HORMONE REPLACEMENT THERAPY AND VASCULAR PROTECTION – THE INFLUENCE OF OESTROGEN ON THE ENDOTHELIUM

ANN CAROLYN DUNCAN

DOCTOR OF MEDICINE

UNIVERSITY OF GLASGOW
FACULTY OF MEDICINE
DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY

July 2001

ABSTRACT

Cardiovascular disease is the leading cause of death and an important cause of morbidity in postmenopausal women, the incidence being particularly high in the West of Scotland. Retrospective studies strongly suggest an aetiological link between oestrogen deficiency and cardiovascular risk, and moreover have suggested that mortality can be reduced by up to 50% by the administration of hormone replacement therapy. However, recently reported randomised controlled studies have challenged this view (Hulley et al, 1998). The mechanism by which the apparent cardioprotective benefit occurs is multi-factorial and incompletely understood. Direct effects on the vessel wall particularly at the level of the endothelium, as well as indirect effects on metabolic risk factors such as lipid and carbohydrate metabolism, may play an important role in any underlying protective mechanism. The aim of this project was to study the effect of oestrogen on the vessel wall, more specifically by examining its effect on vascular reactivity and its influence on nitric oxide synthesis and release from the vascular endothelium. *In vivo* studies using forearm venous occlusion plethysmography were used to assess the effect of oestrogen on forearm vasodilator responses, and on the release of nitric oxide from the intact endothelium. To compliment this work, the effect of sex steroids on the growth and function of endothelial cells, and on the synthesis and release of nitric oxide from vascular endothelial cells, was studied *in vitro* using cultured human aortic endothelial cells. The effect of short and long-term oestrogen on endothelial nitric oxide synthase (eNOS) gene expression was studied in cultured cells using northern analysis. No change in forearm blood flow was observed following intra-arterial infusion of 17β-oestradiol, suggesting that oestrogen is not an acute vasodilator in the forearm vasculature. Forearm blood flow responses to N^G-monomethyl-L-arginine, a stereospecific inhibitor of nitric oxide which provides an index of basal nitric oxide synthesis, were unchanged following 4 weeks' of transdermal 17β-oestradiol treatment, suggesting that oestrogen supplementation did not enhance basal nitric oxide release in the forearm vascular bed. In cultured endothelial cells, a significant increase cell number was observed following long term oestradiol treatment. The underlying mechanism for this observation is unclear. Expression of messenger RNA for the eNOS gene was confirmed by northern analysis, however the results of these studies did not demonstrate any change in eNOS mRNA expression following oestrogen treatment, supporting the findings of the in-vivo study. With the exception of the growth studies which yielded interesting results requiring further evaluation, the negative results of the remaining carefully designed and well executed studies, while at variance with the results of other investigators, nevertheless generate interesting new data which appears to be in broad agreement with the albeit limited results of the randomised control studies currently available. From our present knowledge base, there remain many unanswered questions about the true risks and benefits of hormone replacement therapy, and a better understanding of the mechanisms contributing to any beneficial effects will lead to better prescribing practices and may ultimately lead to improvements in womens' health.

CONTENTS

CHAPTER 2: OVERVIEW OF METHODS

2.1 Forearm venous occlusion plethysmography

2.2 Cell culture techniques

2.3 Molecular techniques

CHAPTER 5: STUDY THREE

The effect of oestradiol on the growth characteristics of human aortic endothelial cells in culture

CHAPTER 6: STUDY FOUR

Determination of eNOS expression in cultured human aortic endothelial cells following oestradiol treatment

ACKNOWLEDGEMENTS

This work was supported financially by the Chest, Heart and Stroke Scotland Association (plethysmography studies), the British Menopause Society (cell culture and molecular studies) and the Nuffield Hospital Group who provided the Department of Obstetrics and Gynaecology with a grant towards my salary.

I would like to thank Dr Mary-Ann Lumsden, Senior Lecturer, Department of Obstetrics and Gynaecology, University of Glasgow, for her supervision during the preparation of this thesis, and for her detailed appraisal of this manuscript. Thanks also to Professor A Dominiczack and Professor JMC Connell, Department of Medicine and Therapeutics, University of Glasgow, for their guidance and helpful comments.

I am also grateful to Dr J Petrie, Senior Lecturer, Department of Medicine, University of Glasgow, for his assistance with the plethysmography studies, to Dr A Devlin, Post-Doctoral Research Fellow, Department of Medicine and Therapeutics, University of Glasgow, for assistance with the cell culture techniques, and to Dr J Brosnin, Lecturer, Department of Medicine and Therapeutics, University of Glasgow for her guidance with the molecular techniques.

I would also like to thank Dr I Montgomery, Department of Physiology, University of Glasgow, for characterising the endothelial cells using immunocytochemistry. Thanks also to Dr R Bass, Research Fellow, Department of Obstetrics and Gynaecology, University of Cambridge, who performed the oestrogen receptor studies on behalf of Dr S Charnock-Jones. I am grateful to the consultants of the Department of Gynaecology, Western Infirmary, Glasgow for allowing me to recruit their patients, and of course to the patients themselves who participated with these studies.

I would like to acknowledge my family for their continued support following my decision to pursue a medical career, in particular my mother Margaret Duncan, to whom I am immensely grateful.

Finally, I wish to dedicate this thesis to my father Alexander Duncan, who sadly died before it was completed.

DECLARATION

Ethical approval

Written ethical approval for recruitment of patients into the clinical studies was granted by the West Glasgow Hospitals University NHS Trust Ethical Committee.

Experimental work

The experimental work described in this thesis was performed by the author with the exception of the immunocytochemical characterisation of the endothelial cells which was kindly performed by Dr I Montgomery, Department of Physiology, University of Glasgow, and the oestrogen receptor studies which were performed by Dr R Bass, Research Fellow, Department of Obstetrics and Gynaecology, University of Cambridge on behalf of Dr S Charnock-Jones.

PUBLICATIONS

A Duncan, H Lyall, R Roberts, M Perera, J Petrie, J Connell, MA Lumsden. (1999) The effect of HRT on insulin sensitivity in healthy postmenopausal women. Journal of Clinical Endocrinology and Metabolism; 84 (7): 2402-07

A Duncan, M Brosnan, J Petrie, A Devlin, A Dominiczak, J Connell, MA Lumsden. (1998) Cardioprotection and oestradiol: is it a nitric oxide mediated effect? (Abstract) Journal of Human Hypertension; 12 (supplement): 5-6

A C Duncan. (1998) Oestrogen and the endothelium. (Abstract) Journal of the British Menopause Society; 4 (S1): 17

A Duncan, H Lyall, R Roberts, M Perera, J Petrie, J Connell, MA Lumsden. (1998) The effect of HRT on insulin sensitivity in healthy postmenopausal women. (Abstract) Journal of the British Menopause Society; 4 (S1): 25

A Duncan, MA Lumsden. (1998) HRT and the cardiovascular debate. (Review) British Journal of Sexual Medicine; Jan/Feb: 18-20

LIST OF FIGURES

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

1.1 Background

1.1.1 Introduction

In 1868 the life expectation for a newborn female infant was 40 years (Felstein, 1973). By 1995 the female life expectation in Scotland had risen to 77.6 years, and by the year 2025 almost one quarter of the population will be aged 60 or over, and a higher proportion of this group will be female (Health in Scotland, 1995). Thus, most women are living at least one third of their lives after reproductive function has ceased.

The climacteric, derived from the Greek 'klimakter' (the rung of the ladder), is a normal ageing phenomenon in women which reflects the gradual transition from the reproductive to the non-reproductive phase of life, and represents a period of physical and mental transition from fertility and normal ovarian function to infertility and ovarian failure. The menopause or last menstrual period, which is derived from the Greek 'menos' (month) and 'pausis' (to stop), is defined by the World Health Organisation as 'the permanent cessation of menstruation resulting from the loss of ovarian follicular activity'. It forms part of the climacteric process and marks the end of ovulation. In Western society cessation of menses occurs at a median age of 50 years, however the climacteric occurs over a ten-year period from the mid-forties onwards.

A vast increase in the total number of women passing through the climacteric period during the last century, has transformed the menopause from being a subject peripheral to medical interest, to one which has become central to clinical practice and about which there is considerable debate both in the medical and lay press. With the increasing longevity now seen in women the clinical features of the climacteric are more readily appreciated by physicians and there is better understanding of the underlying endocrine physiology.

1.1.2 Historical perspectives

In the late 17^{th} and early 18^{th} centuries with changing political and economic events around Europe a new stability emerged. This, accompanied by an abatement in the plagues which regularly devastated the continent, led to a rise in the expectation of life, both at birth and during adulthood. Many women, particularly those from the upper

classes, were experiencing the climacteric and some were reaching the menopause and beyond. However the climacteric changes were perceived to rob these women of their sexuality, and usually marked the start of the decline in their status.

The first English reference to the features associated with the climacteric period appeared in a guide for women published in 1927 by an anonymous physician (Wilbush, 1988). In 1857 a physician named Edward Tilt wrote the first English book devoted to climacteric disorders. He was the first to suggest that not only the menopause but also the symptoms and disturbances of the climacteric, were due to ovarian involution (Wilbush, 1980). Nevertheless, his work had little impact at this time, most probably because of its perceived unimportance as climacteric patients seldom complained. Women in the climacteric generally accepted their fate as an act of nature, and with their offspring as their compensation, did not feel demoted or diminished. However, by the turn of the last century Europe was deep in economic depression, families became smaller (partly due to economic considerations and partly due to the greater availability of contraceptive methods), children increasingly moved away from their home towns to follow economic opportunities, and women found themselves alone with their role in family life decimated. Unable to accept their position they often became ill. Concurrently the number of women who reached and passed the menopause continued to increase, and the largely middle and lower-middle classes became the principal sufferers. Thus, there was a renewed interest in the whole dimension of the menopause at the beginning of the 20^{th} century.

The early part of the century saw a great upsurge in the study of the endocrinology of the climacteric and the menopause. Nineteen twenty-three marked the discovery of oestrogens in follicular fluid and thereafter, there was rapid isolation of the various ovarian hormones such that by the mid 1930's they were being therapeutically assayed in North America and Germany (Wilbush, 1988). The 1940's and 1950's saw a great increase in experimental and therapeutic hormone therapy including treatment of climacteric symptoms with oestrogen. This marked a new era in womens' health and has shaped the recent developments in the menopausal milieu and hormonal replacement therapy (HRT).

1.2 Steroid hormones

1.2.1 Introduction

The steroid hormones are a group of small and phylogenetically very old molecules, with strikingly similar structures which are based on a common structural subunit - the perhydroxycyclopentanephanthrene molecule (figure 1), consisting of four fused hydrocarbon rings - three 6-C rings and one 5-C ring (Birkhauser, 1996).

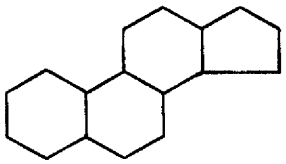


Figure 1.1 – Basic steroid structure

They are classified according to the number of carbon atoms (C) thus: C-21 steroids (pregnane nucleus) - progesterone, cortisol, and aldosterone; C-19 steroids (androstane nucleus) - testosterone, and androstendione; C-18 steroids (oestrone nucleus) - oestrogen (figure 1.2), (Birkhauser, 1996).

1.2.2 Steroid hormone biosynthesis

Cholesterol is the common pre-cursor of all steroid hormones and is the basic molecule for biosynthesis. The cholesterol used for steroid hormone biosynthesis is derived principally from dietary sources entering the steroid producing cells via the bloodstream, although with the exception of the placenta, all molecules involved in steroidogenesis can also synthesise cholesterol de-novo from acetate (figure 1.3) (Chard & Lilford, 1995).

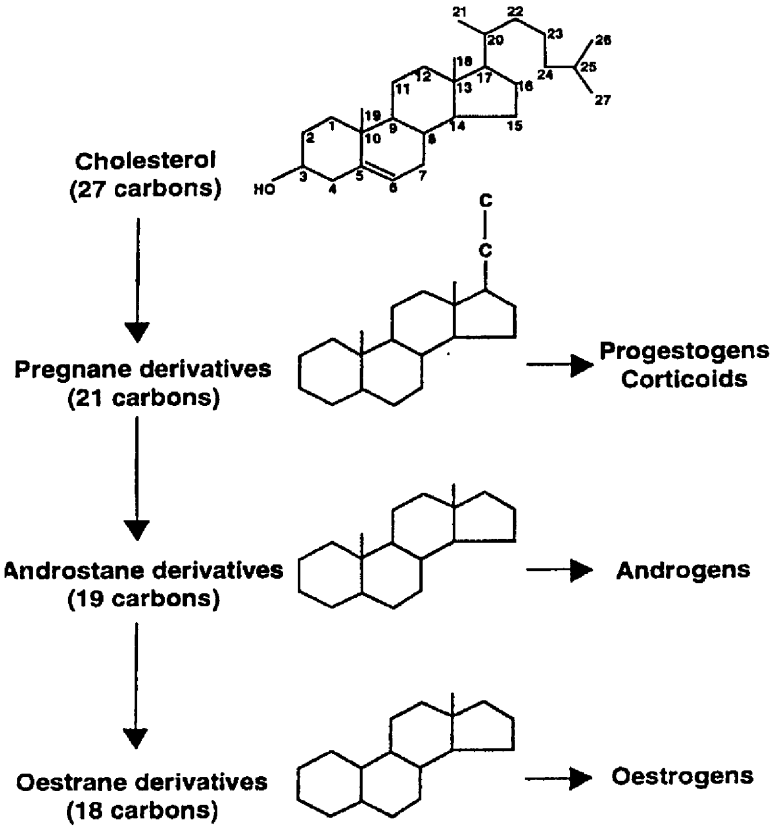


Figure 1.2 – Steroid hormone structure (adapted from Birkhauser, 1996)

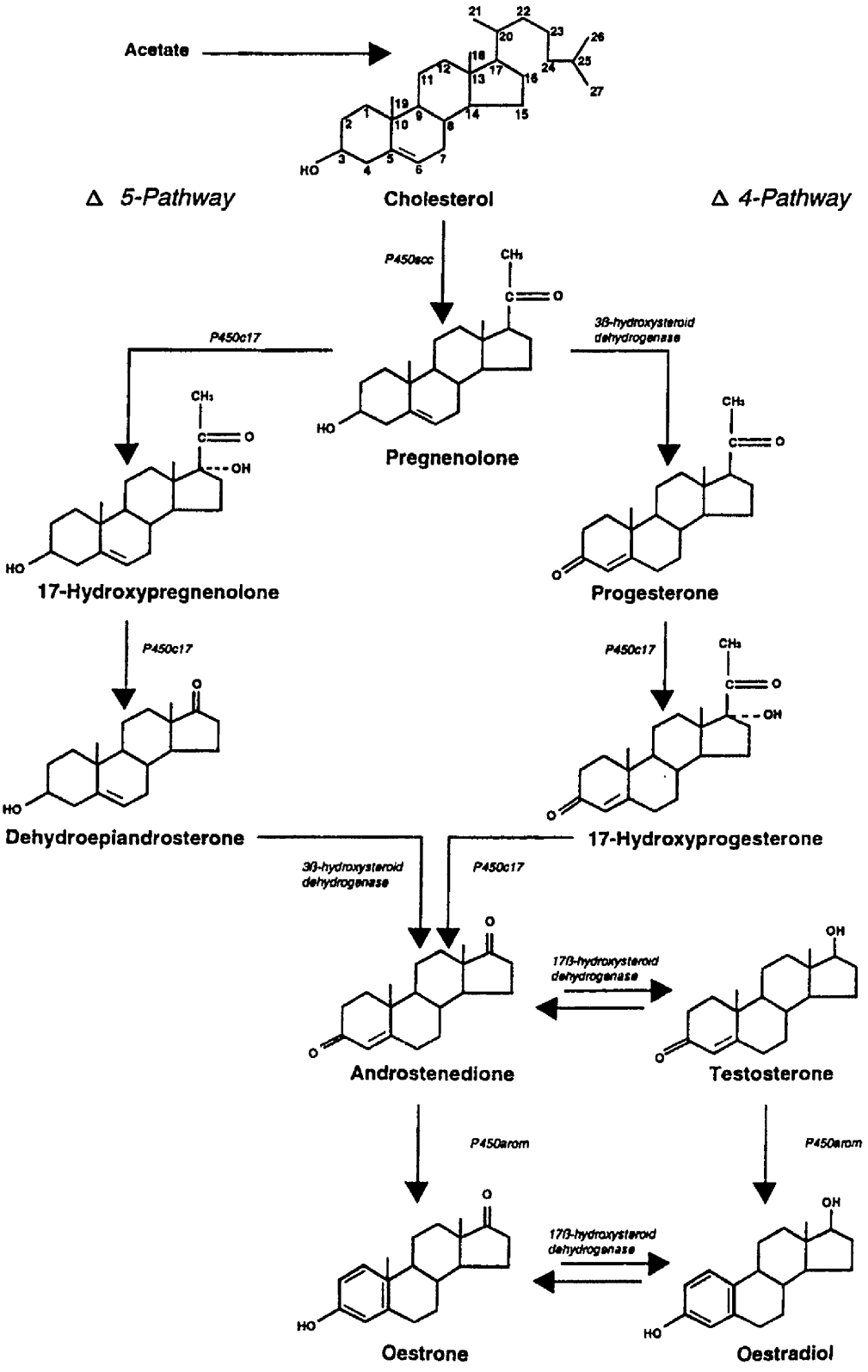


Figure 1.3 – Steroid hormone biosynthesis (adapted from Birkhauser, 1996)

All of the steps in steroidogenesis from cholesterol to pregnenolone are mediated by a single enzyme known as P450scc, which is controlled by a single gene on chromosome 15 (Speroff, Glass & Kase, 1994). This reaction, the rate limiting step in the steroid synthetic pathway, occurs within the mitochondria by hydroxylation at positions C20 and C22, and is followed by cleavage of the side chain. Thereafter, in all steroid-producing organs, steroidogenesis follows one of two basic pathways, either the pregnenolone or Δ-5 pathway via pregnenolone and dehydroepiandrostenedione, or the progesterone (Δ-4) pathway via progesterone and 17-hydroxyprogesterone (Jeffcoate, 1992).

1.2.3 Sex steroids

The sex steroids are a subgroup of the steroid hormones and are classified into three groups according to the number of carbon atoms as previously described.

The ovary possesses the full capability of every steroid-secreting endocrine organ and is thus able to synthesise all three classes of natural sex steroids namely progestogens, androgens and oestrogens, however since the ovaries are deficient in 21-hydroxylase and 11-hydroxylase enzymes, they are therefore incapable of producing glucocorticoids and mineralocorticoids (Birkhauser, 1996). Natural progesterone is secreted by the ovary, principally by the corpus luteum. In the female androgens are produced by the ovaries and the adrenal glands. Ovarian androgens are synthesised by the theca cells and pass into the granulosa cells where they are aromatised to oestrogens. The initial growth of the primordial follicle is not hormone-dependent and is not controlled by gonadotrophins. Later, follicular growth is dependent on follicle-stimulating hormone (FSH), as is oestradiol secretion. FSH induces its own receptors on the cell membrane of granulosa cells. Theca cells are dependent on luteinising hormone (LH) and are responsible for the synthesis of androgens. These diffuse into the granulosa cell layer, where the FSH-dependent aromatase (P450arom) reaction converts them into oestrogens (Erikson, 1986).

During the fertile period, oestradiol is the dominant oestrogen, secreted mainly by the ovary. Androstendione and testosterone are synthesised by theca cells (under the control of LH) and then pass to the granulosa cells, where they are aromatised (under FSH control) to oestrone and oestradiol (Birkhauser, 1996). After the menopause, the main

oestrogen source is peripheral or acyclical aromatisation of androgens to oestrone in adipose tissue, skin, and muscle (Chard & Lilford, 1995). Oestrone is the dominant oestrogen after menopause, circulating mainly in the reversible form of oestrone sulphate, which is 90% bound to albumin. Oestradiol binds specifically (60%) to sex hormone binding globulin (SHBG) which has a high affinity for testosterone and oestradiol and non-specifically (38%), to albumin which has a low affinity for sex steroids (Rosner, 1990). Only 2-3% of oestradiol circulates freely (i.e. unbound), and is able to diffuse across cell membranes. Oestrone, oestrone-sulphate, and oestriol have a much lower affinity to SHBG than oestradiol but have a greater affinity for albumin. Alterations in SHBG levels change the concentration of free oestradiol and therefore its bioavailability. The production rate of oestrogens represents the sum of glandular secretion and extraglandular (peripheral) conversion from androgens to oestrogens, although the secretion rate represents only the direct (i.e. glandular) ovarian secretion of oestrogens. Circulating oestrone and oestradiol are in constant equilibrium. Their main metabolite is oestriol through hydroxylation at position sixteen (D-ring). Hydroxylation at positions two or four (A-ring) results in catecholoestrogens, which are involved in the control of gonadotrophin-releasing hormone (GnRH) secretion through modulation of several neurotransmitters (Birkhauser, 1996). Conjugation of oestrogens to water-soluble sulphates and glucuronides in the kidney and liver permits their rapid urinary and biliary excretion, although 80% of the oestrogens eliminated in bile are reabsorbed from the gut via the enterohepatic circulation (Jeffcoate, 1992).

1.2.4 Exogenous steroids

The pharmacological actions of exogenous steroids are in essence similar to the physiological actions of the endogenous hormones, particularly when they are administered as replacement therapy. In ovarian failure, oestrogen replacement therapy is the most appropriate treatment for the associated hypo-oestrogenic symptoms, with the aim of the therapeutic regimen being to provide the most effective treatment with the fewest side effects.

Oestrogens can be administered orally or parenterally, for example percutaneously, transvaginally or subcutaneously as implants. The biochemical and pharmacological effects of exogenous oestrogens will thus be dependent on the route of administration.

Natural oestrogens (oestradiol, oestrone and oestriol) have the most appropriate profile for HRT. When administered orally, pure oestrogens are metabolised by the intestinal mucosa thus limiting their oestrogenic activity (Kuhl, 1990). The absorbed steroid is then rapidly transferred to the liver via the portal venous system where further metabolism occurs thus inactivating the steroid before it reaches the systemic circulation ('first-pass' effect). In part, this can be overcome by the use of micronised oestrogen but because of the first-pass effect oral oestrogen must be given in higher doses than parenteral therapy in order to achieve symptom relief.

The use of synthetic steroids and conjugated equine oestrogens to some extent overcomes the problems associated with natural oral oestrogens. Conjugated equine oestrogens (CEE) are prepared from pregnant mares' urine and contain principally sodium oestrone sulphate, equilin sulphate and 17α-dihydroequilin. The oestrone sulphate is then absorbed in its natural form but is then hydrolysed to oestrone and oestradiol (Davey, 1995). The synthetic steroids, the most important of which is ethinyloestradiol, are rapidly absorbed from the intestine but undergo little metabolism in the liver. Unlike natural oestrogens however, they do not bind with SHBG but are converted into a sulphate resulting in the formation of a reservoir of hormone. On a weight-for-weight basis, the potency of synthetic oestrogens is considerably greater than that of natural oestrogens (Davey, 1995).

The use of parenteral oestrogen preparations is particularly helpful when oral therapy causes gastrointestinal upset and where bypassing first-pass hepatic metabolism is desirable. Percutaneous delivery systems are perhaps the most widely used parenteral route of administration, in particular transdermal oestrogen patches. These can administer oestrogen at controlled rates of 0.025 – 0.1mg/day depending on the surface area of the patches and steady-state concentrations can be achieved by changing the patches once or twice weekly, ensuring that the site of application is changed regularly to avoid any skin irritation. A variety of preparations are currently available using either the original reservoir type or the more recent matrix patches. Both oestrogen and combined oestrogen and progestogen patches are now available. Percutaneous creams or gels are increasingly popular in Europe and are gaining some popularity in the United Kingdom. A once daily dose of gel containing 3g oestradiol appears to give good symptom relief, however the daily application of the gel requires good patient compliance particularly as it is sticky when first applied. Subcutaneous oestradiol

implants (50–100mg) avoid the need for daily patient compliance and give good symptomatic relief for up to six months. The implants can easily be inserted in an outpatient clinic under local anaesthetic, and have the advantage of enabling simultaneous insertion of a testosterone implant for relief of lethargy and loss of libido.

Like natural oestrogen, pure progesterone must be given orally in a micronised form. However, unlike different oestrogen formulations which have strikingly similar effects when given in equipotent doses, progestogens have significantly different effects depending on whether they are oestrane, gonane or pregnane derivatives (Davey, 1995). Oestrane and gonane derivatives such as levonorgestrel and norethisterone are more potent and have more androgenic effects than pregnane derivatives such as medroxyprogesterone acetate (MPA). With the exception of MPA 150mg and norethisterone 200mg which are high dose progestogens used as injectable contraceptives, progestogens are otherwise generally administered orally although norethisterone can be administered topically.

The principal use of progestogen therapy in HRT preparations is to counteract the potential adverse effects of oestrogen on the endometrium, with the dose of progestogen being kept to the minimum required to prevent endometrial hyperplasia and the choice of progestogen being limited to the one with the least adverse effects. In patients in whom there is an absolute contraindication to oestrogen therapy, single agent unopposed progestogens may offer some relief of menopausal symptoms if adequate doses are used, for example a minimum of 20mg MPA per day (Schiff et al, 1980; Lobo et al, 1984) and in the case of norethisterone bone protection also (Mandell et al, 1982; Abdalla et al, 1985).

As it has been reported that the use of continuous unopposed oestrogen results in an increased incidence of both endometrial hyperplasia and endometrial carcinoma, the importance of using combined oestrogen/progestogen preparations (either as monthly or three monthly cyclical preparations or as combined continuous regimens) in non-hysterectomised women has been established (Gambrell, 1978; Grady et al, 1995, Weiderpass et al, 1999). Recent evidence however has challenged the view that the addition of a progestogen eliminates the excess risk for endometrial carcinoma. Long cycle HRT regimens (progestogen administered following twelve weeks of oestrogen administration) appear to be associated with an increased incidence

of both endometrial hyperplasia and carcinoma compared with monthly cyclical HRT regimens (Bjarnason et al, 1999). Compared with non-users, postmenopausal women who use combined therapy of oestrogen and long cycle progestogen (greater than ten days per month) may also have an increased risk of endometrial cancer, although this risk appears to be much smaller that for unopposed oestrogen or long cycle regimens (Beresford et al, 1997, Gruber et al, 1999). The use of combined continuous HRT regimens does however appear to confer endometrial protection. (Bertone & Weiderpass, 2000; Sturdee et al, 2000) Although the risk of developing endometrial cancer is increased with certain HRT regimens, the risk of death from this type of endometrial cancer does not increase proportionately. Oestrogen associated endometrial cancer is thought to be a less aggressive variant than spontaneously occurring disease, and is more likely to be diagnosed earlier in women taking oestrogen, which may account for the better survival rates reported.

Early estimates of the HRT-related risk of breast cancer were reported as a relative risk (RR) of 1.1 for all oestrogen use, suggesting a 10% increase in risk relative to no oestrogen use (Henderson, Paganini-Hill and Ross, 1991) However recent analysis of pooled data from 51 epidemiologic studies on breast cancer in HRT users, reported an overall 14% increase in RR with ever-use of HRT. The excess risk was largely confined to current and recent users, in whom the relative risk increased by 2.3% for each year of use. Overall, short-term use of oestrogen (1-2 years duration) was not associated with an increased risk of breast cancer, however a small increased risk of breast cancer related to longer duration of HRT use was identified (47 cases/1000 women using HRT for five years compared with 45/1000 in non-users) (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). Comparable findings have been reported in a large epidemiological study from Sweden (Magnusson et al, 1999), and by the Iowa Women's Health Study, a prospective study of HRT and various types of invasive carcinoma. In the above studies the majority of women (over 80% in the 1997 meta-analysis) were using oestrogen-only preparations. The effect of addition of progestogens on the incidence of breast cancer has recently been evaluated in a number of studies (Ross et al, 2000; Schairer et al, 2000). The results of these studies are in broad agreement that the risk of breast cancer is significantly increased beyond that associated with oestrogen alone following addition of a progestogen. Moreover, it appears that the risk is greater with continuous combined HRT than with opposed cyclical preparations. In spite of the increase in incidence of breast cancer an increase in

breast-cancer related mortality does not appear to be associated with HRT use, and indeed may in fact be better in HRT users which may be a reflection of earlier diagnosis. Among 984 women diagnosed with breast cancer, those who had previously used HRT had significantly longer survival overall than those who had never used HRT (RR 0.78) (Jenstrom et al, 1999). Furthermore it appears that the clinical and biological characteristics of breast cancer in users of HRT are more favourable than in non-users (Salmon et al, 1999).

The issue of HRT use in women previously treated for breast and gynaecological cancers is becoming an increasingly important area of interest. A review of the literature for studies of HRT in women treated for gynaecological malignancies, concluded on the basis of circumstantial evidence that women with stage I or II endometrial cancer could be offered HRT without increasing the risk of disease recurrence of death (Burger et al, 1999). With regard to breast cancer and subsequent HRT use there is evidence to suggest that HRT does not appear to increase breast cancer events (Vassilopoulou-Sellin et al, 1999). Nevertheless, in breast cancer survivors it would be prudent to use the lowest effective dose of oestrogen and arrange careful monitoring.

Recent observational studies have also shown a slightly increased risk (2- to 4-fold) of deep venous thrombosis and of pulmonary embolism in women currently taking HRT particularly on initiation of therapy (Daly et al, 1996; Grodstein et al, 1996 & Jick et al, 1996). These findings were supported by the findings of the HERS study (Heart and oestrogen-progestin replacement study) in which an increased risk for venous thromboembilism among women taking HRT was noted (Hulley et al, 1998). An interim report from the ongoing Women's Health Initiative (WHI) in the United States has reported similar findings (WHI, 2000). Furthermore it appears that the risk of such events is not uniform across the population. The risks are greatly increased in the presence of thrombophilias particularly if there are multiple risks (Lowe et al, 2000). Accordingly, women with proven thrombophilic defects should be counselled about this increased risk prior to commencing HRT.

1.3 Female reproductive physiology

1.3.1 Introduction

Reproductive function is controlled by the hypothalamic-pituitary-gonadal axis which functions as an integrated unit, but consists of a complex series of connections and feedback loops between the higher centres of the central nervous system and the target end organs (Browning, 1988).

The hypothalamus is composed of neural tissue and lies in the lower part of the lateral wall of the third ventricle, extending from the optic chiasma anteriorly to the mamillary bodies posteriorly. It is connected to higher centres by afferent fibres from parts of the frontal lobes and medial aspects of the temporal lobes, and by efferent fibres which pass to higher cortical areas via the thalamus. Inferiorly, it is connected to the pituitary gland by the pituitary stalk (Jeffcoate, 1992). The hypothalamus produces pituitary regulating hormones which pass down the pituitary portal system to regulate the production of pituitary trophic hormones by the anterior pituitary. The hypothalamic regulatory hormones are a diverse group of peptide hormones and include: GnRH or luteinising hormone-releasing hormone (LH-RH), corticotrophin-releasing hormone (CRH), growth hormone-releasing hormone (GH-RH), thyrotrophin-releasing hormone (TRH), growth hormone-release inhibiting hormone (somatostatin) and prolactin-inhibiting factor (PIF) or dopamine. In addition vasopressin and oxytocin released from the hypothalamic nuclei pass via the neural connections to the posterior pituitary where they are stored.

The mode of production of the regulating hormones is a neurosecretory mechanism. The hypothalamic nerve cells act both as neurones and endocrine cells. The hormones are synthesised in the cytoplasm of the neurone, pass along the nerve axon to the nerve terminal, from which they are released into the hypophyseal portal blood vessels passing to the anterior pituitary. The production of the hormones is under both tonic and cyclic control by the hypothalamus, the former being responsible for the constant day-to-day production of gonadotrophins, while the latter for the LH and FSH surges which lead to ovulation. Tonic control is principally under negative feedback control, whereas cyclic control is by a positive feedback mechanism. The secretion of hypothalamic peptides is controlled both by the higher neural centres, which receive input from

external stimuli, particularly of emotional origin, and by the feedback loop mechanisms (Jeffcoate, 1992).

In the adult male, the pituitary gland weighs approximately 500mg; it is slightly larger in women and increases in size during pregnancy. The pituitary gland lies within the sella turcica of the sphenoid bone situated below the hypothalamus with the cavernous sinuses lying laterally and the intercavernous sinuses lying anteriorly and posteriorly, and is divided into two regions with distinctive functions reflecting their differing embryological origin. The anterior pituitary or adenohypophysis arises from Rathke's Pouch, an upward invagination of the ectoderm of the roof of the primitive pharynx. The posterior pituitary or neurohypophysis arises as a similar downgrowth of neuro-ectoderm from the third ventricle in the floor of the midbrain. The hypothalamus lies immediately above the pituitary gland connected to it by the pituitary stalk. The blood supply of the pituitary gland is derived from the inferior and superior hypophyseal arteries which originate from the internal carotid arteries. The posterior lobe is supplied directly by branches of the inferior hypophyseal arteries. Branches of the superior hypophyseal arteries supply the hypothalamus and the pituitary stalk. The anterior lobe of the pituitary has no direct arterial supply and receives its blood from a capillary venous plexus known as the pituitary portal system (Jeffcoate, 1992).

The anterior pituitary secretes a variety of important trophic hormones. These include the glycoproteins FSH, LH (gonadotrophin hormones) and thyroid stimulating hormone (TSH), and the peptide hormones growth hormone (GH), adrenocorticotrophin hormone (ACTH) and prolactin. The anterior lobe of the pituitary gland is devoid of neural elements but consists of three distinct groups of cells, characterised by their staining with haemotoxylin and eosin, into chromophils (acidophils and basophils) and chromophobes. The chromophobes are thought to be reserve or resting cells capable of developing into acidophils and basophils. The chromophil cells are responsible for producing GH and prolactin (acidophils), TSH, LH and FSH (basophils) (Chard & Lilford, 1995).

FSH and LH are glycoproteins consisting of two subunits - α and β. The α subunit is the same in all pituitary glycoproteins but the β subunits differ giving each hormone its specificity. These hormones have an important combined action on the ovary, and in the hormonal control of the menstrual cycle.

Thyroid stimulating hormone is a glycoprotein which stimulates the thyroid gland to secrete two iodine-containing hormones, thyroxine (T_4) and tri-iodothyronine (T_3) which have an important role stimulating oxygen consumption, heat production and glucose mobilisation. In young children, thyroxine is essential for adequate growth and maturation.

Growth hormone is a protein hormone produced by the α cells of the anterior pituitary under the influence of GHRH and somatostatin. It is responsible for growth in children, and with other factors, for control of carbohydrate metabolism (via insulin antagonism) and lipid metabolism (principally lipolysis). It is secreted in short bursts mostly in the early nocturnal period, but secretion can be stimulated by stress, hypoglycaemia and inhibited by glucose and corticosteroids.

Prolactin is a protein hormone with a similar chemical structure to GH and human placental lactogen (HPL). Like GH, it is produced by the α cells of the anterior pituitary, however the use of specialised staining techniques has shown that it arises from a different subgroup of cells from GH. Prolactin is the only anterior pituitary hormone which is inhibited rather than stimulated by hypothalamic factors, principally by prolactin inhibiting factor or dopamine, which maintains the hormone in a state of tonic inhibition. The primary action of prolactin is on the breast, in the initiation of lactation.

Adrenocorticotrophic hormone is a polypeptide which stimulates the adrenal cortex (zona glomerulosa and zona fasciculata) to produce corticosteroids, notably cortisol which are involved in the control of protein, carbohydrate and lipid metabolism.

The posterior pituitary comprises numerous glial cells called pituicytes, which give the gland its distinct white appearance. These cells are concentrated principally in the main part of the gland but to a lesser extent are also found in the infundibulum. The remainder of the gland is composed of a multitude of nerves connecting the gland to specific hypothalamic nuclei by a rich plexus of nerves in the interior of the pituitary stalk (Jeffcoate, 1992).

The posterior pituitary produces two hormones namely oxytocin and antidiuretic hormone (ADH). The hormones synthesised in the nerve endings of the paraventricular

and supraoptic nuclei combine with protein carrier molecules (neurophysins) and are transported down the nerve axons within the pituitary stalk to the posterior lobe where they are stored in vesicles prior to release.

Both oxytocin and ADH are nonapeptides and have similar structures. ADH has its primary action on the kidney where it increases the permeability of the renal collecting system to water, and is secreted in response to alterations in plasma volume and osmolality. Oxytocin has two main actions - stimulation of the uterus and stimulation of the myoepithelial cells of the breast to cause milk ejection. It is released into the bloodstream as a result of neurogenic stimuli notably from the uterus and breast.

1.3.2 General principals of feedback control

Physiologic hormone control levels in the blood are maintained by a variety of homeostatic mechanisms which entail precise signalling between the hormone-secreting gland and the target organ. Negative feedback control (figure 1.4) is commonly employed, especially in the hypothalamic-pituitary target gland systems (Jennett, 1989)

A hypothalamic releasing hormone stimulates the synthesis and release of an anterior pituitary hormone, which in turn stimulates the production of the target organ hormone. High levels of the latter inhibit the system by decreasing hypothalamic releasing hormone synthesis and action, or by decreasing secretion of pituitary releasing hormones (long feedback loops), while low levels result in the system being activated both at hypothalamic and pituitary levels. Short loop negative feedback mechanisms exist between the pituitary and hypothalamus (in response to high levels of pituitary hormones), and an ultrashort feedback system appears to exist with self-inhibition of hypothalamic regulating hormones as their production rises (Jeffcoate, 1992). In some systems, notably the hypothalamic-pituitary-gonadal axis, there are additionally long positive feedback control mechanisms mainly between the target organ and the pituitary, but there may be a direct hypothalamic effect also (Jennett, 1989).

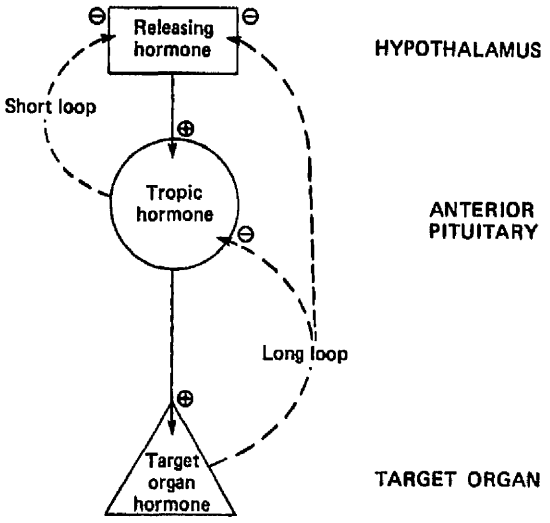


Figure 1.4 – General principals of feedback control

1.3.3 The hypothalamic-pituitary-gonadal axis

In the adult female, the control of reproductive function is a complex process dependent upon input from higher cerebral centres, and feedback from the ovaries (figure 1.5).

During the reproductive years GnRH is released in a pulsatile manner, dependent on the phase of the menstrual cycle. On reaching the anterior pituitary, the release of GnRH stimulates the synthesis and release of the gonadotrophins FSH and LH. Follicle stimulating hormone stimulates follicular development, which occurs in several phases: recruitment, selection and dominance (Shaw, 1997). During each cycle, many follicles are recruited from the pool of primordial follicles, however only one of these will be destined to ovulate. The granulosa cells of the selected follicles acquire FSH receptors and respond by proliferation, differentiation and increased aromatase activity resulting in oestrogen secretion. The rising levels of oestradiol exert a negative feedback effect at the hypothalamic-pituitary level resulting in a fall in FSH and a reduction in aromatase activity in all but the largest follicle. This dominant follicle has more granulosa cells and therefore more FSH receptors, and hence it is able to maintain its growth in spite of the low FSH levels. The continued oestradiol production in the dominant follicle accounts for the rise in serum oestradiol levels seen in the late

follicular phase of the cycle, and results in activation of a positive feedback mechanism inducing a sudden surge and release of LH (and to a lesser extent FSH), resulting in ovulation within twenty-four hours of this peak (Shaw, 1997). A temporary fall in oestrogen production that occurs with ovulation inactivates the positive-feedback mechanism. Following ovulation, LH induces the theca-granulosa cells of the collapsed follicle to transform into lutein cells, which synthesise oestrogen and progesterone, which then feedback to the hypothalamus and pituitary gland. The rising levels of oestrogen and progesterone initially inhibit FSH release, but in the absence of pregnancy the levels fall releasing the negative inhibition, and initiating a new cycle (Shaw, 1997).

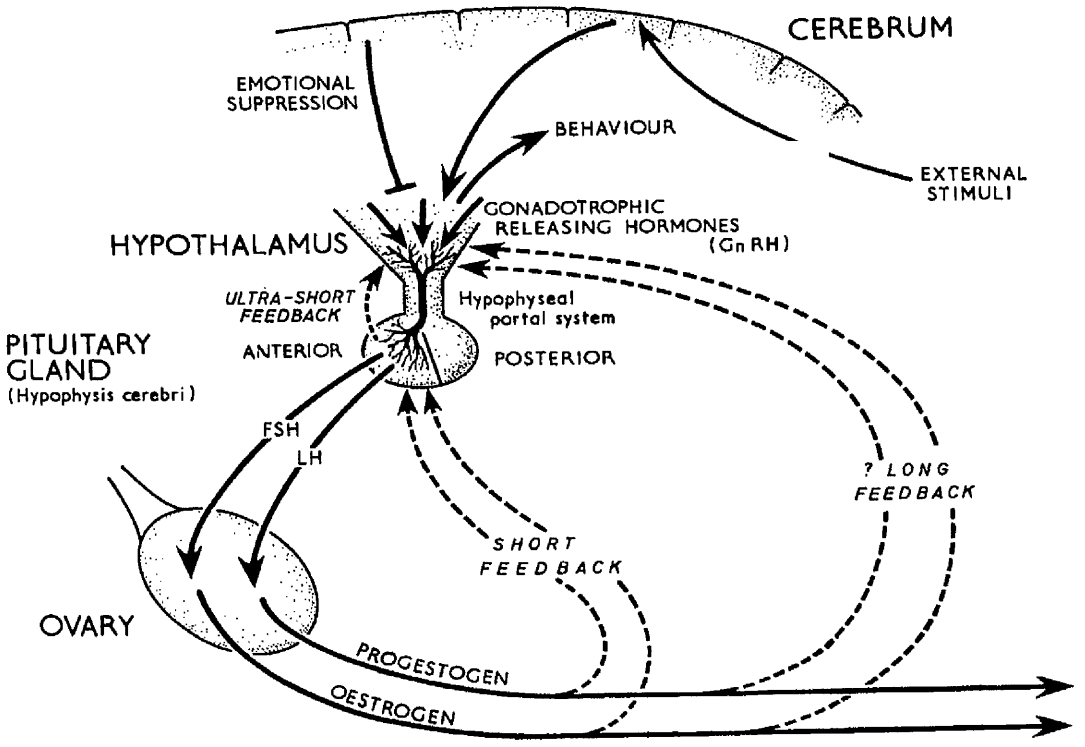


Figure 1.5 - Hypothalamic-pituitary-gonadal axis (Llewellyn-Jones, 1990)

1.3.4 The endocrinology of the perimenopause and menopause

In some women ovarian function ceases abruptly, however in general immediately prior to the menopause as a result of anovulatory cycles, there is decreased oestradiol secretion, decreased or absent progesterone secretion and a compensatory hyperactivity of the hypothalamus and pituitary gland.

The menopause is essentially an endocrinopathy characterised by ovarian failure and resulting in oestrogen deficiency. The mechanism underlying the menopause appears to be the disappearance of ovarian follicles (Faddy & Gosden, 1995). The process starts before birth, continues throughout the reproductive years, and eventually ceases during the perimenopausal period (Sherman, West & Korenman 1976). Human primordial germ cells separate from the somatic cells at an early stage of embryogenesis, migrating to the genital ridge, where they rapidly multiply to reach a maximum of seven million in each ovary around the fifth month of intrauterine life. Thereafter, multiplication ceases and the primordial follicles begin to regress leaving approximately two million follicles remaining at birth, of which fewer than 0.02% will ovulate during the reproductive phase of life. The remaining follicles will continue to degenerate throughout life, the rate of loss accelerating immediately prior to the menopause, which occurs when the number of follicles has fallen below a critical number (Richardson, Senikas & Nelson, 1987), and insufficient oestradiol is secreted to produce adequate endometrial proliferation.

From around the age of 35 years onwards, the primordial follicles which remain in the ovary become more resistant to stimulation by gonadotrophins so that in the latter stages of menstrual life the cycles become anovulatory and irregular. This ovulation failure or deficiency in corpus luteal function causes an absent or reduced progesterone secretion. Initially these anovulatory cycles are isolated occurrences, but become increasingly more frequent with increased periods of unopposed oestrogen secretion perimenopausally. The resulting fall in plasma oestradiol levels triggers the negative feedback hypothalamic-pituitary feedback mechanism resulting in an increase in FSH secretion and consequently in LH secretion also. Follicle stimulating hormone levels appear to increase from the late thirties onwards and may be related to the exponential increase in the follicular loss occurring at this time.

Immediately after the menopause the normal negative feedback mechanism of oestrogen on the higher brain centres is maintained and the reduced plasma oestradiol results in increased hypothalamic – pituitary activity causing plasma FSH and LH levels to increase rapidly to reach a maximum around two or three years after the menopause. Thereafter, the feedback mechanism appears to become reset at a lower level and both GnRH and gonadotrophin levels fall progressively over the next two decades despite the low oestradiol levels.

Gonadal glycoprotein molecules such as inhibins or activins also contribute to the synthesis and/or release or FSH. There are two major inhibin species, A and B, which comprise a dimeric structure consisting of a common α-subunit and one of two different β-subunits, β_A and β_B. Activins are dimers of the β-subunits (Burger, 1992). Inhibins appear to suppress the synthesis and/or secretion of FSH secretion by direct pituitary action, whereas the activins are probably more important in regulation of autocrine and paracrine activities, paradoxically stimulating, rather than inhibiting FSH. Inhibin levels decrease with age to undetectable levels postmenopausally. Additionally, there is considerable fluctuation during the menstrual cycle (McLachlan et al, 1987). Oestradiol levels per se are remarkably constant throughout life, suggesting that there may be a role for serum inhibin in FSH regulation during the follicular phase of the menstrual cycle as a function of increasing age. One possible explanation is that follicular exhaustion leads to decreased inhibin production resulting in a corresponding rise in FSH. Since the ovarian granulosa cells are the principal source of inhibin, the decreasing inhibin levels are consistent with the decline in primordial follicles.

During reproductive life the main circulating oestrogen is oestradiol which is produced cyclically with low levels in the early follicular phase, a peak in the late follicular phase and a second peak in the luteal phase of the cycle. Oestrone is produced in smaller amounts cyclically by the ovary, but also continuously by peripheral conversion of androstenedione. Postmenopausally, the predominant circulating plasma oestrogen changes from ovarian produced oestradiol to oestrone produced from extra-ovarian sources (Chard & Lilford, 1995). Plasma oestriol levels in postmenopausal women are similar to premenopausal women in the early follicular phase of the cycle, but are considerably less than late follicular phase levels. The plasma oestradiol:oestrone ratio therefore falls postmenopausally. The efficiency of conversion of androstenedione to oestrone increases considerably with increasing age, although the biological activity of

the resulting circulating pool of oestrogen remains low since the potency of oestrone is approximately one-tenth of that of oestradiol. Oestriol is a low potency oestrogen produced in large amounts primarily during pregnancy.

To some extent, the climacteric and the menopause could also be considered to be a progesterone deficiency state. Postmenopausally, the plasma progesterone and 17-hydroxyprogesterone levels are reduced as a consequence of anovulation and corpus luteal failure, although there is continued background progesterone production, almost exclusively of adrenal origin.

In Westernised industrialised societies the median age of the menopause is consistently reported as 50 years, the median rather than mean age being used to describe the distribution since it is negatively skewed (Lindquist, 1982). Women who smoke experience the menopause up to two years earlier than non-smokers, and moreover the effects of smoking appear to be dose related (Baron, Adams & Ward, 1988; Longcope & Johnstone, 1988).

1.4 Cardiovascular disease and the menopause

1.4.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death and an important cause of disability in Western society. The well-recognised male excess in CVD has often obscured the fact that it is also the commonest cause of death in women, however the demography of the disease clearly differs between the sexes. The incidence of CVD in women prior to the menopause is lower than that in age-matched men, however after the menopause the incidence is similar to that in men, suggesting that women are relatively more protected from CVD until after the menopause when a steep rise in the incidence of CVD is observed (table 1.1).

Although men and women share many risk factors for CVD including the non-modifiable factors such as age, family history and genetic predisposition, and the so-called modifiable risk factors such as cigarette smoking, hypertension, dyslipidaemias and diabetes, the menopause appears to be an additional risk factor for women.

1.4.2 Cardiovascular disease epidemiology in women

Evidence from retrospective observational studies comparing the incidence and severity of CVD in men and women has shown, almost without exception, that the incidence of CVD in premenopausal women is much lower than in men of similar age (Castelli WP, 1984; Barrett-Connor & Bush, 1991; Wenger, Speroff & Packard, 1993). The association of premature menopause with increased cardiovascular risk (Colditz et al, 1987), and correspondingly of late menopause with reduced risk, (Persson et al, 1997) lend further support to this argument. Moreover, with the exception of the Framingham data (Gordon et al, 1978), there is an abundance of evidence in the literature that HRT has a favourable influence on cardiovascular morbidity and mortality (Stampfer & Colditz, 1991; Grodstein & Stampfer, 1995, Barrett-Connor, 1996).

Observational data is of course subject to the criticism that it is likely to be biased and the interpretation of these observational data has recently been questioned, and is now the subject of considerable debate. It is argued that if age-specific mortality rates are plotted on a semi-logarithmic scale, the exponential increase with age appears to be

remarkably constant in both sexes, with no sudden change in the rate in women around the time of the menopause and no apparent closure of the gender gap (Newton, 1998; Tunstall-Pedoe, 1998). This debate is likely to continue until the results of adequately powered long term randomised controlled studies are available, although early evidence from the HERS study would tend to favour the arguments of Tungstall-Pedoe and Newton. Despite the obvious limitations of the observational data, it is a widely held belief that the undoubted trend towards cardioprotection seen in the vast majority of studies published would refute the idea that this is an entirely spurious observation.

Age	Sex	Ischaemic heart disease	Stroke	Cancer (all causes)
15-34	M	1.6	1.3	6.1
	F	2.6	3.7	16.8
35-44	M	15.2	4.2	16.7
	F	3.7	6.9	43.4
45-64	M	30.9	5.7	32.7
	F	17.2	7.3	45.2
65-74	M	31.7	8.8	32.6
	F	25.1	10.7	34.7
>75	M	26.2	13.2	23.1
	F	23.3	19.3	16.0

Table 1.1 – Cause of death by age and sex (expressed as % total deaths) (Health in Scotland, 1995)

1.5 Pathophysiology of atherosclerosis

1.5.1 Introduction

Atherosclerosis is a complex disease of multiple aetiology resulting from a series of molecular and cellular changes arising from within the vessel wall amid a variety of genetic and environmental influences. In the 19th century there were two main hypotheses to explain the pathogenesis of atherosclerosis. The 'incrustation' hypothesis of von Rokitansky proposed in 1852 and subsequently modified by Duguid in 1946 (cited by Fuster, 1992), suggested that intimal thickening resulted from fibrin deposition with subsequent organisation by fibroblasts and secondary lipid accumulation. The 'lipid hypothesis' proposed by Virchow in 1856 (cited by Fuster, 1992), suggested that the lipid which accumulated in the arterial wall was a transudate of blood lipid which subsequently formed complexes with acid mucopolysaccharides. Lipid then accumulated in the arterial walls because the mechanisms of lipid deposition dominated over those of removal. These two hypotheses have subsequently been integrated into a more complex 'response to injury' theory proposed by Ross (Ross, 1986).

1.5.2 Atherosclerotic lesions

Atherosclerosis begins in the first decade of life with the appearance of fatty streaks, the first grossly visible evidence of this disease (Strong, 1992). The term fatty streak or Type II lesion refers to the appearance of yellow, slightly raised areas that can be seen as dots or streaks on gross inspection of the endothelial surface of large arteries. Fatty streaks appear microscopically in the intima as slightly raised areas composed of fat-filled cells known as foam cells. Both macrophage-derived and smooth muscle cell-derived foam cells can be seen, although macrophage foam cells are thought to be the first to appear and predominate in fatty streaks (Stary et al, 1995). Cholesterol esters are the primary lipid of fatty streaks and at this stage, most are present as droplets within macrophage foam cells. The extracellular space does however also contain minute lipid droplets and vesicules which may represent the initial changes that lead to fatty streak formation (Simionescu & Simionescu, 1993).

Up to about 20 years of age, the frequency of fatty streaks in the aorta and coronary arteries is similar for all populations, regardless of the prevalence of atherosclerosis and

the rate of coronary heart disease in that population as adults (McGill, 1968). Thus, the development of fatty streaks may be a normal response that initially protects the arterial wall from the effects of accumulating plasma lipoproteins, but which ultimately provides the foundation of a pathologic process that is accelerated in the presence of excess environmental risk factors.

Some fatty streaks appear to progress to more advanced atherosclerotic lesions. Intermediate or Type III lesions have been identified in atherosclerotic arteries from human and animal models which appear to bridge the gap in the continuum between fatty streaks and the more complex lesions. Macrophages continue to predominate in intermediate lesions, but smooth muscle cells are present in greater numbers. There is more extracellular lipid, and small pools of lipid droplets can be seen aligned along layers of smooth muscle cells. These lesions appear to progress to atheromatous (Type IV lesions) and ultimately to fibroatheromatous lesions (Type V lesions), which together represent the first of the clinically dangerous advanced lesions. True atheromatous lesions are characterised by a dense extracellular lipid core and both macrophages and smooth muscle cells are present, with and without lipid droplets, along with some lymphocytes and occasional mast cells. There is often a proteoglycan-rich layer between the lipid core and the endothelium, but at this stage there may be little evidence of a fibrous cap. Type V lesions are very similar, with the exception that there is an increase in fibrous tissue, particularly collagen, over the lipid core, and complicated features such as calcification and fibrosis often are seen. The Type IV and V lesions can restrict the lumen of the artery by up to about 50%, but are not occlusive. Nevertheless, these types of lesions represent the most clinically dangerous of the atherosclerotic plaques because of their susceptibility to plaque rupture and thrombosis (Brown et al, 1993). Eccentric Type IV and V atherosclerotic plaques that are rich in macrophage foam cells at the periphery of the lesion appear most susceptible to rupture. Type VI lesions are often referred to as complicated plaques because of the presence of disruptions in the integrity of the endothelial surface, such as ulceration, calcification, and thrombosis.

1.5.3 Cellular events in atherosclerosis

The hallmark of atherosclerosis is the accumulation of cholesterol, particularly cholesterol esters in the arterial wall. This occurs initially in macrophage foam cells, however as the lesions become more severe, cholesterol esters and cholesterol monohydrate crystals are also found extracellularly. Many of the subsequent changes associated with more severe atherosclerotic lesions can be attributed to macrophage foam cells and their secretory products. The origin of the cholesterol that accumulates in the atherosclerotic plaque is the plasma lipoproteins (Dayton & Hashimoto, 1970). It is now known that plasma lipoproteins normally pass from the blood into the arterial wall. As long as endothelial cells remain intact, their junctions are too tight to allow molecules much larger than albumin to pass between cells. Because lipoproteins are much larger than albumin, these normally pass through endothelial cells by a process of transecytosis via plasmalemma vesicles to the subendothelial compartment (Simionescu & Simionescu, 1993). This process is independent of lipoprotein receptors and functions for all lipoproteins. Because of this, the absolute amount of lipoprotein transported into the arterial wall is largely dependent on the concentration of lipoproteins in the blood.

Atherosclerosis does not occur uniformly in all arteries or regions of the same artery but instead occurs focally at specific sites, such as the proximal main coronary arteries, abdominal aorta, bifurcations and branch points of arteries. This has led to speculation that this site-specific predilection may be caused by localised damage of endothelial cells by haemodynamic factors, resulting in greater entry of plasma lipoproteins at these sites. Although not much is known about the nature of these early events, several investigators have identified lipoprotein-like particles from the arterial wall of experimental animals with altered physiochemical properties that may represent the form by which lipoprotein cholesterol first accumulates in the intima before being taken up by macrophages (Simionescu & Simionescu, 1993). This is also consistent with the initial or type I lesion that includes the appearance in the intima of microscopically and chemically detectable lipid-containing vesicles and associated small isolated groups of macrophage foam cells (Stary et al, 1995). The extracellular lipid vesicles resemble the lipoprotein-like particles from the arterial wall of cholesterol-fed animals. One explanation for these observations is oxidation of the lipid trapped within the intima resulting in chemical signals being sent to the overlying endothelial cells causing expression of adhesion molecules that bind monocytes and T-cells (Berliner et al,

1990). Monocyte adhesion is probably one of the earliest morphologically detectable cellular events in the pathogenesis of atherosclerosis (Joris et al, 1983), however it is not clear whether oxidation of extracellular lipoprotein-like vesicles represents the only trigger for monocyte recruitment into the artery wall. The principal monocyte adhesion molecules on endothelial cells include intercellular adhesion molecules (ICAM-1&2), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1) (Faruqui & Dicorleto, 1993). Leucocyte recruitment is a complex process that includes a cell rolling phase followed by adhesion to endothelial cells via some of the previously described adhesion molecules, and ultimately migration down a chemoattractant gradient (Lasky, 1992). This process has characteristic features of a chronic inflammatory response. Consistent with this, neutrophils are not generally seen in atherosclerotic plaques. The reason for this cellular specificity appears to be the type of endothelial adhesion molecules which are expressed. In addition to the generation of adhesion molecules on endothelial cells, the oxidation of lipids in lipoproteins results in the production of chemoattractant molecules that attract monocyte macrophages to specific sites.

Smooth muscle cells and smooth muscle derived foam cells are also found in atherosclerotic plaques, although are usually found at a later stage in plaque development than macrophages (Ross, 1986). Macrophages appear early in the pathogenesis of atherosclerosis. Included in the many proteins secreted by macrophages are a number of growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and chemoattractants such as monocyte chemotactic protein-1 (MCP-1) (Ross, 1993). The secretion of these factors probably represents the principal stimulus for recruitment of smooth muscle cells from the media of the artery into the intima, where they are stimulated to proliferate. The smooth muscle cells of atherosclerotic lesions exist primarily in a synthetic phenotype in which they express genes for a number of growth-regulatory molecules and cytokines (Libby et al, 1988), and additionally can synthesise extracellular matrix proteins (Ross, 1993). Smooth muscle cells may also have an important role in stabilising atherosclerotic plaques by virtue of their role in formation of a fibromuscular cap. Smooth muscle cells produce a variety of collagen subtypes which accumulate in atherosclerotic plaques, as well as proteoglycans found in the extracellular matrix and on the surface of cells (Wight, 1989). The proteoglycans, by virtue of their ability to bind some lipoproteins more avidly than others, are thought to play an important role in mediating the flux of plasma lipoproteins through the arterial wall and also perhaps the length of time a lipoprotein

spends in the arterial wall. This could be important relative to susceptibility to oxidation or any other change that would cause the lipoprotein to be recognised for uptake by macrophages. Proteoglycans may also mediate cell proliferation and the binding of lipoproteins to macrophages and smooth muscle cells (Wight, 1989). Smooth muscle cells can accumulate cholesterol esters to become foam cells and are seen in types IV, V, and VI lesions, although the precise mechanisms of smooth muscle foam cell formation are unknown. One potential mechanism is via active scavenging of lipids with subsequent formation of lipid inclusion bodies (Wolfbauer et al, 1986).

Macrophage proliferation in atherosclerotic plaques has been recognised and this in part accounts for necrotic debris found in atherosclerotic lesions (Rekhter & Gordon, 1995). It also has been shown that dead macrophages are phagocytosed by other macrophages (Lewis, Taylor & Jerome, 1985), and that they have the potential to migrate out of the atherosclerotic plaque, possibly carrying lipids and other plaque constituents after phagocytosing debris, (Gerrity, 1981) and thus may have a potential role in preventing severe atherosclerotic lesions from forming.

1.6 Cardiovascular disease mechanisms and vascular protective effects of oestrogen

1.6.1 Introduction

Since CVD rarely affects women before the menopause, and the incidence appears to accelerate after the menopause, oestrogen deficiency has been strongly implicated in the aetiology of the disease in women and suggests that oestrogen replacement therapy will decrease subsequent risk of CVD.

Loss of ovarian function at the menopause with a consequent decrease in plasma oestrogen levels, results in a number of detrimental effects on cardiovascular risk factors including both direct arterial effects and adverse effects on metabolic risk factors such as plasma lipids, insulin sensitivity, body fat distribution and haemostasis. Oestrogen replacement therapy appears to have the capacity to reverse the negative effects of the menopause on some or all of these cardiovascular risk factors. A clearer understanding of the mechanisms whereby oestrogen exerts its cardiovascular benefits, will allow optimisation of hormone replacement therapy to achieve maximum benefit, whilst eliminating unwanted side effects.

1.6.2 Oestrogen receptors

The presence of oestrogen receptors in vascular tissues has been inferred from a number of animal studies which have demonstrated specific binding of oestrogen to vascular cells (Colburn & Buonassisi, 1978; Nakao et al, 1981; Horwitz & Horwitz, 1982). Oestrogen, like all steroid hormones, classically acts by binding to nuclear receptors and initiating a sequence of events leading to modulation of gene expression.

The oestrogen receptor belongs to a nuclear receptor superfamily, a group of ligand-activated transcription factors (Mangelsdorf et al, 1995). Steroid hormone receptors consist of a hypervariable N-terminal domain that contributes to the transactivation function; a highly conserved central domain responsible for specific DNA binding (steroid response elements), dimerisation, and nuclear localisation, and a C-terminal domain involved in ligand binding and ligand-dependent transactivation function. Following binding of oestrogen to the receptor, resulting in homodimerisation of the

receptor and activation of the oestrogen response elements and transcriptional co-activators, there is alteration in the level of transcription of specific genes, leading to altered messenger ribonucleic acid (mRNA) synthesis and ultimately to altered protein synthesis.

Until recently, only a single major form of the oestrogen receptor (ER-α) had been identified (Green et al, 1986; Greene et al, 1986). However, a second protein encoding a separate subtype (ER-β) has now been detected although the significance of this observation is not fully appreciated but may explain the selective actions of oestrogens in a variety of target tissues (Kuiper et al, 1996; Mosselmann, Polman & Dijkema, 1996; Venko, Rankin & Vaughan,1996). Both ER-α and ER-β bind oestradiol with similar high affinity, however the relative tissue distributions of the receptor subtypes appear less uniform (Enmark et al, 1997).

Oestrogen receptors have been identified in myocardial, vascular smooth muscle cells and endothelial cells in both humans and animals (Orimo et al, 1993; Karas, Patterson & Medelsohn, 1994; Bayard et al, 1995). Additionally, heterogeneity of oestrogen receptor distribution between vascular beds in male and female animals, and altered oestrogen receptor expression between normal and atherosclerotic vessels has been reported (Knauthe et al, 1996; Losordo et al, 1994). These observations suggest that the cardioprotective effects of oestradiol may in part be mediated via altered oestrogen receptor expression in the vasculature.

1.6.3 Metabolic risk factors

1.6.3.1 Lipids

An association between high circulating levels of cholesterol and an increased incidence of CVD is well established. Low density lipoproteins (LDL) form a large portion of total plasma cholesterol in most individuals, and increased concentrations are positively correlated with an increased risk of CVD. Conversely, high density lipoproteins (HDL) are inversely associated with cardiovascular risk with high levels conferring cardioprotection. (Miller, 1987) Hypertriglyceridaemia is also a risk factor for CVD, and appears to be a particularly important risk factor in postmenopausal women (Bengstsson et al, 1993).

LDL cholesterol comprises of various subclasses which differ in size and structure. Lipoprotein (a) is an LDL which contains two distinct apolipoproteins, apolipoprotein B and apolipoprotein (a), the latter being cited as an independent marker for ischaemic cardiovascular disease with high levels being associated with an increased risk (Utermann, 1989). Lipoprotein (a) is atherogenic largely because of its propensity for retention into the arterial wall as it binds readily with arterial proteoglycans and also enhances LDL retention (Dahlen, Ericson & Berg, 1978; Yashiro, O'Neil & Hoff, 1993). LDL particle size varies and can be classified into subgroups accordingly. Small dense LDL particles (subtype B) appear to be clinically important due to their strong association with cardiovascular disease (Austin et al, 1988). The clearance of LDL from the circulation is via hepatic receptors which operate at near saturation and thus if LDL levels are high the process of clearance will be slow (Krauss, 1991). Like LDL, HDL cholesterol exists in various subtypes of which the HDL_2 subfraction is considered to be the most beneficial. HDL participates in reverse cholesterol transport removing cholesterol from the tissues then returning it to the liver where it is catabolised by the action of hepatic lipase and excreted.

The changes in lipids associated with the menopause are all in a direction consistent with increased cardiovascular risk. With ageing, total cholesterol, LDL cholesterol and triglyceride levels all increase while HDL cholesterol levels fall, however at the menopause these changes are more marked and the detrimental change in lipid profile is greater (Stevenson, Crook & Godsland, 1993). Other adverse changes reported include an increase in lipoprotein (a) (Meilahn et al 1991) and a shift towards smaller more dense LDL particles (Campos, McNamara & Wilson, 1988).

Approximately 50% of the cardioprotective benefit attributed to HRT is thought to be as a result of beneficial effects on lipid profiles (Stampfer & Colditz, 1991). The effects of HRT on lipids and lipoproteins will depend on the type of oestrogen, its dose and its route of administration. In addition, the oestrogenic effects will be modified if progestogens are used in combination with oestrogen. Independent of the type of steroid or the dose of oestrogen, there is sustained reduction in total cholesterol with long term oestrogen therapy (Whitcroft et al, 1994). This action is primarily via a reduction in LDL cholesterol by upregulation of apolipoprotein B receptors and is most efficient with oral administration. Additionally, protection against lipoprotein oxidation (Sack, Rader & Cannon, 1994) and improvements in postprandial clearance of potentially

atherogenic lipoprotein particles has been reported with oestrogen use (Westerveld et al, 1995). Oral oestrogens increase HDL cholesterol, particularly the protective HDL_2 subfraction, however transdermal preparations appear to have a less marked effect (Crook et al, 1992). The effect of HRT on triglycerides is dependent on both the type and route of administration. Conjugated equine oestrogens increase triglycerides (Crook et al, 1992), and other oral oestrogens may exert similar effects (Crook et al, 1997). Conversely transdermal oestradiol causes a reduction in triglycerides (Crook et al, 1992). The effect of progestogens on lipid profiles is largely dependent on the androgenicity of the progestogen used, but overall does not appear to negate the beneficial effects of oestrogens.

1.6.3.2 Carbohydrate metabolism

Diabetes is strongly associated with cardiovascular disease. Additionally, diabetic women have a higher incidence of cardiovascular disease than diabetic men (Abbott et al, 1987), suggesting that women are more adversely affected by diabetes and thus that the effects of sex steroids on glucose and insulin metabolism may be significant. Impaired glucose tolerance resulting from reduced sensitivity of target tissues to insulin-mediated glucose uptake is associated with an approximate doubling in the risk of ischaemic heart disease (Jarrett, McCartney & Keen, 1982; Fuller et al, 1983), and it has been proposed that insulin resistance and hyperinsulinaemia are pivotal disturbances in the aetiology of coronary heart disease in non-diabetic subjects (Reaven, 1988) either directly by promoting atherogenesis (Stout, 1990) or by adversely affecting other cardiovascular risk factors such as dyslipidaemias (Ley, Lees & Stevenson, 1992) or hypertension.(Ferrannini et al, 1987).

Glucose metabolism deteriorates with increasing age (Stevenson et al, 1994) and is also influenced by the menopause although it is difficult to determine changes due to the menopause from changes attributed to age alone. Glucose and insulin profiles of pre- and postmenopausal women are similar, however there is evidence suggesting changes in insulin metabolism in postmenopausal women with an increase in insulin sensitivity, a decrease in non-insulin dependent glucose disposal, plasma C-peptide response and pancreatic insulin secretion (Walton et al, 1993).

Studies of insulin sensitivity in postmenopausal women using hormone replacement therapy are complicated by the use of different methods of measurement and experimental design and in consequence their results are not uniform. Some of the discrepancies may however be due to the route of hormone administration. Oral administration of oestrogen is associated with a decrease in insulin sensitivity (Barrett-Connor & Laakso, 1990) while transdermal preparations appear to have either a neutral or beneficial effect (Cagnacci et al, 1992; Duncan et al, 1999).

1.6.3.3 Body fat distribution

Although obesity itself is a risk factor for CVD, there is additionally an association between body fat distribution and cardiovascular risk that is independent of obesity. Premenopausal gynoid or peripheral fat distribution is associated with low cardiovascular risk, however android or central body fat distribution found postmenopausally is associated with increased cardiovascular risk (Ley, Lees & Stevenson, 1992). This apparent redistribution of fat in women after the menopause therefore predisposes to an increased cardiovascular risk and is suggestive of a role for oestrogen in its aetiology. Additionally there is evidence that HRT prevents an increase in abdominal fat and redistributes it to the more favourable gynoid distribution with relatively little effect on the overall total body fat. (Haarbo et al, 1991)

1.6.3.4 Haemostasis

Thrombogenesis has a pivotal role in the pathogenesis of atherosclerosis and haemostatic factors have an important role in this process. The haemostatic system exists in a state of dynamic equilibrium which is achieved by a complex interplay between coagulation and fibrinolysis. Haemostatic regulation occurs at the endothelial surface and exhibits continuous baseline activity. Reduced baseline fibrinolytic activity and increased baseline coagulatory activity are both implicated in the development of CVD.

The menopause appears to have a variety of effects on haemostasis although it is difficult to separate these effects from ageing alone. The pro-coagulatory factors Factor VII (F VII) (Scarabin et al, 1990) and plasma fibrinogen (Folsom et al, 1991) increase predisposing to an increased risk of arterial thrombosis due to hypercoagulability.

Plasminogen activator inhibitor-1 (PAI-1), the principal intra- and extra-vascular inhibitor of fibrinogen activity is also increased (Heinrich et al, 1991). The pro-fibrinolytic factors anti-thrombin III and plasminogen also increase. The overall effect is that the balance between coagulation and fibrinolysis is not significantly altered.

The effect of HRT on haemostasis is complex and the magnitude of the effect observed will depend on the type and dose of the preparation as well as the mode of delivery. Oral oestrogen therapy is associated with an increase in both F V11 antigen and activated F VII levels (Cushman[1] et al, 1999; Vehkavaara et al, 2001). However, a reduction in plasma fibrinogen levels towards premenopausal values can be achieved by oestrogen administration (Writing group for the PEPI trial, 1995; Cushman[2] et al, 1999). A reduction in PAI-1 levels with oral oestrogen (either alone or in combination with a progestogen) has consistently been reported (Scarabin et al, 1993; Vehkavaara et al, 2001), although similar reductions have not been observed with transdermal oestrogen use (Koh et al, 1997; Vehkavaara et al, 2001). Antithrombin III levels also fall in a dose dependent fashion with oral oestrogen (Kroon, Silfverstolpe & Tengborne 1994). A reduction in tPA in association with oral oestrogen may impair fibrinolysis and thus promote an adverse pro-coagulatory shift (van Baal et al, 2000; Giltay et al, 2000).

The liver is an important site for the manufacture and metabolism of coagulation factors. Evidence suggests that the effect of transdermal HRT on haemostatic variables is less significant than oral oestrogen therapy and it is suggested that this is due to the reduced first pass hepatic metabolism which occurs with transdermal preparations (Kroon, Silfverstolpe & Tengborne 1994). Although not yet proven, it may be that transdermal preparations reduce the risk of deep venous thrombosis.

1.6.3.5 Inflammatory Mediators

C-Reactive protein (CRP) is a mediator of inflammation and a strong predictor of cardiovascular events (Ridker et al, 2000). Increased levels of CRP have been reported following unopposed oestrogen use (Cushman[1] et al, 1999) and similarly following combined therapy with conjugated oestrogen and MPA (Walsh et al, 2000). Again there appears to be a differential response relating to the mode of delivery, with no apparent effect on CRP levels with the transdermal route of administration (Vehkavaara et al, 2001). Elevated plasma homocysteine levels are also a marker of increased risk of

CVD. Recent evidence suggests that both opposed and unopposed HRT regimens may reduce fasting homocysteine levels (van Baal et al, 2000; Walsh et al, 2000).

1.7 Vascular reactivity

1.7.1 Introduction

Sex steroids are vasoactive substances which have systemic vascular effects that are independent of effects of oestrogen on the reproductive vascular system. Genomic dependent mechanisms are thought to be involved in long term vascular protection following chronic exposure to oestrogen, however acute responses in the vasculature are unlikely to be mediated by genomic mechanisms as they occur very rapidly. A number of mechanisms have thus been suggested to explain the direct vascular effects of oestrogen including direct effects on vascular smooth muscle resulting in relaxation and indirect effects via the endothelium.

1.7.2 Endothelium independent mechanisms of smooth muscle relaxation

Animal and human studies have shown that oestrogen can induce direct relaxation of blood vessels in the coronary vasculature via direct smooth muscle relaxation independently of the endothelium (Jiang et al, 1991; Mugge et al, 1993; Chester et al, 1995). A number of potential mechanisms to explain these observations have been proposed including inhibition of the release of, or response to, vascular constricting factors such as endothelin (Mueck et al, 1993; Polderman et al, 1993) and angiotensin II (Cheng & Gruetter, 1992) or via a calcium antagonistic mechanism. (Jiang et al, 1991; Jiang et al, 1992; Han et al, 1995).

1.7.3 Endothelium dependent mechanisms of smooth muscle relaxation

1.7.3.1 Introduction

The endothelium is a monolayer of cells lining the intimal surface of the vasculature. It has a pivotal role in the modulation of vasomotor tone primarily by elaboration of a variety of substances, the most important of which is nitric oxide (NO).

In 1980 Furchgott identified that endothelial cells had an obligatory role in the vascular relaxation to the vasodilator acetylcholine (ACh) (Furchgott & Zawadzki, 1980). He proposed that endothelial muscarinic receptors liberated a substance which activated the

underlying vascular smooth muscle cells when activated by ACh, and referred to this substance as endothelial derived relaxing factor (EDRF). Six years after this initial discovery Furchgott suggested that EDRF might be NO, and within a year it was confirmed that the release of NO could account for the bioactivity of EDRF (Palmer, Ferrige & Moncada 1987; Ignarro et al 1987).

Nitric oxide is a ubiquitous, naturally occurring gaseous molecule found in a wide variety of cell types and organ systems. It is a small diatomic free radical with a very short half-life and is thus highly reactive combining readily with other free radicals. It is soluble in many solvents, although its solubility in water is low, and can diffuse relatively easily across biological membranes. In biological systems it decomposes very rapidly to nitrate and nitrite. The very short half-life of NO and its high reactivity suggests a likely role as a local messenger transferring messages within and between individual cells.

1.7.3.2 Nitric oxide synthase enzymes

Nitric oxide is synthesised via a five electron oxidation of a terminal guanidinium nitrogen on the amino acid L-arginine. The reaction, which is catalysed by a nitric oxide synthase (NOS) enzyme, is both oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) dependent and yields the co-product L-citrulline in addition to NO (Figure 1.6). The physiological actions of NO are mediated by activation of soluble guanylate cyclase and subsequent increase in the concentration of cyclic guanosine monophophate (cGMP) in the target cells (Ignarro, 1991).

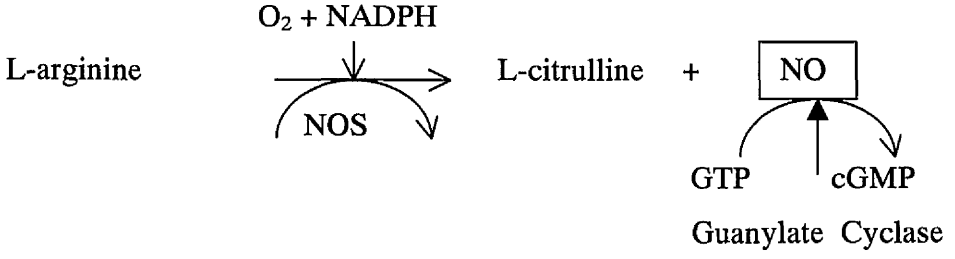


Figure 1.6 – Biosynthesis of nitric oxide

Nitric oxide synthase is a haem-containing enzyme with a sequence similar to cytochrome P-450 reductase. Several isoforms of NOS are now known to exist, two of which are constitutive (endothelial and neuronal) and calcium and calmodulin dependent, and one of which is inducible by immunological stimuli independently of calcium. The genes for these enzymes have been localised to chromosome 7 (endothelial or eNOS), chromosome 12 (neuronal or nNOS) and chromosome 15 (inducible or iNOS). Across species any one of the three isoforms is highly conserved with 85-90% sequence homology (Knowles & Moncada, 1994). In contrast however, the three isoforms share only 50-55% sequence identity with each other, although in certain regions of the proteins there is high sequence identity particularly in the domains that contain binding sites for various cofactors. These subtle but significant structural differences probably account for differences between the isoforms such as localisation (eNOS – membrane; nNOS and iNOS – cytosolic) and affinity for calcium and calmodulin binding (Cooke & Dzau, 1997). The three NOS isoforms were originally named after the tissues in which they were first cloned and characterised. The constitutive NOS (cNOS) was first identified in the vascular endothelium and in the brain, spinal cord and peripheral nervous system, and have been designated the terms eNOS and nNOS respectively. The iNOS isoform is expressed only after activation of cells by infective or inflammatory mediators. It can be expressed in many cell types including vascular smooth muscle, cardiac muscle, and immune cells. As this is an inducible enzyme it produces substantially greater amounts of NO than the other isoforms. All three isoforms have now been identified in other tissues. The eNOS isoform being present in platelets, (Radomski, Palmer & Moncada, 1990) certain areas of the brain (Dinerman et al, 1994) and in cardiac tissue both myocardial and endocardial, while the nNOS isoform has been identified in respiratory tract epithelium (Kobzik et al, 1993) and in skeletal muscle (Kobzik et al, 1994). Although in general eNOS and nNOS are considered constitutive enzymes, under certain circumstances the expression of eNOS and nNOS is inducible. Shear stress up-regulates the expression of eNOS (Kanai et al, 1995), and pregnancy and exogenous oestrogen treatment induce both eNOS and nNOS expression (Weiner et al, 1994). Although iNOS is regulated by cytokines and induced during the immune response, in certain circumstances it can also be constitutively expressed, the enzyme having been identified in fetal tissues, (Baylis et al, 1994) in human bronchial epithelium and alveolar macrophages (Kobzik et al, 1993) and rat kidney (Mohaupt et al, 1994) in the absence of immune activation.

1.7.3.3 Role of nitric oxide

Nitric oxide plays a diverse role in many physiological functions. Its formation in vascular endothelial cells, in response to chemical stimuli and to physical stimuli such as shear stress, maintains vasodilator tone essential for regulation of blood flow and pressure (Moncada, Palmer & Higgs, 1989; Vanhoutte, 1989; Luscher, 1991; Vane, Anggard & Botting, 1990). Nitric oxide produced by the endothelium also inhibits platelet aggregation and adhesion, inhibits leucocyte adhesion and modulates smooth muscle cell proliferation, (Moncada & Higgs, 1993) and is thought to retard the development of atherosclerosis. Nitric oxide is synthesised in neurones of the central nervous system, where it acts as a neuromediator with a variety of physiological functions (Garthwaite, 1991; Synder & Bredt, 1992). In the peripheral nervous system, NO is released from many nerve endings which mediate some forms of neurogenic vasodilatation in addition to regulating gastrointestinal, respiratory and genitourinary functions (Rand, 1992; Synder & Bredt, 1992). In addition, NO is generated in copious amounts during activation of the immune response (Nathan & Hibbs, 1991; Nussler & Billiar, 1993). Initially this was observed in activated macrophages where it contributes to their cytotoxicity against tumour cells, bacteria, viruses and other pathogenic microorganisms.

1.7.3.4 Oestrogen and nitric oxide

Evidence that the L-arginine / nitric oxide pathway differs between the sexes and that its function may be modulated by oestradiol, has suggested a potential contribution of endothelial nitric oxide to the cardioprotective mechanism of oestradiol.

Basal release of NO from endothelium-intact aortic rings from female rabbits is greater than that from males (Hayashi et al, 1992). Similar results have been reported from studies of spontaneously hypertensive rats in which inhibition of NO synthesis by L-arginine analogues potentiated agonist induced contractions that were more sustained in aortic segments from female than male rats (Kauser & Rubanyi, 1993). In guinea pigs, an *in-vivo* study demonstrated an increase in NOS activity and NO production in heart, kidney and skeletal muscle tissue after five days of oestradiol treatment in females compared with ten days of treatment in males (Weiner et al, 1994).

Atherosclerosis impairs ACh mediated vascular responses in oestrogen deficient female monkeys, however long term oestrogen therapy protects against these impaired responses (Willliams, Adams & Klopfenstein, 1990). Acute responses to oestrogen in female monkeys with coronary artery disease have also been reported (Williams et al, 1992). In human studies using postmenopausal women with proven coronary artery disease oestrogen attenuates (Reis et al, 1994) or abolishes (Gilligan, Quyyumi & Cannon, 1994) Ach-induced coronary artery vasoconstriction when administered acutely, although has little direct effect. Furthermore, the coronary artery response to ACh after exposure to oestradiol appears to be gender dependent (Collins et al, 1995). Improvements in endothelial dependent vasodilation in the forearm vasculature of postmenopausal women following direct intra-arterial infusion of oestradiol have been reported, but the effects were not sustained by systemic transdermal 17β-oestradiol administration (Gilligan et al, 1995). Following short-term oestrogen deprivation impaired forearm vasodilator responses have been observed, however these effects are readily reversed by subsequent oestrogen replacement (Pinto et al, 1997). Similarly in the forearm vasculature of perimenopausal women, enhanced basal but not ACh-induced NO release following oestrogen supplementation has been reported (Sudhir et al, 1996).

A number of *in-vitro* studies using cultured endothelial cells have reported that oestrogen administration appears to stimulate NO production (Schray-Utz, Zeiher & Busse, 1993; Hayashi et al, 1995; Hishikawa et al, 1995), although there is considerable species variation and the mechanism for this up-regulation is unclear. Two candidate mechanisms exist. Increased expression of nitric oxide synthase (Kleinhart et al, 1998) or inhibition of production of superoxide anions, the principal inactivator of NO (Arnal, et al, 1996).

1.7.4 Endothelial cell proliferation

Oestradiol may also influence endothelial cell regeneration and angiogenesis. It appears to promote neovascularisation, as well as migration, proliferation and differentiation of endothelial cells (Morales et al, 1995). The ability of oestradiol to augment basic fibroblast growth factor-induced angiogenesis is lost in transgenic mice lacking functional oestrogen receptors (ER-α), providing evidence for receptor dependence

(Johns et al, 1996). Oestradiol may also stimulate expression of endothelial growth factors such as fibroblast or vascular endothelial growth factors (Sheweiki et al, 1993).

1.8 Aims and objectives of thesis

1.8.1 Introduction

Observational studies allow inferences and associations to be drawn from data, but they clearly do not establish cause and effect. In the absence of data from randomised controlled studies, the consistency of the data from observational studies of HRT and its effect on cardiovascular disease, was supportive of a protective role for oestrogen. However, after twenty years of observational studies the first clinical trials examining the role of HRT in cardioprotection are emerging (Hulley et al, 1998) and suggest that the preventative effect of HRT in primary and secondary heart disease is equivocal. Although a number of biologically plausible mechanisms have been proposed to explain the cardioprotective potential of oestrogen, whether these changes result in clinical benefits remains uncertain.

Like much of the research carried out in this area, the work presented in this thesis was based on the assumption that the consistency of the data from observational studies inferring a cardioprotective role for oestrogen, would be reproducible in basic science studies and that ultimately this would translate into a clinical benefit. In particular, this work sought to examine an expanding area of research which in the late 1990's was perceived as an important potential cardioprotective mechanism worthy of further investigation both from basic science and clinical perspectives.

1.8.2 Aims

The aim of this project was to examine the vascular effects of oestrogen to test the hypotheses that:

1. Oestradiol modulates vascular reactivity
2. Changes in vascular reactivity may be due to a NO mediated effect
3. Oestradiol stimulates endothelial cell proliferation

The following studies aimed to examine the vascular effects of oestradiol using bilateral venous occlusion plethysmography to determine whether oestradiol had acute vasodilator effects in the forearm vascular bed of healthy postmenopausal women, and

to determine whether HRT potentiated basal endothelial nitric oxide synthesis or release. To compliment this *in-vivo* work, endothelial cell responses to oestrogen were also examined at a molecular level by determining eNOS messenger ribonucleic acid (mRNA) expression in cultured human aortic endothelial cells (HAEC) using northern analysis. The oestrogen receptor status of the cells used in these studies was established using the polymerase chain reaction (PCR). Additionally, the effect of oestradiol treatment on endothelial cell proliferation, which is perceived to be of importance in atherosclerosis in vascular remodelling and repair, was examined *in-vitro* by studying the growth characteristics of cultured HAEC following addition of oestradiol.

CHAPTER TWO

OVERVIEW OF METHODS

2.1 Forearm Venous Occlusion Plethysmography

2.1.1 Introduction

Forearm venous occlusion plethysmography (FVOP) is a technique for measuring forearm blood flow (FBF), which is used to study forearm vascular haemodynamics and control mechanisms. The original technique which was described in the late 19th century, used awkward water jackets to estimate changes in forearm volume. More recently following the advent of mercury-in-silastic gauges; coupled with the development of electrical calibration techniques and the use of computerised chart recorders, it has become a widely used research method. The advantage of the technique is that it allows vessels to be studied in their normal physiological environment, and enables assessment of the effect of vasoactive mediators on resistance vessel tone following direct infusion via the brachial artery. Adopting a bilateral approach with simultaneous measurements in both arms allows the measurements in the experimental arm to be adjusted for changes in the control (non-infused) arm, the results being expressed as a ratio of the two measurements.

2.1.2 Principal of forearm venous occlusion plethysmography

The principal of the technique is such that if venous return from the arm is obstructed while arterial inflow continues unimpeded, the forearm swells at a rate proportional to the rate of arterial inflow (Whitney, 1953). The result is a measure of total FBF expressed per unit volume of the forearm (ml/100ml forearm/minute), the main bulk of which corresponds to blood flow through skeletal muscle, the remainder being flow through the skin (Barcroft et al, 1943; Cooper et al, 1955). Since blood flow in the hand is primarily through the skin and there are large numbers of arterio-venous shunts present, it is necessary to exclude the hands from the circulation (Whitney, 1953; Sorrol, Walsh & Whelan, 1965). This is achieved by inflating a wrist cuff to suprasystolic pressures. Forearm blood flow is then determined from the gradient of the resulting plethysmograph trace recorded on the chart recorder according to the equation of Whitney (Whitney, 1953).

2.1.3 Calculation of forearm blood flow

In these studies blood flow measurements were derived from the mean of five sequential recordings. The gradients were calculated from data points by acquiring their co-ordinates using MacChart Software (AD Instruments, London), and the information pasted into a customised spreadsheet (Excel, Microsoft Corporation, USA). For all subjects, each set of forearm blood flow recordings was analysed twice and the mean values used for the overall analysis. Data were expressed as a percentage change in forearm blood flow ratio from baseline (Benjamin et al, 1995; Petrie et al, 1998). Unless otherwise indicated, results are expressed as means ± standard deviations. Statistical significance was assessed using the Student's t-test with 95% confidence intervals. A p-value of less than 0.05 was considered significant.

2.1.4 Plethysmography technique

Subjects were positioned supine on a bed with both arms supported on foam blocks, in a quiet, dimly lit, temperature controlled (25°C) room. Paediatric arterial occlusion cuffs (Hokanson SC5, PMS Instruments, Maidenhead, Berkshire) were placed around the wrists and inflated to suprasystolic pressure (200 mmHg) for three minutes during each set of measurements. Collecting cuffs (Hokanson SC10, PMS Instruments) which were placed around the upper arms were inflated (40 mmHg) and deflated cyclically after fifteen seconds. A commercially available air source (Hokanson AG101, PMS Instruments) connected to a rapid cuff inflator (Hokanson E20, PMS Instruments), enabled rapid cuff inflation to be achieved. Mercury-in-silastic strain gauges (Hokanson, PMS Instruments) connected to the plethysmograph were placed around each forearm at the widest diameter. A strain gauge two centimetres smaller than the widest diameter was chosen. The distance from the olecranon to the position of the largest circumference was noted to allow for standardisation of the strain gauge position at each visit. After positioning, the strain gauges were calibrated for the chart recorder used (MacLab, AD Instruments, London), and test readings obtained.

A 27gauge unmounted steel needle (Cooper's Needleworks, Birmingham) was inserted into an epidural cannula and sealed in position with dental wax. The patency of the cannula was ascertained and the system was flushed with heparinised saline to maintain patency. The cannula was then inserted into the brachial artery of the non-dominant arm

following infiltration with local anaesthetic (1% Lignocaine), and a constant infusion of normal saline (1ml/min) was commenced.

For each set of measurements, blood flow recordings (i.e. during inflation of upper arm cuffs) commenced forty-five seconds after wrist cuff inflation, and continued at intervals over the remaining one hundred and thirty-five seconds. The upper arm cuffs were inflated via a trigger switch under the operator's control, but deflated automatically after fifteen seconds.

At the start of each study, thirty minutes of test measurements were made to allow for acclimatisation to the inflation and deflation of the wrist and upper arm cuffs, and to allow for return to the resting state. Two further sets of recordings ten minutes apart were then made in order to determine the baseline forearm blood flow. Solutions of test drugs of increasing incremental doses which, were prepared by the sterile production unit of the hospital pharmacy, were then infused intra-arterially at a constant rate of 1ml/min.

On the evening prior to the study, women were asked to fast from midnight, although they were allowed to drink water on the morning of the study. They were asked to abstain from driving on the morning of the study and arrangements were made for them to be brought to the unit by a relative or by taxi.

On arrival the subjects were familiarised with the staff, surroundings and equipment. Measurements of height and weight were made to enable calculation of body mass index (BMI), and baseline measurements of pulse and blood pressure were recorded. Venous blood was taken for routine biochemistry (including lipid and hormone profiles) and haematology screening tests, and a resting electrocardiograph was performed.

2.2 Cell culture techniques

2.2.1 Initiation and maintenance of cultures

Primary cell cultures can be initiated from normal (adult), embryonic and malignant tissues from which they are aseptically collected by dissection. The simplest method of culture is then to suspend the chopped material in serum containing medium and add to a tissue culture flask. The pieces of tissue then adhere to the substratum and cells migrate out from the cut ends. Alternatively the pieces of tissue can be immobilised in a plasma clot. The commonest way to initiate cultures from solid tissues is to disaggregate the tissue into single cells either mechanically or enzymatically (Folkman, Haudenschild & Zetter, 1979; Ryan, 1984).

After establishing the primary culture, the culture medium is replaced twenty-four hours after initiation from single cells, since cells which have not adhered are likely to be non-viable. Thereafter the medium is changed every two or three days.

Cells which have reached confluence are subcultured into fresh flasks and medium. This involves rinsing the flask with buffered saline, then adding trypsin to remove the cells from the substratum. To prevent further trypsin activity once the cells have detached, a trypsin neutralising or inhibiting agent is added. The cells are then separated by centrifugation, washed in medium, counted, and seeded into fresh medium at an appropriate cell density determined by the cell doubling time.

2.2.2 Counting cells

A cell count gives a measure of the status of the culture at a given time point, and is essential when subculturing or when assessing the effects of experimental treatment on cells. In general the cell count is expressed as number of cells per millilitre of medium. A number of methods for quantifying cells are available and these include the haemocytometer, the Coulter counter and flow cytometry.

A haemocytometer or counting chamber is a simple and reliable method of determining the cell count. It consists of a glass slide engraved with grids of a known area, onto which a glass cover slip of precise thickness is placed so that when correctly placed, the

volume of liquid over the grid is known. The number of cells present can be determined from the corresponding grid areas. A refinement of this technique is to incorporate a viability test in which a dye is used to stain non-viable cells (dead cells are permeable and will take up the stain whereas living cells will exclude certain stains from crossing their membrane) and allows them to be excluded from the final cell count (McAteer & Davis, 1994).

The Coulter counter (Coulter Electronics Limited) is an electronic particle counting device which allows particles of different size to be counted. Cells suspended in saline are drawn through an orifice by the application of a vacuum. This results in a change in the electrical resistance to a current flowing through the orifice causing a series of pulses which is counted. Cell debris may also generate pulses and may lead to inaccurate counts, although most instruments have a threshold control allowing only pulses from whole cells to be counted (McAteer & Davis, 1994).

A flow cytometer is an instrument in which cells are made to flow in single file through an orifice where they intersect a laser beam. The cells will cause light to be scattered and may fluoresce if stained with an appropriate dye. An optical sensor makes multiple measurements on the cells as they pass through the machine and the information measured is transferred to a computer for analysis. Light scatter, fluorescence emission and optical density can all be measured making this a particularly useful and flexible method of cell analysis (Watson & Erba, 1992).

2.2.3 Characterisation of cultured cells

Cells in culture are not readily distinguishable by appearance alone. Most cells have either a fibroblastic or epithelial morphology when adherent, or a common rounded appearance if non-adherent. Thus it can be difficult, on the basis of appearance alone, to determine if the cells being cultured are the correct ones, or if they have been contaminated by another cell line. A number of methods are available for identifying the cells and the culture parameters. These include determining the cell growth, the cell cycle time, characterisation of the cells by staining and deoxyribonucleic acid (DNA) analysis.

Cell division, which leads in an increase in cell numbers, results in the growth of a culture. The rate of growth of a culture under a given set of conditions can be a useful method of characterising a culture. Cell growth can be determined from the absolute cell number determined from the cell count or by determining the cell proliferation using growth curves (McAteer & Davis, 1994).

A typical growth curve for cells seeded from subculture (figure 2.1) consists of four distinct phases: lag, log (exponential), plateau (stationary) and decline. In the lag phase (lasting 1-24 hours) the growth is negligible and will be dependent on the cell line and cell condition prior to culture. In the log phase, there is exponential growth that can be used to determine the cell doubling time. When adherent cells become confluent division slows down and the cell growth is balanced by the cell death. If the medium is insufficient, cell death eventually exceeds cell growth and the cell number declines.

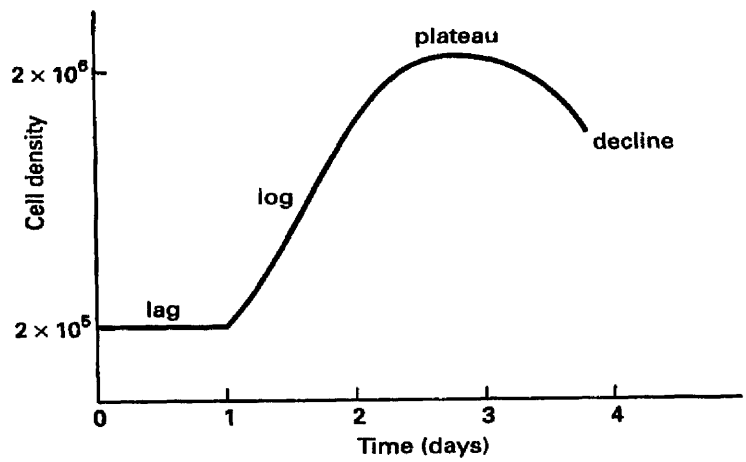


Figure 2.1 - Growth curve

The cell cycle is defined as the time interval between divisions (McAteer & Davis, 1994). It is divided into a number of phases (figure 2.2): G_1^0 (gap), S (DNA synthesis), G_2 (Gap 2 – resting phase), M (mitosis). The length of the cell cycle varies between cultures, as does the length of the individual phases.

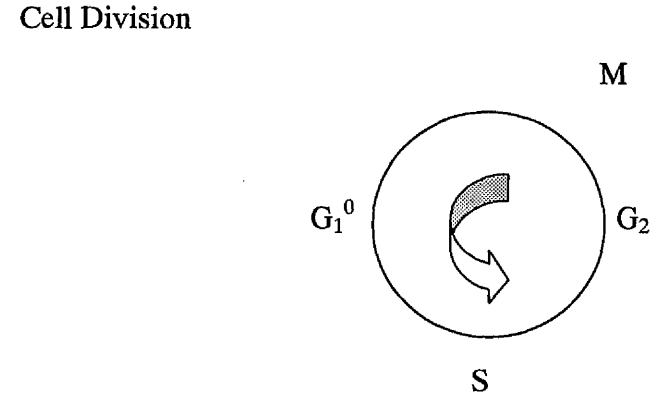


Figure 2.2 - The cell cycle

An asynchronous culture is pulsed with tritiated thymidine and examined by autoradiography for radiolabelled mitosis. Cells which are first into labelled mitosis are those which were in the S phase at the time of the pulse. The time interval between removal of the pulse of radioactivity and the detection of the labelled mitosis represent the growth phase (G_2 + M). The cell cycle time is represented by the interval between the midpoint of the first and second rising curves (figure 2.3).

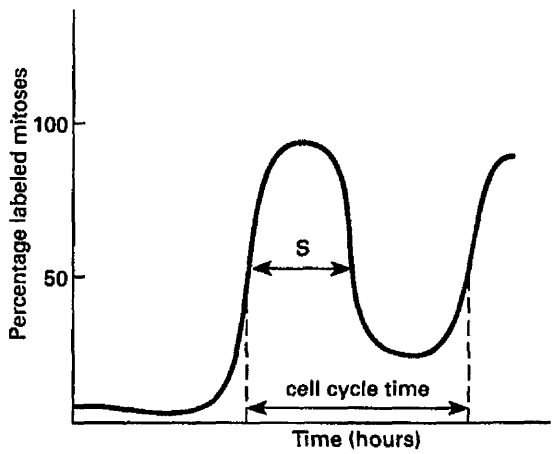


Figure 2.3 - The cell cycle time

Immunocytochemistry is the localisation of antigens in or on cells by the use of a specific antibody to that antigen. The antibody is then labelled by some means in order to permit identification. A variety of labelling techniques are available including fluorochrome, radioisotopes and biotin/avidin. The staining may be carried out in a number of ways. Direct staining is a one-stage process in which the cell is incubated with the labelled antibody. In contrast, indirect staining is a two stage process in which unlabelled antibody is first incubated with the cell. In the second stage, the antibody is located with a labelled anti-immunoglobulin. This method is more sensitive although there may be a higher background. For immunofluorescence the antibody is labelled with a fluorochrome which is a molecule that is capable of absorbing light of short wavelength and emitting light of longer wavelength. A further refinement of this technique is double staining to locate two different antigens at the same time. The two antibodies should be conjugated to fluorochromes whose emission spectra are far apart such as Texas red and fluorosceine isothiocyanate (FITC) (Dawson, 1992).

2.2.4 Human aortic endothelial cells

The cell culture experiments described in this thesis were performed using HAEC which were purchased from Clonetics Inc (UK Supplier TCS Biologicals, Buckinghamshire). Provided the supplier's recommended protocols, medium and reagents were used, the cells were certified for use through at least fifteen population doublings (approximately three passages).

The cells, which had been obtained from a healthy thirty-four year old caucasian female road traffic accident victim, were shipped cryopreserved in dry ice at the third passage (the cells were cryopreserved in endothelial growth medium supplemented with 10% v/v fetal bovine serum and 10% v/v dimethyl sulfoxide (DMSO) as a cryopreservation solution to improve cell viability and seeding efficiency on thawing). Prior to shipping the cells were performance tested and confirmed negative for human immunodeficiency virus (HIV) and Hepatitis B & C viral infections (PCR DNA negative), mycoplasma, yeast, bacterial and fungal infections. Additionally the cells tested positive for Factor VIII (von Willibrand factor) antigen and for uptake of di-acetylated LDL, but tested negative for smooth muscle α actin.

2.2.4.1 Preparation of Medium

Endothelial cell growth medium is provided by Clonetics as a Bulletkit® consisting of a basal medium (EBM) and individual growth factors conveniently packaged as single use aliquots (Singlequots®). The endothelial basal medium was stored at 4°C in a cold room, and the supplements stored frozen at -20 °C until required. The fully supplemented solution was stored under refrigeration at 4°C.

The following components were supplied with a Bulletkit®:

Endothelial basal medium (modified MCDB 131) – 500ml
Epidermal growth factor (10ng/ml) – 0.5ml
Bovine brain extract (3mg/ml containing 10μg/ml heparin) – 2ml
Fetal bovine serum (2%) – 10ml
Gentamicin (50mg/ml) / Amphoteracin-B (50mg/ml) – 0.5ml
Hydrocortisone (1mg/ml) – 0.5ml

A complete bottle of endothelial growth medium was prepared thus. The supplements were rapidly thawed at 37°C and transferred with the basal medium to a sterile field. The contents were then mixed thoroughly by gentle inversion. The external surfaces of the vials and the EBM bottle were then decontaminated by wiping with 70% v/v ethanol. Each cryovial was then aseptically opened and the entire contents added to the basal medium using sterile Pasteur pipettes. The cryovials were then carefully rinsed out with some of the medium to ensure recovery of the entire volume of the cryovial. The complete medium was then thoroughly mixed and appropriately labelled.

Before use, it was recommended that the medium was equilibrated for temperature and CO_2 and thus pre-prepared flasks were placed in a humidified incubator at 5% CO_2 and 37°C for at least thirty minutes. This was thought to be particularly important in ensuring a gentle recovery of cells after thawing.

2.2.4.2 Thawing and inoculating cells

A seeding density of 2500 cells/cm^2 and a volume of 1ml of growth medium per 5cm^2 surface area of flask was recommended by Clonetics. Cells were initially recovered into

25 cm^2 flasks. Five millilitres of complete medium was added to the number of flasks required (see below) and the flasks placed in the CO_2 incubator for thirty minutes. As unvented flasks were used, the caps were loosely applied (tightened then loosened by a half turn). Each flask was labelled with the passage number, cell type, strain number and date thus:

P4, HAEC, 2241, 10/12/96

The number of flasks required was determined using the following calculation:

$$\frac{\text{No of cells available}}{\text{Recommended seeding density}} = cm^2 \text{ that can be plated}$$

$$\frac{\text{Maximum no } cm^2 \text{ of cells platable}}{\text{Growth area of flask}} = \text{No of flasks}$$

Cells were removed from liquid nitrogen, placed on dry ice, and transported to the sterile field. The vial was then wiped with ethanol, and the cap opened slightly to release pressure then retightened. The vial was immediately transferred to the 37°C water bath and the bottom half dipped in the water, care being taken to ensure that the cap did not become submerged in the water. The vial was thawed over two minutes (thawing cells for more than 3 minutes results in less than optimal results) while gently agitating the contents. The cryovial was then removed, dried and transferred to the sterile field (where the previously prepared equilibrated flasks were ready to be seeded), rinsed with 70% v/v ethanol and dried. The contents were gently agitated using a pipette and the desired volume of cells pipetted into the culture flasks. The cells were carefully placed onto the culture medium surface in an arc thus spreading the cells over the surface, then gently mixed with the medium in the flask by a swirling action.

The growth medium was changed the following day within 24 hours to remove residual DMSO and unattached cells. Thereafter the medium was changed on alternate days, although the cells were inspected daily. Care was taken to ensure that the medium was aspirated from the opposite side of the flask to which the cells were attached and similarly, the fresh medium was added to the same side.

As the cells became more confluent, it was necessary to increase the volume of medium according to the following protocol:

Confluence	Feeding volume
<25% confluence	1ml/5cm^2
25-45% confluence	1.5ml/5cm^2
>45% confluence	2ml/5cm^2

2.2.4.3 Subculturing human aortic endothelial cells

The cells were subcultured on reaching 70-90% confluence since allowing the cells to become over-confluent resulted in irreversible contact inhibition and loss of cell viability.

The old medium was firstly aspirated from the tissue culture flask. The cells were then rinsed with 2-3ml of room temperature Clonetics Hepes Buffered Saline Solution (HBSS) which neutralises any traces of protein from the medium which would otherwise inactivate the trypsin. This was then aspirated and 3ml of Clonetics Trypsin/EDTA solution which had been thawed to room temperature was added (3ml/25cm^2). After gently rocking the flask to ensure that all the cells had come into contact with the trypsin solution, the flask was examined under the microscope until around 90% of the cells had rounded up, the entire process taking 5-6 minutes. Finally the flask was firmly tapped to detach the cells from the surface of the flask. After release of the cells, the entire process was arrested by addition of Clonetics trypsin neutralising solution (TNS) (3ml/25cm^2). The cells were then transferred to a sterile universal container before centrifuging at 2000rpm (4°C) for 5 minutes. (IEC Centra – 7R, International Equipment Company, Dunstable, UK) The supernatant was then decanted and discarded, and the pellet resuspended in 4ml of fresh growth medium. After flicking the container to dislodge the pellet, the cells were mixed by triturating with a fine tipped Pasteur pipette before counting in a Coulter counter to determine the yield. The cells were then seeded into new flasks according to the seeding density described above. The medium was then changed after 24 hours and thereafter at 48 hour intervals until the next passage (70-90% confluence).

2.2.4.4 Preparation of cells for storage in liquid nitrogen

After harvesting the cells as described above, the cells were resuspended in a cryopreservation medium consisting of complete endothelial medium and DMSO (ratio 9:1; 1ml DMSO in 9ml complete endothelial cell growth medium). The cells were then frozen in a stepwise fashion thus: 30 minutes at –20°C, 24 hours at –70°C prior to transfer to liquid nitrogen.

2.3 Molecular Techniques

2.3.1 Introduction

Ribonucleic acid is a macromolecular structure comprising of a polymeric sugar (ribose) 'backbone' linked to the nucleotide bases adenosine, cytosine, guanine and uridine. It exists predominantly as a single stranded polymer and within the eukaryotic cell is found in three forms: ribosomal (rRNA), transfer (tRNA) and messenger (mRNA), (Strachan & Reid, 1996). Ribosomal RNA is the most abundant species comprising 80-90% of the total, with tRNA making up 5-10%. In contrast mRNA comprises only 1-5% of the total and varies considerably in size. Messenger RNA is transcribed from the antisense coding strand of genomic DNA and its sequence is therefore equivalent to that of the sense complementary DNA sequence. In common with DNA, RNA possesses complementary base-pairing properties. This facilitates the analysis of RNA immobilised on filters and labelled with specific cDNA, cRNA or oligonucleotide probes, and is an important component of many techniques in molecular biology.

It is of paramount importance in the analysis of RNA to rapidly and efficiently protect it against the action of endogenous and exogenous ribonucleases (RNAses) which are present in all living cells and throughout the general laboratory environment (Chabot, 1994), and also to protect it against physical and chemical damage during manipulation. This is particularly important during the extraction and purification of RNA from tissues and cells but also applies to maintaining valuable stocks of RNA.

There are several well-characterised classes of RNAses, which can broadly be divided into either endo- or exo-nucleases. They are cofactor-dependent and are very resistant to destruction even at high temperatures (>90°C) and across wide-ranging pH's. Laboratory glassware and plastics are usually contaminated with RNAses. Therefore, it is necessary to set aside and treat equipment that is required and label for RNA use only. Glassware can be baked at 250°C for 4hours, 180°C overnight or soaked in 0.1% v/v diethyl-pyrocarbonate (DEPC) followed by autoclaving. Some plastics and electrophoresis equipment cannot be autoclaved and should therefore be soaked overnight in 0.1% v/v DEPC and then rinsed thoroughly with RNAse-free water. Where possible, the use of sterile disposable plasticware (e.g. pipettes, centrifuge tubes) is

advisable and the use of filtered pipette tips can protect samples from RNAses present on pipette barrels. Disposable plastic gloves should be worn at all times to prevent contamination when handling items (Sambrook, Fritch & Maniatis, 1989).

Prior to autoclaving, 0.1% v/v DEPC should be added to most autoclavable reagents, dispersed by thorough mixing, then left to stand overnight. Solutions containing Tris do not benefit from DEPC treatment because it is inhibited by Tris. Non-autoclavable reagents, such as those containing sucrose, can be sterilised by filtering through a sterile 0.2μm filter.

2.3.2 RNA extraction

There are a number of methods for the preparation of total cellular RNA, which broadly involve cell lysis separation of RNA from DNA, proteins and other macromolecules and finally precipitation and concentration of RNA (Grabowski, 1994). The phenol-chloroform method of extraction is probably the commonest rapid method in use today. It utilises the chaotropic and RNAse inhibiting properties of guanidinium isothiocyanate and the acidic nature of water-saturated phenol, to extract both DNA and protein, leaving RNA soluble in the aqueous phase. RNA is then carefully transferred to another tube (avoiding contact with the precipitated DNA at the interphase with the phenol) and precipitated by mixing with one volume isopropanol (propan-2-ol) at –20°C for at least one hour. At this stage the RNA can be stored at –20°C without risk of degradation. The whole sample or aliquots can then be centrifuged, the RNA pellet washed with 70% ethanol and resuspended in RNAse-free water (DEPC treated) on ice and stored at –70°C (Sambrook, Fritch & Maniatis, 1989).

2.3.3 RNA quantification and visual assessment of integrity

Assessing the integrity of RNA and the presence of any genomic DNA contamination requires ethidium bromide stained agarose gel electrophoresis of each sample alongside a standard RNA pool. Undegraded RNA is represented by the appearance of two distinct 28S and 18S ribosomal bands. Smearing between the two bands reflects limited degradation. The absence of ribosomal bands and smearing in the region of the loading dye indicates complete degradation. The appearance of bands larger than the 28S rRNA

band reflects genomic DNA contamination, necessitating re-purification (Grabowski, 1994).

RNA yield can be accurately quantified spectrophotometrically by measuring the optical density (OD) of an aliquot of each RNA sample in solution at its maximum absorbance wavelength, i.e. 260nm. An OD value of one represents an RNA concentration of 40μg/ml. Measuring the OD at 280nm can assess the level of protein contamination. A 260/280nm ratio of < 1.5 indicates considerable contamination, necessitating re-extraction.

2.3.4 Analysis of RNA

A number of techniques are now available for analysing both the size and abundance of different RNA transcripts. Despite the advent of powerful techniques such as RNAse protection assays and the polymerase chain reaction, northern analysis or blotting has remained the standard method for detection and quantification of mRNA.

2.3.4.1 Northern analysis

Northern blotting is widely used to determine both the size and abundance of different RNA transcripts (Alwine, Kemp & Stark, 1977). It requires the use of relatively large amounts of total RNA (15-50μg) or poly (A-) mRNA (1-10μg) per lane which can be compared to standard markers of known size. The RNA is electrophoresed through an agarose gel (1-1.5% depending on the size of the target RNA) in electrophoresis buffer at 80-100 mAmps for 3-6 hours depending on the resolution of homologous RNA species required. The RNA is transferred either by capillary blotting in a high ionic strength buffer, or more rapidly by electroblotting, to a positively charged or uncharged nylon membrane, immobilised covalently by UV crosslinking, (or non-covalently by baking at 80°C for 3 hours) and stored damp in plastic wrap at 4°C until required for probing. To orientate the location of samples on the filter it is important to use an indelible marker pen or a scalpel to clearly identify on which side the RNA is immobilised, and to identify the top and bottom of the filter. Specific RNA species can then be detected by hybridisation with a labelled probe. If radiolabelled probes are used, the intensity and position of the signal assessed densitometrically gives an indication of the size and concentration of the RNA. To improve resolution and accurately determine

the size of the target mRNA species it is necessary to use conditions which will destroy secondary structures (i.e. heating RNA to 65°C for 10 minutes, then snap cooling on ice prior to loading of the gel) (Farrell, 1993).

2.3.4.2 RNAse protection assay

The RNAse protection assay (RPA) utilises highly specific single-strand (ss) cRNA probes or riboprobes, which form specific duplexes with the target mRNA. This results in high sensitivity and is thus useful for quantitation of very low levels of target RNA, but increases the risk of developing non-specific binding. Target mRNA is mixed with the riboprobe in hybridisation buffer and the RNA denatured before incubating at 45-50°C for 8-12 hours. Any RNA not hybridised is then digested with RNAse A (attacks pyrimidine residues) and RNAse T_1 (attacks guanosine residues) at room temperature. The reaction is then halted and after addition of a carrier tRNA the duplex RNA is salt/ethanol precipitated, resuspended and denatured prior to electrophoresis (Higgins & Hames, 1994).

2.3.4.3 Polymerase chain reaction (reverse transcription)

This is the most sensitive technique by which a specific RNA species can be detected, and under the correct conditions quantified also. The principal of the technique is that by combining first strand DNA synthesis by 3'antisense oligonucleotide-directed reverse transcription and subsequent PCR amplification of this DNA using both the antisense and a 5'sense primer, yields many copies of double-strand DNA product from a single RNA transcript. The product can then be visualised on an ethidium bromide stained agarose gel (Brown, 1995).

2.3.5 Probe Technology

Nucleic acid probes are a widely used tool in molecular biology having numerous applications including detection of quantitative and qualitative changes in gene expression, gene amplification and deletion, chromosomal translocations, point mutations and detection of the presence of new genetic sequences in cells. The role of the probe is to hybridise to any complimentary sequence in hybridisation reaction

mixture. A probe has a label incorporated into the molecule which will enable its detection (localisation and quantification) at the conclusion of the experiment.

Nucleic acid probes consist of either DNA or RNA molecules and can be either homogeneous or heterogeneous in nature. A homogeneous probe preparation contains only one type of probe molecule, whereas a heterogeneous probe consists of mixtures of two or more sequences which may or may not be closely related nucleotide sequences. DNA probes consist of a variety of molecules including cloned double-stranded cDNA or genomic DNA sequences and oligonucleotide probes. Ribonucleic acid probes include cloned cRNA and oligonucleotide probes. Both RNA and DNA probes produce hybrids with the bound RNA, however the RNA:RNA hybrids bind more avidly and are therefore more stable than DNA:RNA hybrids. Deoxyribonucleic acid probes can therefore be more readily stripped from the membrane to allow repeated probing (Hames & Higgins, 1988).

Nucleic acid probes can be isotopically or non-isotopically labelled by several different means and separated from unincorporated label in order to minimise background signals. Radioisotopes in general use are ^{32}P, ^{35}S, ^{3}H but their choice depends on the requirements for sensitivity, resolution and speed of development. For filter hybridisations, ^{32}P gives high sensitivity and sufficient resolution, but ^{35}S remains the isotope of choice for probes used in in-situ hybridisation and in filter hybridisations where high resolution but not detection sensitivity is required. Non-radioactively labelled probes are generally less sensitive for filter hybridisations, but are more frequently used in in-situ hybridisation. They also have a number of advantages over radio-labelled probes including improved safety during synthesis and use, and the ability to prepare probes in batches, increased probe stability and detection in minutes rather than weeks. Non-isotopic labels include biotin, digoxigenin and fluoroscein, all of which are larger molecules than radiolabels, which therefore precludes incorporation into probes at high specific activity. Detection systems include secondary antibodies against digoxigenin or biotin which may be conjugated to enzyme reporters such as horseradish peroxidase, alkaline phosphatase or to chromogens and fluoro-chromogens (Hames & Higgins, 1988).

The most commonly employed methods of internally labelling double-stranded cDNA probes are nick translation, random priming and polymerase chain reaction; kits for

which are commercially available. Nick translation uses DNA polymerase I (DNAse I) to randomly introduce nicks into the backbone of the double-stranded DNA and insert cDNA to expose 3'-OH groups from which DNA-dependent DNA polymerase I incorporates deoxyribonucleic acid triphosphates (dNTPs) sequentially in the 5'-3' direction. Concurrent 5'-3' exonuclease activity removes dNTPs from the 5'end. Random priming is a type of primer extension in which a mixture of small oligonucleotide sequences, acting as primers, anneal to a heat denatured double-stranded template. The annealed primers ultimately become part of the probe itself, because the Klenow fragment of DNA polymerase extends the primers in the 3'direction incorporating the label. Random priming appears to work significantly better with linearised DNA molecules. The PCR is a useful method for probe synthesis as it requires very little starting template and the synthesised product is of uniform length. In the presence of an appropriate precursor, molecules are labelled continuously as they are being synthesised, thus ensuring a high degree of label incorporation (Higgins & Hames, 1994).

In comparison with DNA probe synthesis, there are relatively few methods for labelling RNA probes. *In vitro* transcription is the commonest method currently in use and enables large quantities of efficiently labelled probes of uniform length to be generated. Antisense and sense cRNA's are transcribed *in vitro* from a cDNA template using bacteriophage polymerases. The transcription vector is first linearised at a site just downstream from the insert to yield a blunt end (5'). An antisense cRNA complimentary to the non-coding DNA strand and sense RNA complimentary to the coding strand is then synthesised from a bacteriophage promotor located upstream of the DNA insert. A number of kits are now commercially available to facilitate *in vitro* transcription of RNA probes.

2.3.6 Detection and quantification of RNA

Autoradiography is a simple and reliable photochemical technique in which the capture of ionising radiation and photons by a film placed in direct contact with a radioactive source provides a relatively permanent visual record of the decay events associated with unstable radioisotopes (Jones, Qiu & Rickwood, 1994).

In Northern blot analysis of gene expression a piece of high-speed medical X-ray film (e.g. Kodak X-OMATIC AR, Kodak Limited, UK) is placed in direct contact with the plastic wrapped filter membrane (this avoids the filter drying out). This technique is referred to as direct exposure. During direct film exposure, energy is adsorbed in the silver-halide grains in the film, with the release of electrons. The resulting negatively charged areas attract the positively charged silver ions, forming an atom of metallic silver. Exposure of the X-ray generates an invisible precursor to the visible image manifested when the film is immersed in a suitable photographic developing solution. The formation of the visible autoradiographic image can be done manually or by machine, the latter method having the advantage of being developed according to a standardised process. The final developed image may be assessed by visually or more accurately using densitometry.

Autoradiographic detection of ionising radiation can be enhanced by the use of image intensifying screen cassettes which are cassettes with screens consisting of a polyester base coated with inorganic phosphors. Low temperature autoradiography (i.e. -70 °C to -80 °C) greatly enhances efficiency when using image intensifying screens hence the cassettes are stored frozen at -70 °C. Before developing the cassette is allowed to warm to room temperature.

The duration of exposure of the film will be dependent on the apparent signal strength which can be assessed by placing a Geiger counter probe over the filter to determine the activity on the filter. A high activity will require a much shorter exposure time, although it is often useful to do an overnight exposure in order to assess the extent of background and non-specific hybridisation. Subsequent exposure times can be derived on the basis of the image obtained from this test exposure.

The signal density on an autoradiograph can be quantified by using a scanning densitometer. The autoradiographic film is placed on a scanner and scanned over a defined area of the densitometer grid system and the information obtained is then transferred to a computer software package. The position of the molecular weight standards can provide useful information regarding the size of the bands. An attempt to quantify the signal can be made by outlining the band to be measured, selecting a volumetric integration of the area defined and expressing the signal in relation to the background density.

CHAPTER THREE

STUDY ONE

ACUTE EFFECTS OF OESTRADIOL ON FOREARM BLOOD FLOW IN HEALTHY POSTMENOPAUSAL WOMEN

3.1 Method

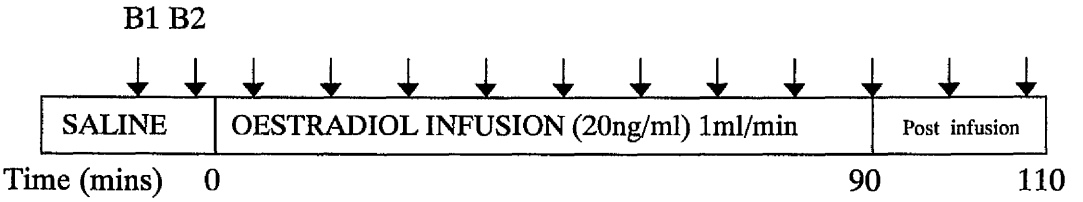
The technique of forearm venous occlusion plethysmography previously described was used to determine the acute effect of an intra-arterial infusion of soluble 17β-oestradiol on the forearm blood flow responses in a group of healthy postmenopausal women.

3.1.1 Recruitment Criteria

The women who participated in this study were recruited either via the gynaecology department of The West Glasgow Hospitals University NHS Trust, Glasgow, or in response to a request for volunteers in an article published in the Glasgow University Newsletter. All women gave written informed consent to participate in the study, which was approved by the ethics committee of the West Glasgow Hospitals University NHS Trust. All the women recruited had no personal history of CVD, hypertension or diabetes, no contra-indications to oestrogen, and were deemed to be fit on routine physical screening which included a pre-study electrocardiograph which was within normal limits. None of the women were smokers, and only one woman was a previous HRT user, although had not used HRT in the preceding twelve months.

3.1.2 Study Protocol

The study protocol adopted is shown in figure 3.1. Subjects were positioned on the bed as previously described in chapter 2.1.4. After thirty minutes of test measurements, to allow for return to the resting state, two measurements at ten-minute intervals were used to calculate the baseline forearm blood flow. An infusion of 20ng/ml of soluble 17β oestradiol (Clinalfa, Switzerland) was infused at a constant rate of 1ml/min for ninety minutes. Blood flow measurements were made at ten-minute intervals during the infusion. Two further measurements at ten and twenty minutes post infusion were also made. To determine the local circulating oestradiol levels, the venous effluent from the infused arm was sampled from a subgroup of women at the end of the study.



↓ = Measurement of forearm blood flow, B1 = Baseline 1, B2 = Baseline 2

Figure 3.1 - Protocol for Study 1

A summary measure of forearm blood flow during the final thirty minutes of the oestradiol infusion was calculated for individual subjects and compared with the initial baseline measurements. An overall comparison of the results was then made using a 2-sample Student's t-test with 95% confidence intervals.

3.2 Results

Fifteen women were recruited to the study initially. One subject withdrew after initial counselling. Six subjects were withdrawn due to difficulty cannulating the brachial artery at the start of the study, or due to the needle becoming dislodged during the course of the study. All women with whom we had difficulty with the initial cannulation except one, agreed to return for a second visit. In each case the cannulation was not successful at the subsequent visit.

Eight women, aged between 52 and 66 years, successfully completed the study. The mean duration from the menopause was 8.4±4.21 years. One woman had undergone total abdominal hysterectomy with ovarian conservation for menorrhagia eleven years earlier at the age of 41 years, the remaining seven women had undergone a natural menopause. Only one woman had previously taken HRT, but not in the preceding twelve months. At the time of entering the study, all women were biochemically postmenopausal, with a circulating oestradiol level <50pmol/L, and a mean FSH level of 102.5±25.00 IU/L.

All women who participated were normotensive (mean blood pressure 133/71), non-obese (body mass index 25.2±2.20 kg/m^2) with a mean cholesterol level of 6.4±1.10, and were also non-smokers.

The results of this study are detailed in appendix 2, and summarised in tables 3.1 & 3.2, and in figure 3.2. These data have a 95% power to exclude a 13% acute vasodilator effect of a supraphysiological concentration of oestradiol in the forearm resistance vessels of a group postmenopausal women, although the original protocol aimed to exclude a 10% effect.

The mean forearm blood flow ratio at baseline was 1.12±0.34, and during the final thirty minutes of the infusion was 1.07±0.30 (95%CI –0.30, +0.40, p=0.76). The mean local circulating oestradiol level measured in a small subgroup of women at the end of the infusion was 2740 pmol/L.

Subject	FBF Ratio (baseline)	FBF Ratio (final thirty minutes of oestradiol infusion)
1	0.74±0.14	0.72±0.05
2	1.39±0.08	1.35±0.13
3	0.82±0.00	0.95±0.09
4	1.07±0.03	0.96±0.12
5	0.70±0.07	1.13±0.05
6	1.37±0.23	0.67±0.08
7	1.26±0.10	1.24±0.19
8	1.60±0.05	1.53±0.10

Table 3.1 – Mean FBF ratios at baseline and during final 30 minutes of oestradiol infusion (summary data for individual subjects)

Time (min)	% Change in FBF ratio (mean±SD)
10	-0.69±17.1
20	2.10±36.3
30	2.57±23.2
40	2.40±28.7
50	-5.41±25.0
60	-4.40±34.7
70	-2.70±33.5
80	5.10±40.9
90	2.20±32.2
100	-4.60±33.5
110	-12.30±34.4

Table 3.2 – Percentage change in FBF ratio from baseline during oestradiol infusion (pooled data for all subjects)

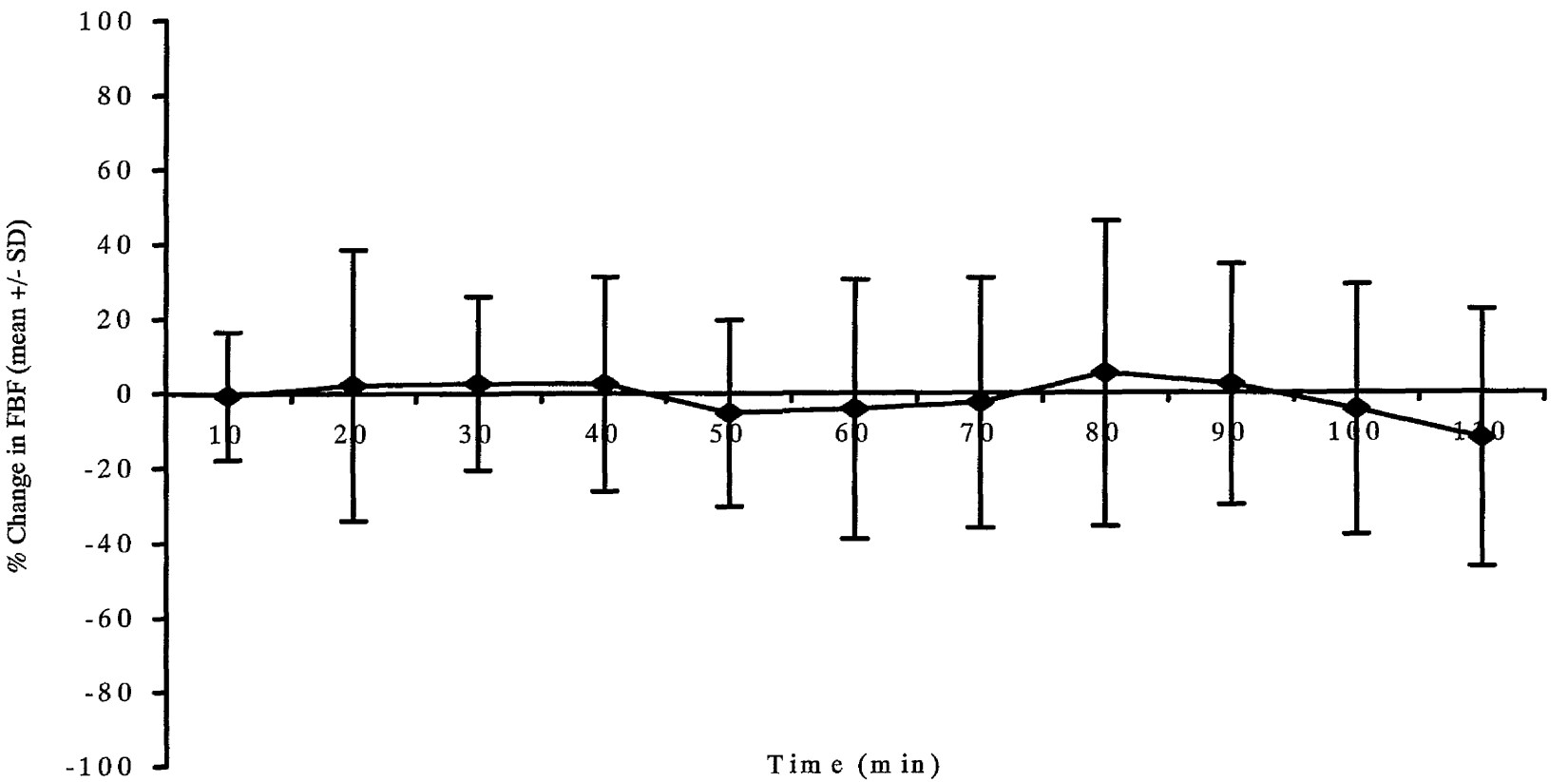


Figure 3.2 - Percentage Change in FBF ratio from baseline during 90 minute oestradiol infusion (pooled data all subjects)

3.3 Discussion

Sex steroids are vasoactive hormones which profoundly influence vascular tone in the reproductive system. In addition to their dominant role in reproductive physiology, they also influence the vasculature of many other systems, including the cardiovascular system. Direct effects of oestrogen on blood flow and arterial tone have previously been reported in both animal and human studies using a variety of species and vascular beds, and such effects of oestrogen on the vasculature may have an important influence on cardiovascular physiology and health.

Early animal studies reported that oestrogen induced dilation of small blood vessels in the ear of ovariecomized rabbits (Reynolds & Foster, 1940). A reduction in peripheral vascular resistance in non-pregnant oophorectomized ewes following oestrogen administration has also been documented (Magness & Rosenfeld, 1989). Relaxation of isolated perfused human umbilical vessels in response to oestrogen (Silva de Sa & Meirelles, 1977), and vasodilatory responses to 17β-oestradiol in pre-contracted rabbit coronary arteries (Raddino et al, 1986) was also suggestive of a direct effect of oestrogen on blood vessels. However, these early pharmacological studies used significantly higher oestrogen concentrations than those produced endogenously or given exogenously as replacement therapy, and failed to identify specifically which vascular cells were responding to oestrogenic stimulation. To evaluate the stereospecificity of the rapid vascular effects of oestradiol, more controlled studies using other steroids, non-steroidal oestrogens and anti-oestrogens will be necessary.

Further evidence for an acute vasodilatory effect of oestrogen has been acquired from a number of in-vivo human studies. Doppler velocimetry studies have shown the oestrogen administration increases blood flow to the vulva and thenar eminence (Sarrel, 1989). Blood flow impedance in the internal carotid artery was assessed in twelve postmenopausal women using Doppler ultrasound and colour flow imaging, before and after oestrogen treatment. Pre-treatment, there was a linear association between the internal carotid artery pulsatility index (blood flow impedance downstream to the point of sampling) and menopausal age, suggesting that low circulating oestradiol levels are associated with increased blood flow impedance. However, after nine weeks of transdermal oestrogen administration, there was a significant reduction in the pulsatility index from the baseline pre-treatment level (Gangar et al, 1991). Acute administration of

sublingual 17β-oestradiol to postmenopausal women resulted in an increase in forearm blood flow and a reduction in vascular resistance when compared to placebo (Volteranni et al, 1995). A beneficial effect of acute oestrogen administration on myocardial ischaemia in postmenopausal women with angiographically proven coronary artery disease, was observed after administration of sublingual oestrogen (1mg). This study was a double-blind placebo controlled cross-over study, which examined the effect of oestradiol on exercise induced myocardial ischaemia in eleven women with chronic stable angina. Both total exercise time and the time to 1mm ST segment depression, which was taken as an indicator of myocardial ischaemia, was increased after taking sublingual oestradiol forty minutes prior to treadmill exercise, and was attributed to either a direct coronary relaxing effect, peripheral vasodilation or to a combination of both (Rosano et al, 1993).

It has been suggested that these rapid vasodilatory effects of oestrogen are mediated independently of the classic genomic pathway of steroid action, which involves receptor activation and gene transcription. There is increasing evidence suggesting that steroid hormones can bind to cell membranes and induce rapid cellular events within a few minutes of exposure (Moura & Worcel, 1984; Wehling, 1994). Indeed, the presence of membrane oestrogen receptors is thought to be partly responsible for the rapid vasodilatory effects of oestrogen, acting via rapid intracellular signalling pathways (Farhat , Abi-Younes & Ramwell, 1996).

A direct vasodilating effect of oestrogen was not demonstrated in this study as no significant difference in the mean forearm blood flow of the control to the infused arm following infusion of water soluble 17β-oestradiol was observed. The results shown in figure 3.2, expressed as the percentage change in the forearm blood flow over baseline (where a positive value indicates vasodilation and a negative value vasoconstriction), clearly demonstrate no specific trend, although the spread of these data suggests that there is considerable inter-individual variation between these subjects.

The apparent lack of vasodilation observed in the human forearm in this study suggests that, if present, any vasodilator effect of oestradiol in this tissue is likely to occur over a longer time period and may therefore be mediated via genomic mechanisms. As supraphysiological concentrations of oestradiol were achieved and the infusion was continued for a duration close to the maximum achievable in studies of this nature, it

would appear that the vasodilator effects of oestradiol are either tissue and/or species specific, or that it is not a direct vasodilator.

In this study water soluble oestradiol was purposely used as other studies examining the acute effects of intra-arterial oestradiol on the vasculature have previously employed ethanol (a known vasodilator) as a dilutant which may have prejudiced their results (Gilligan et al, 1994).

CHAPTER FOUR

STUDY TWO

EFFECT OF 17β-OESTRADIOL ON BASAL ENDOTHELIAL NITRIC OXIDE RELEASE

4.1 Method

Using the technique of forearm venous occlusion plethysmography previously described in section 2.1.4, forearm blood flow measurements were used to examine the effect of transdermal 17β-oestradiol on basal endothelial nitric oxide synthesis or release by measuring the responses to L-NMMA.

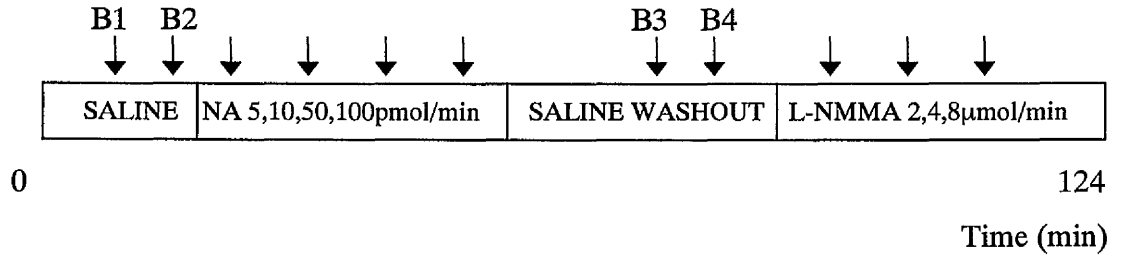
4.1.1 Recruitment criteria

The study was conducted as an unblinded 'before and after' study. Approximately half the women were recruited post-operatively; the remainder had responded to an article on 'HRT and heart disease' published in the local press. In the post-operative women, the study was timed to start at three to six weeks postoperatively, to allow for stabilisation of gonadotrophins and other metabolic processes (Thorrell et al, 1994). All women gave written informed consent to participate in the study, which was approved by the ethics committee of the West Glasgow Hospitals University NHS Trust.

In keeping with the previous study we sought non-obese healthy women, with no personal history of cardiovascular disease, hypertension or diabetes, no contra-indications to oestrogen, and who were deemed to be fit on routine physical screening. All women had a pre-assessment electrocardiograph which was within normal limits. Women taking regular medications with the exception of HRT were excluded, however all women who had previously been using HRT were asked to discontinue their HRT for four weeks prior to participating in the study.

4.1.2 Study protocol

Subjects attended for two identical study day's four weeks' apart. On the first study day the women had not used HRT in the preceding four weeks. A second study day was arranged after four weeks of transdermal 17β-oestradiol (Fematrix 80, Solvay Healthcare Limited, Southampton). The study protocol adopted is illustrated in figure 4.1.



B1 – B4 = Baseline 1-4

↓= Measurement of forearm blood flow

Figure 4.1 - Protocol for study 2

Subjects were positioned on the bed as previously described. After thirty minutes of test measurements, to allow for return to the resting state, two measurements at ten-minute intervals were used to calculate the baseline forearm blood flow. Intra-arterial infusions of ascending doses of noradrenaline (NA) (5,10,50,100 pmol/min), a control vasoconstrictor, were each infused for a period of seven minutes. Blood flow measurements were recorded three minutes after each infusion commenced, over a period of three minutes. Following a thirty-minute washout period during which a saline infusion was re-commenced, two further baseline measurements were recorded. In the remaining part of the study N^G monomethyl-L-arginine (L-NMMA), a stereospecific inhibitor of NOS which gives an index of basal NO synthesis, was infused following the same protocol at 2,4,8 μmol/min.

Mean blood flow responses for each dose of NA and L-NMMA were determined for individual subjects as previously described. An overall summary measure was then calculated for each dose of agent used, and the responses before and after oestradiol treatment compared using a 2-sample Student's t-test with 95% confidence intervals.

4.2 Results

Nineteen women were recruited to the study. Three withdrew after the initial counselling visit, prior to commencing the study. A further six women had to be withdrawn as a result of failed arterial cannulation. Where arterial cannulation failed on the second visit (i.e. post oestrogen) the study was abandoned, however if the arterial cannulation failed at the initial baseline visit, the women were asked to return for a further attempt. All women except one, who had a needle phobia, attended for a second visit, although subsequent attempts to cannulate were also unsuccessful.

Ten healthy non-obese, (BMI 24.8±4.7 kg/m^2) normotensive (mean blood pressure 124/70), women aged between 35 and 60 years, all of whom were non-smokers, completed the study. Three women had undergone a natural menopause (mean duration from the menopause 7.0± 3.46years), and all had been taking HRT prior to taking part in the study (Premarin or Trisequens). One woman had previously undergone total abdominal hysterectomy with ovarian conservation at the age of 45 years for fibroids. The remaining seven women were recruited postoperatively from the gynaecology ward, following total abdominal hysterectomy and bilateral salpingo-oopherectomy for benign gynaecological disease. The circulating oestradiol levels at baseline indicated biochemical postmenopausal status in all subjects at the time of entering the study. The mean circulating oestradiol level during the treatment cycle was 243±170pmol/L.

The results of this study are detailed in appendix 2, and are summarised in tables 4.1 & 4.2, and in figures 4.2 & 4.3. Responses to NA were similar before and after treatment and no statistically significant differences were apparent (95%CI –28.2, +7.20, p=0.23). Similarly, no statistically significant differences in the vasoconstrictor responses to L-NMMA were observed before and after oestradiol treatment (95%CI –31.2, +4.8, p=0.14).

Noradrenaline infusion	FBF Ratio (pre)	FBF Ratio (post)
5pmol/min	-10.67±17.86	+4.04±21.06
10pmol/min	-6.02±19.97	-3.50±18.87
50pmol/min	-7.62±27.21	-4.79±23.62
100pmol/min	-13.00±43.20	+1.18±29.51

Table 4.1 - Mean summary data for NA infusion before and after oestradiol treatment

L-NMMA infusion	FBF Ratio (pre)	FBF Ratio (post)
2μmol/min	-13.84±14.52	-3.55±26.67
4μmol/min	-30.87±12.06	-20.31±18.38
8μmol/min	-18.30±37.90	-23.95±23.78

Table 4.2 - Mean summary data for L-NMMA infusion before and after oestradiol treatment

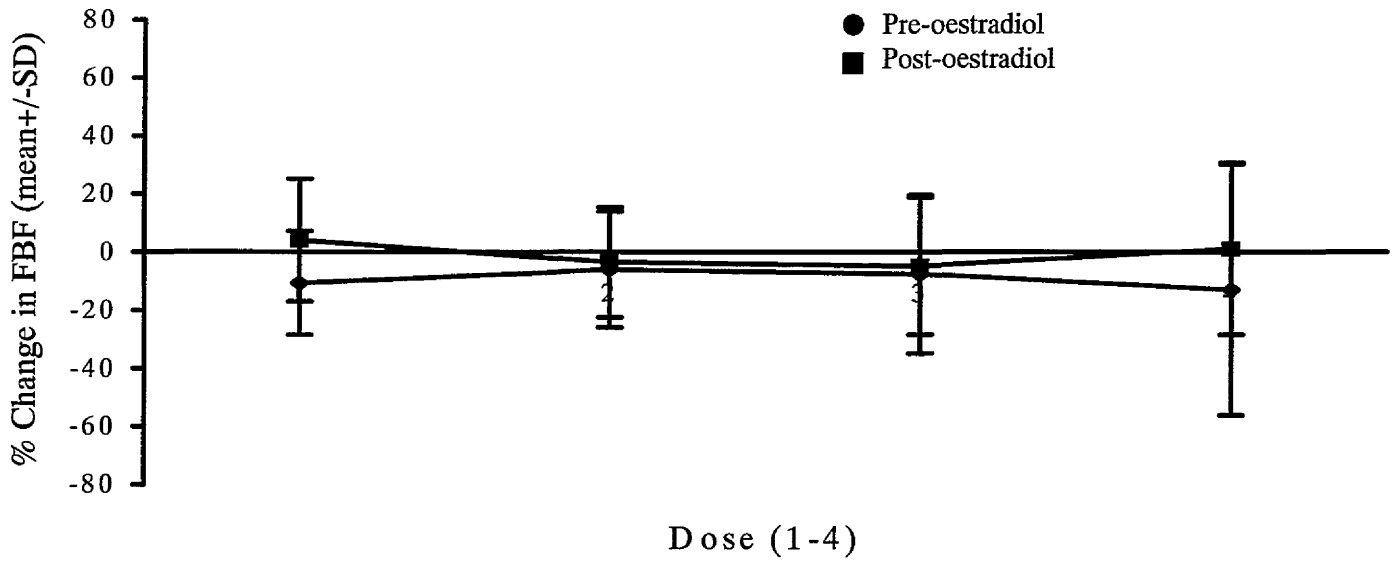


Figure 4.2 - Mean summary data for noradrenaline infusion before and after oestradiol

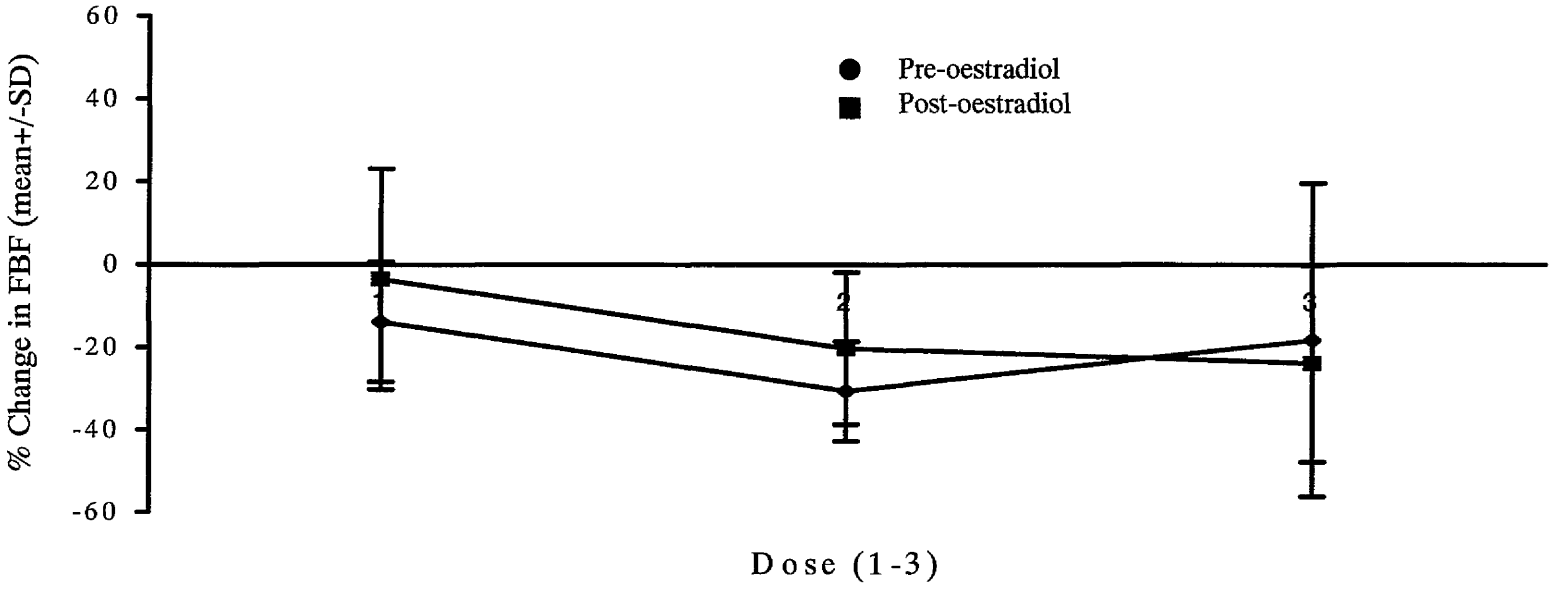


Figure 4.3 - Mean summary data for L-NMMA infusion before and after oestradiol

4.3 Discussion

It is well recognised that vascular smooth muscle cell relaxation, causing blood vessel dilatation, occurs as a result of the release of a number of vasoactive factors by endothelial cells in response to a variety of chemical and physical stimuli including ACh, histamine, serotonin, blood flow and shear stress (Vanhoutte, 1989). One of the principal vasoactive mediators thought to be released as a consequence of this, is NO. Atherosclerosis is associated with impaired endothelial-dependent relaxation, and it is thought that this may be as a consequence of impaired production or release of NO. There is increasing evidence from both animal and human work to suggest that oestrogen stimulates secretion of NO, and may therefore protect the vasculature by facilitating the vasodilator responses in both normal and atherosclerotic arteries.

Basal release of NO from the vasculature is generally greater in vessels from female animals than in those from males, suggesting that female sex hormone status is important in regulation of basal vasomotor tone (Hayashi et al, 1992). A cyclical variation in expired NO production in women, with levels peaking in the middle of the menstrual cycle, is also highly suggestive of an influence of gonadal hormones on the synthesis and release of NO (Kharitonov et al, 1994).

A number of *in vitro* animal studies using a variety of species and vascular beds, have shown that oestrogen induces endothelium-dependent relaxation of vessels. Oestrogen facilitates the normal vasodilator response to ACh in rabbit femoral arteries (Gislard, Miller & Vanhoutte, 1998) and in porcine coronary vessels (Bell et al, 1995). Acute (Williams, Adams & Klopfenstein, 1990) and chronic (Williams et al, 1992) oestrogen administration attenuates or reverses the vasoconstriction effect of ACh in ovariectomised cynomolgus monkeys with coronary atherosclerosis.

In humans, there is also a suggestion of a role for the endothelium in the mediation of oestrogen-induced vessel relaxation. Studies of the coronary vasculature have shown that oestrogen attenuates or abolishes ACh-induced vasoconstriction when administered acutely in postmenopausal women with angiographically proven coronary artery disease (Reis et al, 1994; Collins et al, 1995). Similar responses to acute intracoronary 17β-oestradiol have been reported in postmenopausal women in healthy coronary vessels (Gilligan et al, 1994). The response of coronary arteries is also gender dependent with

evidence of 17β-oestradiol-induced modulation of ACh-induced coronary artery responses of female but not male atherosclerotic coronary arteries (Collins et al, 1995). Studies of the peripheral vasculature, most commonly the forearm, have also reported beneficial effects of oestrogen on vascular reactivity. A study of forty postmenopausal women, (of whom half had risk factors for vascular dysfunction) in whom forearm vascular responses to the endothelium dependent vasodilator ACh were studied before and during intra-arterial infusion of oestradiol, demonstrated that oestrogen potentiated the vasodilator response to ACh in both healthy and potentially diseased blood vessels (Gilligan, Quyyumi, Cannon, 1994). Similar findings have been reported in a recent plethysmography study which examined changes in forearm blood flow responses induced by intra-arterial infusion of ACh at baseline, one month after bilateral oophorectomy, and after three months of oestrogen replacement therapy (50μg transdermal oestradiol) in a small group of women (ten subjects) (Pinto et al, 1997). Endogenous oestrogen deprivation following bilateral oophorectomy resulted in a reduction in acetylcholine induced vasodilation compared with baseline, but this was subsequently restored by oestrogen supplementation. In contrast to these findings, a small randomised double blind placebo controlled study also examining forearm blood flow using venous occlusion plethysmography, showed no evidence of any alteration in the vasodilator responses to ACh following oestrogen supplementation (2mg oestradiol valerate daily for eight weeks duration) (Sudhir et al, 1996). However, enhanced vasoconstrictor responses to the nitric oxide synthase inhibitor L-NMMA was observed, suggesting that there was enhanced basal NO release in the forearm vasculature of perimenopausal women following oestrogen supplementation.

The results of this study do not support the hypothesis that transdermal oestrogen results in an improvement in basal endothelial NO synthesis in the forearm vasculature of healthy postmenopausal women. These data exclude a 10% absolute increase in basal NO release in the forearm vasculature following transdermal oestradiol treatment. This contrasts with the findings of other investigators (Sudhir et al, 1996). One possible explanation for differences between the studies may be the route of administration of oestrogen.

Transdermal oestrogen avoids any first pass metabolism, whereas oral oestradiol passes from the gut directly to the liver via the portal circulation giving high local concentrations, which profoundly affect hepatic metabolism. Hence any indirect effects

of oestrogen on endothelial function mediated by changes in serum lipids will be much greater. Thus, although this study did not demonstrate an effect of transdermal oestrogen in the forearm vascular bed, it clearly does not exclude an effect of oestrogen on the forearm vasculature if delivered by an alternative route, or indeed an effect on a different vascular bed when delivered by the same route.

Results from other studies have also argued against a role for NO in oestradiol mediated cardioprotection. One study, conducted in castrated apolipoprotein-E deficient mice (which develop early atherosclerosis), demonstrated that oestradiol prevented fatty-streak formation despite inhibition of NO by N^G nitro-L-arginine methyl ester (L-NAME), suggesting that the anti-atherosclerotic effect of oestradiol is independent of NO (Elhage et al, 1997).

Interestingly in this study, no clear dose response effect to NA was observed, although the doses used were similar to those used in previous studies by our group in healthy male subjects and appeared adequate during pilot studies. The variability observed is unlikely to be due to the experimental conditions as this part of the study was performed prior to the L-NMMA infusions, which displayed less variability and a dose response element. Similar findings have been reported by another group who observed blunted vasoconstrictor responses to NA in postmenopausal women, suggesting that this is perhaps a true biological response (Kneale et al, 1997).

This study was designed as a randomised double-blind placebo controlled study, however there was considerable difficulty in recruiting patients to a study of this nature as most women approached postoperatively expected to commence HRT prior to discharge from hospital and were unwilling to have their HRT withheld for up to three months. The study protocol was therefore adapted to a 'before and after' study to facilitate recruitment.

Although the procedure was generally well tolerated, the failure rate of intra-arterial cannulation was unusually high in this study compared to that encountered in previous studies in young healthy male volunteers conducted by our group. As assistance was sought from Dr J Petrie, Senior Lecturer, Department of Medicine and Therapeutics, University of Glasgow, who had considerable experience in performing arterial cannulation in male subjects, this was not felt to be related to the experience of the

operator, but rather that it was a feature of this particular sample group, and may be a reflection of different arterial physiology in this group.

CHAPTER FIVE

STUDY THREE

THE EFFECT OF OESTRADIOL ON THE GROWTH CHARACTERISTICS OF HUMAN AORTIC ENDOTHELIAL CELLS IN CULTURE

5.1 Methods

5.1.1 Establishing human aortic endothelial cells in culture

Using the protocols previously described in chapter 2.2.4, the cells were initially plated into seven small tissue culture flasks ($25cm^2$). After reaching confluence, several flasks of cells were harvested, transferred to cryovials, and stored in liquid nitrogen for future use. The remaining flasks were subcultured into medium and large flasks to provide an adequate stock of cells for experimental purposes. Any surplus cells were subsequently harvested and stored in liquid nitrogen to maintain adequate stocks.

5.1.2 Characterising human aortic endothelial cells

After re-establishing the cells in culture, in order to clarify the nature of the cells and confirm that they were indeed endothelial cells, immunocytochemistry was performed on several flasks of cells. Assistance with this technique was kindly provided by Dr I Montgomery, Department of Physiology, University of Glasgow.

After fixing in methanol, specimens were washed several times in Tris buffered saline (TBS). Non-specific binding sites were blocked with normal serum from the animal supplying the second antibody (anti-rabbit raised in a goat). The primary antibody (anti human Von Willebrand factor raised in a rabbit) was then applied to the slides which were placed at room temperature in a darkened moist chamber overnight. The slides were then washed several times with TBS, before applying the second antibody conjugated with Texas Red for several hours in a moist darkened chamber at room temperature. The slides were then rinsed in several washes of TBS, followed by a short immersion in running tap water. The slides were then mounted in a non-fluorescent mounting media (Immuno-mount). The finished slides were then examined with epifluorescence using a Zeiss Axiophot Microscope (Carl Zeiss) at x197 magnification. Photomicrographs were taken using Fuji Provia film (Fuji).

5.1.3 Growth studies

As the HAEC's used in this project had not previously been cultured within this laboratory, two preliminary experiments was set up to ascertain the behaviour and

growth characteristics of the cells, and to ensure that oestradiol would not be cytotoxic to the cells.

5.1.3.1 Preliminary growth experiments

In the first experiment half of the cells were exposed to a water-soluble preparation of 17β-oestradiol (10nM) (Sigma-Aldrich), with the remaining untreated cells acting as a control population. The cell growth in both groups was monitored on days four, six, eight, eleven and fourteen, by harvesting and counting the cells as previously described. A second experiment was performed to determine the cell growth in relation to different oestradiol preparations. In this experiment the cells were exposed to 10nM and 10pM concentrations of 17β-oestradiol and ethinyl oestradiol (Sigma-Aldrich). The cell growth on day four and day twelve was determined and compared with untreated controls.

On the basis of these pilot studies, it was decided to treat the cells with two doses of 17β-oestradiol (10nM and 10pM) and to harvest the growth curves on days four, eight and twelve. These doses of oestradiol were chosen as they are representative of the extremes of the physiological range and as similar concentrations had been reported by other investigators (Venkov, Rankin & Vaughan, 1996).

5.1.3.2 Growth curves

Four principal experiments were performed. All experiments were performed under identical standardised conditions using cells form the third to eighth passages. A total of twenty seven small flasks were required per experiment (three sample days examining three sample groups, with three flasks per group).

Briefly, cells were seeded in complete endothelial growth medium into small flasks at a density of 60,000 cells/flask. After 24 hours the medium was removed and replaced with serum free medium (standard EBM with antibiotic supplements only). After a further 24 hours the medium was replaced with fully supplemented medium with or without 17β oestradiol (10nM or 10pM). The following day a number of flasks were harvested as described. Thereafter, the remaining flasks were fed and harvested on alternate days until day twelve according to the protocol summarised in table 5.1.

DAY 1	Inoculation
DAY 2	Serum Deprive
DAY 3	Feed ± Treat
DAY 4	**1st Harvest**
DAY 5	Observe
DAY 6	Feed ± Treat
DAY 7	Observe
DAY 8	**2nd Harvest**
DAY 9	Observe
DAY 10	Feed ± Treat
DAY 11	Observe
DAY 12	**3rd Harvest**

Table 5.1 – Growth curve protocol

A direct comparison of the mean cell counts for the control and oestrogen treated groups at each time point (day 4, 8 & 12) was determined for the individual experiments using simple 2-sample t-tests with 95% confidence intervals.

5.2 Results

Figure 5.1 shows a photomicrograph of the human aortic endothelial cells used in this project after staining with FITC and viewing at x196 magnification. These cells demonstrate a characteristic flattened epithelial-type cell morphology.

The results of the preliminary experiments are detailed in appendix 2, and are summarised in figures 5.2 & 5.3. The results of these studies demonstrated that it was possible to obtain reproducible cell growth using this cell population. Figure 5.2 shows that after the initial lag phase there is an exponential growth phase followed by tailing off of growth after fourteen days. The studies also indicated that the oestrogen preparations used were well tolerated by the cells. The results of these preliminary experiments were not incorporated into the final analysis of the results.

Four identical growth curve experiments were performed. Where possible, three measurements were taken per flask. The results of the individual growth curve are detailed in appendix 2, and summarised in figures 5.4-5.7. The results of the fourth experiment (figure 5.7) were excluded from the final analysis as the cell growth was erratic particularly in the control cells, and it was considered that this experiment might compromise the overall results and conclusions. Interestingly, the timing of this experiment coincided with a malfunction of the CO_2 incubator, which may have prejudiced the results of this particular experiment.

Each experiment demonstrated a trend towards increased cell growth with time which was independent of treatment. The only exception to this was seen in growth curve one, in which there was a failure in proliferation of the oestradiol (10pM) treated cells by day twelve. It was considered that this was a spurious observation, as it was not replicated in subsequent experiments. In general, the cell proliferation was enhanced by oestradiol in a time and dose dependent fashion.

No statistically significant differences between the growth in the control or oestrogen treated cells was observed on day four and eight. However, following treatment with 10nM 17β-oestradiol a statistically significant increase in cell growth was consistently observed in each experiment (growth curve 1 – P = 0.039, 95% CI –0.277, -0.0008;

growth curve 2 – P = 0.007, 95% CI –0.141, -0.029; growth curve 3 – P = 0.013, 95% CI –0.0537, -0.0082).

Although the individual measurements in each experiment were almost normally distributed, there was considerable variation in the absolute cell counts between the individual experiments which tends to skew the overall data set making pooling the data unreliable. However, this can be overcome by performing a log transformation of the data which then allows simple parametric analysis using 2-sample t-tests, with 95% confidence intervals. Again a statistically significant difference is seen following 10nM 17β-oestradiol treatment at day twelve (P = 0.026, 95% CI –0.63, -0.04).

Figure 5.1 - Photomicrograph of human aortic endothelial cells stained with fluorosceine isothiocyanate and viewed with Zeiss Axiophot Microscope at x196 magnification

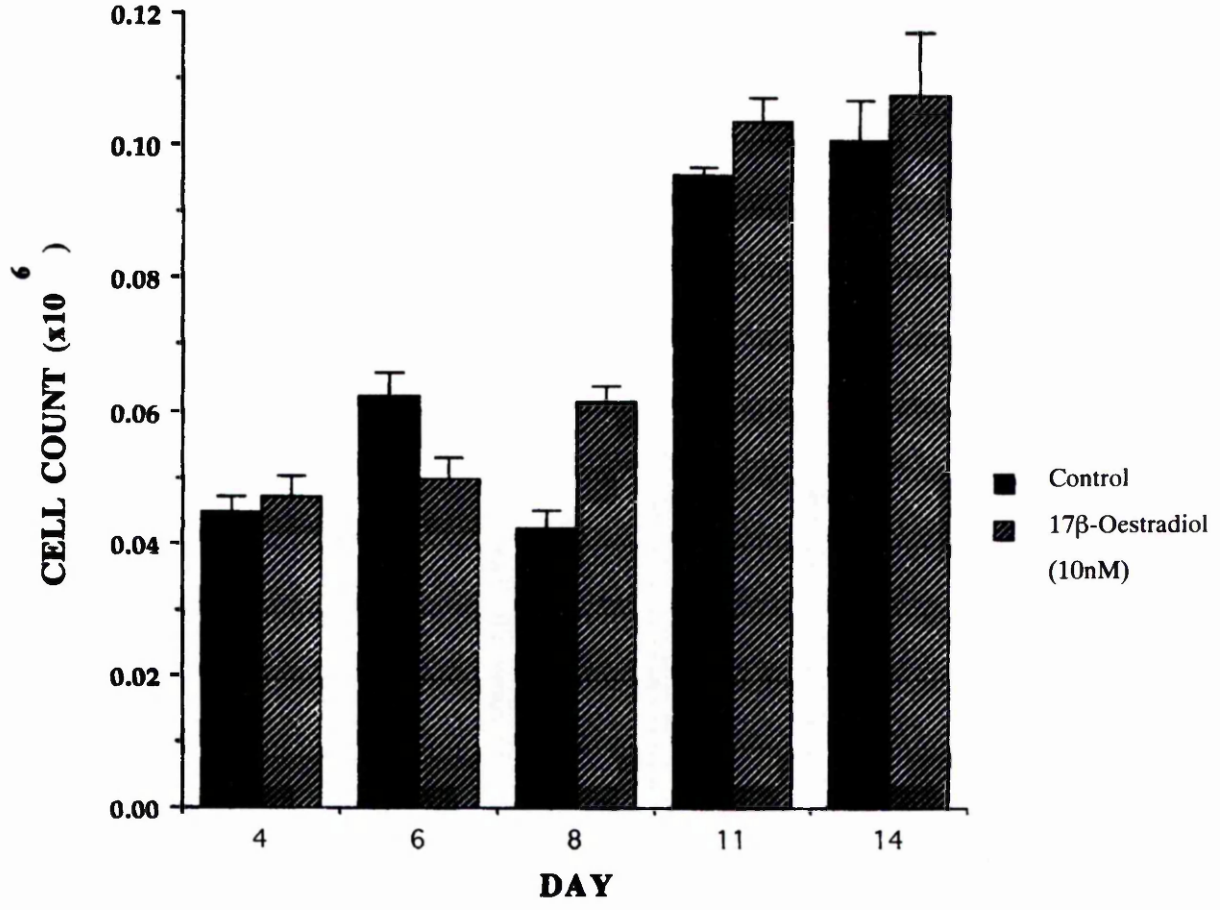


Figure 5.2 - Pilot study 1

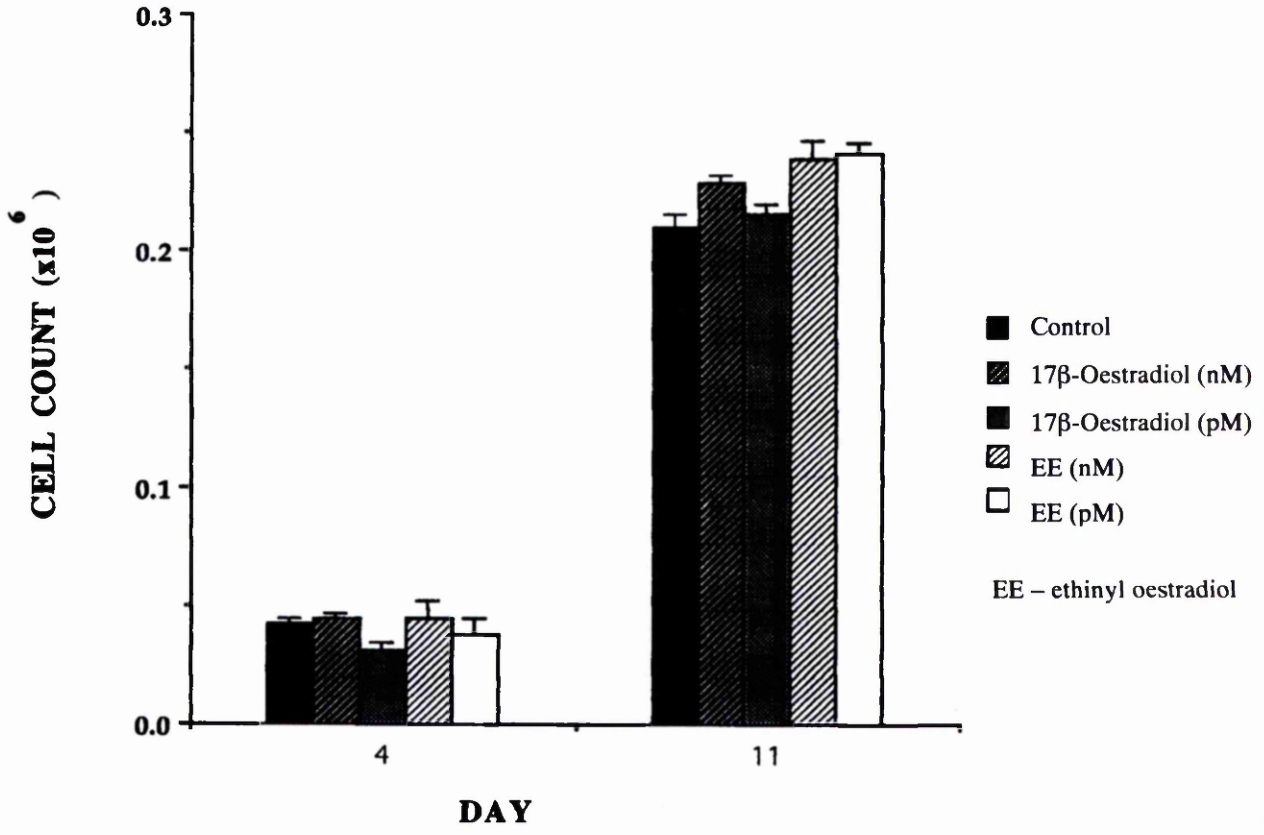


Figure 5.3 – Pilot study 2

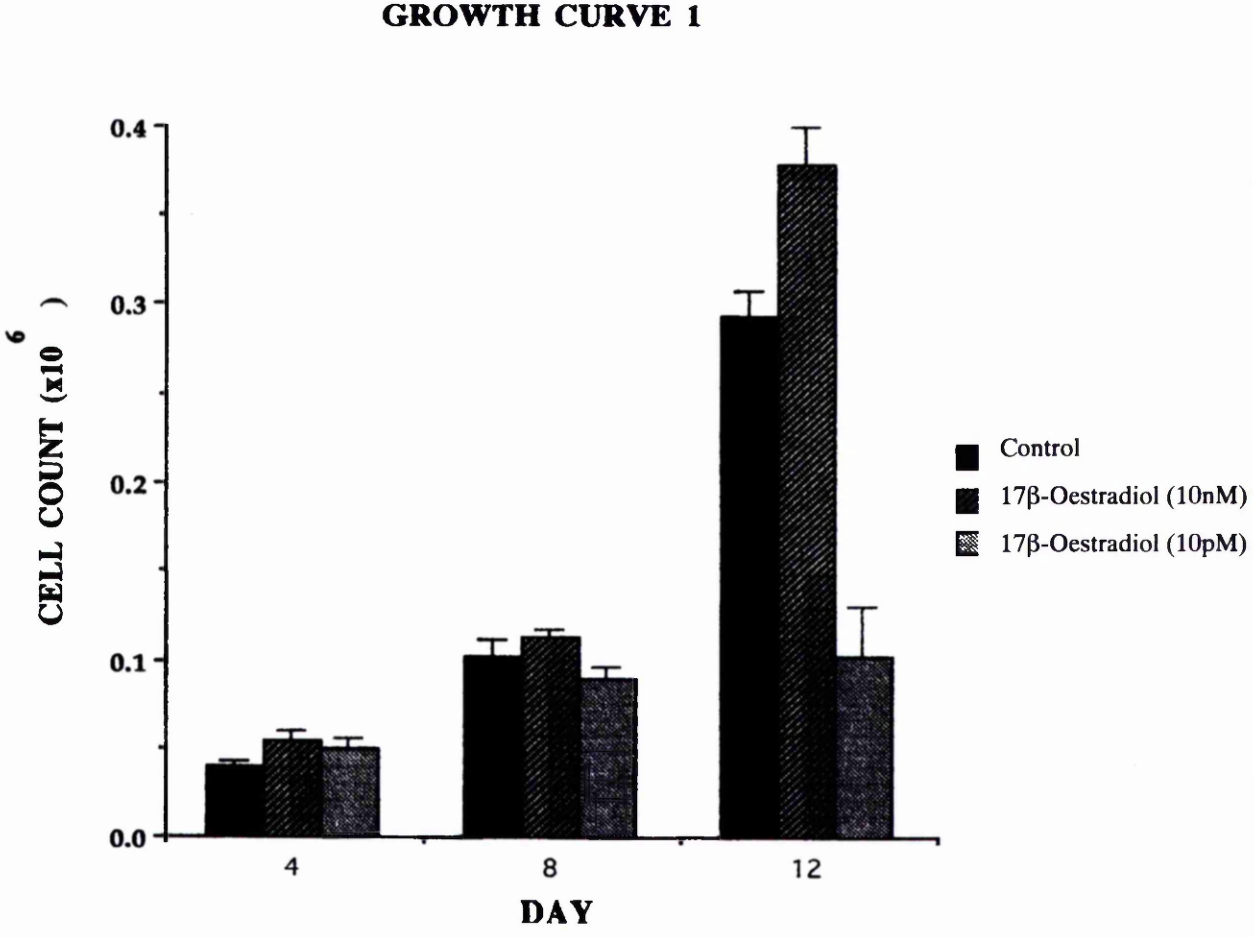


Figure 5.4 – Growth curve 1

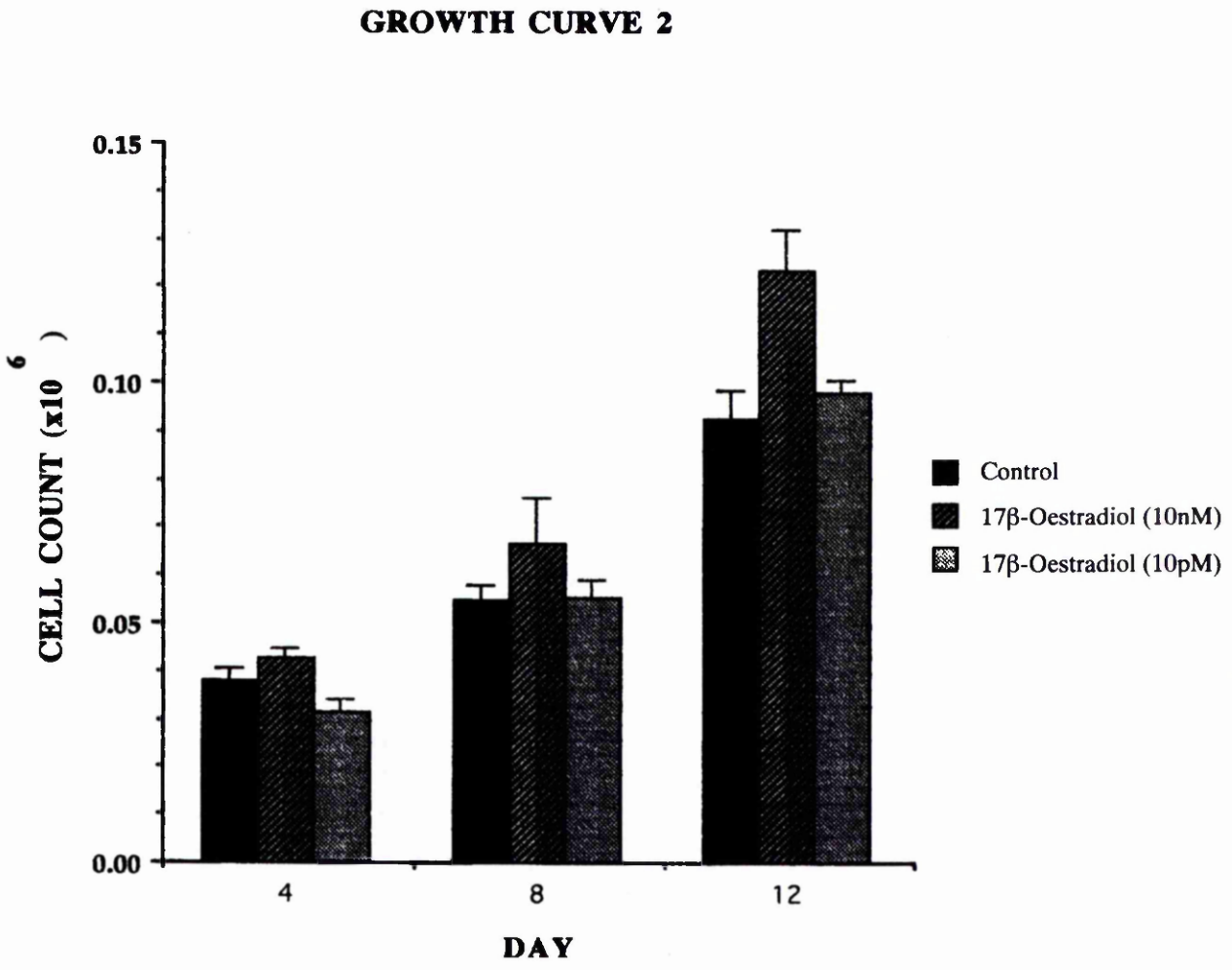


Figure 5.5 – Growth curve 2

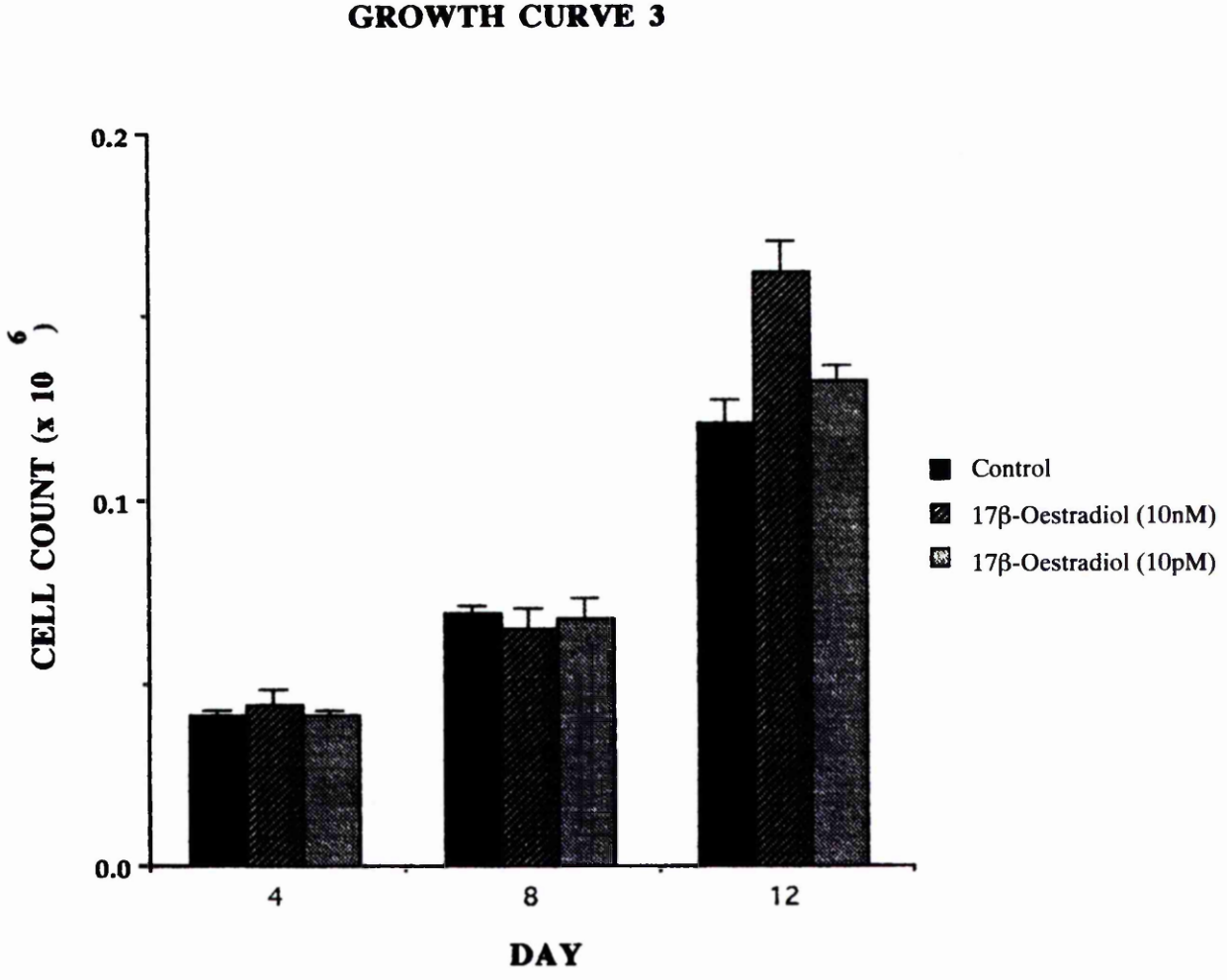


Figure 5.6 – Growth curve 3

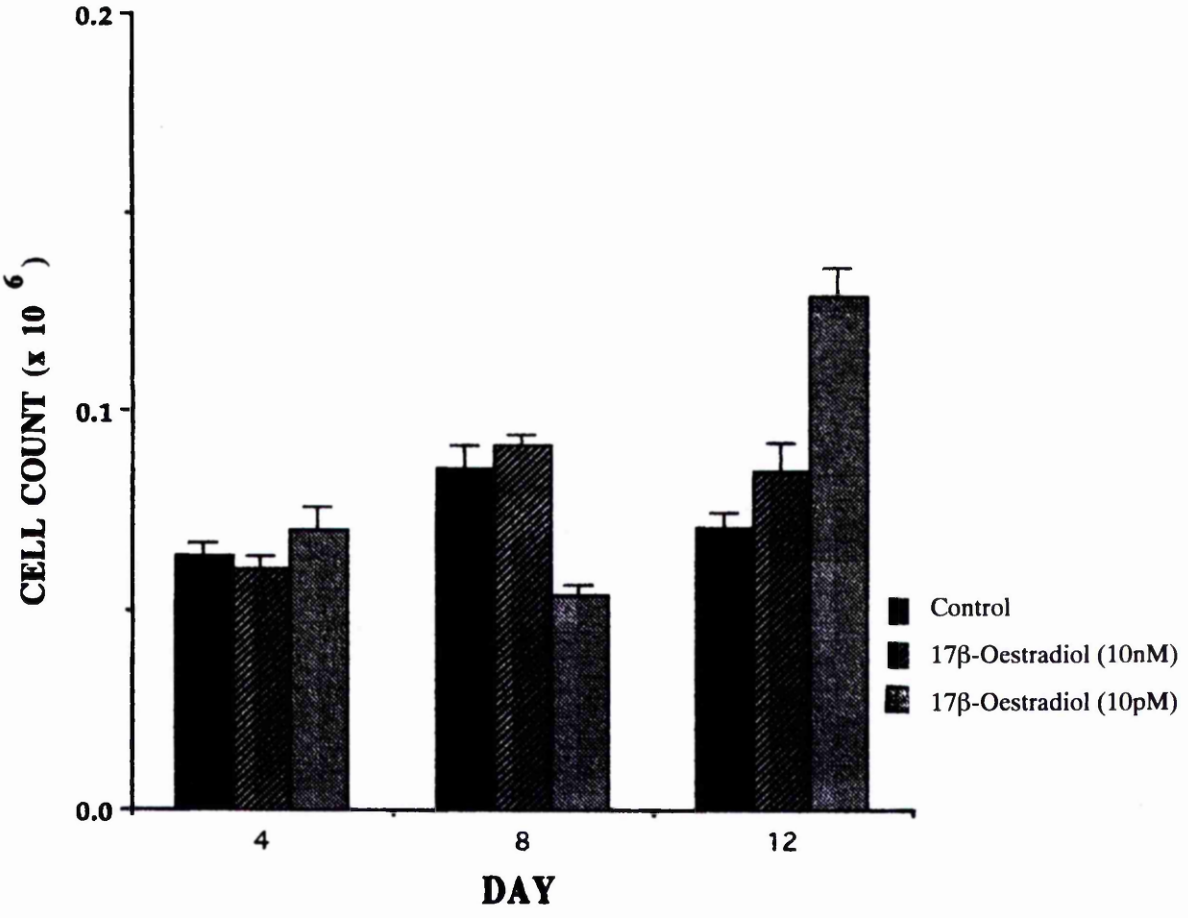


Figure 5.7 – Growth curve 4

5.3 Discussion

In the past there was considerable difficulty in establishing and maintaining primary human cell lines in culture, however the recent introduction of commercially available sources of human cells has generated renewed interest in the field of human tissue culture research. The cell culture work described in this thesis was developed from cells obtained commercially due to their relative ease of availability, and due to constraints of time.

Endothelial cells are now known to express functional oestrogen receptors. Studies of human aortic and umbilical vein endothelial cells and bovine aortic endothelial cells, confirmed that these cells expressed an abundance of mRNA for the classical oestrogen receptor, and furthermore demonstrated using electrophoretic mobility shift assays that each endothelial cell type contained a protein which reacted with anti-oestrogen receptor monoclonal antibodies specific to different domains of the oestrogen receptors (Venkov, Rankin & Vaughan, 1996).

Endothelial 'injury' is thought to be the key event in the initiation of atherogenesis and the development of atherosclerosis (Ross, 1993), and thus protection of the endothelium from toxic insults could represent a potential mechanism for protection against atherosclerosis. Furthermore, stimulation of endothelial cell growth and prevention of smooth muscle cell migration and proliferation should be of benefit for restoration of normal vascular function.

In cell culture experiments, 17β-oestradiol promotes proliferation of human umbilical vein endothelial cells (Morales et al, 1995), although other studies using the same cell type have either detected no effect (Miller et al, 1994) or only a very weak effect of a stable oestrogen analogue (Corvazier et al, 1984). Similarly, there have been inconsistencies reported when using cultured bovine aortic endothelial cells with reports of enhanced (Piotrowicz et al, 1995) and reduced (Drummond et al, 1996) endothelial cell proliferation in response to 17β-oestradiol treatment. These differences are probably a reflection of differences in culture conditions, species variations and variations dependent on different vascular beds, as well as the assays used for evaluation. Although readily available and more easily established in culture, human umbilical vein endothelial cells may behave very differently from endothelial cells

derived from adult tissue and for this reason were not used in this work. Similarly, there is debate as to whether the endothelial cell responses to oestrogen are conserved across the species and therefore it was decided to use human cells in these studies. Accepting the limitation that endothelial cell behaviour may not be uniform across different vascular beds within the same species, human aortic endothelial cells were used for the purpose of these studies.

The results of this study confirm that high dose oestrogen treatment is associated with a significant increase in cell number after sustained treatment. However, as this was not a functional study, it does not explain the potential underlying mechanism. Two candidate mechanisms exist. Firstly, oestrogen may generate an increase in cell number by directly stimulating cell growth by the release of growth promoting factors. A recent study showing that oestrogen increased wound healing of the endothelial cell monolayer and enhanced the ability of endothelial cells to organise into a tubular network (equated to angiogenesis), would support this argument (Morales et al, 1995). Alternatively, it has been suggested that oestrogen may be acting as a survival factor preventing apoptosis, although to date only one study has reported this as a plausible mechanism (Spyridopoulos et al, 1997). In this study the authors examined the potential role of oestrogen as a survival factor by examining the ability of oestrogen to inhibit cytokine induced (Tumour Necrosis Factor α (TNF-α)) programmed cell death in human umbilical vein endothelial cells. Their results indicated that oestrogen treatment resulted in a dose-dependent receptor-mediated inhibition of TNF-α induced endothelial cell apoptosis.

As the increase in cell numbers seen appears to be a time dependent effect it is therefore suggestive of an underlying genomic mechanism. Oestrogen may regulate cell proliferation through alteration of gene expression and synthesis of protein involved in the regulation of the cell cycle (Brown et al, 1984; Dubick, Dembiski & Shiu, 1987). Numerous genes have been identified in vascular tissues as potential targets for oestrogen regulation, including the prostaglandin cylo-oxygenase and prostaglandin synthase (Chang et al, 1980), collagen and elastin synthase (Cembrano et al, 1960; Wolinsky, 1972) as well as NOS (Weiner et al, 1994).

Transdermal oestrogen avoids any first pass metabolism, whereas oral oestradiol passes from the gut directly to the liver via the portal circulation giving high local concentrations, which profoundly affect hepatic metabolism. Hence any indirect effects of oestrogen on endothelial function mediated by changes in serum lipids will be much greater. Thus, although this study did not demonstrate an effect of transdermal oestrogen in the forearm vascular bed, it clearly does not exclude an effect of oestrogen on the forearm vasculature if delivered by an alternative route, or indeed an effect on a different vascular bed when delivered by the same route.

Results from other studies have also argued against a role for NO in oestradiol mediated cardioprotection. One study, conducted in castrated apolipoprotein-E deficient mice (which develop early atherosclerosis), demonstrated that oestradiol prevented fatty-streak formation despite inhibition of NO by N^G nitro-L-arginine methyl ester (L-NAME), suggesting that the anti-atherosclerotic effect of oestradiol is independent of NO (Elhage et al, 1997).

Interestingly in this study, no clear dose response effect to NA was observed, although the doses used were similar to those used in previous studies by our group in healthy male subjects and appeared adequate during pilot studies. The variability observed is unlikely to be due to the experimental conditions as this part of the study was performed prior to the L-NMMA infusions, which displayed less variability and a dose response element. Similar findings have been reported by another group who observed blunted vasoconstrictor responses to NA in postmenopausal women, suggesting that this is perhaps a true biological response (Kneale et al, 1997).

This study was designed as a randomised double-blind placebo controlled study, however there was considerable difficulty in recruiting patients to a study of this nature as most women approached postoperatively expected to commence HRT prior to discharge from hospital and were unwilling to have their HRT withheld for up to three months. The study protocol was therefore adapted to a 'before and after' study to facilitate recruitment.

Although the procedure was generally well tolerated, the failure rate of intra-arterial cannulation was unusually high in this study compared to that encountered in previous studies in young healthy male volunteers conducted by our group. As assistance was sought from Dr J Petrie, Senior Lecturer, Department of Medicine and Therapeutics,

University of Glasgow, who had considerable experience in performing arterial cannulation in male subjects, this was not felt to be related to the experience of the operator, but rather that it was a feature of this particular sample group, and may be a reflection of different arterial physiology in this group.

CHAPTER SIX

STUDY FOUR

DETERMINATION OF eNOS EXPRESSION IN CULTURED HUMAN AORTIC ENDOTHELIAL CELLS FOLLOWING OESTRADIOL TREATMENT

6.1 Method

6.1.1 Extraction and assessment of mRNA from cultured human aortic endothelial cells

Untreated (control) cells and cells which had been treated with 10nM or 10pM 17β-oestradiol for either twenty-four hours or seven days were used for these studies. Total RNA was extracted from the cells using RNAzol B (Biogenesis Ltd, Poole) according to their protocol. Within a laminar-flow hood (Bio Mat II Medical Air Technology Limited, Manchester), medium was extracted from the flasks using an aspirator system and discarded. The base of the tissue culture flask was covered with RNAzol B, and the flask gently agitated by rocking the flask back and forth to dislodge the cells. The volume of RNAzol B required was dependent on the size of the flask: 8ml/175cm^2, 6ml/80 cm^2, and 4ml/25 cm^2. After harvesting the flask contents were transferred to a sterile plastic Falcon tube using a sterile 10ml pipette. A 1/10 volume of chloroform (i.e. 800μl, 600μl or 400μl depending on the size of the flask) was added to the Falcon tube which was vigorously shaken for fifteen seconds. The cells were then transferred on ice to a centrifuge and centrifuged for five minutes at 2500rpm (4°C). Glass Corex tubes which had previously been soaked in 3% w/v hydrogen peroxide, wrapped in silver foil, then baked at 220°C to destroy any RNAses, were labelled for each sample. After centrifuging (IEC Centra-7R), there was separation into three phases. The upper aqueous phase, which contains the RNA, was carefully removed using a sterile fine-tipped Pasteur pipette taking great care not to disturb the interphase containing DNA which separates the two layers. An equal volume of isopropanol (propan-2-ol) was then added to the Corex tube which was then covered with Parafilm (3M Products), mixed by inversion several times, and left to stand on ice for a minimum of fifteen minutes or alternatively was left overnight at -20^{0}C to allow precipitation. Following precipitation, the RNA was pelleted by centrifuging at 10,000rpm (4°C) in a Beckman centrifuge (Model J2-21, Beckman) for fifteen minutes. The supernatant was then discarded and the pellet, which was visible as a white smear on the inside of the Corex tube, was washed with 2ml of 70% w/v ethanol then re-spun at 10,000rpm for a further fifteen minutes at 4^{0}C. The supernatant was then removed using a sterile 1000μl pipette (plugged tip), and left to dry on ice. At this stage extreme care was taken to ensure that as much of the ethanol as possible was allowed to evaporate without allowing the RNA to dry out, in order to maximise the yield of RNA. The RNA pellet was then re-

solubilised in DEPC water (100µl or 50µl depending on the size of the flask from which the RNA was extracted) and centrifuged briefly for several minutes at 1000rpm. Finally, the contents were transferred to previously sterilised Eppendorf tubes and stored at -70^{0}C. The integrity of the RNA was determined by ethidium bromide stained agarose gel electrophoresis.

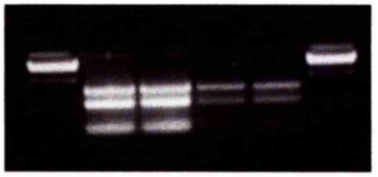
6.1.2 Preparation of an agarose gel

An agarose gel was prepared by dissolving a known amount of agarose, measured in grams, in a given volume of 1x tris-acetate buffer (1xTAE). A 1% gel was prepared by dissolving 1g of agarose in 100ml of 1xTAE for a standard gel (10cm), or 0.5g in 50ml for a small gel (5cm). A 2% gel was prepared by dissolving 2g in 100ml etc. The solution was then microwaved on high power for one to two minutes until boiling, then transferred to a fume hood where ethidium bromide was added (2µl), the solution mixed thoroughly and left to cool until hand hot. The gel was then poured into a gel tray, a comb inserted and the gel was left to set for at least thirty minutes. Once set, the comb was removed and the gel tray placed in the gel tank with 1xTAE buffer, sufficient buffer being added to submerge the gel completely.

6.1.3 Preparation of RNA for electrophoresis

Samples of total RNA (2µl) were prepared in Eppendorfs for electrophoresis after addition of 3µl dH_2O and 1µl 6x loading dye (agarose gel blue). An appropriate control sample, for example Lambda Hindi III (λHIII), was also run on the gel to ascertain that the gel was run correctly.

After preparation of the gel as described above, the wells were loaded with appropriately prepared samples. Extra care was taken while loading each well to ensure equal and even loading. The gel tank (Hybaid) was then connected to an electrical power source (EPS 200/1000, Pharmacia Biotech) and run at 100V for thirty to forty minutes. Once completed, the power source was disconnected and the gel carefully transferred to an ultraviolet illuminator (Chromato-vue, Mod TM-20) to allow visualisation. Good quality RNA can be clearly visualised as three distinct bands (figure 6.1).



Lane 1 2 3 4 5 6

Lane 1 & lane 6 – molecular weight markers (λH III)

Lane 2 – control

Lane 3 – control

Lane 4 – 17β-Oestradiol (10nM)

Lane 5 – 17β-Oestradiol (10pM)

Figure 6.1 – Resolution of RNA transcripts by agarose gel electrophoresis

6.1.4 Quantification of RNA

RNA yield was quantified spectrophotometrically using an Ultraspec 2000, UV/Visual Spectrophotometer, Pharmacia Biotech.

The machine was switched on approximately fifteen minutes before it was required for use to enable it to perform a self-calibration test. A standard 1ml cuvette provided with the machine was rinsed with distilled water (dH_2O), then 1ml of dH_2O was added to the cuvette which was placed in the spectrophotometer. The machine was then set to RNA mode, with the background correction off, and references set for the blank (dH_2O). A 1µl sample of RNA was then added to the cuvette, the sample run and the results noted. This was repeated three times for each sample and the mean calculated. For each µl sample, the concentration is expressed in µg/µl. The results were expressed as follows:

OD_{260}	OD_{280}	Ratio (OD_{260}/ OD_{280})	Concentration (µg/µl)

To avoid cross contamination between samples, the cuvette was thoroughly rinsed with dH_2O between each sample.

6.1.5 Synthesis of human eNOS cDNA probe

A human eNOS probe was prepared from a stock plasmid, pHu NOS endo PM831221, which was obtained courtesy of Dr Philip Marsden, University of Toronto, Canada (Marsden et al, 1992).

The concentration of the stock eNOS plasmid DNA was 5μg/μl. This was diluted to a concentration of 1μg/μl with TE buffer (1μl = 5μg therefore 1μl diluted to 5μl corresponds to 1μg/μl).

Diluted stock human eNOS plasmid DNA was thus used to prepare digests of cut (1μl plasmid DNA (1μg), 1μl EcoR1, 1μl 10x buffer, 7μl dH_2O) and uncut (1μl plasmid DNA (1μg), 9μl TE buffer) fragments. The cut and uncut fragments were then run on an agarose gel to assess the quality of the digest. A 1% agarose gel was prepared as previously described, and run at 100V for 45 minutes. The gel was loaded thus: Lane 1 (control) 4μl λHIII; Lane 2 (uncut fragment) 10μl uncut, 2μl Tris; Lane 3 (cut fragment) 10μl cut, 2μl Tris.

Having established that the above mini-digest was satisfactory, the process was repeated for the main digest using 10μl (50μg) of plasmid DNA. The main digest was prepared as follows - cut fragment (10μl plasmid DNA, 5μl EcoR1, 10μl Buffer H, 75μl dH_2O); uncut fragment (1μl plasmid DNA, 9μl TE buffer). The cut fragment was allowed to digest overnight at 37°C. The following morning a 1% agarose gel was prepared and loaded thus: Lane 1 (control) 4μl λHIII; Lane 2 (uncut fragment) 10μl uncut, 2μl Tris; Lane 3 (cut fragment) 10μl cut, 2μl Tris.

The gel was initially run at 100V for forty-five minutes to determine if the digest had been satisfactory. The remainder of the DNA digest was then loaded onto the gel and allowed to run over several hours. On completion, the required band was cut from the gel and placed in a polythene membrane previously rinsed with dH_2O, and the bottom was sealed with a clip. One micro-litre of 1xTAE was added to the membrane which was sealed with a second clip, and placed in a gel tank and run for twenty minutes. The gel was then visualised under ultraviolet light to ensure that the band had completely resolved into the TAE. The liquid was then decanted into two Eppendorfs (0.5ml each)

and an equal volume of phenol was added (0.5ml). The Eppendorfs were then whirlimixed (Fisons Whirlimixer, Loughborough) for thirty seconds prior to centrifugation for four minutes in a bench-top microfuge (Eppendorf Mod 5415C). The top layer was then removed using a fine tipped Pasteur pipette, transferred to a sterile Eppendorf tube and a further 0.5ml phenol/chloroform added, before re-spinning in the microfuge for a further four minutes. This step was then repeated, before adding 50μl of 3M Sodium Acetate (1/10 volume) and 1000μl of 100% ethanol (2x volumes). The digest was then transferred to a –20°C freezer for forty-eight hours. The digest was then removed from the freezer and transferred to a pre-cooled (4°C) centrifuge and spun at 1400 rpm for fifteen minutes (Eppendorf Mod 5402). The supernatant was removed and the pellet washed with 70% v/v ethanol prior to re-spinning for a further five minutes. The supernatant was then decanted and the pellet allowed to dry in air for twenty minutes. The pellet was then re-solubalised in 25μl of buffer and refrigerated overnight prior to quantification. The fragment was quantified using a DNA spectrometer (Hoeffer Dyna Quant 200), and the concentration adjusted to 40ng/μl with TE buffer. The quality of the DNA fragment isolated and the completeness of the digest was then determined by running by running a small aliquot on a 1% agarose gel, with an appropriate DNA standard and a control (λHIII).

The above method of probe synthesis was also used to manufacture a GAP-DH probe. This had previously been prepared within the laboratory and was kindly made available for use by Dr J Brosnin, Lecturer, Department of Medicine and Therapeutics, University of Glasgow.

6.1.6 Northern Blotting

6.1.6.1 Preparation

The gel tank, comb and tray were soaked in 3% w/v hydrogen peroxide (H_2O_2) for one hour, thoroughly rinsed in DEPC treated water and allowed to dry inverted on a clean paper towel. The barrels of all pipettes were cleaned with 3% w/v H_2O_2 then covered with plastic film. Finally the surrounding bench area was prepared by thoroughly cleaning the working area with 3% w/v H_2O_2 solution and clearly indicating that it had been prepared as such.

6.1.6.2 RNA Sample preparation

The required amount of RNA (10μg) was precipitated on ice thus: 10μl RNA (1μl = 1μg), 3 volumes 100% ethanol (30μl), 1/5 volume sodium acetate (2μl), and added to appropriately labelled sterile Eppendorf tubes, which were transferred to a microfuge and spun briefly before being transferred to a –20 °C freezer overnight to allow precipitation.

The samples were transferred to a pre-cooled (4 °C) centrifuge and spun on maximum speed (14000 rpm) for fifteen minutes taking care to orientate the tubes correctly in the centrifuge (hinge of lid orientated to outside edge ensured pellet formation in line with hinge) as this aided subsequent identification of the RNA pellet. The supernatant was then removed and the pellet washed with 180μl of 70 % w/v ethanol before re-spinning at 4 °C for a further five minutes. With the supernatant removed the pellet was then left to stand to allow to dry for fifteen minutes, before the pellet was resolubilised in 3.7μl of DEPC H_2O. To avoid obtaining a low yield of RNA, care had to be taken to ensure that the pellet was free of ethanol prior to resolubilising and that it had properly re-dissolved. 12.3μl of fresh glyoxal solution was then added to the RNA, and the samples transferred to a dry heating block (Griffin Dri-Block Unit, Griffin & George Ltd, London) at 50 °C for one hour. After heat denaturing, the samples were then transferred to ice for snap cooling before adding 4μl of 5x RNA loading dye. After briefly spinning in a microfuge the samples were immediately loaded on to a previously prepared agarose gel.

6.1.6.3 Preparation and running an RNA gel

A 1.2% agarose gel containing (0.1M $NaPO_4$ buffer) was prepared by dissolving 1.6g of electrophoresis grade agarose in 120ml DEPC H_2O by microwaving, and adding 13.3 ml of 0.1M $NaPO_4$ once cooled to 50 °C. The gel was then poured in the RNA designated gel tray and the comb added. To prevent leakage of the gel the gel tray was carefully sealed with autoclave tape. The gel was allowed to stand for at least 30 minutes to set prior to loading.

The gel tank (Hybaid Ltd) used for RNA work differs from conventional gel tanks in that it has the facility to re-circulate the buffer whilst the gel is running to prevent pH changes. The previously prepared gel was immersed in 1L of 10mM $NaPO_4$ tank running buffer, and carefully loaded with the prepared samples. A space was left between the molecular weight marker lane and the first sample to enable the marker lane to be cut off later and stained to confirm that appropriate transfer has taken place. The gel was run for four to six hours at 100V to allow adequate resolution of the RNA samples, i.e. until the RNA had migrated approximately 10-12cm from the loading wells. Once completed the RNA could be transferred to an appropriate membrane.

6.1.6.4 Capillary transfer of RNA

The RNA was transferred from the gel to a positively charged nylon membrane (Hybond N^+, Amersham International plc,) using an upward capillary transfer system.

Three Whatman paper 'bridges' (Whatman Labsales Ltd) were cut to the same width and approximately twice the length of the gel being blotted. A further nine pieces of Whatman paper were cut to the same size as the gel. A piece of membrane was cut to 1mm less than the gel size, taking great care not to damage the membrane or handle it excessively. The 'bridges' were soaked in 20x SSC and laid over the base support smoothing out any air bubbles and ensuring both ends were in contact with the transfer buffer (20x SSC). The gel was then placed over the base support with the RNA surface uppermost. For orientation purposes the top left-hand corner of the gel was carefully removed using a scalpel blade. The membrane which had been pre-wet with 2x SSC, was the placed over the gel and the corner trimmed to match the gel. Any air bubbles were carefully smoothed out and the edges of the gel sealed with Parafilm to prevent

'short-circuiting'. Six of the previously cut pieces of Whatman paper were soaked in 2x SSC and placed on top of the filter and a further three dry pieces were placed on top of these before placing a 3-4cm thick pile of absorbent tissues on top of the Whatman paper stack. Finally a glass plate was placed on the top to support a 500g weight. After confirming that capillary transfer was taking place the transfer set up was left overnight. This required approximately 1L of transfer buffer.

After disassembling the transfer apparatus, the blot was air dried on a piece of clean Whatman paper for thirty minutes, then baked in an oven at 80°C for sixty minutes, before ultra-violet cross linking in a UV stratifier crosslinker (UV Stratalinker 1800, Stratagene). At this stage membranes were carefully stored dry prior to hybridisation.

To confirm that transfer has taken place the marker lane was carefully cut off and stained using methylene blue (0.04% w/v) in 0.5M sodium acetate, then rinsed in distilled water several times before air drying for 10 minutes. Visible bands confirmed that transfer had taken place (figure 6.2).

Figure 6.2 – Confirmation of transfer of RNA

6.1.7 Prehybridisation and hybridisation of membranes

The prehybridisation/hydridisation solution was made according to the following protocol: Deionised formamide (20.0ml), 20x SSPE (10.0ml), 100x Denhards solution (2.0ml), 10% w/v SDS (2.0ml), 0.4M EDTA (pH=8) (0.1ml), DEPC H_2O (5.9ml).

6.1.7.1 Prehybridisation

This solution described above was made up in a 50ml conical tube (Falcon), and 20ml added to the hybridisation bottle (Techne) and pre-warmed to 42°C prior to addition of the membrane for probing. Before adding the membrane to the hybridisation bottle, it was submerged in a tray of 6x SSC for five minutes. Extreme care had to be taken when transferring the wet membrane to the hybridisation bottle, to prevent damage to the membrane. The membrane was then prehybridised for a minimum of four hours at 42°C in a hybridisation oven (HBI Hybridisation Oven, Techne,).

6.1.7.2 Preparation of probe

During the prehybridisation step, a probe was prepared using a random priming kit (Gibco, Life Technologies). GAP-DH and eNOS probes were prepared in sterile Eppendorf tubes as follows:

	GAP-DH	eNOS
Probe	1μl (50ng/μl)	1.25μl (40ng/μl)
DEPC H_2O	22μl	21.75μl

Thereafter the preparation of the two probes was identical and they were heated to 100°C for five minutes before snap cooling on ice. The following reagents from the random priming kit were then added: dATP (2μl), dGTP (2μl), dTTP (2μl), random primer buffer (15μl). The Eppendorf tubes were then placed in a microfuge and briefly spun for a few seconds, before being transferred on ice to the radioactive hood for addition of the radiolabelled (^{32}P, 18.5mbq, α emmision) dCTP (5μl) and the Klenow fragment (1μl). The probe was then carefully mixed in the radioactive hood, and left to stand for at least one hour prior to checking the incorporation of the probe.

In duplicate, 1µl of the probe was carefully spotted on to a small piece of DE 81 filter disc (Whatman Labsales Ltd), allowed to dry, then placed in a scintillation counter tube. The tube was then placed in a perspex box and transferred to the scintillation counter (Tri-Carb 2100 TR, Packard Instruments). The scintillation tube was then removed from the perspex box once in the scintillation counter, transferred to a counting rack and run on a pre-set program. The activity in counts per minute (cpm) was noted. The tube was then removed from the counter and returned to the radioactive hood where a sequence of washings were undertaken thus: 5x washes Na_2 HPO_4; 2x washes d H_2O (removes salt from filter); 2x washes 100% ethanol (dehydrates filter). Care was taken to dispose of the washes in an appropriate receptacle for handling radioactive waste. The filter was then left to air dry in the hood for around ten minutes prior to transfer back to the scintillation counter for re-counting. It is important to allow the filter to dry before counting since moisture has a deleterious effect on the count. The incorporation of the probe was thus calculated from the two measurements and expressed as a percentage:

Count 1 (pre-wash) 1164357 cpm

Count 2 (post-wash) 946291 cpm

$$\% \text{ Incorporation } \frac{946291}{1164357} \times 100 = 81\%$$

A percentage incorporation above 50% was deemed satisfactory for the purposes of northern blotting.

6.1.7.3 Hybridisation

The probe was then transferred to a dry block and heated to 100 °C to denature the probe, taking care to ensure that the probe was appropriately shielded from other laboratory personnel. It was then removed from the heating block, snap cooled on ice and immediately added to the pre-warmed hybridisation solution. The prehybridisation solution was then decanted off and discarded, and the hybridisation solution added. The blot was then returned to the hybridisation oven at 42°C and left overnight to hybridise.

The following day the hybridisation solution was decanted off and disposed of in a radioactive sink in the radioactive hood. The blot was then rinsed according to the following regime: 2x rinses room temperature; 2x SSPE (0.1% SDS); 2x washes 15

minutes, 65 °C 2x SSPE; 2x washes 10 minutes, 65 °C 1x SSPE; 2x washes 30 minutes, 65 °C 1x SSPE

The early washes were generally carried out in the hybridisation bottles, although the final washes were usually performed in flat plastic tubs which were manually agitated intermittently to aid the washing process. This also enabled the blot to be assessed between washes by listening to the radioactivity with a Geiger counter (Series 900 Mini-monitor Mini-Instruments Ltd), as this provided a useful indicator of the quality of the blot and the need for further high stringency washes in the presence of high background noise.

After the final wash, the blot was carefully wrapped in cling film and exposed to radiographic film overnight. Once developed, the blot was re-exposed to film for a variable period of time dependent on the signal intensity and signal-to-noise ratio.

Care was taken to ensure that the blots were never allowed to dry out, and thus after probing they were stored in the freezer at -20 °C until requiring to be re-probed.

6.1.7.4 Quantification of blots

After developing, the blots were scanned into a phosphoimager (Biorad Molecular Imager FX, Biorad Instruments, UK), and quantified densitometrically as previously described in chapter 2.3.6.

6.1.8 Determination of oestrogen receptor status

The oestrogen receptor status of the HAEC was determined using RT-PCR. The work was undertaken by Dr Rosemary Bass on behalf of Dr Steve Charnock-Jones, Department of Obstetrics and Gynaecology, University of Cambridge, who has considerable expertise in this area of reproductive endocrinology and who kindly agreed to colloborate. Full details of their methodology have been published previously (Maclaren et al, 1996). In brief, RT-PCR was carried out using 2.6μg of RNA with primers to specifically amplify ERα and ERβ cDNA's. (Primer 1 – GGAGACATGAGAGCTGCCAA and primer 4 – TCATCATGCGGAACCGAGAT were used for the first round and primer 2 – CCAGCAGCATGTCGAAGATC and primer 3 – CTTTGGCCAAGCCCGCTC for the second round). Primers for ERβ were

designed from the published sequence (Primer 1 – TTACAGCATTCCCAGCAATG primer 2 – GAACCTGGACCAGTAACAG) and the amplified product was verified by cloning and sequencing.

6.2 Results

Cells between passages three and six were used for experiments and the results of five separate experiments performed under identical conditions are presented.

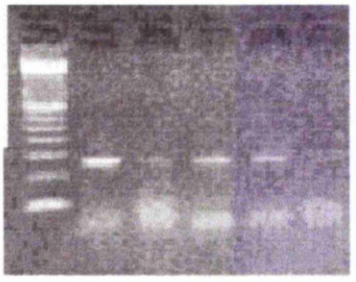
The presence of mRNAs encoding both classical ERα and ERβ in these HAEC was confirmed by RT-PCR. Figures 6.3 & 6.4 show samples of the PCR products for oestrogen receptors α and β obtained.

Using northern blotting, GAPDH and eNOS signals were readily detectable and quantifiable. Figure 6.5 shows a typical radiograph of a northern blot after probing for GAPDH and eNOS genes.

After quantification of the radiographic images, no significant change in eNOS gene expression was observed following oestradiol treatment. This was independent of the dose or duration of treatment. (Control 0.496 ± 0.24; 17β-oestradiol (nM, 24h) 0.422 ± 0.24, p=0.64; 17β-oestradiol (nM, 7d) 0.348 ± 0.17, p=0.30; 17β-oestradiol (pM, 24h) 0.240 ± 0.13, p=0.11; 17β-oestradiol (pM, 7d) 0.290 ± 0.08, p=0.13).

Oestrogen receptor α

L1 L2 L3 L4 L5 L6



Samples loaded left to right – lanes 1-6

Lane 1 – molecular weight marker

Lane 2 - control

Lane 3 - 17β-Oestradiol (10pM, 24h)

Lane 4 - 17β-Oestradiol (10pM, 7d)

Lane 5 - 17β-Oestradiol (10nM, 24h)

Lane 6 - 17β-Oestradiol (10nM, 7d)

Figure 6.3 – PCR products for oestrogen receptor α

Oestrogen receptor β

L1 L2 L3 L4 L5 L6

Samples loaded left to right – samples 1-6

Lane 1 – molecular weight marker

Lane 2 - control

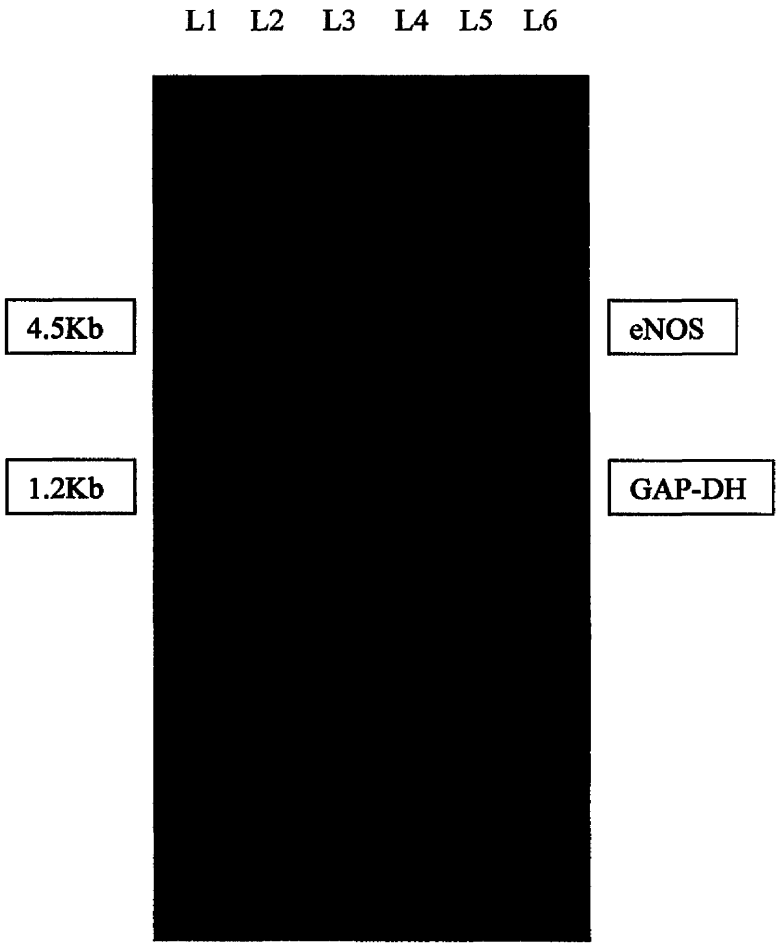
Lane 3 - 17β-Oestradiol (10pM, 24h)

Lane 4 - 17β-Oestradiol (10pM, 7d)

Lane 5 - 17β-Oestradiol (10nM, 24h)

Lane 6 - 17β-Oestradiol (10nM, 7d)

Figure 6.4 – PCR products for oestrogen receptor β



L1 – control, L2 – 10nM 17β-Oestradiol (24h), L3 – 10nM 17β-Oestradiol (7d)

L4 – control, L5 – 10pM 17β-Oestradiol (24h), L6 – 10pM 17β-Oestradiol (7d)

Figure 6.5 – Autoradiograph of northern blot probed for eNOS and GAP-DH signals

6.3 Discussion

This study was designed to parallel the *in-vivo* study discussed in chapter 4. The results of this study showed no evidence of an up regulation of eNOS gene expression in cultured aortic endothelial cells following exposure to oestrogen, and thus do not support the hypothesis that oestrogen is acting via a receptor mediated action to enhance basal endothelial NO expression in the human endothelium. These results were consistent with the results of the *in-vivo* study.

The presence of mRNA's for both ERα and ERβ receptors in the endothelium was confirmed by RT-PCR. Confirmation of the presence of oestrogen receptor is important, as it suggests that the negative findings reported do not reflect low or absent oestrogen receptor expression..

These results contrast with the findings of other investigators who have previously reported an up-regulation of eNOS by oestradiol in cultured endothelial cells. An up-regulation of eNOS protein levels in response to 17β-oestradiol has been observed in human aortic endothelial cells using Western blotting (Hayashi et al, 1995). In this study, the level of eNOS protein was determined after short (8h) and long (48h) term pre-treatment of cells prior to measurement of calcium stimulated (ionomycin) NO production. Similar findings have been observed in human umbilical vein endothelial cells and bovine aortic endothelial cells (Hishikawa et al, 1995). These investigators reported an increase in eNOS protein using Western blot analysis following oestrogen pre-treatment for a minimum of eight hours, an effect which was inhibited by addition of the oestrogen receptor antagonist tamoxifen. These effects were consistent in both of the cell populations studied. These findings suggest an involvement of the classic oestrogen receptor (ERα), a hypothesis which is supported by observation that knock-out mice lacking ERα have reduced NO production (Freay et al, 1995). More recently, another group demonstrated that oestrogens increase transcription of the human eNOS gene and has suggested that this may be mediated as a consequence of enhanced activity of transcription factor Sp 1 (Kleinert et al, 1998). This study examined eNOS mRNA expression in human endothelial cells following incubation with 17β-oestradiol and 17α-oestradiol using RNAse protection assays. They also examined eNOS protein levels using western blotting techniques.

Recently, it has also been argued that increased bioactive nitric oxide levels in response to oestrogen do not occur due to enhanced expression of NOS, but rather that they are as a consequence of inhibition of superanion production (Arnal et al, 1996). This study reported that treatment of cultured bovine aortic endothelial cells with ethinyl oestradiol had no effect on either the activity or expression of eNOS. They did however demonstrate an increased superoxide anion production correspondingly increased expression of mRNA for superoxide dismutase (SOD).

Other studies have also argued against the potential role of NO in explaining the non-lipid-mediated component of HRT cardioprotection. Elhage reported that treatment of castrated apolipoprotein E-deficient mice (which develop early atherosclerosis) with oestradiol appeared to prevent atherosclerotic fatty streak formation and this effect continues to be observed despite inhibition of NOS using L-NAME suggesting that the anti-atherosclerotic effect of oestradiol was independent of NO (Elhage et al, 1997).

CHAPTER SEVEN

DISCUSSION

7.1 Introduction

The high prevalence and significant morbidity and mortality associated with cardiovascular disease in women, particularly after the menopause, has promoted interest the possibility of a hormonally based aetiological link for the disease. However, despite the compelling evidence of cardiovascular benefit associated with oestrogen use in observational studies and a number of potential biologically plausible mechanisms to explain this effect, uncertainty about the effects of HRT remain.

Observational studies of HRT and its effect on cardiovascular disease are not controlled clinical studies, and are not uniform in terms of type or dose of oestrogen and current use, past use or non-use of oestrogen. Observational studies enable the reader to make inferences or associations from the data but not to determine cause or effect. Since the observational data on oestrogen and cardiovascular benefit were so consistent for so long, it was assumed that the results of the clinical trials would be supportive of the clinical effects of oestrogen. However, recently completed clinical trials do not clearly support the protective effects of oestrogen indicated in the observational studies.

In 1998, the Heart and Oestrogen Replacement Study (HERS) reported its findings (Hulley et al, 1998). This study was a secondary prevention study in 2736 women with established coronary heart disease. It found no significant differences between the women in the control and experimental groups with the primary end-point of the study being the occurrence of non-fatal myocardial infarction or death from coronary heart disease. It did however report an early increase in coronary events in the HRT group, which tapered off after two years and was balanced by a decline over the next two years. A possible explanation for the increase in cardiovascular events observed is that there is an early thrombotic risk associated with a delayed anti-atherogenic benefit or that certain subgroups of women are more susceptible to an early thrombotic risk. Another secondary prevention trial, the Estrogen Replacement and Atherosclerosis trial (ERA) is due to be reported shortly and early indications suggest that the results will be in broad agreement with the HERS study. The Women's Health Initiative (WHI) is a primary prevention trial with over twenty-seven thousand women. This study initiated in 1994 which will be due to report its findings in 2005, recently announced that during the first two years of the study, a small increase in the number of myocardial infarctions, strokes and thromboses had been observed in the women taking active

hormones, although the investigators speculate that this may disappear with time (WHI Update 2000, NIH).

7.2 Discussion

The studies reported in this thesis fall into two broad categories with two purely clinical studies and two laboratory based projects.

The clinically based studies used the technique of FVOP to study the forearm vascular haemodynamics in two groups of postmenopausal women, to establish firstly whether oestradiol modulated vascular reactivity and secondly to determine if changes in vascular reactivity were due to a NO mediated effect. The laboratory based studies sought to compliment these studies by determining the endothelial cell responses to oestrogen at a molecular level by examining eNOS mRNA expression using northern analysis and by directly measuring cell proliferation.

7.2.1 Forearm venous occlusion plethysmography studies

The technique of FVOP with intra-arterial cannulation is complex requiring considerable skill to master the technique. Within the department, most of the previous experience of this technique had been obtained in healthy young male volunteer subjects, and although the procedure was generally well tolerated by the women who took part in these studies (apart from discomfort associated with immobility and with the insertion of the intra-arterial needle) it was felt that overall this subject group was technically more challenging, with an unusually high failure rate which could perhaps be attributed to different arterial physiology in this group.

In study one, an acute vasodilator response to intra-arterial infusion of water-soluble oestradiol was not observed in the human forearm. This indicates that if present, any vasodilator effect in this vascular bed is likely to occur over a longer period time, possibly via the classic genomic pathway of steroid action.

The results of the second study did not support the original hypothesis that transdermal HRT causes an increase in basal endothelial NO synthesis in the forearm vasculature as determined by measuring the response to L-NMMA. Most other studies of this nature

have used oral oestradiol preparations such as oestradiol valerate or CEE which may explain the negativity of these results, and does not preclude an effect of oestrogen on the forearm vasculature if delivered by an alternative route, or indeed, an effect on a different vascular bed if delivered by the same route.

Both of these studies were carefully designed to minimise the inclusion of patients with known arterial disease, as the arterial physiology of such patients will be very different. The results of these studies are further strengthened by adopting a bilateral approach, and expressing the results with respect to the control arm.

7.2.2 Growth studies

As these experiments were the first to be performed in the laboratory using HAEC there was considerable interest in the success of establishing and maintaining these cells.

After characterising (to confirm endothelial nature), then observing the growth characteristics of the cells by undertaking pilot studies, standard growth curve studies were performed to determine the cell proliferation in response to oestrogen exposure. The results of these studies confirmed that prolonged exposure to pharmacological doses of 17β-oestradiol resulted in a significant increase in absolute cell number, either as a direct consequence of stimulating cell growth or by prevention of cell death and thus prolongation of cell life. Further studies are indicated to clarify the underlying mechanism.

7.2.3 eNOS mRNA expression

Expression of mRNA for the eNOS gene was confirmed by northern analysis, however the results of these studies did not support the hypothesis that oestrogen up-regulates eNOS mRNA expression.

The presence of mRNA for both ERα and ERβ was confirmed by RT-PCR. Clarifying the oestrogen receptor expression was important as it suggested that the negative findings were not a consequence of an absence of the receptor. This raises the question that there is an alternative mechanism to explain the presence of increased NO levels in response to oestrogen, which have been reported. One possible explanation is that there

is an inhibition of superoxide anions resulting in reduced clearance of nitric oxide. This may be manifest by increased expression of the SOD gene as this is the principal inactivator of superoxide anions.

7.3 Future studies

The work presented in this thesis has hopefully provided a framework from which to build a number of future studies.

Unfortunately, FVOP is a highly invasive technique which requires acquisition of considerable skill, however recent advances in ultrasound technology have provided an alternative method for assessing vascular reactivity *in vivo* and may ultimately form the basis of future work.

Having ascertained that it is possible to establish and maintain commercially available human aortic endothelial cells in culture, will allow the planning of future studies involving several key areas of interest. Firstly, to determine the basal and stimulated rates of NO production by the cells, using a NO microsensor. Secondly, if NO levels are enhanced in response to hormonal stimulation, to determine if this is due to inhibition of superoxide anion production rather than enhanced eNOS production. This will be done by direct assessment of superoxide anion production using a superoxide assay developed within the group, and by assessment of SOD mRNA expression using northern analysis. In addition it is intended to develop the RNA work further, in particular to establish RT-PCR and RNAse protection assays within this area of work.

The results of the growth studies provided interesting data on the effect of oestrogen on absolute cell number. However, the underlying mechanism to explain the observations made is not clear from studies of this nature. It is therefore proposed that a study is undertaken to examine the cell cycle to establish whether this effect is due to a true increase in proliferation or if it is due to a reduction in apoptosis.

7.4 Conclusion

With the exception of the growth studies which yielded interesting results requiring further evaluation, the negative results of the remaining carefully designed and well executed studies, while at variance with the results of other investigators, nevertheless generate interesting new data which appears to be in broad agreement with the albeit limited results of the randomised control studies currently available.

It appears therefore that the paradox of coronary risk and the menopause is an issue which has not been fully resolved, and there remain many unanswered (and perhaps also unasked) questions which continue to fuel the HRT / cardiovascular debate. The results of the forthcoming large-scale randomised controlled studies are therefore awaited with interest. Hopefully, these results will complete our understanding of this complex issue and enable us to offer women advice based on the evidence, rather than on an association from observational studies.

REFERENCES

Abbott WG, Lillioja S, Young AA, Zawadzki JK, Yki-Jarviner H, Christin L, Howard BV. (1987) Relationships between plasma lipoprotein concentrations and insulin action in obese and non-obese hyperinsulinaemic populations. Diabetes; 36: 897-904

Abdalla HI, Hart DM, Lindsay R, Leggate I, Hooke A. (1985) Prevention of bone mineral loss in postmenopausal women by norethisterone. Obstetrics and Gynaecology; 66: 789-92

Alwine JC, Kemp DJ, Stark G. (1977) Method for detection of specific RNA's in agarose gels by transfer to diazobenzylmethyl-paper and hybridisation with DNA probes. Proceedings of the National Academy of Science USA; 74: 5350-54

Arnal JF, Clamens S, Pechet C, Negre-Salvayare A, Allera C, Giorobibi JP, Salvayare R, Bayard F. (1996) Ethinyloestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. Proceedings National Academy Science USA; 93: 4108-13

Austin M, Breslow J, Hennekens C, Buring JE, Willett WC, Krauss RM. (1988) Low density lipoprotein subclasses and risk of myocardial infarction. Journal of American Medical Association; 26: 1917- 21

Barcroft H, Bonnar W, Edholm O, Effro S. (1943) Sympathetic vasoconstrictor tone in human skeletal muscle. Journal of Physiology; 102: 21-31

Baron J, Adams P, Ward M. (1988) Cigarette smoking and other correlates of cytologic oestrogen effect in postmenopausal women. Fertility Sterility; 50 (5): 766-71

Barrett-Connor E. (1996) The menopause, hormone replacement and cardiovascular disease: the epidemiologic evidence. Maturitas; 23: 227-34

Barrett-Connor E, Bush TL. (1991) Oestrogen and coronary heart disease in women. Journal of American Medical Association; 265: 1861-67

Barrett-Connor E, Laakso M. (1990) Ischaemic heart disease risk in postmenopausal women: effects of oestrogen on glucose and insulin metabolism. Arteriosclerosis; 10: 531-34

Bayard F, Clemens S, Meggatto F, Blaes N, Delsol G, Faye JC. (1995) Oestrogen synthesis, oestrogen metabolism and functional oestrogen receptors in rat arterial smooth muscle cells in culture. Endocrinology; 136: 1523-29

Baylis SA, Weiner CP, Moncada S, Charles IG. (1994) In-vivo expression of inducible nitric oxide synthase in the human fetus. Abstract presented at First International Conference on Biochemistry and Molecular Biology of Nitric Oxide, Los Angeles, California

Bell DR, Rensberger HJ, Koritnik DR, Koshy A. (1995) Oestrogen pretreatment directly potentiates endothelium-dependent vasorelaxation of porcine coronary arteries. American Journal of Physiology; 268: H377-83

Bengstsson C, Bjorkelund C, Lapidus L, Lissner L. (1993) Associations of serum lipid concentrations and obesity with mortality in women: 20 year follow up of participants in prospective population study in Gothenburg, Sweden. British Medical Journal; 307: 1385-88

Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. (1995) Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension; 25: 918-23

Beresford S, Weiss N, Voigt L, McKnight B. (1997) Risk of endometrial cancer in relation to use of oestrogen combined with cyclic progestagen therapy in postmenopausal women. Lancet; 349: 458-61

Berliner J, Territo M, Sevanian A, Ramis S, Kim JA, Barshad B, Esterson M, Fogelman AM. (1990) Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. Journal Clinical Investigation; 85: 1260-66

Bertone E, Weiderpass E. (2000) Low dose oestrogens and endometrial cancer. J British Menopause Society; 6: 89-92

Birkhauser MH. (1996) Chemistry, physiology and pharmacology of sex steroids. Journal Cardiovascular Pharmacology; 28 (supplement 5): S1-13

Bjarnason K, Cerin A, Lindgren R, (1999) Adverse endometrial effects during long cycle hormone replacement therapy. Scandinavian Long Cycle Study Group. Maturitas; 32: 161-70

Brown A, Jeltsch JM, Roberts M, Chambon P. (1984) activation of pS_2 gene transcription is a primary response to oestrogen in the human cancer cell line MCF-7. Proceedings of the National Academy of Science USA; 81; 6344-48

Brown B, Zhao X, Sacco D, Albers J. (1993) Atherosclerosis regression, plaque disruption, and cardiovascular events: a rational for lipid lowering in coronary artery disease. Annual Review of Medicine; 44: 365-76

Brown TA (Ed). (1995) Gene Cloning: an introduction (3^{rd} Ed), Chapman and Hall, London

Browning M. (1988) In: BDS Textbook of Physiology (11^{th} Ed). Emslie-Smith D, Paterson CR, Scratchard T, Read NW (Eds). Churchill Livingstone, Edinburgh; Chapter 24

Burger CW, Van Leeuwen FE, Scheele F, Kenemans P. (1999) Hormone replacement therapy in women treated for gynaecological malignancy. Maturitas; 32: 69-76

Burger HG. (1992) Inhibin. Reproductive Medicine Review; 1: 1-20

Cagnacci A, Soldani R, Carriero PL, Paoletti AM, Fioretti P, Melis G. (1992) Effects of low dose transdermal 17β-oestradiol on carbohydrate metabolism in postmenopausal women. Journal of Clinical Endocrinology and Metabolism; 74: 1396-1400

Campos H, McNamara J, Wilson P, Ordavas JM, Schaefer EJ. (1988) Differences in low density lipoprotein subfractions and apolipoproteins in premenopausal and postmenopausal women. Journal Clinical Endocrinology and Metabolism; 67: 30-35

Castelli WP. (1984) Epidemiology of coronary heart disease; the Framingham Study. American Journal of Medicine; 76: 4-12

Caulin-Glaser TL, Sessa W , Sarrel P, Bender J. (1994) The effect of 17β oestradiol on human endothelial cell nitric oxide production. Circulation research; 90: 1-30

Cembrano J, Lillo M, Val J, Mardones J. (1960) Influence of sex difference and hormones on elastin and collagen in aortas of chickens. Circulation Research; 8; 527-33

Chabot B. (1994) In: RNA processing – a practical approach. Higgins SJ, Hanes BD (Eds), IRL Press Oxford; Chapter 1

Chang WC, Nakao J, Orimo H, Murota SI. (1980) Stimulation of prostaglandin cyclo-oxygenase and prostacyclin synthase activities by oestradiol in rat aortic smooth muscle cells. Biochemical and Biophysical Acta; 620: 472-82

Chard T, Lilford R (Eds). (1995) Basic sciences for obstetrics and gynaecology (4^{th} Ed). Springer, London; Chapter 4

Cheng DY, Gruetter CA. (1992) Chronic oestrogen alters contractile responsiveness to angiotensin II and norepinephrine in female rat aorta. European Journal of Pharmacology; 215: 171-76

Chester AH, Jiang C, Borland JA, Jacoub MH, Collins P. (1995) Oestrogen relaxes human epicardial coronary arteries through non-endothelial dependent mechanisms. Coronary Artery Disease; 6: 417-22

Colburn P, Buonassisi V. (1978) Oestrogen binding sites in endothelial cell cultures. Science; 201: 817-19

Colditz GA, Willet WC, Stampfer MJ, Rosner B, Speizer FE, Hennekens CH. (1987) Menopause and risk of coronary heart disease in women. New England Journal of Medicine; 316: 1105-10

Collaborative Group on Hormonal Factors in Breast Cancer. (1997) Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 104,411 women without breast cancer. Lancet; 350: 1047-59

Collins P, Rosano GMC, Sarrel PM, Ulrich L, Adamopoulos S, Beale CM, McNeill J, Poole-Wilson PA. (1995) 17β-oestradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. Circulation; 92: 24-30

Cooke JP, Dzau VJ. (1997) Nitric oxide synthase: Role in the genesis of vascular disease. Annual Review of Medicine; 48: 489-509

Cooper K, Edholm O, Mottram R. (1955) The blood flow in skin and muscle of the human forearm. Journal of Physiology; 128: 258-67

Corvazier E, Dupuy E, Dosne AM, Maclouf J. (1984) Minimal effect of oestrogen on endothelial cell growth and production of prostacyclin.Thrombosis Research; 34: 303-10

Crook D, Cust M, Ganger K, Worthington M, Hillard T, Stevenson J. (1992) Comparison of transdermal and oral oestrogen/progestin hormone replacement therapy: effects on serum lipids and lipoproteins. American Journal of Obstetrics and Gynaecology; 166: 950-55

Crook D, Godsland I, HullJ, Stevenson J. (1997) Hormone replacement therapy with dydrogesterone and oestradiol -17β: effects on serum lipoproteins and glucose tolerance. British Journal of Obstetrics and Gynaecology; 104: 298-304

Cushman[1] M, Legault C, Barrett-Connor E, Stefanick ML, Kessler C, Judd HL, Sakkinen P, Tracy RP. (1999) Effect of postmenopausal hormones on inflammation-sensitive proteins. Circulation; 100: 717-22

Cushman[2] M, Meilahn EN, Psaty BM, Kuller LH, Dobs AS, Tracy RP. (1999) Hormone replacement therapy, inflammation and haemostasis in elderly women. Arteriosclerosis, Thrombosis and Vascular Biology; 19: 893-99

Dahlen G, Ericson C, Berg K. (1978) In vitro studies of the interaction of Lp(a) lipoprotein and other serum lipoproteins with glycosaminoglycans. Clinical Genetics; 14: 36-42.

Daly E,Vessey MP, Hawkens MM, Carson JL, Gough P, Marsh S. (1996) Risk of venous thromboembolism in users of hormone replacement therapy. Lancet; 348: 977-80.

Davey DA. (1995) In: Dewhurst's Textbook of Obstetrics and Gynaecology for Postgraduates (5th Ed). Whitfield CR (Ed), Blackwell Science, Oxford; Chapter 41

Dawson M. (1992) In: Cell Culture – Labfax. Butler M, Dawson M (Eds), BIOS, Oxford; Chapters 2 & 3

Dayton S, Hashimoto S. Recent advances in molecular pathology. (1970) Experimental and Molecular Pathology; 13: 253-68

Dinerman JL, Dawson TM, Schell MJ, Snowman A, Synder SH. (1994) Endothelial nitric oxide synthase localised to hippocampal pyramidal cells; implications for synaptic plasticity. Proceedings of the National Academy of Sciences USA; 91: 4214-18

Drummond AE, McPherson SJ, Laslett A, Hearn M. (1996) Application of chromogenicbioassay procedure for the measurement of the proliferation of endothelial cells in vitro under the influence of the effects of steroid hormones and growth factors. Journal of Biochemical and Biophysical Methods; 31: 123-34

Dubick D, Dembiski TC, Shiu RPC. (1987) stimulation of *c-myc* oncogence expression associated with oestrogen-induced proliferation of human breast cancer cells. Cancer Research; 47: 6517-21

Duncan AC, Lyall H, Roberts RN, Petrie JR, Perera MJ, Monaghan S, Hart DM, Connell JMC, Lumsden MA. (1999) The effect of oestradiol and a combined oestradiol/progestogen preparation on insulin sensitivity in healthy postmenopausal women. Journal of Clinical Endocrinology and Metabolism; 84: 2402-07

Elhage R, Bayard F, Richard V, Holvoet P, Duverger N, Fievet C, Arnal JF. (1997) Prevention of fatty streak formation of 17β-oestradiol is not mediated by the production of nitric oxide in apolipoprotein-E deficient mice. Circulation; 96: 3048-52

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Fried G, Nordenskjold M, Gustafsson J. (1997) Human oestrogen receptor β-gene structure, chromosomal localisation, and expression pattern. Journal of Clinical Endocrinology and Metabolism; 82: 4258-65

Erikson GF. (1986) An analysis of follicle development and ovum maturation. Seminars in Reproductive Endocrinology; 4: 233

Faddy M, Gosden R. (1995) A mathematical model of follicle dynamics in the human ovary. Human Reproduction; 10 (4): 770-75

Farhat MY, Abi-Jounes S, Ramwell PW. (1996) Non-genomic effects of oestrogen and the vessel wall. Biochemical Pharmacology; 51(5): 571-6

Farrel RE (Ed). (1993) RNA methodologies – a laboratory guide for isolation and characterisation (1st Ed), Academic Press, London

Faruqui R, Dicorleto P. (1993) Mechanisms of monocyte recruitment and accumulation. British Heart Journal; 69: S19-29

Felstein I. (1973) The Menopause. British Journal Sexual Medicine; 1(5): 19-24

Ferrannini E, Buzziogli G, Bonadoma R, Giorico MA, Oleggini M, Graziadei L, Pedronelli R, Brandi L, Bevelqucqne S. (1987) Insulin resistance in essential hypertension. New England Journal of Medicine; 317: 350-57

Flokman J, Haudenschild C, Zetter BR. (1979) Long-term culture of capillary endothelial cells. Proceedings of the National Academy of Science USA; 76: 5217

Folsom AR, Wu KK, Davis CE, Conlan MG, Sorlie PD, Szklo M. (1991) Population correlates of plasma fibrinogen and Factor VII, putative cardiovascular risk factors. Atherosclerosis; 91: 191-205

Freay AD, Korach KS, Lubahn D, Rubyani GM. (1995) Circulation; 92: 1-106

Fuller JH, Shipley MJ, Rose G, Jarrett RJ, Keen H. (1983) Mortality from coronary heart disease and stroke in relation to degree of glycaemia: the Whitehall study. British Medical Journal; 287: 867-70

Furchgott R, Zawadzki J. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle cells by acetylcholine. Nature; 288: 373-76

Fuster V, Badimion L, Badimon J, Chesebro J. (1992) The pathogenesis of coronary artery disease and the acute coronary syndromes. New England Journal of Medicine; 326: 242-50

Gambrell RD. (1978) The prevention of endometrial cancer in postmenopausal women with progestagens. Maturitas; 1: 99-106

Ganger KF, Vyas S, Whitehead MI, Crook D, Meire H, Campbell S. (1991) Pulsatility index in internal carotid artery in relation to transdermal oestradiol and time since menopause. Lancet; 338: 839-42

Garthwaite J. (1991) Glutamate, nitric oxide and cell signalling in the nervous system. Trends in Neurosciences; 14: 60-67

Gerrity R. (1981) The role of the monocyte in atherogenesis. American Journal of Pathology; 103: 191-200

Gilligan DM, Badar DM, Panza JA, Quyyumi AA, Cannon RO. (1994) Acute vascular effects of oestrogen in postmenopausal women. Circulation; 90: 786-91

Gilligan DM, Badar DM, Panza JA, Quyyumi AA, Cannon RO. (1995) Effects of oestrogen replacement therapy on peripheral vasomotor function in postmenopausal women. American Journal of Cardiology; 75: 264-68

Gilligan DM, Quyyumi AA, Cannon RO. (1994) Effects of physiological levels of oestrogen on coronary vasomotor function in postmenopausal women. Circulation; 89: 2545-51

Giltay EJ, Gooren LJ, Emeis JJ, Kooistra T, Stehouwer CD. Oral but not transdermal administration of oestrogen lower tissue-type plasminogen activator levels in humans without affecting endothelial synthesis. Arteriosclerosis Thrombosis Vascular Biology; 20: 1396-403

Gislard V, Miller V, Vanhoutte PM. (1988) Effect of 17β oestradiol on endothelium-dependent responses in the rabbit. Journal of Pharmacology and Experimental Therapeutics; 244: 19-22

Gordon T, Kannal WB, Hjortland MC, McNamara PM. (1978) Menopause and coronary heart disease. The Framingham Study. Annals of Internal Medicine; 89: 157-61

Grabowski PJ. (1994) In: RNA processing – a practical approach. Higgins SJ, Hanes BD (Eds), IRL Press Oxford; Chapter 2

Grady D, Gebretsadik T, Kerlikanske K, Ernster V, Petitti D. (1995) Hormone replacement therapy and endometrial cancer risk: a meta analysis. Obstetrics Gynaecology; 85: 304-13

Green S, Walter P, Kumar V, Krust A, Bornet JM, Argos P, Chambon P. (1986) Human oestrogen receptor cDNA: sequence, expression and homolgy to v-erb-A. Nature; 320: 134-39

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. (1986) Sequence and expression of human oestrogen receptor complimentary DNA. Science; 231: 1150-54

Grodstein F, Stampfer M. (1995) The epidemiology of coronary heart disease and oestrogen replacement in postmenopausal women. Progress in Cardiovascular Disease; 20: 47-63

Grodstein F, Stampfer MJ, Goldhaber S, Manson JE, Colditz GA, Speizer FE, Willett WC, Hennekens CH. (1996) Prospective study of exogenous hormones and risk of pulmonary embolism in women. Lancet; 348: 983-87

Gruber DM, Wagner G, Kurz C, Sator M, Huber J. (1999) Endometrial cancer after combined hormone replacement therapy. Maturitas; 32: 161-70

Haarbo J, Marslew U, Godfredson A, Christiansen C. (1991) Postmenopausal hormone replacement therapy prevents central distribution of body fat after the menopause. Metabolism; 40: 1323-26

Hames BD, Higgins SJ (Eds). (1988) Nucleic acid hybridisation – a practical approach. IRL Press, Oxford

Han SZ, Karaki H, Ouchi Y, Akishita M, Orimo H. (1995) 17β-oestradiol inhibits Ca^{2+} influx and Ca^{2+} release induced by thromboxane A_2 in porcine coronary artery. Circulation; 91: 2619-26

Hayashi T, Fukuto JM, Ignarro LJ, Chaudhuri G. (1992) Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. Proceedings of the National Academy of Science USA; 89: 11259-63

Hayashi T, Yamada K, Esaki T, Kuzuya M, Satakes S, Ishikawa T, Hidaki H, Iyuchi A. (1995) Oestrogen increases endothelial nitric oxide by a receptor mediated system. Biochemical Biophysical Research Communications; 214: 847-55

Health in Scotland, 1995. HMSO.

Heinrich J, Sandkamp M, Kokott R, Schulte H, Assmann G. (1991) Relationship of lipoprotein (a) to variables of coagulation and fibrinolysis in a healthy population. Clinical Chemistry; 37: 1950-54

Henderson BE, Paganini-Hill A, Ross RK. (1991) Decreased mortality in users of oestrogen replacement therpy Arch Internal Medicine; 1991: 75-78

Higgins SJ, Hames BD (Eds). (1994) RNA processing: a practical approach (Vols 1&2). IRL Press, Oxford

Hishikawa K, Nakaki T, Marumo T, Suzuki H, Kato R, Saruta T. (1995) Up-regulation of nitric oxide synthase by oestradiol in human aortic endothelial cells. FEBS Letters; 360: 291-93

Horwitz KB, Horwitz LD. (1982) Canine vascular tissues are targets for androgens, oestrogens, progestins and glucocorticoids. Journal of Clinical Investigation; 69: 750-58

Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff V. (1998) Randomized trial of oestrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Journal of the American Medical Association; 280: 615-613

Ignarro L, Buga G, Wood K, Byrns R, Chaudhuri G. (1987) Endothelium derived relaxing factor produced and released from artery and vein is nitric oxide. Proceedings of the National Academy of Science USA; 84: 9265-69

Ignarro LJ. (1991) Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. Blood Vessels; 28: 67-73

Jarrett RJ, McCartney P, Keen H. (1982) The Bedford survey ten year mortality rates in newly diagnosed diabetics, borderline diabetics and normoglycaemic controls and risk indices for coronary heart disease in borderline diabetics. Diabetologia; 22: 77-84

Jeffcoate SL. (1992) In: Basic Science in Obstetrics and Gynaecology (2nd Ed). De Sweit, Chamberlain G (Eds), Churchill Livingstone, Edinburgh; Chapter 7

Jennett S (Ed). (1989) Human Physiology (1st Ed), Churchill Livingstone, Edinburgh; Chapter 16

Jenstrom H, Frenander J, Ferno M, Olsson H. (1999) Hormone replacement therapy before breast cancer diagnosis significantly reduces the overall death rate compared with never-use among 984 breast cancer patients. Br J Cancer 80; 1453-58

Jiang C, Poole-Wilson PA, Sarrel PM, Mochizuki S, Collins P, Macloed KT. (1992) Effect of 17β-oestradiol on contraction, Ca^{2+} current and intracellular free Ca^{2+} in guinea-pig isolated cardiac myocytes. British Journal of Pharmacology; 106: 739-45

Jiang C, Sarrel PM, Lindsay DC, Poole-Wilson PA, Collins P. (1991) Endothelium independent relaxation of rabbit coronary artery by 17β-oestradiol in-vitro. British Journal of Pharmacology; 101: 1033-37

Jick H, Derby LE, Myers MW, Vasilakis C, Newton KM. (1996) Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal oestrogen. Lancet; 348: 981-83

Johns A, Freay AD, Fraser W, Korach KS, Rubanyi GM. (1996) disruption of oestrogen receptor gene prevents 17β-oestradiol induced angiogenesis in transgenic mice. Endocrinology; 137: 4511-13

Jones P, Qiu J, Rickwood D (Eds). (1994) RNA isolation and analysis (1st Ed), Bios, Oxford

Joris I, Zand T, Nunnari J, Krolikowski F, Majno G. (1983) Studies on the pathogenesis of atheroscelosis. American Journal of Pathology; 116: 341-58

Kanai AJ, Strauss HC, Truskey GA, Crews AL, Cranfeld S, Malinski T. (1995) Sheer stress induces ATP-independent transient nitric oxide release from vascular endothelial cells, measured directly with a porphyrinic microsensor. Circulation Research; 77: 284-93

Karas RH, Patterson BL, Medelsohn ME. (1994) Human vascular smooth muscle cells contain functional oestrogen receptors. Circulation; 89: 1943-50

Kauser K and Rubanyi G. (1993) Gender differences in EDRF/NO release from rat aorta. Circulation; 88: 179

Kharitonov SA, Logan-Sinclair RB, Busset CM, Shinebourne EA. (1994) Peak expiratory nitric oxide differences in men and women: relation to menstrual cycle. British heart Journal; 72: 243-5

Kleinert H, Wallerath T, Euchenhofer C, Ihrig-Biedert I, Li H, Fostermann U. (1998) Oestrogens increase transcription of the human endothelial NO synthase gene. Hypertension; 31: 582-88

Knauthe R, Diel P, Hagele-Haratung C, Engelhaapt A, Fritzemeir K. (1996) Sexual diamorphism of steroid hormone receptor messenger ribonucleic acid expression and hormonal regulation in rat vascular tissue. Endocrinology; 137: 3220-27

Kneale BJ, Chowienczyk PJ, Cockcroft JR, Coltart DJ, Ritter JM. (1997) Vasoconstrictor sensitivity to noradrenaline and N^G-monomethyl-L-arginine in men and women. Clinical Science; 93: 513-18

Knowles RG, Moncada S. (1994) Nitric oxide synthase in mammals. Biochemical Journal; 298: 249-58

Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stamler JS. (1993) Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localisation. American Journal of Respiratory Cell and Molecular Biology; 9: 371-77

Kobzik L, Reid MB, Bredt DS, Stamlar JS. (1994) Nitric oxide in skeletal muscle. Nature; 372: 546-48

Koh K, Mincemoyer R, Bui MN, Csako G, Pucino F, Guetta V, Waclawiw M, Cannon R. (1997) Effects of hormone replacement therapy on fibrinolysis in postmenopausal women. New England Journal of Medicine; 336 (10): 683-690

Krauss R. (1991) The tangled web of coronary risk factors. American Journal of Medicine; 90 (supplement 2A): 36-41

Kroon UB, Silfverstolpe G, Tengborne L. (1994) The effects of transdermal oestradiol and oral conjugated equine oestrogens on haemostasis variables. Thrombosis and Haemostasis; 34: 457-62

Kuhl H. (1990) Pharmacokinetics of oestrogens and progestogens. Maturitas; 12: 171-97

Kuiper GGJM, Enmark E, Pelro-Huikko M, Nilsson S, Gustaffson JA. (1996) Cloning of a novel oestrogen receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Science USA; 93: 5925-30

Lasky L. (1992) Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science; 258: 964-69

Lewis J, Taylor R, Jerome W. (1985) Foam cell characteristics in coronary arteries and aortas of white carneau pigeons with moderate hypercholesterolaemia. Annals of New York Academy of Science; 454: 91-100

Ley CJ, Lees B, Stevenson JC. (1992) Sex and menopause associated changes in body fat distribution. American Journal of Clinical Nutrition; 55: 950-54

Ley CJ, Swan J, Godsland IF, Walton C, Crook D, Stevenson JC. (1994) Insulin resistance, lipoproteins, body fat and haemostasis in non-obese males with angina and normal or abnormal coronary angiograms. Journal of the American College of Cardiologists; 23: 377-83

Libby P, Warner S, Salomen R, Birinyi L. (1988) Production of platelet derived growth factor-like mitogen by smooth muscle cells from human atheroma. New England Journal of Medicine; 318: 1493-98

Lindquist O. (1982) Influence of the menopause on ischaemic heart disease and its risk factors and on bone mineral content. Acta Obstetrics and Gynaecology Scandinavia; 110 (supplement): 7-32

Llewellyn-Jones D. Fundemantals of Obstetrics and Gynaecology Vol II, 5th Ed. Faber & Faber, London

Lobo RA, McCormick, W, Singer F, Roy S. (1984) DMPA compared with conjugate oestrogens for the treatment of postmenopausal women. Obstetrics and Gynaecology; 63: 1-5

Longcope C, Johnstone C. (1988) Androgen and oestrogen dynamics in pre- and postmenopausal women: a comparison between smokers and non-smokers. Journal of Clinical Endocrinology and Metabolism; 67: 379-83

Losordo DW, Kearney M, Kim EA, Jekanowski J, Inner JM. (1994) Variable expression of oestrogen receptors in normal and atherosclerotic coronary arteries of premenopausal women. Circulation; 89: 1501-10

Lowe G, Woodward M, Vessey M, Rumley A, Gough P, Daly E. (2000) Thrombotic variables and risk of idiopathic venous thromboembolism in women aged 45-64 years. Relationships to hormone replacement therapy. Thrombosis Haemostasis; 83 (4) 530-35

Luscher TF. (1991) Endothelium derived nitric oxide: the endogenous nitrovasodilator in the human cardiovascular system. European Heart Journal; 12 (supplement E) E 2-11.

Magness RR, Rosenfield CR. (1989) Local and systemic effects of 17β oestradiol: effects on uterine and systemic vasodilation. American Journal of Physiology; 256; E 536-42

Magnusson C, Baron JA, Correia N, Bergstrom R, Adami HO, Persson I. (1999) Breast cancer following long-term oestrogen – and oestrogen-progestin-replacement therapy. Int J Cancer; 81: 339-44

Mandel FP, Davidson BJ, Erlik Y, Judd HL, Meldrum DR. (1982) Effects of progestins on bone metabolism in postmenopausal women. J Repro Med; 13: 511-14

Mangelsdorf DJ, Thummel C, Beato M, Herlich P, Schmitz G, Umesono K, Blunberg B, Kastener P, Manuel M, Chambon P, Evens RM. (1995) The nuclear receptor superfamily: the second decade. Cell; 83: 835-39

Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Michel T. (1992) Molecular cloning and characterisation of human endothelial nitric oxide synthase. FEBS Letters; 307 (3): 287-93

Meilahn E, Kuller L, Matthews K, Stein A. (1991) Lp (a) concentrations among pre- and postmenopausal women over time: the Healthy Woman Study. Circulation; 84 (supplement II): 546

Miller ME, Dores GM, Thorpe SL, Akerley WL. (1994) Paradoxical influence of oestrogenic hormones on platelet-endothelial cell interactions. Thrombosis Research; 74 577-94

Miller N. (1987) Associations of HDL subclasses and apolipoproteins with ischaemic heart disease and coronary atherosclerosis. American Heart Journal; 113: 589-97

Mohaupt MG, Elzie JL, Ahn KY, Clapp WL, Wilcox CS, Kane BC. (1994) Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in the rat kidney. Kidney International; 46: 653-65

Moncada S, Higgs EA. The L-arginine nitric oxide pathway. (1993) New England Journal of Medicine; 329: 2002-12

Moncada S, Palmer RM, Higgs E A. (1989) Biosynthesis of nitric oxide from L-arginine: a pathway for the regulation of cell function and communication. Biochemical Pharmacology; 38: 1709-15

Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK, Schnaper HW. (1995) Oestrogen promotes angiogenic activity in human umbilical vein endothelial cells *in vitro* and in a murine model. Circulation; 91: 755-63

Mosselmann S, Polman J, Dijkema R. (1996) ER-β: identification and characterisation of a novel human oestrogen receptor. FEBS Letters; 392: 49-53

Moura AM, Worcel M. (1984) Direct action of aldosterone on transmembrane Na efflux from arterial smooth muscle. Rapid and delayed effects. Hypertension; 6: 425-30

Mueck AO, Seeger H, Korte K, Lippert TH. (1993) The effect of 17β-oestradiol and endothelin I on prostacyclin and thromboxane production in human endothelial cell cultures. Clinical and Experimental Obstetrics and Gynaecology; 20: 203-6

Mugge A, Reidel M, Barton M, Khun M, Lichtlen P. (1993) Endothelium independent relaxation of human coronary arteries by 17β-oestradiol in vitro. Cardiovascular Research; 27: 1939-42

McAteer J, Davis J. (1994) In: Basic cell culture – a practical approach. Davis JM (Ed), IRL Press, Oxford; Chapter 4

McGill H. (1968) Fatty streaks in coronary arteries and aorta. Laboratory Investigation; 18: 560-4

McLachlan RI, Robertson DM, Healy DL, Burger HG, Kretser DM. (1987) Circulating immunoreactive levels during the normal menstrual cycle. Journal of Clinical Endocrinology and Metabolism; 65: 954-61

McLaren J, Prentice A, Charnock-Jones DS, Millicen S, Muller K, Sharkey AM, Smith S. (1996) Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by steroids, Journal of clinical Investigation; 98: 482-89

Nakao J, Chang WC, Murota SI, Orimo H. (1981) Oestradiol binding sites in rat aortic smooth muscle cells in culture. American Heart Journal; 13767: 12336-13364

Nathan CF, Hibbs JB. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Current Opinion in Immunology; 3: 65-70

Newton JN. (1998) The epidemiology of coronary heart disease and HRT; What are the contentious issues? Journal of British Menopause Society; 4 (4): 135-42

Nussler AK, Billiar TR. (1993) Inflammation, immunoregulation and inducible nitric oxide synthase. Journal of Leukocyte Biology; 54: 171-78

Orimo A, Inoue S, Ikegami A, Hosoi T, Akishita M, Ouchi Y, Muramatsu M, Orimo H. (1993) Vascular smooth muscle cells as targets for oestrogen. Biochemical and Biophysical Research Communications 195: 730-36

Palmer R, Ferrige A, Moncada S. (1987) Nitric Oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature; 327: 524-26

Persson I, Bergvist l, Lindgreen C, Yuen J. (1997) Hormone replacement therapy and major risk factors for reproductive cancers, osteoporosis, and cardiovascular disease: evidence of confounding by exposure characteristics. Journal of Clinical Epidemiology; 50: 611-18

Petrie J, Ueda S, Morris A, Elliot H, Connell J. (1998) How reproducible is bilateral venous occlusion plethysmography in man? British Journal of Clinical Pharmacology; 45: 131-39

Pinto S, Virdis A, Ghiadoni L, Bernini G, Lombardo M, Petraglia F, Genazzani AR, Taddei S, Salvetti A. (1997) Endogenous oestrogen and acetylcholine-induced vasodilation in normotensive women. Hypertension; 29 (2): 268-73

Piotrowicz RS, Weber LA, Hickey E, Levin EG. (1995) Accelerated growth and senescence of arterial endothelial cells expressing the small molecular weight heat shock protein HSP 27. FASEB Journal; 9: 1079-84

Polderman KH, Stehouwer CDA, van Kamp GJ, Schalkwijk CG, Cronen J. (1993) Influence of sex hormones on plasma endothelin levels. Annals of Internal Medicine; 118: 429-32

Raddino R, Manca C, Poli E, Bolognesi R, Visiolo O. (1986) Effects of 17β-oestradiol on the isolated rabbit heart. Archives of International Pharmacodynamics; 281: 57-65

Radomski MW, Palmer RM, Moncada S. (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proceedings of the National Academy of Science USA; 87: 5193-97

Rand MJ. (1992) Nitregic transmission, nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. Clinical and Experimental Pharmacology and Physiology; 19: 147-69

Reaven GM. (1988) Role of insulin resistance in human disease. Diabetes; 37: 1595-1607

Reis SE, Gloth ST, Blumenthal RS, Resar JR, Zacur HA, Gerstenblith G, Brinker JA. (1994) Ethinyl oestradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. Circulation; 89: 52-60

Rekhter M, Gordon D. (1995) Active proliferation of different cell type, including lymphocytes, in human atherosclerotic plaques. American Journal of Pathology; 147: 668-77

Reynolds SRM, Foster FI. (1940) Peripheral vascular action of oestrogen observed in the ear of the rabbit. Journal Pharmacology and Experimental Therapeutics; 68: 176-77

Richardson S, Senikas V, Nelson J. (1987) Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion. Journal of Clinical Endocrinology and Metabolism; 65: 1231-37

Ridker PM, Hennekens CH, Buring JE, Rifai N. (2000) C-Reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. New England Journal of Medicine; 342: 836-43

Rosano GM, Sarrel PM, Poole-Wilson PA, Collins P. (1993) Beneficial effects of oestrogen on exercise-induced myocardial ischaemia in women with coronary artery disease. Lancet; 342: 133-36

Rosner W. (1990) The functions of corticosteroid binding globulin and SHBG; recent advances. Endocrinology Review; 11: 80-91

Ross R. (1986) The pathogenesis of atherosclerosis - an update. New England Journal of Medicine; 314: 488-500

Ross R. (1993) The pathogenesis of atherosclerosis - a perspective for the 1990's. Nature; 362: 801-09

Ross RK, Paganini-Hill A, Wan PC, Pike MC. (2000) Hormone replacement therapy and breast cancer. J Natl Cancer Inst; 16; 92(4): 328-32

Ryan US. (1984) Isolation and culture of pulmonary endothelial cells. Environmental Health Perspectives; 56: 103

Sack M, Rader D, Cannon R. (1994) Oestrogen and inhibition of oxidation of low density lipoproteins in postmenopausal women. Lancet; 343: 269-70

Salmon RJ, Ansquer Y, Asselain B, Languille O, Lesec G, Remvikos Y. (1999) Clinical and biological characteristics of breast cancers in postmenopausal women receiving hormone replacement therapy. Oncol Rep; 6: 699-703

Sambrook J, fritsch EF, Maniatis T (Eds). (1989) Molecular cloning: a laboratory manual (2nd Ed). Cold Spring Harbour Laboratory Press, USA

Sarrel PM. (1989) In: Circulation in the female. Ginsberg J (Ed), Parthenon Publishing, UK; Chapter 2

Sayegh HS, Ohara Y, Navas JP, Peterson TE, Dockery S, Harrison DG. (1993) Endothelium nitric oxide synthase regulation by oestrogens. Circulation; 88(4) 1-80 (abstract)

Scarabin PY, Bonithon-Kopp C, Bara L, Malmejac A, Guize L, Samama M. (1990) Factor VII activation and menopausal status. Thrombosis Research; 57: 227-34

Scarabin PY, Plu-Bureau G, Bara L, Bonithon-Kopp, Guize L, Samama MM. (1993) Haemostatic variables and menopausal status: influence of hormone replacement therapy. Haemostasis; 70: 584-7

Schairer C, Lubin J, Troisi R, Sturgeon S, Brinton L, Hoover R. (2000) Menopausal oestrogen and oestrogen-progestin replacement therapy and breast cancer risk. JAMA; 283 (4): 485-91

Schiff I, Tulchinsky D, Cramer D, Ryan KJ. (1980) Oral MPA in the treatment of menopausal symptoms. JAMA; 244: 1443-45

Schray-Utz B, Zeiher AM, Busse R. Expression of constitutive NO synthase in cultured endothelial cells is enhanced by 17β-oestradiol. (1993) Circulation; 88: 1-80 (abstract)

Shaw R. (1997) In: Gynaecology (2nd Ed). Shaw RW, Souter WP, Stanton SL (Eds), Curchill Livingstone, Edinburgh; Chapter 12

Sherman B, West J, Korenman S. (1976) The menopausal transition: analysis of LH, FSH, oestradiol and progesterone concentrations during menstrual cycles of older women. Journal of Clinical Endocrinology and Metabolism; 24(4): 629-36

Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. Journal of Clinical Investigation; 91: 2235-43

Silva de Sa MA, Meirelles RS. (1977) Vasodilating effect of oestrogen on the human umbilical artery. Gynaecological Investigation; 8: 307-13

Simionescu M, Simionescu N. (1993) Proatherosclerotic events: pathophysiochemical changes occurring in the arterial wall before monocyte migration. FASEB Journal; 7: 1359-66

Sorrol G, Walsh T, Whelan R. (1965) A comparison of the effects of intra-arterial and intravenous infusions of angiotensin and noradrenaline on the circulation in man. Clinical Science; 29: 319-26

Speroff L, Glass RH, Kase NG (Eds). (1994) Gynaecologic endocrinology and infertility. Wilkins and Wilkins, Baltimore

Spyridopoulos I, Sullivan A, Kearney M, Isner JM, Losordo D. (1997) Oestrogen receptor-mediated inhibition of human endothelial cell apoptosis. Oestradiol as a survival factor. Circulation; 95: 1505-14

Stampfer M, Colditz G. (1991) Oestrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiological evidence. Preventative Medicine; 20: 47-63

Stary HC, Chandler AB, Dinasmore RE, Fuster V, Glagor S, Insull W, Rosenfield ME, Schwartz CJ, Wagner WD, Wissler RW. (1995) A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. Arteriosclerosis Thrombosis and Vascular Biology; 15: 1512-31

Stevenson J, Crook D, Godsland I. (1993) Influence of age and menopause on serum lipids and lipoproteins in healthy women. Atherosclerosis; 98: 83-90

Stevenson JC, Crook D, Godsland IF, Collins P, Whitehead MI. (1994) Hormone replacement therapy and the cardiovascular system non-lipid effects. Drugs; 47 (supplement 2): 35-41

Stout R. (1990) Insulin resistance and atheroma a 20year perspective. Diabetes Care; 13: 631-34

Strachan T, Reid PR (Eds). (1996) Human Molecular Genetics (1st Ed), Bios, Oxford. Chapter 1

Strong JP. (1992) Atherosclerotic lesions: natural history, risk factors and topography. Archives of Pathology and Laboratory Medicine; 116: 1268-75

Sturdee DW, Ulrich LJ, Barlow DH, Wells M, Campbell MJ, Vessey MP, Neilson B, Anderson MC, Bragg AJ. (2000) The endometrial response to sequential and continuous combined oestrogen-progestogen replacement therapy. British Journal of Obstetrics and Gynaecology; 107 (11) 1392-400

Sudhir K, Jennings GL, Funder JW, Komesaroff PA. (1996) Oestrogen enhances basal nitric oxide release in the forearm vasculature in perimenopausal women. Hypertension; 28: 330-34

Synder WC, Bredt DS. (1992) Biological roles of nitric oxide. Scientific America; 266: 68-71

Thorrell A, Gutniak M, Haggmark T, Ljumkqvist O. (1994) Insulin resistance after abdominal surgery. British Journal of Surgery; 81: 59-63

Tunstall-Pedoe H. (1998) Myth and paradox of coronary risk and the menopause. Lancet; 351: 1425-27

Utermann G. (1989) The mysteries of lipoprotein (a). Science; 264: 904-10

van Baal WM, Emeis JJ, van der Mooren MJ, Kessel H, Kenemans P, Stehouwer CD. (2000) Impaired pro-coagulant balance during hormone replacement therapy. A randomised placebo-controlled 12 week study. Thrombosis Haemostasis; 83: 29-34

Vane JR, Anggard EE, Botting RM. (1990) Regulatory functions of the vascular endothelium. New England Journal of Medicine; 323: 27-36.

Vanhoutte PM. (1989) Endothelium and control of vascular function. Hypertension; 13: 658-67

Vassilopoulou-Sellin R, Asmsr L, Hortobagyi GN, Klein MJ, McNeese M, Singletary SE. (1999) Oestrogen replacement therapy after localised breast cancer: clinical outcome of 319 women followed prospectively. J Clin Oncol; 17: 1482-87

Vehkavaara S, Silveira A, Hakala-Ala-Pietila T, virkamaki A, Hovatta O, Hamsten A, Taskinen M, Yki-Jarvinen H. (2001) Effects of oral and transdermal oestrogen replacement therapy on markers of coagulation, fibrinolysis, inflammation and serum lipids. Thrombosis Haemostasis; 85: 619-25

Venkov CD, Rankin AB, Vaughan DE. (1996) Identification of authentic oestrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. Circulation; 94: 727-33

Volteranni M, Rosanno GM, Coats A, Beale C, Collins P. (1995) Oestrogen acutely increases peripheral blood flow in postmenopausal women. American Journal of Medicine; 99: 119-22

Walsh BW, Paul S, Wild RA, Dean RA, Tracy RP, Cox DA, Anderson PW. (2000) The effects of hormone replacement therapy and Raloxifene on C-Reactive protein homocysteine in healthy postmenopausal women: a randomised controlled trial. Journal of Clinical Endocrinology and Metabolism; 85(1): 214-18

Walton C, Godsland IF, Proudlar AJ, Wynn V, Stevenson JC. (1993) The effect of the menopause on insulin sensitivity, secretion and elimination in non-obese healthy women. European Journal of Clinical Investigation; 23: 466-73

Watson J, Erba E. (1992) In: Animal Cell Culture – a practical approach. Freshney J (Ed). IRL Press, Oxford; Chapter 6

Wehling M. (1994) Novel aldosterone receptors: specificity conferring mechanisms at the level of the cell membrane. Steroids; 59: 160-63

Weiderpass E, Baron J, Adami H, Magnusson C, Lindgren A, Bergstrom R, Correia H, Persson I. (1999) Low-potency oestrogen and risk of endometrial cancer. Lancet; 353: 1824-28

Weiner CP, Lizasoain I, Bayliss SA, Knowles RG, Charles IG, Moncada S. (1994) Induction of calcium dependent nitric oxide synthases by sex hormones. Proceedings of the National Academy of Science USA; 91: 5212-16

Wenger NK, Speroff L, Packard B. (1993) Cardiovascular health and disease in women. New England Journal of Medicine; 329: 247-56

Westerveld H, Kock L, Van Rijn J, Erkelens D, De Bruin T. (1995) 17β oestradiol improves postprandial lipid metabolism in postmenopausal women. Journal of Clinical Endocrinology and Metabolism; 80: 249-53

Whitcroft SI, Crook D, Marsh MS, Ellerington MC, Whitehead MI, Stevenson JC. (1994) Long term effects of oral and transdermal hormone replacement therapies on serum lipid and lipoprotein concentrations. Obstetrics and Gynaecology; 84: 222-26

Whitney R. (1953) The measurement of volume changes in the limbs. Journal of Physiology; 121: 1-27

Wight T. (1989) Cell biology of arterial proteoglycans. Arteriosclerosis; 9: 1-20

Wilbush J. (1980) Tilt EJ and the change of life (1857) 'The only work on the subject in the English language'. Maturitas; 2: 259-67

Wilbush J. (1988) In: The menopause (1st Ed). Studd JW, Whitehead MI (Eds), Blackwell Scientific Publications, Oxford; Chapter 1

Willliams JK, Adams MR, Klopfenstein HS. (1990) Oestrogen modulates responses of atherosclerotic coronary arteries. Circulation; 81: 1680-87

Williams JK, Adams MR, Herrington DM, Clarkson TB. (1992) Short-term administration of oestrogen and vascular responses of atherosclerotic coronary arteries. Journal of the American College of Cardiology; 20: 452-57

Wolfbauer G, Glick J, Minor L, Rothblat G. (1986) Development of smooth muscle foam cells: Uptake of macrophage lipid inclusions. Proceedings of the National Academy of Science USA; 83: 7760-64

Wolinsky H. (1972) Effects of oestrogen and progestogen treatment on the response of the aorta of male rats to hypertension. Morphological and chemical studies. Circulation Research; 30 (3) 341-49

Womens' Health Initiative, Update for 2000. National Institute for Health, USA
Writing group for PEPI trial. (1995) Effects of oestrogen or oestrogen/progestogen regimens on heart disease risk factors in postmenopausal women. JAMA; 273: 1389-96

Yashiro A, O'Neil J, Hoff H. (1993) Insoluble complex formation of lipoprotein (a) with low density lipoproteins in the presence of calcium ions. Journal of Biological Chemistry; 268: 4709-15

APPENDIX 1

SOLUTIONS FOR MOLECULAR BIOLOGY

Solutions for molecular biology

The reagents used in the work described in this thesis were purchased from Sigma Ltd (Poole, Dorset, UK) or from Fischer Scientific Ltd (Loughborough, Leicestershire, UK) unless otherwise stated. Disposable plasticware was purchased from Fischer Scientific Ltd (Loughborough, Leicestershire, UK).

Buffer H

50mM Tris HCl
10nM $MgCl_2$
100nM NaCl
1nM dithiothreitol @ pH 7.5

Deionised Glyoxal (6M solution = 40% w/v)

Stock glyoxal solution was deionised by stirring for one hour with mixed bed resin (5mg resin/100ml glyoxal) until the pH is above 5. The deionised glyoxal was then gassed with oxygen-free nitrogen, split into small single use aliquots and stored at –70°C.

Denhard's solution (100x)

10g Ficoll (20mg/ml)
10g Polyvinylpyrrolidone (20mg/ml)
10g Bovine Serum Albumin (fraction V) (20mg/ml)
dH_20 to 500ml

After preparation the solution was filtered and stored at -70°C.

Diethylpyrocarbonate treated water (DEPC H_2O) – 0.01% DEPC in water
50µl of DEPC was added to 500ml dH_2O, the bottle cap loosened and allowed to stand in a fume hood for several hours before autoclaving. This procedure was undertaken in a fume hood due to the toxicity of DEPC.

Disodium Ethylene Diamine Tetra-acetic Acid (0.5M EDTA)

EDTA	186g
dH_2O	800ml

Sodium hydroxide pellets were dissolved in solution as required to achieve pH 8.0.

Ethanol (70%)

70 ml of 100% ethanol (analar grade) was made up to a final volume of 100ml with dH_2O to give a 70% solution of ethanol.

Ethidium Bromide (10mg/ml)

0.5g of ethidium bromide was added to 50ml of dH_2O, and stirred for several hours to ensure that the dye had completely dissolved. The solution was stored in a container at room temperature, wrapped in aluminium foil.

Formamide

Analar grade formamide from a fresh unopened bottle was split into 20ml aliquots and stored at -70°C until required. Analar grade formamide can be used directly in hybridisations without the need for deionising.

Fresh Glyoxal Solution

DMSO	80µl
0.1M Na Phosphate	16µl
Deionised Glyoxal	27µl

This provided sufficient glyoxal solution for 10 samples.

Gel Loading Dye (6x)

0.25% Bromophenol Blue
0.25% Xylene Cyanol
40% (w/v) Sucrose Solution

Made up to total volume 10ml.

Hydrogen Peroxide (3% H_2O_2)

100ml of 30% H_2O_2 stock solution was diluted to 1L with dH_2O to give a 3% H_2O_2 working solution.

RNA loading buffer (5x)

0.4% w/v bromophenol blue	0.16g
50% v/v glycerol	20ml
0.1M Na-phosphate buffer @ pH7	4ml
dH_2O	16ml

The bromophenol blue was added to a flask and the solutions added to a final volume of 40ml. The flask was then sealed and incubated at 37°C for approximately one hour to dissolve the components, treated with DEPC then autoclaved. The stock was then aliquoted into small volumes and stored at -20 °C.

Sodium dodecyl sulphate (10% SDS)

50g of analar grade SDS (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) was dissolved in 500ml of dH_2O.

Note – due to its potential respiratory irritant effect extreme care must be taken when weighing out this reagent and a suitable facemask should be worn.

Sodium phosphate (0.1M) gel buffer (pH 7)

0.5M NaH_2PO_4 (30g/500ml)	78ml
0.5M Na_2HPO_4 (35.5g/500ml)	122ml
dH_2O	800ml

The solution was then DEPC treated prior to autoclaving.

Sodium phosphate tank running buffer (0.01M)

0.1M $NaPO_4$ (stock solution)	100ml
DEPC H_2O	900ml

Saline sodium citrate buffer (20x SSC) (pH 7)

NaCl	175.3g
Na Citrate	88.2g

The salts were dissolved in 800ml initially and the pH adjusted to 7, before making up to a final volume of 1L. The final solution was autoclaved following DEPC treatment.

SSC 6x

150 ml of 20x SSC was made up to a final volume of 500ml with DEPC treated water to give a final concentration of 6x SSC.

2x SSC

50 ml of 20x SSC was made up to a final volume of 500ml with DEPC treated water to give a final concentration of 2x SSC.

Saline sodium phosphate EDTA buffer (20x SSPE) (pH 7.4)

NaCl	175.32g
NaH_2PO_4	27.60g
Na_2EDTA	7.45g

The salts were dissolved in 800ml of dH_2O, and the pH adjusted to 7.4 before making up to a final volume of 1L. The solution was then DEPC treated and allowed to stand before autoclaving.

SSPE 2x

20x SSPE	40ml
10%SDS	4ml
dH_2O	356ml

The final solution was autoclaved following DEPC treatment.

SSPE 1x

20x SSPE	20ml
10% SDS	4ml
dH_2O	376ml

The final solution was autoclaved following DEPC treatment.

Tris acetate EDTA buffer (50x TAE)

Tris base	242g
Glacial acetic acid	57.1ml
0.5M EDTA (pH 8.0)	100ml

This stock solution was then further diluted with dH_2O to the desired concentration, for example, a 1x solution of TAE was prepared by taking 10ml of 50x stock TAE and diluting it to 500ml.

APPENDIX 2

RESULTS

Results – Study 1

IM	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	3.87	4.38	0.88	
Baseline 2	3.04	4.98	0.61	
Dose 1	3.09	4.42	0.71	-5.34
Dose 2	3.06	3.96	0.78	4.74
Dose 3	3.00	3.86	0.78	4.80
Dose 4	3.20	4.02	0.81	8.60
Dose 5	3.00	4.05	0.75	0.01
Dose 6	2.33	3.84	0.62	-18.10
Dose 7	2.92	4.51	0.64	-13.42
Dose 8	3.28	4.39	0.76	0.98
Dose 9	2.97	4.02	0.75	0.09
Post dose 1	3.01	4.34	0.70	-6.82
Post dose 2	3.00	4.20	0.75	0.70

SC	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	1.94	1.34	1.47	
Baseline 2	2.26	1.74	1.31	
Dose 1	2.14	1.47	1.46	5.39
Dose 2	2.20	1.37	1.62	16.80
Dose 3	1.86	1.18	1.62	16.86
Dose 4	2.03	1.25	1.61	16.77
Dose 5	2.06	1.52	1.38	-0.23
Dose 6	1.94	1.55	1.27	-8.42
Dose 7	2.24	1.59	1.42	2.12
Dose 8	2.16	1.50	1.46	4.94
Dose 9	1.89	1.64	1.16	-16.25
Post dose 1	2.04	1.74	1.22	-11.73
Post dose 2	2.01	1.76	1.16	-17.08

SF	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	9.74	12.70	0.82	
Baseline 2	9.67	12.50	0.82	
Dose 1	9.49	14.19	0.78	-5.30
Dose 2	9.21	15.60	0.66	-20.30
Dose 3	9.64	13.56	0.69	-15.12
Dose 4	9.02	15.94	0.80	-13.80
Dose 5	8.79	11.66	0.68	-16.06
Dose 6	9.74	12.92	0.82	-0.72
Dose 7	9.60	9.68	0.98	4.50
Dose 8	9.30	9.38	1.04	30.01
Dose 9	10.06	14.70	0.99	40.50
Post dose 1	10.34	16.85	0.65	-18.17
Post dose 2				

MY	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	3.85	3.59	1.10	
Baseline 2	4.15	4.00	1.04	
Dose 1	4.15	4.52	0.92	-13.26
Dose 2	4.69	5.36	0.89	-17.06
Dose 3	3.80	3.70	1.04	-2.61
Dose 4	4.79	5.14	0.93	-12.89
Dose 5	5.00	6.16	0.82	-23.62
Dose 6	5.00	6.85	0.75	-31.41
Dose 7	5.61	6.74	0.84	-21.70
Dose 8				
Dose 9	7.70	7.18	1.08	0.48
Post dose 1	7.00	6.54	1.06	-0.44
Post dose 2				

JM	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	2.92	4.73	0.63	
Baseline 2	3.04	4.10	0.77	
Dose 1	3.48	3.60	0.85	21.21
Dose 2	4.03	3.64	1.28	83.52
Dose 3	3.86	3.88	1.02	44.49
Dose 4	3.70	3.94	0.96	37.49
Dose 5	3.84	3.86	1.02	45.17
Dose 6	3.62	3.14	1.15	63.99
Dose 7	4.12	3.48	1.18	68.86
Dose 8	4.61	4.11	1.14	74.68
Dose 9	4.70	4.57	1.06	52.20
Post dose 1	3.78	3.46	1.10	55.74
Post dose 2	2.89	3.34	0.88	24.28

AM	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	1.89	1.28	1.60	
Baseline 2	1.96	2.42	1.14	
Dose 1	2.11	2.18	0.96	-29.20
Dose 2	1.86	2.04	0.93	-31.84
Dose 3	1.51	1.73	0.88	-35.77
Dose 4	1.31	1.64	0.81	-40.80
Dose 5	1.96	2.30	0.86	-37.08
Dose 6	2.34	3.76	0.63	-54.08
Dose 7	1.92	2.57	0.76	-44.90
Dose 8	1.82	3.26	0.56	-59.20
Dose 9	1.92	2.86	0.70	-49.07
Post dose 1	3.66	2.62	0.74	-45.93
Post dose 2	1.64	2.90	0.58	-57.14

MM	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	2.14	1.76	1.15	
Baseline 2	2.40	1.76	1.36	
Dose 1	2.95	2.00	1.53	22.13
Dose 2	2.38	2.04	1.22	-2.34
Dose 3	2.22	1.74	1.29	3.12
Dose 4	2.06	1.25	1.78	41.51
Dose 5	1.83	1.64	1.02	-18.20
Dose 6	2.55	2.19	1.26	0.24
Dose 7	4.44	4.54	0.98	-21.16
Dose 8	2.20	1.57	1.42	13.56
Dose 9	2.09	1.70	1.32	5.60
Post dose 1				
Post dose 2				

JM	FBF: left arm	FBF: right arm	Ratio left/right	% change
Baseline 1	2.58	1.68	1.55	
Baseline 2	2.40	1.46	1.65	
Dose 1	2.25	1.30	1.76	-1.12
Dose 2	1.68	1.36	1.34	-16.41
Dose 3	2.20	1.34	1.68	4.78
Dose 4	2.72	2.06	1.32	-17.48
Dose 5	2.18	1.28	1.70	6.72
Dose 6	1.90	1.42	1.61	13.44
Dose 7	2.42	1.57	1.68	4.56
Dose 8	2.70	1.86	1.46	-2.00
Dose 9	2.16	1.48	1.46	-15.60
Post dose 1				
Post dose 2				

Results – Study 2

SF D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.56	1.93	1.33	
Baseline 2	2.51	1.90	1.33	
NA 5pmol/ml	2.86	2.58	1.12	-15.80
NA 10pmol/ml	2.54	2.28	1.10	-14.85
NA 50pmol/ml	2.40	2.31	1.04	-21.52
NA 100pmol/ml	2.28	2.30	1.00	-24.27
Baseline 3	2.94	2.50	1.18	
Baseline 4	3.08	2.52	1.23	
L-NMMA 2μmol/min	1.91	2.45	0.78	-34.86
L-NMMA 4μmol/min	1.98	2.67	0.74	-38.14
L-NMMA 8μmol/min	1.70	2.48	0.70	-42.32

SF D2	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	3.02	2.67	1.14	
Baseline 2	3.53	2.82	1.32	
NA 5pmol/ml	2.56	2.50	1.02	-16.38
NA 10pmol/ml	2.49	2.34	1.08	-12.04
NA 50pmol/ml	2.96	2.43	1.23	0.41
NA 100pmol/ml	3.19	2.82	1.16	-5.73
Baseline 3	3.26	2.80	1.20	
Baseline 4	3.77	2.75	1.38	
L-NMMA 2μmol/min	3.92	2.66	1.15	-11.08
L-NMMA 4μmol/min	3.74	4.08	0.92	-29.08
L-NMMA 8μmol/min	2.32	3.84	0.60	-52.90

JH D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.26	2.65	0.86	
Baseline 2	3.16	4.00	0.80	
NA 5pmol/ml	2.94	4.65	0.64	-22.98
NA 10pmol/ml	3.09	3.36	0.71	-14.06
NA 50pmol/ml	2.95	3.92	0.76	-8.07
NA 100pmol/ml	3.35	4.43	0.76	-7.47
Baseline 3	1.96	3.40	0.68	
Baseline 4	1.62	2.56	0.80	
L-NMMA 2μmol/min	1.09	1.98	0.54	-20.42
L-NMMA 4μmol/min	0.98	2.24	0.43	-38.47
L-NMMA 8μmol/min	0.84	2.14	0.38	-45.22

JH D2	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.28	3.81	0.60	
Baseline 2	2.08	3.14	0.66	
NA 5pmol/ml	1.73	3.58	0.48	-23.31
NA 10pmol/ml	1.81	3.06	0.59	-6.71
NA 50pmol/ml	2.16	4.16	0.52	-17.65
NA 100pmol/ml	2.19	4.15	0.54	-15.90
Baseline 3	2.00	3.62	0.56	
Baseline 4	2.14	3.71	0.58	
L-NMMA 2μmol/min	1.75	3.67	0.48	-15.63
L-NMMA 4μmol/min	1.28	4.65	0.28	-51.46
L-NMMA 8μmol/min	1.26	3.78	0.34	-41.48

DM D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.54	2.92	0.88	
Baseline 2	2.84	3.35	0.85	
NA 5pmol/ml	2.98	3.90	0.76	-11.42
NA 10pmol/ml	3.08	4.20	0.74	-14.56
NA 50pmol/ml	2.61	4.36	0.66	-30.56
NA 100pmol/ml	2.10	4.54	0.46	-46.42
Baseline 3	3.24	3.96	0.82	
Baseline 4	2.87	3.52	0.82	
L-NMMA 2μmol/min	2.78	4.28	0.66	-20.13
L-NMMA 4μmol/min	2.94	4.52	0.66	-19.88
L-NMMA 8μmol/min	2.90	5.06	0.58	-28.76

DM D2	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	3.68	2.06	1.80	
Baseline 2	3.79	1.84	2.06	
NA 5pmol/ml	3.32	2.04	1.68	-13.06
NA 10pmol/ml	4.60	3.26	1.42	-26.67
NA 50pmol/ml	3.64	2.18	1.68	-12.94
NA 100pmol/ml	3.46	2.14	1.62	-16.23
Baseline 3	3.78	2.22	1.70	
Baseline 4	3.94	2.30	1.78	
L-NMMA 2μmol/min	3.30	2.64	1.28	-26.21
L-NMMA 4μmol/min	3.07	2.23	1.38	-20.38
L-NMMA 8μmol/min	3.99	2.80	1.42	-18.02

PH D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	1.43	1.13	1.26	
Baseline 2	1.29	1.63	0.80	
NA 5pmol/ml	1.11	1.14	0.99	-4.02
NA 10pmol/ml	1.24	1.13	1.23	18.82
NA 50pmol/ml	1.28	1.04	1.32	27.34
NA 100pmol/ml	1.16	1.04	1.17	13.40
Baseline 3	1.94	2.45	0.81	
Baseline 4	2.00	1.57	1.29	
L-NMMA 2μmol/min	1.54	1.32	1.16	10.93
L-NMMA 4μmol/min	1.40	1.54	0.92	-11.66
L-NMMA 8μmol/min	1.70	1.29	1.38	31.26

PH D2	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	1.31	1.36	0.98	
Baseline 2	1.32	1.30	1.02	
NA 5pmol/ml	1.62	1.24	1.30	30.67
NA 10pmol/ml	1.26	1.20	1.06	6.67
NA 50pmol/ml	1.74	1.28	1.36	37.24
NA 100pmol/ml	1.74	1.48	1.17	17.62
Baseline 3	1.87	1.60	1.19	
Baseline 4	1.98	1.78	1.18	
L-NMMA 2μmol/min	2.04	1.70	1.20	2.28
L-NMMA 4μmol/min	2.30	2.87	0.82	-31.16
L-NMMA 8μmol/min	2.04	2.04	0.99	-15.97

LH D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	1.22	2.38	0.52	
Baseline 2	0.86	2.12	0.40	
NA 5pmol/ml	0.72	1.70	0.42	-7.12
NA 10pmol/ml	0.72	1.32	0.54	17.05
NA 50pmol/ml	0.88	1.35	0.65	41.52
NA 100pmol/ml	1.84	2.08	0.89	93.18
Baseline 3	1.64	2.52	0.65	
Baseline 4	1.08	1.68	0.66	
L-NMMA 2μmol/min	0.70	1.54	0.46	-30.59
L-NMMA 4μmol/min	0.56	1.52	0.38	-43.07
L-NMMA 8μmol/min	0.50	2.06	0.24	-63.39

LH D2	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.16	2.17	1.00	
Baseline 2	1.97	2.06	0.96	
NA 5pmol/ml	1.78	2.02	0.90	-7.54
NA 10pmol/ml	1.65	1.98	0.84	-14.12
NA 50pmol/ml	1.84	1.68	1.10	11.88
NA 100pmol/ml	2.66	1.66	1.62	65.06
Baseline 3	1.74	1.97	0.89	
Baseline 4	1.66	1.88	0.90	
L-NMMA 2μmol/min	0.93	1.64	0.57	-36.31
L-NMMA 4μmol/min	0.91	1.68	0.55	-38.92
L-NMMA 8μmol/min	0.63	1.82	0.34	-61.74

PW D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.20	2.46	0.92	
Baseline 2	2.38	2.48	0.96	
NA 5pmol/ml	1.65	2.52	0.66	-29.94
NA 10pmol/ml	1.77	2.73	0.66	-29.58
NA 50pmol/ml	1.42	2.81	0.50	-46.14
NA 100pmol/ml	1.00	2.30	0.44	-53.40
Baseline 3	2.76	1.40	2.04	
Baseline 4	2.52	2.84	0.90	
L-NMMA 2μmol/min	2.24	2.04	1.32	-10.27
L-NMMA 4μmol/min	1.85	2.30	0.82	-44.54
L-NMMA 8μmol/min	1.60	2.48	0.64	-56.18

PW D2	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.44	1.43	1.82	
Baseline 2	2.31	1.52	156	
NA 5pmol/ml	2.31	1.17	2.00	18.04
NA 10pmol/ml	2.40	1.55	1.57	-7.18
NA 50pmol/ml	2.24	1.33	1.72	1.60
NA 100pmol/ml	2.50	1.48	1.83	7.41
Baseline 3	2.10	1.49	1.42	
Baseline 4	2.05	1.54	1.36	
L-NMMA 2μmol/min	2.58	1.72	1.42	2.48
L-NMMA 4μmol/min	1.97	1.82	1.09	-21.67
L-NMMA 8μmol/min	1.96	1.58	1.27	-8.94

MP D1	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.80	3.03	0.90	
Baseline 2	2.82	2.70	1.06	
NA 5pmol/ml	2.79	3.46	0.82	-16.40
NA 10pmol/ml	2.40	3.31	0.75	-23.52
NA 50pmol/ml	2.58	3.72	0.70	-28.46
NA 100pmol/ml	2.53	3.58	0.72	-26.42
Baseline 3	3.24	4.12	0.79	
Baseline 4	3.02	2.30	1.32	
L-NMMA 2μmol/min	2.60	2.60	1.02	-3.96
L-NMMA 4μmol/min	1.82	2.24	0.82	-22.86
L-NMMA 8μmol/min	2.12	2.41	0.93	-12.34

MP D2	FBF: left arm	FBF: right arm	Ratio: left/right	% change FBF ratio
Baseline 1	2.94	6.29	0.48	
Baseline 2	3.42	5.26	0.66	
NA 5pmol/ml	2.70	4.31	0.63	12.38
NA 10pmol/ml	2.78	4.27	0.64	15.14
NA 50pmol/ml	2.26	5.83	0.38	-31.02
NA 100pmol/ml	2.70	4.77	0.57	0.85
Baseline 3	2.78	4.83	0.57	
Baseline 4	3.88	2.98	1.36	
L-NMMA 2μmol/min	3.46	2.38	1.53	58.50
L-NMMA 4μmol/min	2.49	2.62	0.96	-0.47
L-NMMA 8μmol/min	2.46	2.11	1.18	22.22

VH D1	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	2.05	1.16	1.88	
Baseline 2	2.32	1.28	1.84	
NA 5pmol/ml	1.54	1.25	1.28	-31.44
NA 10pmol/ml	1.70	0.88	2.73	-25.74
NA 50pmol/ml	1.70	0.92	1.99	6.70
NA 100pmol/ml	1.56	1.56	1.04	-44.06
Baseline 3	2.14	1.14	2.04	
Baseline 4	2.22	1.60	1.42	
L-NMMA 2μmol/min	1.68	1.25	1.42	-18.02
L-NMMA 4μmol/min	1.88	1.90	1.06	-39.15
L-NMMA 8μmol/min	1.08	1.04	0.88	-47.88

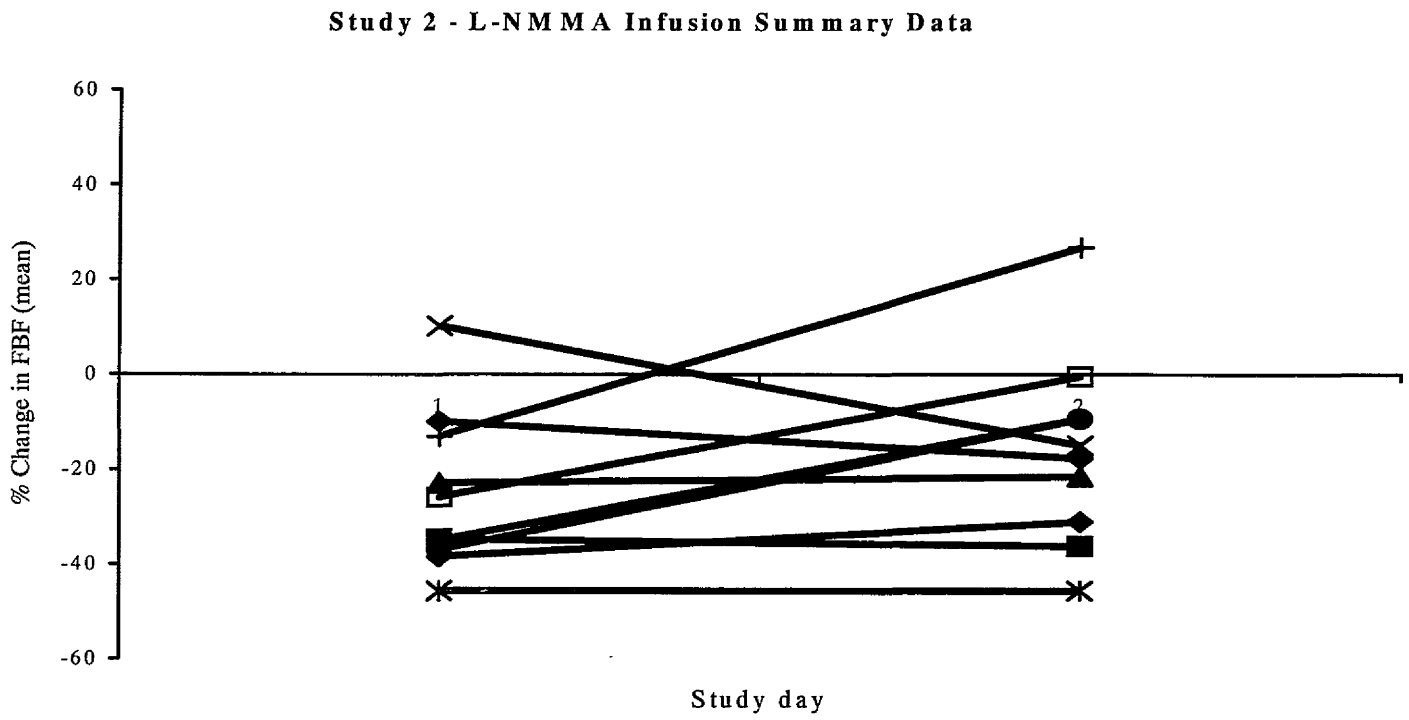
VH D2	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	2.08	1.22	1.72	
Baseline 2	2.16	1.17	1.88	
NA 5pmol/ml	1.86	1.10	1.70	-5.48
NA 10pmol/ml	1.65	1.14	1.54	-31.97
NA 50pmol/ml	1.49	1.20	1.30	-27.74
NA 100pmol/ml	0.98	0.96	1.03	-42.78
Baseline 3	1.46	1.37	1.10	
Baseline 4	1.40	0.86	1.64	
L-NMMA 2μmol/min	1.15	1.00	1.19	-12.44
L-NMMA 4μmol/min	1.02	0.92	1.42	7.97
L-NMMA 8μmol/min	1.22	1.17	1.04	-23.58

MM D1	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	1.44	2.14	0.68	
Baseline 2	1.40	2.49	0.58	
NA 5pmol/ml	1.60	1.98	0.82	29.52
NA 10pmol/ml	1.46	1.88	0.78	24.08
NA 50pmol/ml	1.15	2.24	0.52	-17.98
NA 100pmol/ml	1.12	2.78	0.40	-36.02
Baseline 3	3.20	3.52	0.90	
Baseline 4	1.12	1.70	0.72	
L-NMMA 2μmol/min	1.06	1.57	0.68	-15.80
L-NMMA 4μmol/min	0.89	1.71	0.53	-34.96
L-NMMA 8μmol/min	0.88	1.50	0.60	-26.96

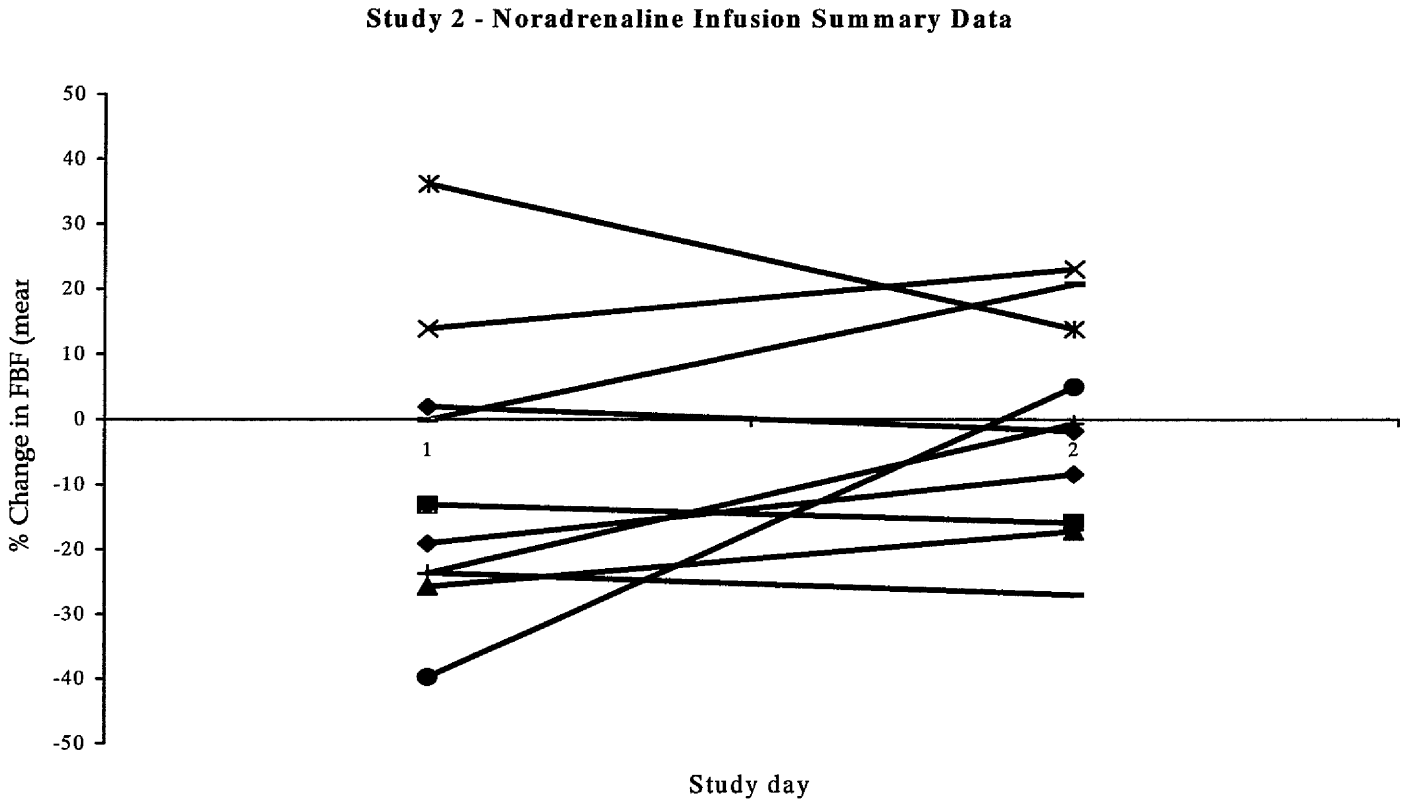
MM D2	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	2.12	1.86	1.15	
Baseline 2	2.54	1.61	1.60	
NA 5pmol/ml	1.89	1.44	1.92	40.58
NA 10pmol/ml	1.75	1.18	1.64	19.96
NA 50pmol/ml	1.89	1.18	1.68	22.17
NA 100pmol/ml	2.78	2.02	1.38	0.30
Baseline 3	2.50	1.76	1.44	
Baseline 4	2.75	1.93	1.48	
L-NMMA 2μmol/min	2.24	1.36	1.72	18.24
L-NMMA 4μmol/min	1.68	1.24	1.40	-3.39
L-NMMA 8μmol/min	1.98	1.70	1.22	-16.16

IC D1	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	0.80	1.40	0.66	
Baseline 2	1.03	1.08	0.98	
NA 5pmol/ml	0.92	1.14	0.84	2.94
NA 10pmol/ml	0.92	1.28	0.84	2.15
NA 50pmol/ml	0.91	1.13	0.82	1.00
NA 100pmol/ml	1.02	1.30	0.82	1.48
Baseline 3	1.26	1.40	0.94	
Baseline 4	1.08	1.54	0.77	
L-NMMA 2μmol/min	1.00	1.22	0.82	4.76
L-NMMA 4μmol/min	1.06	1.52	0.70	-15.94
L-NMMA 8μmol/min	0.86	1.22	0.71	-18.82

IC D2	FBF: left arm	FBF: right arm	Ratio: left/right	%change FBF ratio
Baseline 1	1.48	1.26	1.18	
Baseline 2	1.47	1.24	1.20	
NA 5pmol/ml	1.22	1.00	1.24	4.51
NA 10pmol/ml	1.06	1.19	1.47	21.90
NA 50pmol/ml	1.10	1.48	0.81	-31.88
NA 100pmol/ml				
Baseline 3	2.51	1.64	1.54	
Baseline 4	2.20	1.56	1.45	
L-NMMA 2μmol/min	1.38	1.14	1.26	-15.31
L-NMMA 4μmol/min	1.58	1.32	1.27	-14.92
L-NMMA 8μmol/min	1.48	1.31	1.15	-22.90



% Change in FBF from baseline during L-NMMA infusion – mean summary values for individual subjects on each study day.



% Change in FBF from baseline during noradrenaline infusion – mean summary values for individual subjects on each study day

Results – Study 3

Pilot study 1 – Cell count ($x10^6$)

Flask	C D4	17β nM D4	C D6	17β nM D6	C D8	17β nM D8	C D11	17β nM D11	C D14	17β nM D14
1	0.0311	0.0426	0.0612	0.0492	0.0524	0.0750	0.0956	0.1119	0.1064	0.0973
1	0.0449	0.0428	0.0794	0.0530	0.0504	0.0754	0.0954	0.1180	0.1104	0.1791
1	0.0503	0.0447	0.0540	0.0492	0.0499	0.0461	0.0999	0.1192	0.1364	0.1025
2	0.0382	0.0313	0.0588	0.0384	0.0348	0.0424	0.1001	0.0963	0.0963	0.1182
2	0.0383	0.0418	0.0725	0.0380	0.0323	0.0498	0.0995	0.1055	0.0939	0.0940
2	0.0497	0.0424	0.0720	0.0439	0.0372	0.0684	0.0978	0.0981	0.0792	0.0931
3	0.0484	0.0595	0.0548	0.0640	0.0443	0.0414	0.0907	0.0990	0.0982	0.0919
3	0.0502	0.0605	0.0538	0.0587	0.0442	0.0446	0.0917	0.0920	0.1116	0.1027
3	0.0496	0.0580	0.0546	0.0538	0.0409	0.0445	0.0897	0.0924	0.0744	0.0918

Pilot study 2 – Cell count ($x10^6$)

Flask	C D4	17β nM D4	17β pM D4	EE nM D4	EE pM D4	C D11	17β nM D11	17β pM D11	EE nM D11	EE pM D11
1	0.0394	0.0512	0.0370	0.0668	0.0382	0.2423	0.2370	0.2147	0.2399	0.2340
1	0.0384	0.0398	0.0298	0.0385	0.0318	0.2231	0.2250	0.2151	0.2372	0.2387
1		0.0419	0.0336	0.0299	0.0257	0.2326	0.2241	0.2164	0.2390	0.2503
1		0.0426	0.0246	0.0423	0.0548					
2	0.0393					0.1903				
2	0.0479					0.1742				
2	0.0463					0.1835				
3						0.2096				
3						0.2061				
3						0.2002				
4						0.2187				
4						0.2226				
4						0.2222				

Growth curve 1 – Cell count ($x10^6$)

Flask	C D4	17β nM D4	17β pM D4	C D8	17β nM D8	17β pM D8	C D12	17β nM D12	17β pM D12
1	0.0250	0.0542	0.0404	0.0849	0.1198	0.0823	0.3189	0.3819	0.0586
1	0.0379	0.0479	0.0380	0.0817	0.1149	0.0823	0.2742	0.3794	0.0515
1	0.0326	0.0831	0.0376	0.0653	0.1268	0.0875			0.0595
2	0.0407	0.0469	0.0724	0.0792	0.1246	0.1188	0.2616	0.4813	0.1742
2	0.0373	0.0396	0.0687	0.0993	0.1229	0.1088	0.2486	0.4032	0.1681
2	0.0387	0.0451	0.0718	0.0827	0.1138	0.1003		0.3712	
3	0.0389	0.0709	0.0354	0.1508	0.1014	0.0754	0.3482		
3	0.0433	0.0354	0.0337	0.1423	0.1045	0.0633	0.3213	0.3313	
3	0.0634	0.0631		0.1287	0.0972		0.2873	0.3063	

Growth curve 2 – Cell count ($x10^6$)

Flask	C D4	17βnM D4	17β pM D4	C D8	17β nM D8	17β pM D8	C D12	17βnM D12	17β pM D12
1	0.0526	0.0503	0.0332	0.0572	0.0353	0.0472	0.0815	0.1125	
1	0.0471	0.0381	0.0243	0.0622	0.0415	0.0512	0.1164	0.0925	
1	0.0426	0.0360	0.0274	0.0583	0.0376	0.0370	0.1079	0.1165	
2	0.0326	0.0462	0.0236	0.0582	0.0545	0.0497	0.0795		0.1049
2	0.0320	0.0437	0.0426	0.0583	0.0788	0.0468	0.0735		0.0940
2	0.0336	0.0472	0.0378	0.0692	0.0476	0.0710	0.0672		0.0882
3	0.0344	0.0351	0.0296	0.0481	0.0985	0.0677	0.1104	0.1462	0.1037
3	0.0338	0.0455		0.0410	0.1041	0.0655	0.0968	0.1367	0.0954
3	0.0290			0.0416	0.1007	0.0608	0.0998	0.1368	0.1005

Growth curve 3 – Cell count ($x10^6$)

Flask	C D4	17β nM D4	17β pM D4	C D8	17β nM D8	17β pM D8	C D 12	17β nM D12	17β pM D12
1	0.0431	0.0596	0.0406	0.0725	0.0651	0.0729	0.1484	0.1391	0.1446
1	0.0385	0.0587	0.0413	0.0648	0.0661	0.0632	0.1377	0.1407	0.1475
1	0.0357	0.0585	0.0367	0.0760	0.0580	0.0573	0.1409	0.1398	0.1535
2	0.0398	0.0320	0.0454	0.0665	0.0939	0.0433	0.0956	0.1795	0.1257
2	0.0381	0.0441	0.0396	0.0650	0.0697		0.0963	0.1718	0.1160
2	0.0395	0.0442	0.0409	0.0699			0.1077	0.1470	0.1190
3	0.0456	0.0344	0.0376	0.0771	0.0496	0.0833	0.1252	0.1618	0.1351
3	0.0451	0.0326	0.0397	0.0685	0.0487	0.0766	0.1178	0.1613	0.1250
3	0.0466	0.0334	0.0484	0.0615		0.0775	0.1210	0.2206	0.1259

Growth curve 4 – Cell count ($x10^6$)

Flask	C D4	17β nM D4	17β pM D4	C D8	17β nM D8	17β pM D8	C D12	17β nM D12	17β pM D12
1	0.0705	0.0782	0.0798	0.0653	0.0953	0.0628	0.0860	0.0558	
1	0.0596	0.0672	0.0524	0.0595	0.1035	0.0602	0.0729	0.0600	
1	0.0708	0.0717	0.0636	0.0641	0.0955	0.0668	0.0926	0.0542	
2	0.0441	0.0506	0.0523	0.1017	0.0853	0.0466	0.0674	0.0977	0.1145
2	0.0559	0.0569	0.0677	0.0954	0.0849	0.0522	0.0719	0.0889	0.1118
2	0.0636	0.0617	0.0505	0.0960	0.0822	0.0485	0.0581	0.0975	0.1110
3	0.0743	0.0536	0.0910	0.0974	0.0821	0.0571	0.0679	0.1037	0.1530
3	0.0605	0.0538	0.0924	0.0994	0.0976	0.0473	0.0606	0.1049	0.1404
3	0.0762	0.0529	0.0844	0.0956	0.1005	0.0425	0.0605	0.1057	0.1418