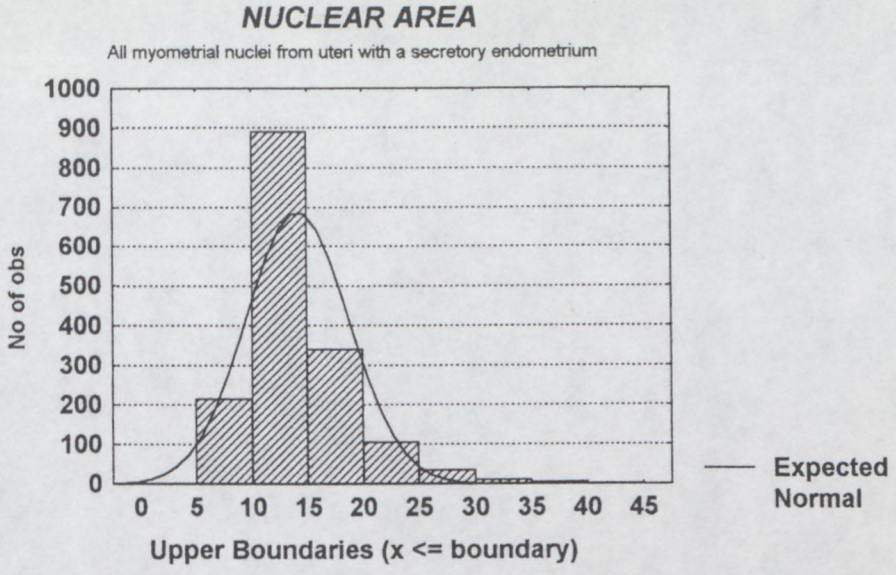

Figure 3.4: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from uteri with a secretory endometrium.

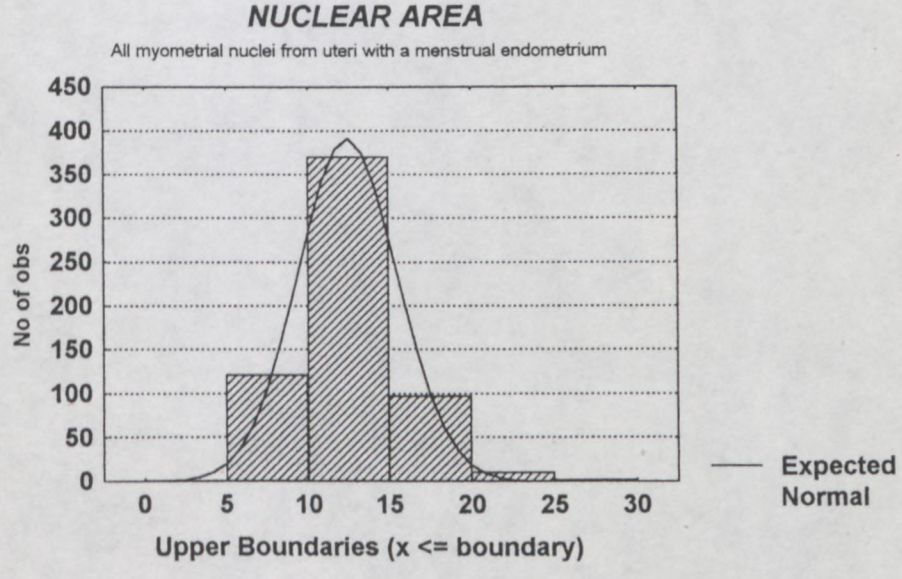

Figure 3.5: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from uteri with a menstrual endometrium.

The nuclear measurements of myometrial nuclei from pre and post-menopausal uteri were also plotted separately and also had normal distributions (Figures 3.6 and 3.7).

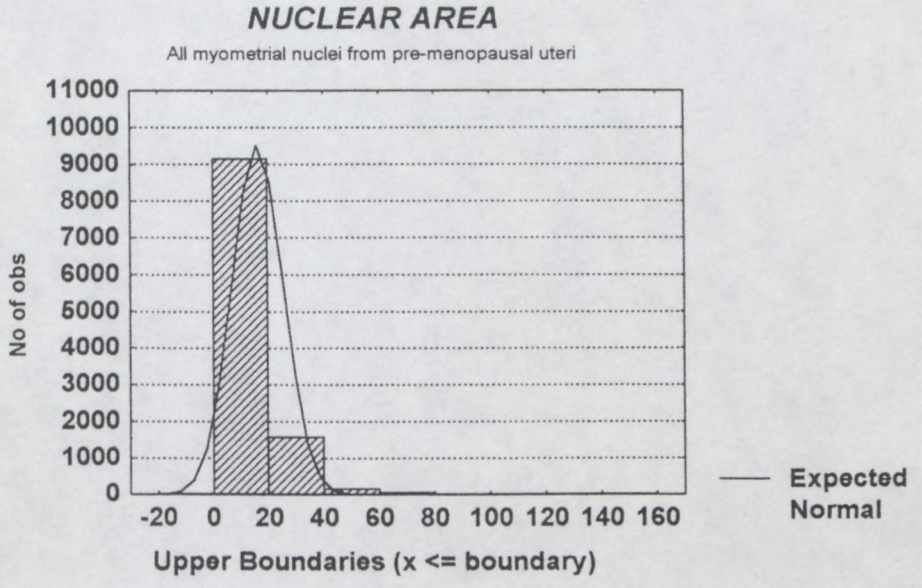

Figure 3.6: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from pre-menopausal uteri.

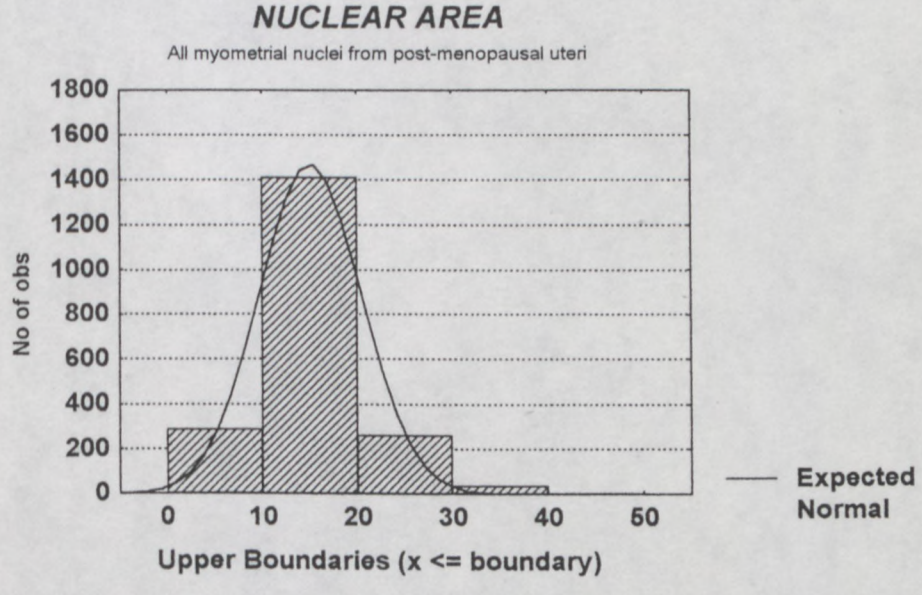

Figure 3.7: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from post-menopausal uteri.

The nuclear measurements from all leiomyomas also demonstrated a normal distribution (Figure 3.8).

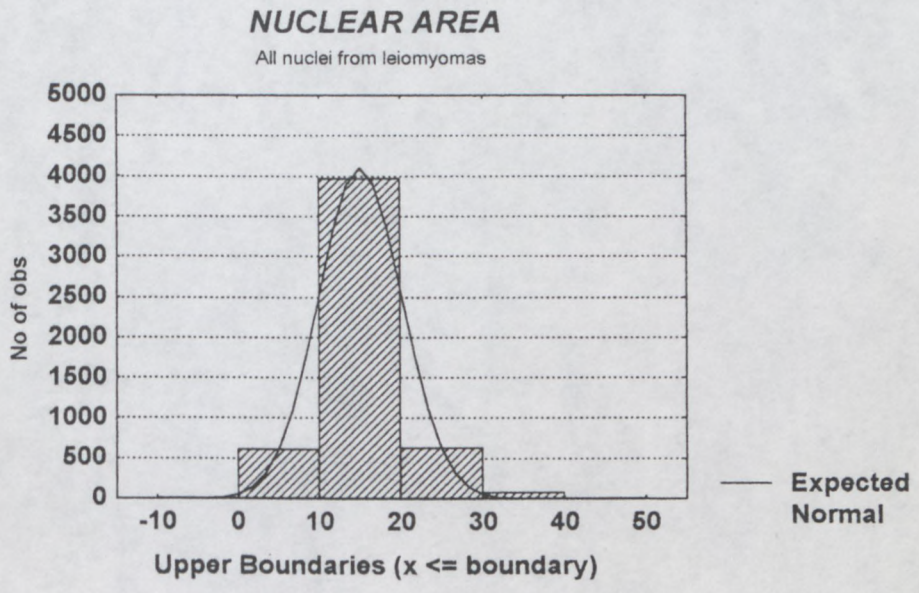

Figure 3.8: Frequency histogram to demonstrate the normal distribution of all leiomyoma nuclei.

Nuclear measurements from pregnant uteri were also shown to demonstrate a normal distribution (Figure 3.9).

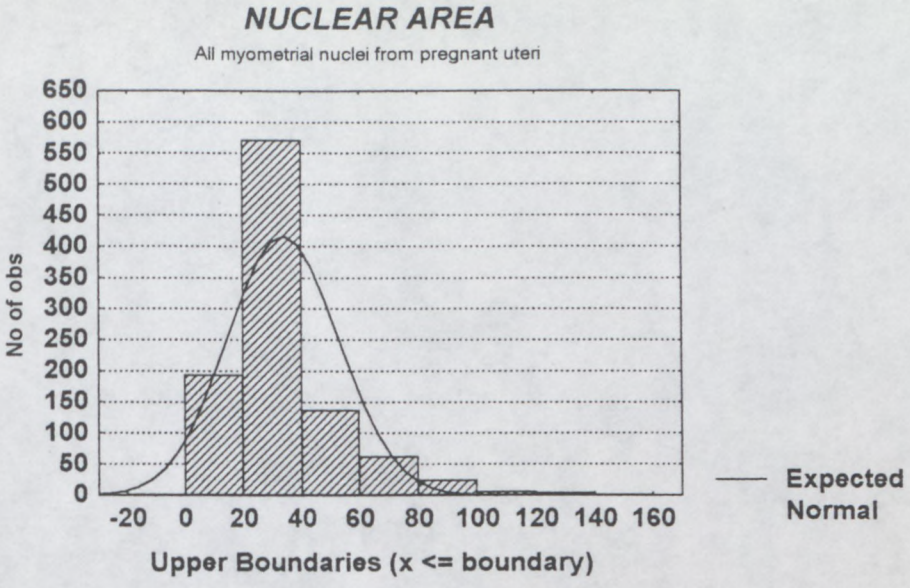

Figure 3.9: Frequency histogram to demonstrate the normal distribution of all myometrial nuclei from pregnant uteri.

3.3 Analysis of nuclear atrophy after the menopause.

One of the questions posed by this study was whether there was a significant size difference between the nuclei from pre and post-menopausal women.

A one-way anova (analysis of variance) test was performed on the data to determine whether there was a significant difference between the two sets of measurements (myometrial nuclei from the uteri of pre and post-menopausal women). This test showed that at a p value < 0.05 there was no significant difference between the myometrial nuclear measurements of pre and post menopausal uteri. A box and whisker plot was performed on these results indicated no significant difference (Figure 3.10).

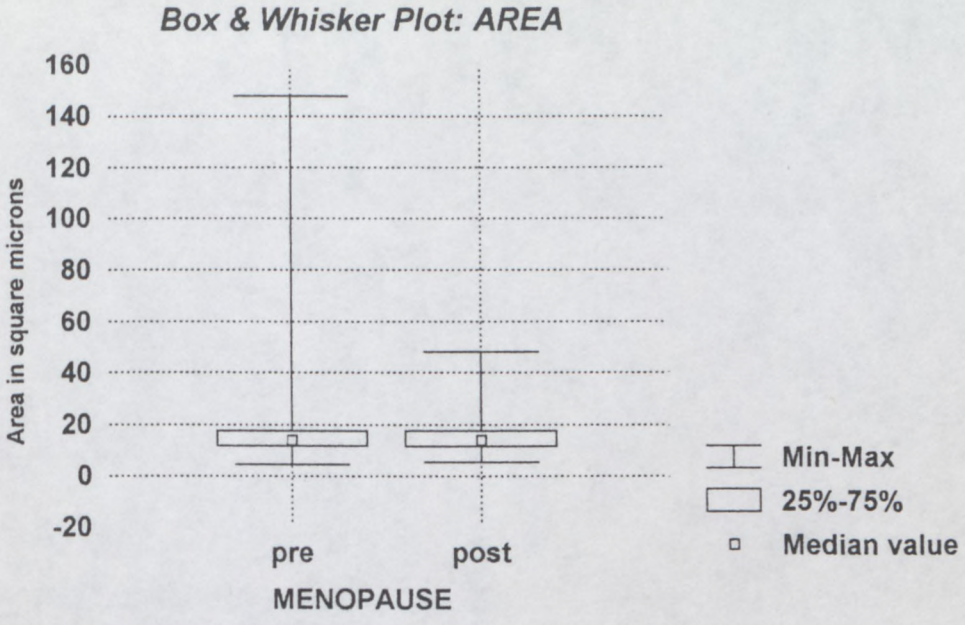

Figure 3.10: A box and whisker plot of myometrial nuclei from pre and post menopausal uteri. There was no significant difference at a p value of <0,05.

The purpose of the analysis of variance (ANOVA) test is to test for significant differences between means. (on line help - Statistica 5).

3.4. Analysis of nuclear size difference during the menstrual cycle.

Anova tests were performed to establish whether there were significant size differences between the nuclei from uteri with proliferative, secretory, menstrual and inactive endometriums. At a *p* value of < 0.05 there was no significant difference in nuclear size during the menstrual phases. A box and whisker plot was performed on these results and the lack of variation is clearly seen (Figure 3.11).

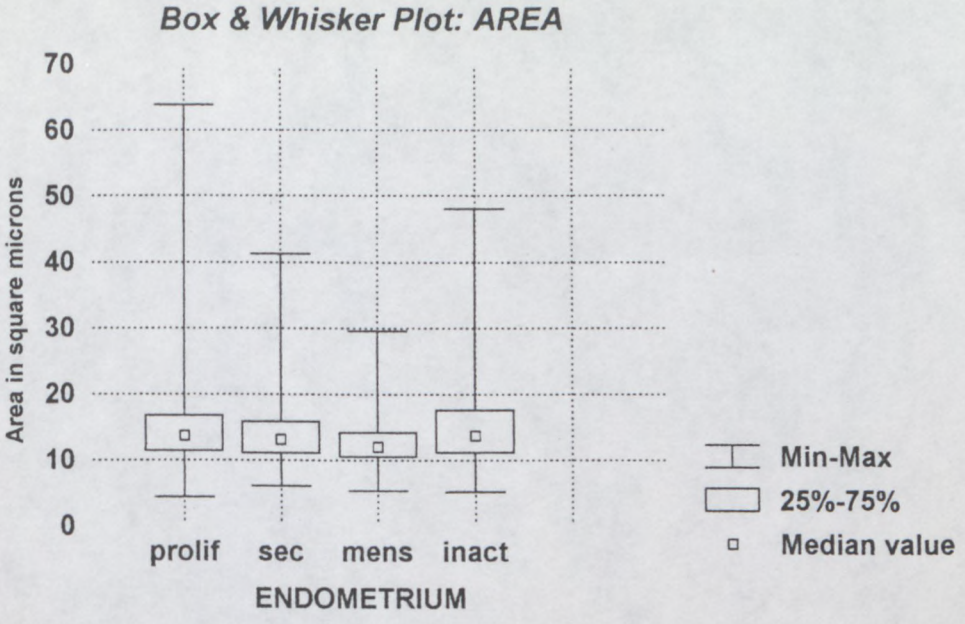

Figure 3.11: A box and whisker plot of nuclei from uteri with proliferative, secretory, menstrual and inactive endometriums. There was no significant difference at a p value of <0,05.

3.5 Analysis of nuclear size difference between fundus and lower segment.

In order to establish whether there are significant differences between myometrial nuclei in the fundus and lower segment of the uterus it was necessary to compare nuclei from the same uterus. A dependent T test was carried out for this analysis. The T test for dependent samples is more sensitive than a T test for independent samples because paired differences are analyzed thus excluding variation in the data that results from unequal base levels of individual subjects. In this study nuclei from the fundus and lower segment of the same uterus were compared against each other. At a *p* value of <0.05 there was no significant difference in nuclear size in these two sites and a box and whisker plot (Figure 3.12) illustrates this.

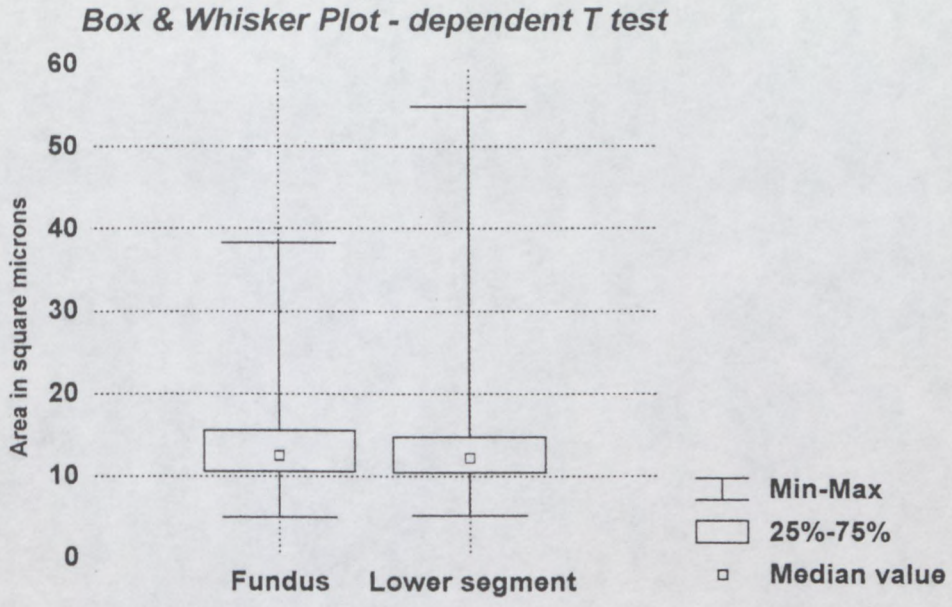

Figure 3.12: A box and whisker plot of myometrial nuclei from fundus and lower segment of all uteri in the study. There was no significant difference at a p value <0,05.

3.6 Comparison between leiomyoma and myometrial nuclear size.

To answer this question dependent paired T tests were performed on the sets of nuclear measurements from normal myometrium and leiomyomas from the same patient. Uteri from all stages of the menstrual cycle were included. At a *p* value of < 0.05 there were significant differences in nuclear size between these two data sets. The leiomyoma nuclei were larger than the nuclei of the normal myometrium. (Figure 3.13).

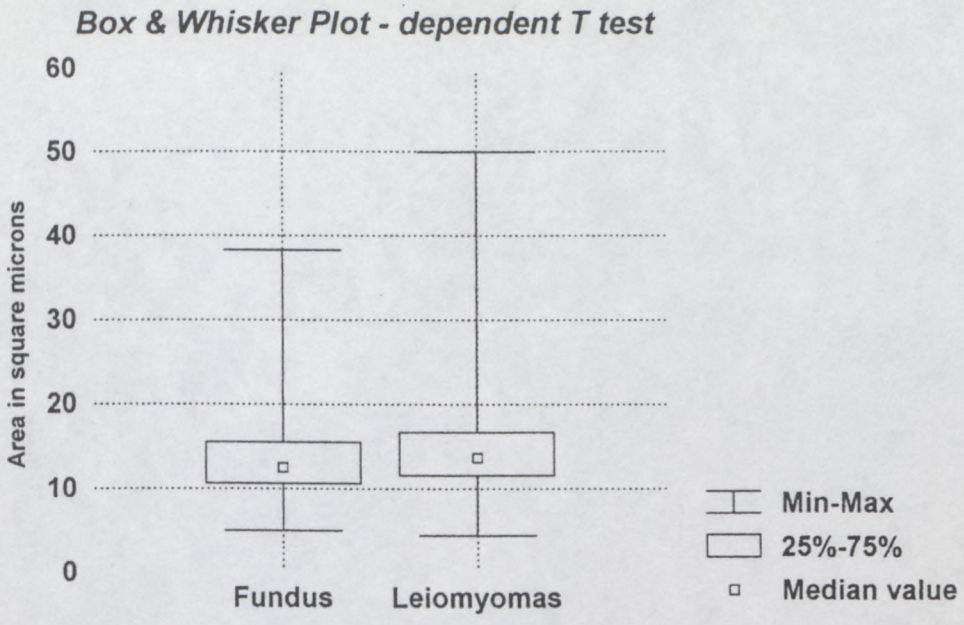

Figure 3.13: A box and whisker plot of nuclei from normal myometrium and leiomyomas. There were significant differences at a p value <0,05.

3.7 Analysis of nuclear hypertrophy during pregnancy.

Independent T tests that were performed to test for statistically significant differences between the myometrium of non-gravid, pre-menopausal, post-menopausal and pregnant uteri found that the nuclei of myometrial cells from pregnant uteri were significantly larger than those from non-gravid uteri. A box and whisker plot was drawn to illustrate this (Figure 3.14).

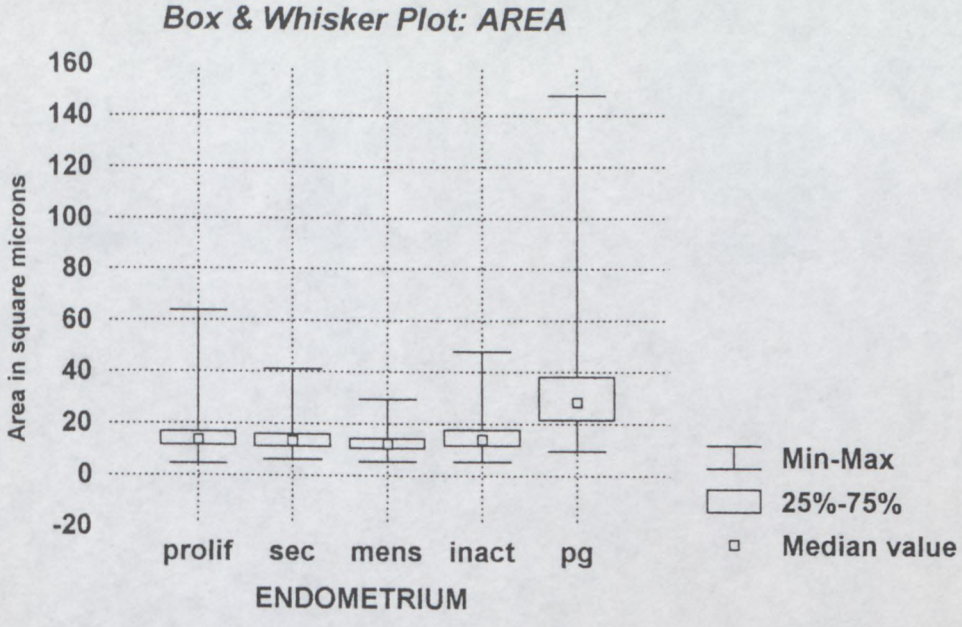

Figure 3.14: A box and whisker plot of nuclei from pregnant uteri and uteri with proliferative, secretory, menstrual and inactive endometriums. There were significant differences at a p value <0,05.

Chapter 4

Discussion

4.1 Introduction

The aim of this study was to measure the nuclei of myometrial cells in the uterus to determine the effect of sex steroids on their size.

4.2 Myometrial nuclear atrophy after menopause

Little variation in size was demonstrated between the nuclei in pre and post menopausal uteri.

In a quantitative investigation of the musculature of the uterus (Schwalm and Dubrauzsky, 1966) reported that the average lengths of uterine cavities of sexually mature women (25 to 45 years) were 6,5 to 9,5 cm and those from the uteri of women between 55 and 84 years were 3,5 to 6 cm. The weights of these uteri also showed great variation; 70 to 100g for the sexually mature uteri and between 35 to 70g for the post-menopausal uteri. This study concludes that during the menopause there is a definite shrinkage of the musculature.

A study by Langlois (1970) also records a decrease in uterine mass after the menopause and that although parity was found to be the primary determinant of uterine weight, after age 49 uterine mass tended to regress back to within the range of nulligravidous patients irrespective of parity.

4.2.1 The influence of sex steroids on the myometrium on post-menopausal uterine atrophy.

Uterine atrophy occurs when there is a reduction or cessation of ovarian hormonal stimulation. In the myometrium this results in a marked decrease in the amount of sarcoplasm present in the smooth muscle cells of the myometrium so that there is very little intervening cytoplasm between adjacent cells. The atrophic myometrium, therefore, appears to be mainly composed of densely packed elongated nuclei (Norris, 1973). This view supports the finding of this study of no change in nuclear size of myometrial nuclei in post-menopausal uteri as the muscle shrinkage of the uterus that accompanies the menopause is reported to be due to a decrease in muscle cell cytoplasm.

4.3 Differences in myometrial nuclear size during the menstrual cycle.

This study found no statistically significant variation in myometrial nuclear size throughout the different phases of the menstrual cycle.

4.3.1 The influence of sex steroids on the myometrium during the menstrual cycle.

Uterine muscle is markedly influenced by the actions of sex steroids that accompany the menstrual cycle. The purpose of this study was to determine the effect of these sex steroids on the nuclear size of myometrial cells. Oestrogen and progesterone are the most directly involved (Norris, 1973). Steroids are heterocyclic lipid compounds. Sex steroids are secreted primarily by the gonads of higher vertebrates, especially mammals. There

are three major groups of sex steroids; progestins, oestrogens and androgens. Steroids are synthesized from the basic unit, acetate. In steroidogenic cells cholesterol is the principal precursor for steriodogenesis. The biosynthesis of sex steroids from cholesterol involves a number of alternate pathways that eventually result in a progressive decrease in the number of carbon atoms attached to the steroid nucleus and alterations of the A ring (Hafez, 1980).

4.3.2 The influence of oestrogen.

Oestrogen causes a pronounced cellular hyperplasia as well as hypertrophy of myometrial cells. The growth response initiated by oestrogen includes the stimulation of RNA polymerase II and I, stimulation of glucose and phospholipid metabolism, an increase in protein synthesis, an increase in cell division and an increase in water uptake by uterine cells (Norman and Litwack, 1987). Hillier (1990) reports that treatment of hypophysectomized immature female rats with exogenous oestrogen stimulates granulosa cells, decreases mitosis, increases gonadotrophin receptor levels and amplifies follicular responsiveness to exogenously administered gonadotrophin. At the cellular level oestrogen augments FSH-induced expression of the regulatory subunit RIIβ of type II cAMP-dependent protein kinase and steroidogenic enzymes P450aro and P450scc. Oestrogen also stimulates expression of inhibin α- and βB subunit mRNA's and augments FSH-induced inhibin production by cultured rat granulosa cells (Hillier, 1990).

In women oestradiol is the major oestrogen secreted by the ovary. During the second half of the follicular phase of the menstrual cycle most of this oestradiol is produced by the ripe follicle. During this luteal phase of the

cycle most of the ovarian oestradiol is produced by the corpus luteum (Hafez, 1980).

The action of oestradiol is not necessarily restricted to an increase of DNA dependent RNA synthesis by these cells. The administration of oestradiol can stimulate adenyl cyclase activity. It is probable however, that this response occurs in different cell types and involves a primary action of oestrogen different from that leading to endometrial proliferation. Oestrogen can also stimulate an increase in uterine cyclic GMP but the significance of this is unclear. It is also thought that steroids act at the cytoplasmic level to alter enzyme activities (Hafez, 1980).

4.3.3 The influence of progesterone.

Progesterone is the most active progestin and is secreted primarily by the corpus luteum. It causes hyperplasia and hypertrophy of the myometrium when acting on a uterus previously stimulated by oestrogen (Norris, 1973).

The concentrations of steroids circulating in plasma are a resultant of many interrelated factors, including gonadal and adrenal secretion of the steroids, peripheral conversion of one steroid to another, relative binding to plasma proteins, metabolism and conjugation by liver, kidney and other tissues, biliary and urinary excretion and resorption and distribution into different body pools (Hafez, 1980).

Plasma concentrations suggest that progesterone is secreted in much larger quantities than oestradiol. Most progesterone is produced by corpora lutea but some is synthesized by maturing follicles. In the plasma a large portion

of each sex steroid is bound to albumin, a plasma protein with a low affinity and high capacity for steroids. Another portion is bound at one or more specific proteins. Oestradiol binds to a high affinity "sex-steroid" binding globulin while progesterone binds to corticosteroid-binding globulin. Blood concentrations of a steroid hormone are effectively a function primarily of the concentrations of the free (unbound) steroid, and this pool is governed partly by the concentrations of the high affinity plasma-binding protein. Administration of oestradiol results in an increase in the concentration of sex steroid-binding globulin (Hafez, 1980)

4.3.4 Sex steroid receptors

Each hormone of the female genital tract acts through its own specific receptor. Sex steroids bind to high affinity, soluble cytoplasmic receptor proteins present in the highest concentrations in target tissues. Strong but reversible binding to these intracellular proteins is an early step in the action of steroids and appears to be responsible for the uptake of steroids by target tissues. Next, the cytoplasmic steroid-protein complex is transported to the nucleus. Once within the nucleus, the steroid protein complex appears to bind to highly specific acceptor regions of the genome. These appear to serve as a template for a species of RNA that has many characteristics of messenger RNA. This synthesis of new RNA leads to the production of specific proteins (Hafez, 1980).

Peptide hormones are charged molecules and so cannot cross the cell membrane; in these cases the receptor is a component of the plasma membrane, with the ligand (hormone) binding site of the receptor sticking out from the cell. Steroid hormones however, are all synthesized from

cholesterol and so are relatively soluble in the cell membrane. Therefore, steroid receptors are found inside the target cell. (Lowe and Fox, 1992).

The results of the present study suggest that in spite of the hypertrophy of the muscle cells induced by the action of oestrogen, the nuclei of these cells do not change in size under the influence of this hormone. From the menstrual phase to the proliferative phase where there is a rapid increase in blood oestrogen levels there is an accompanying increase in the size of myometrial muscle fibres. This study however found no change in the size of the nuclei of these muscle cells for this period.

In the secretory phase increasing progesterone levels in addition to the oestrogen already present causes further hypertrophy of the myometrial muscle. Again, no evidence of a concomitant increase in nuclear size was found in the present study during this phase of the menstrual cycle.

4.4 Differences in nuclear size of myometrial cells in the fundus and lower segment.

This study found that there was no significant difference in nuclear size between fundus and lower segment. No evidence could be found in the literature that this has been investigated although Schwalm and Dubrauszky (1966) did study the percentage muscle content and found that the percentage of musculature was considerably less in the lower segment of the uterus (28% in the fundus and 15% in the lower segment) although no mention was made of nuclear size. The finding of this study that there is no significant difference in nuclear size between these two sites indicates that

the influence of oestrogen and progesterone is uniform across the whole myometrium.

4.5 Differences in size between leiomyoma and myometrial nuclei.

This study found that there was a statistically significant difference in the sizes of leiomyoma nuclei and the nuclei of normal myometrium and that leiomyoma nuclei were larger than normal myometrial nuclei.

4.5.1 The influence of sex steroids on leiomyomas

There is conflicting evidence in the literature as to whether leiomyomas behave differently as a result of varying levels of sex steroids. A study by Brandon *et al* (1995) investigated whether increased expression of progesterone receptor in leiomyomas is a consequence of over expression of functional oestrogen receptor (ER) that results in increased end-organ sensitivity to oestradiol and a subsequent

In contrast to the research by Soules and McCarthy (1982) the study by Brandon *et al* (1995) suggested that the oestrogen signal transduction pathway may be enhanced in uterine leiomyomas. Soules and McCarthy (1982) found that increased ER levels in human uterine leiomyomas were similar to those in normal myometrium although they did agree that the amount of ER in leiomyomas does vary with the menstrual cycle and parallels changes found in the myometrium. The study by Brandon *et al* (1995) was the first to examine ER and mRNA levels as well as protein amount and function. Their data indicated that both transcription of the ER gene and translation of the protein are enhanced in leiomyomas.

4.5.2 The correlation of sex steroid levels in leiomyomas and normal myometrium

Brandon *et al* (1995) also state that although oestrogen levels are not elevated in women with leiomyomas, there are alterations in oestrogen metabolism in the tumour itself. Concentrations of oestrogen, estrone and their sulphates are higher in leiomyomas than in myometrium; the inactivation of oestrogen via estrone is impaired and leiomyomas have elevated aromatase cytochrome P_{450} compared to adjacent myometrium (Brandon *et al*, 1995).

Farber *et al* (1972) also found that ER concentrations in human uterine leiomyomas were significantly higher than levels in corresponding normal myometrial tissues. They concluded that leiomyomas bind approximately 20% more oestradiol per milligram of cytoplasmic protein than normal myometrium from the same uterus.

Papers that found a strong correlation between hormonal levels in myometrium and leiomyomas include a study by Soules and McCarthy (1982) that investigated the relationship between ovarian steroid hormones and uterine leiomyomas as manifested by quantitative and qualitative changes in cytoplasmic steroid receptors. They found that uterine leiomyomas contain oestrogen and progesterone receptor in quantities comparable to those found in normal uterine tissue. In addition they found that the levels of oestrogen and progesterone receptor in leiomyomas demonstrated the same quantitative and qualitative cyclic changes

throughout the menstrual cycle that are found in endometrium and myometrium (Soules and McCarthy, 1982).

A paper by Yo Ho Shek *et al* (1987) to investigate the expression of proteins in uterine leiomyomas found two groups of tumour-associated protein, P34 (34K) and P56 (56K) in uterine leiomyomas. These two groups of proteins showed enhanced expression in the tumours compared to normal myometrium. In the uterine leiomyomas these proteins were found more frequently during the proliferative phase of the menstrual cycle than during other menstrual phases implying that the syntheses of these proteins are related to the cyclic variation of intrinsic sex hormones in the tumours particularly oestrogen which is a predominant sex hormone during the proliferative phase. This study found that P34 and P56 are expressed mainly during the proliferative phase and disappear in the nonproliferative phases. The expression of P34 and P56 occurs when the intrinsic oestrogen level increases which suggests a correlation between the expression of tumour-associated proteins and oestrogen level. Other sex-hormonal regulation of the expression of P34 and P56 cannot be excluded. For example, progesterone may be a suppressing factor for P34 and P56 expression since progesterone levels are decreased during the proliferative phase. Yo Ho Shek *et al* (1987) suggested that P34 and P56 may contribute to the growth of leiomyomas. If this is true and given that P34 and P56 are correlated to the menstrual cycle, uterine leiomyomas may grow cyclically. In their study leiomyomas from perimenopausal or post-menopausal patients did not express P34 and P56 leading to the supposition that the tumours may stop growing when the level of sex hormone decreases.

A paper by Chrapusta *et al* (1990) also reported no significant menstrual cycle-related change in leiomyomas or normal myometrium and emphasized that the reactivity of uterine leiomyomas to oestrogens and or progestins may be related to the histological features of the tumours since they found that ER levels in normal myometrium and cellular leiomyomas but not in usual leiomyomas tended to be higher in the follicular phase than in the luteal one. They also reported however that PR levels in cellular leiomyomas, usual leiomyomas and normal myometrium were significantly lower in the luteal than in the follicular phase.

4.6 Myometrial nuclear hypertrophy during pregnancy

This study found a statistically significant difference between myometrial nuclei in non-gravid uteri and those from gravid uteri.

No evidence could be found in the literature that a study has been done on the size of myometrial nuclei in the pregnant uterus but Norris *et al* (1973) report that the myometrial cell increases in size from 5μm x 90μm at the beginning of pregnancy to approximately 10μm x 800μm at term. Our study confirms that this increase in cell size is accompanied by an increase in nuclear size.

The degree of hypertrophy is a function of the amount and duration of stretch caused by pregnancy. When this stretch is combined with oestrogen or oestrogen and progesterone the myometrial hypertrophy is accentuated. Therefore the considerable increase in cell number and cell size during pregnancy results from the combined actions of oestrogen, progesterone and chronic stretch (Norris *et al*, 1973). Presumably the increase in nuclear size would be mainly due to the increased levels of oestrogen.

4.7 Further investigations.

Further investigations could include a detailed study of the sex steroid levels, especially oestrogen and progesterone, in blood samples taken at the time of hysterectomy.

Additional studies could also be carried out using a fully automatic image analysis system instead of the semi-automatic system used in this project.

Chapter 5

Conclusion

5.1 Introduction

The conclusions of the main study to investigate the effect of sex steroids on the nuclear size of these myometrial cells in the uterus are as follows:

5.2 Nuclear measurement

The aims of measuring myometrial and leiomyoma nuclei in this study were:

1. To ascertain whether myometrial nuclei atrophy after the menopause.
2. To determine whether there are significant myometrial nuclear size changes during the menstrual cycle.
3. To determine whether there are size differences between myometrial nuclei from the fundus and lower segment.
4. To investigate size differences between nuclei from leiomyomas and normal myometrium in the same patient.
5. To ascertain whether myometrial nuclei hypertrophy during pregnancy.

5.2.1 Nuclear atrophy after the menopause.

This study found no significant variation between nuclear measurements from pre and post-menopausal women and therefore concluded that in the patients observed for this study myometrial nuclei did not atrophy after the menopause.

5.2.2 Nuclear size differences during the menstrual cycle.

This study found no statistically significant variation between nuclear measurements from uteri with proliferative, secretory, menstrual or inactive endometriums. It was concluded that in the uteri used in this study there were no nuclear size differences during the menstrual cycle.

5.2.3 Nuclear size differences between fundus and lower segment.

No statistically significant differences were demonstrated between nuclear measurements from the fundus and lower segment in this study.

5.2.4 Nuclear size differences between normal myometrium and leiomyomas.

Dependent T tests demonstrated significant size differences between nuclei from the normal myometrium and leiomyomas in the same patient; those from leiomyomas being larger. This could be the result of a steroid stimulus to which normal myometrium is refactory or some other neoplastic stimulus.

5.2.5 Nuclear hypertrophy during pregnancy.

A statistically significant difference was found between the nuclei of non-gravid pre and post-menopausal uteri and pregnant uteri. It was found that those nuclei from the myometrium of pregnant uteri were significantly larger than those from non-gravid uteri. This is probably in keeping with normal physiological influences on the cells of pregnant uteri and may be the consequence of either steroid influence or stretch.

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Appendix

Summary of means.

Nuclear area measurements in square microns. Transversely sectioned myometrial nuclei on H+E under 100x objective

Endometrium:

Proliferative	14,55
Secretory	14.1
Menstrual	12.44
Inactive	15.01
Pregnant	33,72

Site:

Fundus	17,21
Lower segment	13,0
Leiomyoma	15,94

Pre/Post-Menopausal:

Pre-menopausal	16,1
Post-menopausal	15,08