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DOCTORAL THESIS

IMPACT OF BAZEDOXIFENE ON ENDOTHELIAL HEALTH MARKERS

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CERTIFY

That under their direction **D. Darya Dudenko Lozenko**, graduate in Medicine and Surgery from University of Rovira i Virgili, has successfully completed the present thesis, entitled

IMPACT OF BAZEDOXIFENE ON ENDOTHELIAL HEALTH MARKERS

And has been awarded the degree of Doctor of Medicine.
In witness thereof, they affix their signatures hereunder.

Valencia, 12 of july, 2019

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This Doctoral Thesis has international standing due to completion of research stays in prestigious European centers.

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ABBREVIATIONS

AF	activation function site
BZA	bazedoxifene
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
c-Src	tyrosine-protein kinase
cGMP	cyclic guanosine monophosphate
cDNA	complementary DNA
CVD	cardiovascular disease
CHD	coronary heart disease
CEE	conjugated equine estrogens
CRP	C-reactive protein
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
CNS	central nervous system
CT	cycle threshold
CI	confidence interval
dNTPs	deoxyribonucleotide triphosphate
DBD	DNA binding domain
DVT	deep vein thrombosis
DMSO	dimethyl sulfoxide-based
E1	estrone
E2	estradiol
E3	estriol
E4	estetrol
ER	estrogen receptor
ET	estrogen therapy
ERE	estrogen-response element
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ECs	endothelial cells
FMD	flow-mediated dilation
FSH	follicle-stimulating hormone
FMP	final menstrual period
FBS	fetal bovine serum

FDR	false discovery rate
GRER	protein G located in rough endoplasmic reticulum
GEE	generalized estimating equations
HRT	hormone replacement therapy
HICs	high-income countries
HDL	high-density lipoprotein
HUVECs	human umbilical vein endothelial cells
HUAECs	human umbilical artery endothelial cells
LDL	low-density lipoproteins
LBD	ligand binding domain
MAPK	mitogen-activated protein kinases pathway
MMPs	matrix metalloproteinases
MPA	medroxyprogesterone acetate
MEM	minimum essential medium
NO	nitric oxide
NOS	nitric oxide synthase
NHS	Nurses' Health Study
Pl3K/Akt	phosphatidylinositol 3-kinase/Akt pathway
PGI2	prostacyclin
PBS	phosphate buffered saline
RANKL	receptor activator of the nuclear factor kB-ligand
RIPs	receptor interacting proteins
SERM	selective estrogen receptor modulator
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TSEC	tissue selective estrogen complex
TGFβ	tumor growth factor β
TXA2	thromboxane
TC	total cholesterol
VTD	venous thromboembolic disease
VSMC	vascular smooth muscle cell
VTE	venous thromboembolic events
WHO	World Health Organization
WHI	Women's Health Initiative

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SUMMARY

Atherosclerosis and its most significant clinical repercussion, cardiovascular disease (CVD), is the leading cause of morbidity and mortality in postmenopausal women, with a substantial global public health impact as a consequence. Vascular endothelial dysfunction, which is potentiated by decreasing estrogen levels in the menopausal transition period, is an early hallmark of developing CVD. The action of estrogens has been widely investigated in many critical areas of atherogenesis, due to the presence of estrogen receptors in the vascular system. Estrogens can have both positive and negative effects on the vascular wall depending on the stage of atherosclerosis disease. The estrogen response is mediated by different mechanisms, involving either genomic pathways requiring ligand-activated transcription with posterior modulation of protein synthesis, or non-genomic pathways, mainly affecting enzyme activity and rapid signaling cascades.

The vigorous debate surrounding the risk/benefit profile of estrogen in the development and timing of CVD in postmenopausal women is still ongoing. Evidence that the progestin component of hormone therapy increases breast cancer risk or even attenuates the potential beneficial effects of estrogens has been paralleled by a decline in the use of hormone therapy in postmenopausal women in recent years. This data, along with problems associated with long-term use of bisphosphonates, has shifted the focus of recent research to other

molecules capable of activating estrogen receptors, leading to transcriptional activity in some tissues but not in others. Selective estrogen receptor modulators (SERMs) and more recently, tissue selective estrogen complex (TSEC) have been developed as alternatives to hormonal replacement therapy (HRT).

Previous experimental studies have shown the capacity of estrogens and second-generation SERM raloxifene to promote endothelial cell regrowth and improve cell survival rate in the early stages of atherosclerosis disease. Clinical trials involving bazedoxifene or conjugated estrogens plus bazedoxifene have shown cardiovascular safety in healthy postmenopausal women. However, one research group working with an atherosclerotic animal model in monkeys demonstrated that therapeutic application of bazedoxifene had no effect on atherosclerosis. What is more, bazedoxifene completely impeded the antiatherosclerotic effects of estrogen treatment in the mentioned study. To date no report has been published of bazedoxifene's effect on vascular cell proliferation and the mechanisms involved using an *in vitro* model.

This study was designed to evaluate the effect of bazedoxifene on the proliferation of human umbilical arterial endothelial cells (HUAEC) compared with estradiol, raloxifene and the combination of bazedoxifene plus estradiol. Additionally, we assessed whether the process was associated with changes in the protein and gene expression of key cell

cycle regulators such as cyclin A, cyclin B, cyclin D1 and $p27^{Kip1}$ in both fresh and cryopreserved endothelial cells.

HUAEC were obtained from umbilical cords from healthy women at time of delivery in a Hospital Clinico of Valencia maternity ward. Umbilical cords were processed under specific conditions in the laboratory of the Pediatrics, Obstetrics, and Gynecology Department in the Faculty of Medicine. Primary culture and subcultures were carried out until sufficient endothelial cells were obtained for the study. Cells were synchronized at the G0 and G1 cell cycle phases, and active treatments were applied. Cell proliferation was measured using the Colorimetric Cell-Proliferation Kit II (XTT). Western blot experiments measured changes in protein expression and quantitative real-time polymerase chain reaction tested for gene expression alterations.

At increasing doses bazedoxifene alone showed moderately enhanced increased cell proliferation, a similar increase to estradiol and raloxifene. No significant results on proliferation were found after concurrent cell treatment with bazedoxifene plus estradiol. Immunoblotting assays detected an isolated increase in cyclin A and $p27^{Kip1}$ expression after treatment with estradiol, even though no significant changes in gene expression were associated with bazedoxifene, estradiol, raloxifene, or bazedoxifene plus estradiol for any cyclins. Analysis of the previous cryopreserved status of endothelial cells revealed positive changes in the protein content of almost all

cyclins compared to the fresh endothelial cells. In contrast, no significant trend was observed in their RNA profile.

In a broader perspective, the results of this study show for the first time the capacity of bazedoxifene to promote endothelial cell proliferation in an *in vitro* model. This beneficial effect has been corroborated in the literature for its predecessors estradiol and raloxifene, as is also confirmed in our work. The bazedoxifene-induced improvement in cell proliferation may facilitate endothelial regeneration and repair and thus contribute to the recovery of vascular functions during the early stages of atherosclerosis development. This observation turns the spotlight on bazedoxifene as a potential therapeutic target in CVD prevention when administered to women during the initial postmenopausal period.

Although our results were inconclusive for the gene and protein expression of cell cycle key regulators such as cyclin A, cyclin B, cyclin D1 and $p27^{Kip1}$ associated with bazedoxifene treatment, further experimental studies are nonetheless warranted. These should include the full panel of key regulators in cell cycle machinery to confirm whether other regulatory proteins play a role in the proliferative process.

1. INTRODUCTION

1. 1. Risk of cardiovascular disease in postmenopausal women

Changes in population dynamics are steadily increasing life expectancy, and it is estimated that roughly a third of woman's life will be spent beyond the menopausal transition. CVD seems to reflect the long-term consequences of aging and estrogen deficiency, causing a relative impact on women's welfare, morbidity, and mortality, in addition to a significant economic impact on healthcare service.

1.1.1. Prevalence and incidence of CVD

CVD remains the leading cause of death globally, even though the incidence of cardiovascular disease and death has declined markedly in a high-income countries (HICs) over the last two decades, being cancer responsible for twice as many deaths as CDV, according to a new report from the Prospective Urban Rural Epidemiology (PURE) study (1). Coronary heart disease (CHD), stroke, and venous thromboembolic disease (VTD) are the principal clinical endpoints of CVD. Approximately 45% of deaths are attributed to CVD in Europe, and 31% in the United States. This rate has decreased by up to 15% over the last ten years, but it is still the most common cause of mortality overall (2), (3). According to the latest European Cardiovascular Disease Statistics data, the group of circulatory system diseases remained the leading cause of death for women in Spain, followed by tumors and respiratory disorders. Including all its forms, CVD is responsible for approximately 115,000 deaths in Spain each year (3).

A similarly strong gender pattern was found regarding both prevalence and clinical presentation of CVD. The prevalence of CVD in women of reproductive age is lower than in men, rising substantially when women lose their reproductive capacity and ovarian hormonal production on reaching menopause (Figure 1) (2). If we evaluate the prevalence of CVD by age group, men under 60 years old show a higher frequency of CV events, while after that numbers tend to equalize, and women are typically around four years older than men at onset (≈75 years compared with 71 years).

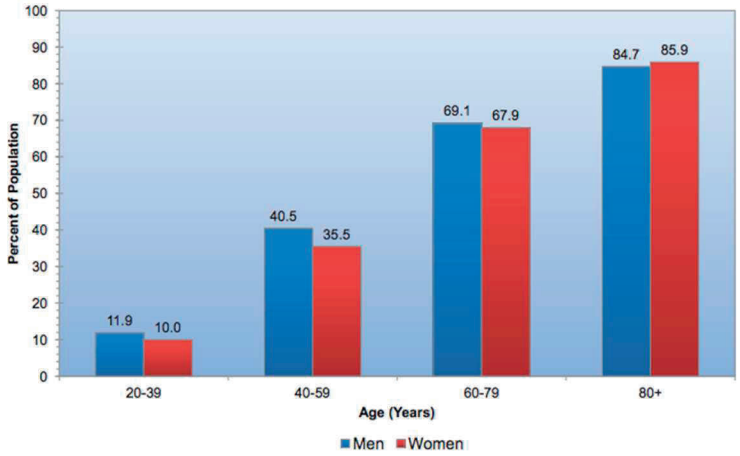


Figure 1. Prevalence of cardiovascular disease in adults by age and gender group. These include data on coronary heart disease, heart failure, stroke, and hypertension taken from American National Health and Nutrition Examination Survey: 2009–2012. *Data taken from American National Health and Nutrition Examination Survey: 2013–2016 (2).*

Most often the problem lies not only with a lower perception of CV risk in initially asymptomatic post-menopausal women, but also with

delayed diagnosis and an oncoming acute CV event. The differences in risk between men and women could be related to the possible influence of sex hormones, as many observational studies have demonstrated a protective effect for estrogens against CVD in premenopausal women.

To tackle this disease approaches are based around modification of the especially modifiable risk factors, and early diagnosis through advances in imaging techniques. Similarly, investigation into the pathophysiology of CVD and a better understanding of how sexual hormones act in this dysfunction opens up a promising field in terms of early diagnosis and appropriate treatment.

1.1.2 Clinical aspects of menopause

Hormone levels changes occurring during the menopausal transition, particularly the decline in quality of estrogens, can manifest clinical symptoms, which are generally divided into short-term and long-term consequences. Short-term symptoms are irregular bleeding, vasomotor signs (hot flashes and night sweats), local symptoms (skin changes, vulvovaginal atrophy), and even psychological symptoms (depressed mood, anxiety, irritability, lack of energy, etc.).

Around 75% of postmenopausal women will suffer hot flashes and night sweats, which constitute the most common and most frequent symptoms of menopause, related both to endothelial dysfunction and estrogen deprivation. Their frequency and duration are variable (from a few seconds to an hour), more frequent during the night, and are often

accompanied by palpitations and a sense of anxiety. They may appear a few years before menopause and last up to about five years, although their intensity decreases with time.

The genitourinary syndrome of menopause (GSM), consisting of the combination of symptoms such as thinning of the epithelium, urogenital atrophy, decreased vaginal and cervical secretions, dyspareunia, itching, dryness, and burning, occurs at the urogenital level as a result of estrogen deficiency. GSM is common, affecting around 50% of postmenopausal women. Its clinical manifestations can impact negatively on the quality of women´s lives, including sexual relationships and emotional well-being.

Long-term effects are characterized by two fundamental problems, osteoporosis and CVD; the latter is the focus of our work. Given that sex hormones contribute to bone homeostasis, their decrease may lead to a loss of bone mass, at both cortical and trabecular bone level. A high rate of bone remodeling occurs across menopause, leading to an increase in the resorption mediated by the osteoclasts, which are greater in number; the osteoblasts, although also in greater quantity, are not able to compensate. This situation will lead to a higher risk of bone fractures, basically vertebral and hip fractures, which have high morbidity and mortality in elderly patients.

Due to the decrease during menopause in the circulating level of estrogens, with a comparatively increased androgen concentration, body fat distribution changes, with an increase in visceral adiposity. There are

also alterations in lipid regulation with a marked pro-atherogenic profile, hypertension and insulin resistance development (4). All these changes represent independent risk factors for developing diseases such as diabetes mellitus type II, metabolic syndrome or CVD.

These changes are concurrent with an increase in proinflammatory cytokines. Chronic inflammation is a significant factor in atherosclerosis and atherothrombosis.

1.1.3 Predicting CV risk factors

Regardless of the origin of the atherogenesis-inducing factors, the first structure affected is the endothelial cell layer, due to its privileged position, with sensor and effector architecture. Impairment in endothelial function can be due to multiple causes such as high blood pressure, diabetes mellitus, genetic alterations, free radicals produced by tobacco or even microorganisms such as Chlamydia pneumoniae or Herpes virus (5). An extensive case-control study (INTERHEART) evaluating the effect of modifiable risk factors associated with acute heart events in both sexes in 52 countries worldwide, found that smoking was the leading preventable risk factor, followed by hypertension, abnormal lipid concentration, abdominal obesity, diabetes, excessive alcohol intake and lack of regular physical activity, all together responsible for 90% of the risk of an initial acute myocardial infarction (6). However, the traditional Framingham risk score and other similar assessment models that take into account major CV risk factors (cigarette smoking, hypertension,

hypercholesterolemia, diabetes mellitus, and age) fail to predict nearly 50% of CV events in asymptomatic patients (7). Defining the origin and the magnitude of particular points in the development of CVD is a primary focus in preventing future clinical events.

There is increasing scientific evidence supporting the hypothesis that vascular endothelial dysfunction is a pivotal promoter in the atherosclerosis and thrombosis processes, and independently predicts acute cardiovascular events in patients with and without established CVD (8), (9). Asymptomatic women are more likely to have a higher occurrence of microvascular lesions than men, especially during the transitional period when sex hormone levels change dramatically (10). Indeed, the main concern is that a significant proportion of post-menopausal women with a silent form of atherosclerosis are classified within low/medium CVD risk groups, allowing it to progress to irreversible chronic disease. It is therefore crucial to develop strategies addressed at improving diagnosis, prognosis, and treatment in these women (11).

To date no specific biomarkers of endothelial damage have been identified, owing to their tissue and vascular region variability, which precludes reliable systematic screening. The invasive approach to endothelial function using quantitative angiography and intravascular ultrasound has the highest clinical value, but is severely limited by the aggressiveness of the methods (12). During recent decades other non-invasive techniques have been investigated, such as measuring flow-

mediated dilation (FMD) in the brachial artery, to evaluate the health of the pan arterial wall and add prognostic information on CV risk (7).

Another independent factor effecting CV risk is menopause, as was revealed in the Framingham Heart Study, where a post-menopausal woman had a two-fold higher incidence of CV events in comparison with a pre-menopausal woman of the same age (13). Estrogen deficiency is involved negatively not only in the cardiovascular system but also in a decrease in serum androgen levels, which is associated with deleterious effects on endothelial function and advanced stage of atherosclerosis (14). Taking this into account, it is noteworthy that the availability of sex steroids in women´s blood could affect the particular of CVD profile, meaning that women with either artificial or early menopause have a notably higher risk of CVD (15).

Data from the large prospective and observational study SWAN (Study of Women's Health Across the Nation) including women of different ethnic groups during the first 12 months of post-menopausal amenorrhea showed that the main change in CVD risk factors occurs at the level of cholesterol metabolism, including total cholesterol, low-density lipoprotein cholesterol (LDL) and apolipoprotein B, which increase exponentially through this period. Note that these particles change their conformation, becoming denser, smaller and atherogenic (16). A smaller population of the SWAN was studied to determine how different patterns of circulating estradiol (E2) and follicle-stimulating hormone (FSH) during the years around the final menstrual period

(FMP) influence the risk markers of atherosclerosis after menopause. The group of women with low levels of E2 after menopause and lower FSH rise after their FMP may be more protected from flourishing atherosclerosis. However, women with high levels of E2 after menopause and medium or high FSH rises were associated with greater atherosclerotic risk (17). It is still unclear if these hormone pathway findings reflect an endogenous response to atherosclerosis development over time or a result from direct harmful actions of elevated levels of E2, but they could change future views of the transitional period of menopause based today mainly on clinical concepts.

1.2. Estrogens and cardiovascular disease

1.2.1 Biological mechanisms of estrogen action

Estrogens play an essential role in the regulation of normal physiology, aging, and many disease states. Therefore, advancing knowledge of estrogen signaling pathways is of great importance, both to interpret their role at the level of different organs and tissues and to understand the symptoms that cause their deficit, as occurs at menopause.

Estrogens are steroid sex hormones produced in the adrenal glands and ovaries, predominantly in women before menopause. Four active forms can be highlighted: estrone (E1), estriol (E3), estetrol (E4) (18), which is secreted only during pregnancy, and estradiol (E2) which

is the most prevalent in the organism. It is well known that the cellular response to estrogen is mediated by the existence of at least three types of cellular receptors, also expressed in the cells of the CV system. The ′classical′ nuclear estrogen receptors α (ERα) and β (ERβ) reflect the genomic pathway because of their ligand-activated transcription factor action; the membrane-associated plasma receptors, located at the caveolae level, are principally responsible for rapid estrogen effects due to a non-genomic pathway (19).

Both classical isoforms of ER share a conventional structure, which contains five different domains and six regions (Figure 2). They perform their functions independently while simultaneously interacting with each other:

- The A/B Domain, located in the amino terminus, is responsible for driving the activation function site 1, AF1, and is the site of interaction of other transcription factors.
- The C Domain, DNA-binding domain, acting on specific sequences in the target genes, known as estrogen-response elements (ERE). The result of the binding between ER to ERE affects gene expression by modulating transcriptional events.
- The D Domain is a region that gives flexibility to the ER for conformational changes and allows its movement towards the nucleus after its synthesis.

- ❖ The E Domain, the ligand-binding domain. The activating function site 2, AF-2, which is considered essential for specific ligand recognition because it allows the ER to be transcriptionally active in a selective and explicit manner.
- ❖ The F Domain, located on the carboxy-terminus, is dispensable in the transcription of estrogens, but it modulates the antiestrogenic activity and the amount of ligand bound to the receptor.

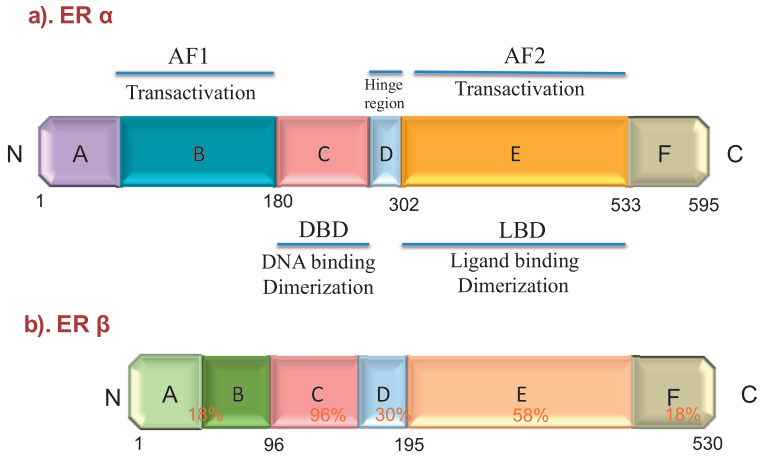


Figure 2. The diagram represents estrogen receptors alpha and beta.
a) The corresponding structural domains of ERα are represented with their main functions. The number of amino acids corresponding to the locations of some domains is detailed below.
b) In addition to the number of amino acids of ERβ, the percentage of homology between molecules is shown for each domain.
DBD: DNA binding domain; LBD: ligand binding domain; AF-1: activation function 1; AF-2: activation function 2.
Modified from MacGregor, JI et al (20).

Although most tissues express both ER subtypes, their distribution seems to be tissue-specific. ERα is predominant in the female reproductive system and the mammary glands, although it is also expressed in testicles, liver, and kidney. ERβ is present in granulosa cells of the ovary, bone, central nervous system, and prostatic tissue (21). The presence of both types of receptors has been identified by different techniques in the cardiovascular system, including endothelial cells, smooth muscle cells, and fibroblasts, although their contribution in the regulation of vascular effects remains the target of several lines of research (22). On the one hand, an impaired vascular function has been described as a consequence of the absence of ERα in a mice model; therefore its presence is probably critical for the protective estrogenic effect in the atherosclerotic process (23). On the other hand, ERβ's broader distribution in the organism is well known, as it its increased overexpression in areas of vascular damage, coronary calcification and atherosclerosis in both women and men (24), (25). In conclusion, both ERα and ERβ may have different roles in the vascular wall, and both their physiological and their pathological involvement in the cardiovascular system is an issue under investigation (26).

The ´classical´ model of estrogen receptor signaling establishes that ERα and ERβ act as ligand-activated transcription factors. Each ligand can induce a different conformation of the receptor, resulting in multiple cell effects. After binding, estrogen receptors interact with ERE in the promoter region of target genes. The corresponding genes are then

activated, transcribed to mRNA and lead finally to the synthesis of specific proteins. This process usually requires at least 1-2 hours after starting hormone treatment (19).

Moreover, the ligand-receptor complex may interact with a set of proteins called RIPs (Receptor Interacting Proteins), coactivators and coreceptors, which act as intermediate markers between the ER and the transcriptional machinery. RIPs have a broad function. On the one hand, they are capable of directing the AF1 areas of the target, enabling different degrees of access to the transcriptional machinery. On the other hand, RIPs can establish a link with other co-regulatory proteins in such a way that their differing concentration affects the final cellular response.

In contrast to the tardy actions of estrogens mediated by the genomic pathway, estrogens can also produce effects in a few seconds or minutes without requiring the intranuclear transcription process directly. This is because estrogens may also bind to plasma membrane-associated subpopulations of classic ERs or a distinct membrane-associated receptor, thereby activating a variety of rapid intracellular signaling cascades (27). Therefore, it should be noted that the final cellular response is given by the complex interaction of transcriptional or "genomic" and non-transcriptional effects. Some authors report that activating signaling cascades could affect the transcription process, either through co-activating elements, by phosphorylation of the receptor or other transcription factors, influencing the final gene expression (28), (29). "Non-genomic" is therefore an inappropriate term for a pathway

where changes occur in transcription, so a new term, "membrane-initiated steroid signaling" is proposed, based on the principle that these actions are generated from second messengers and organized kinases, mainly in the cell membrane.

This phenomenon was discovered and described for the first time in 1942 by Dr. Hans Seyle after observing effects immediately after the administration of certain steroids, in contrast to the impact of delayed duration (hours or days) known at that time. Later on, in 1967 it was observed that estrogen also caused the rapid release of cyclic AMP (cAMP) and immediate changes in calcium concentration in endometrial cells of the rat uterus (30), and specific binding sites for this hormone on the surface of these cells were identified (31). They showed that membrane ERs on the surface of these cells activate signal cascades, which facilitates the permeability of compounds mediated by hormones and the ability to transport E2 into endocytic vesicles to the nucleus where they exert their function.

One of the best reported rapid actions of estrogen occurs in endothelial cells where E2 can stimulate the production of cyclic guanosine monophosphate (cGMP) and nitric oxide synthase (eNOS), in addition to activating kinases involved in cell cycle regulation (19), (32), (33), (34), (35). The arteries of the cardiovascular system respond to rapid non-genotropic actions of the kinase family, causing the reduction of ischemic damage mediated by NO-dependent vasodilation (36), (37). E2 also inhibits Ca2+ channels in the vascular smooth muscle, which

results in improved heart perfusion (38). There are many more examples of this type of actions, that have been gaining increasing importance, constituting the focus of around 1000 publications to date.

In certain cell types, ERs are located in the plasma membrane at the level of invaginations called caveolae, associated with large protein complexes promoting efficient signaling. The steroid actions initiated in the membrane appear to trigger many intracellular signaling pathways, including mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) activation of ion channel fluxes, generation of G protein-coupled receptor-mediated second messengers and stimulation of growth factor receptors (33).

More recent studies have revealed that membrane-bound estrogen receptor or GRER (formerly known as GPR30) is an essential mediator of the rapid cellular effects of estrogen throughout the body. This protein G was later shown to be located in the rough endoplasmic reticulum, therefore was termed as GRER, and to bind specifically to estrogen. As a result of the discovery of selective synthetic ligands for GRER, both agonists and antagonists, as well as the use of GRER knockout mice, several facets of this pathway signaling are being investigated and significant insights have been made into GRER functions at the cellular, tissue and organism level (39), (40). Adenylate cyclase and c-Src are the two effector pathways activated after the estrogen agonist binds with the GRER. Both routes are involved in activating mitogen-activated protein

kinases (MAPK) and PI3K that can lead to the transactivation of genes, even ones without EREs.

As mentioned before, a membrane receptor mediates these actions, but its origin is still unresolved. It could be migration of the nuclear receptors themselves or others linked to the hormones. Studies carried out support the hypothesis that the activation of signaling cascades against the same hormone depends on the receptors of different cellular compartments and the specific cell environment (41).

Figure 3 provides a summary of estrogen receptor signaling mechanisms.

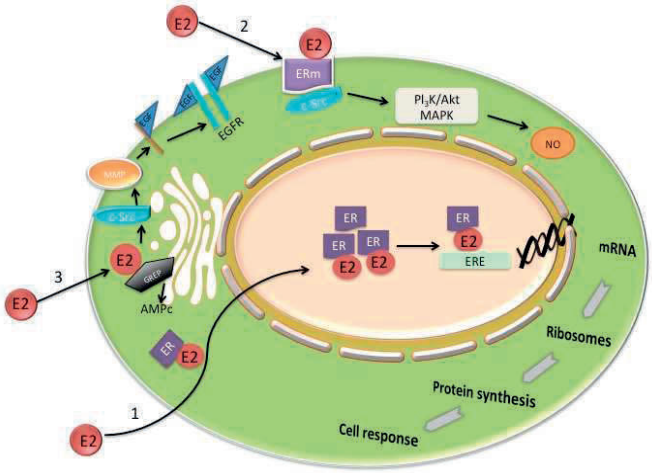


Figure 3. Diagram of the different signaling pathways through the estrogen receptor.

1) The classic intracellular receptor pathway. When the E2 enters the cell and binds to its ER, the complex formed translocates and migrates to the nucleus. The heterodimer interacts with ERE, transcribing to mRNA and leading finally to the synthesis of specific proteins that influence cell behavior.
2) Estrogens can activate a subpopulation of ERs in the cell membrane (ERm) which trigger signaling proteins such as c-Src, a critical point in the activation of fast pathways through PI3K/Akt and MAPK.
3) E2 can also be incorporated into the GRER receptor bound to the G protein that is located in the endoplasmic reticulum. GRER activates effector molecules such as cAMP and c-Src. The last mentioned molecule can stimulate MMPs and favor the release of Epidermal Growth Factor (EGF) through EGFR. Some of the signaling cascades are activated at the same time, such as the activation of PI3K/Akt and MAPK and, consequently, the release of NO in the endothelial cell.

ER: estrogen receptor; ERE: elements of estrogen response; Pl_3K/Akt: phosphatidylinositol 3-kinase/Akt pathway; MAPK: mitogen-activated protein kinases pathway; GRER: distinct membrane receptor; cAMP: cyclic adenosine monophosphate; c-Src: tyrosine-protein kinase; MMPs: matrix metalloproteinases; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; NO: nitric oxide.
Modified from Meyer, MR (42).

1.2.2. Impact of estrogen effects on development of atherosclerosis lesion.

In the pathogenesis of atherosclerosis different mechanisms involved have shown estrogenic sensitivity. Under physiological conditions, the endothelium regulates vascular tone by liberating vasodilator substances such as nitric oxide (NO) and prostacyclin (PGI2), avoiding adhesion and migration of cells from the blood into arterial wall, expression of their receptors, and proliferation of smooth muscle, thus keeping the composition of surrounding extracellular matrix intact (12). The progression of atheromatous plaques may be favored both by changes in lipid metabolism and by alterations in the capacity of the endothelium to release biological mediators.

The direct effect of estrogen loss combined with other major CV risk factors, together contribute to endothelial cell injury by inducing impaired NO bioavailability and significant permeability. This mainly leads lipoprotein deposits to oxidize into sub endothelial space or so-called fatty streaks. When vascular wall damage becomes persistent, it can lead to stable or unstable plaque formation, and become a major trigger for many stages of CVD. Growing evidence has reported that the effect of estrogens on the vascular vessels depends on how advanced the atherosclerosis is. The effect of estrogens on early atherogenesis could be neutral or even beneficial due to their capacity to promote NO release, improve plasma lipids levels and maintain the integrity of intimal cells (43).

When atherosclerosis is advanced, the diseased cells of the arterial wall lose the ability to express ER in number and function. As a consequence, insufficient estrogen-induced vasodilatation is noted. The detrimental action of estrogen on unstable plaques is due in part to greater matrix metalloproteinase (MMP) expression and activation of hemostatic parameters, particularly platelets, greatly favoring fibrous cap instability, plaque rupture and onset of clinical events (Figure 4).

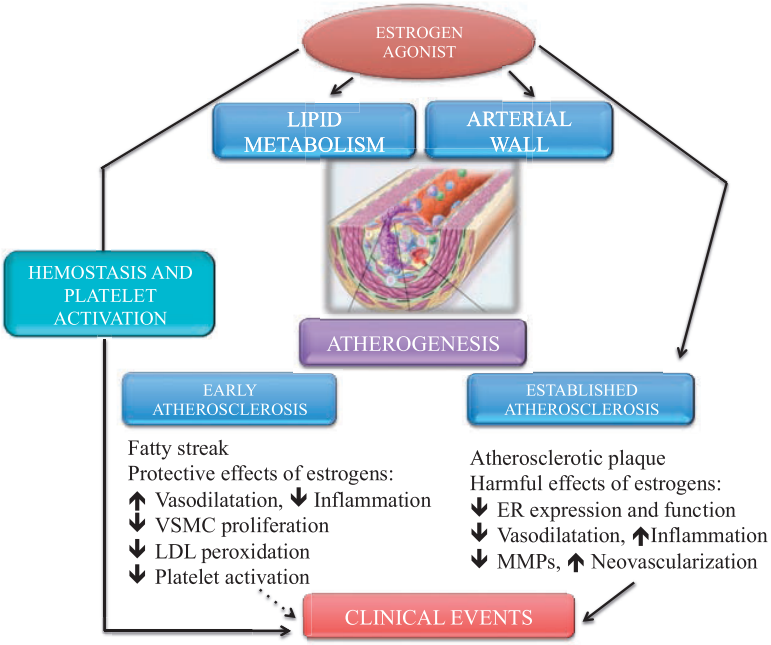


Figure 4. Diagram of atheromatous lesion development showing different protective effects of estrogens or estrogen-based therapies in early atherogenesis and their harmful effects in established atherosclerosis.
VSMC: vascular smooth muscle cell; LDL: low-density lipoprotein; ER: estrogen receptor; MMPs: matrix metalloproteinase.
Modified from Newson, L (43).

1.2.3. Menopausal hormone therapy. Historical background.

The protective effect of hormones has long been emphasized, and the role of estrogen therapy (ET) on the cardiovascular system has been broadly studied. The use of estrogens to improve climacteric symptomatology began in the early 1940s in the United States as a result of obtaining conjugated estrogens from pregnant mares' urine, and in response to the book "Feminine forever" published by Robert A. Wilson in 1968. A similar thing had happened in Europe with the synthesis of the main circulating physiological hormone secreted by the female gonad, 17 β-estradiol. The beneficial effects of estrogen therapy have been proven not only in improving women's quality of life, vulvovaginal atrophy and climacteric symptoms, but also in reports that estrogen provides more significant benefits, among which are protection of the cardiovascular system, memory and cognitive processes, bone safety, skin, and even dental health.

Numerous observational studies in the late twentieth century have reported that postmenopausal women who received long-term treatment with ET and HRT with estrogens and progestogens showed a lower risk for osteoporotic fractures, cardiovascular diseases and even Alzheimer's disease (44). The most extensive observational study, the Nurses' Health Study (NHS), strongly supported a protective cardiovascular benefit of HRT after menopause. More specifically, the women in the study had more than 40% risk reduction of CV events and a 30% decrease in

mortality due to CV causes (45). However, the benefits suggested in the observational studies were not supported by randomized, double-blind, placebo-controlled clinical trials in either primary prevention, Women's Health Initiative (WHI) or secondary prevention, Heart Estrogen/Progestin Replacement Study (HERS), which instead raised severe doubts about its safety (46), (47).

The WHI combined a set of randomized controlled trials and an observational study initiated in 1991, all of which were postulated to address strategies for preventing CVD, breast cancer, colorectal cancer and osteoporotic fractures in postmenopausal women. The hormone therapy trial had two arms and included asymptomatic women aged 50-79 with an average age of 63 years.

A first arm recruited 16,608 women with an intact uterus, who were randomized to receive conjugated equine estrogens (CEE) plus synthetic medroxyprogesterone acetate (MPA) or placebo. After a follow-up of just over five years, the study had to be terminated earlier than planned due to early reports indicating a higher incidence of breast cancer and an increased risk of cardiovascular events (coronary heart disease, pulmonary embolism, and stroke) in this combined estrogen and progesterone arm, specifically in women who had experienced more than 20 years of menopause at baseline (46).

The second arm of the study included 10,739 women with hysterectomy who were randomized to CEE alone or placebo with an almost 7-year mean follow-up (48). That trial showed no overall

cardiovascular benefit, but in women with less than ten years since baseline menopause, there was a relative tendency to coronary protection even if this result wasn't significant, due to insufficient participant numbers (Figure 5).

WHI 2002 → CEE + MPA vs Placebo	
Years since menopause	Relative risk of coronary heart disease (95% IC)
<10	0.88 (0.54-1.43)
10-19	1.23 (0.85-1.77)
>20	**1.66** (1.14-2.41)

Delayed onset of HRT: ↑ risk

WHI 2004 → CEE alone vs Placebo	
Years since menopause	Relative risk of coronary heart disease (95% IC)
<10	**0.48** (0.20-1.17)
10-19	0.96 (0.64-1.44)
>20	1.12 (0.86-1.46)

Early onset of HRT: ↑ protection

Figure 5. Coronary risk observed in three age groups since onset of menopause in two study arms of the Women's Health Initiative.
CEE: conjugated equine estrogens; MPA: medroxyprogesterone acetate; HRT: hormone replacement therapy.
Modified from Rossouw, JE et al (46).

Despite these findings, the long-term follow-up data reported in 2013 by the same study group showed no significant increase in cardiovascular events or mortality in ether intervention arms compared with placebo, regardless of the women's age or the timing of HRT initiation (49).

The negative message about HRT transmitted by early reports from the WHI study precipitated confusion among practitioners about the adverse effects and risks associated with the therapy. Notwithstanding, certain limitations in the trial design cast doubt on the discrepancy between animal/observational studies and randomized controlled trials. These include the older age of women in the clinical trial (average age 63 years), the late timing of initiation (especially in postmenopausal women with an average of ten years after menopause onset), and the high mean body mass in these women compared with the observational studies. The increased risk of CV events in these women could be due to the age of the women included in the study, whose blood vessels had already been exposed to an aging process, probably with underlying asymptomatic atheromatous plaques.

In the HERS study, with a similar design to WHI, the 2,763 selected patients had a known CHD. After a mean of 4.1 years, there was no significant difference in the number of cardiovascular events in the HRT arm versus placebo, so it also ended earlier than expected (47).

Taking this data into account, there are three mechanisms by which estrogens or estrogen-based therapies could be harmful to the cardiovascular system: they can change lipid composition by increasing triglyceride levels, can result in a prothrombotic effect on the coagulation cascade and lastly, estrogens administered orally, but not those administered transdermally, show proinflammatory effects including increased synthesis of C-reactive protein (CRP) (50). The higher risk

associated with oral compared with transdermal estradiol shows that the different of drug administration routes follow diverse metabolic pathways (transdermal, oral, or intravenous with or without progesterone) as well as the dosage.

A new theory reported from the latest studies refers to a 'window of opportunity' based on the 'timing hypothesis' of HRT in cardiovascular disease.

1.2.4. Cardiovascular timing hypothesis

The ´window of opportunity´ refers to the period when estrogen replacement therapy provides a favorable risk/benefit ratio for the CV system. This phenomenon generally occurs when HRT treatment is initiated before the age of 60 years or when it is taken within ten years of onset of menopause. This definition is supported by data accumulated from numerous studies reporting the time-dependent action of estrogens on the atherosclerosis process. A large multicenter randomized study called The Kronos Early Oestrogen Prevention Study (KEEPS) reported a neutral impact on CV risk markers such as intima-media thickness and coronary calcium score in women taking HRT in their early menopause (51). Furthermore, the Danish Osteoporosis Prevention Study, which included women with similar characteristics, revealed a significantly reduced risk of mortality, heart failure, or myocardial infarction (52). An even more recent study is the Early versus Late Intervention Trial with

Estradiol performed in 643 postmenopausal women, who were randomized to receive hormonal treatment, and stratified according to time since menopause (53). The ´early´ or less than six years since menopause group showed a significantly slower rate of atherosclerosis progression.

These findings support that the timeframe of HRT initiation influences the progression of cardiovascular disease, and is thus able to play a crucial role in primary prevention therapy (Figure 6). Although nowadays HRT is not prescribed to prevent cardiovascular events, the European Menopause and Andropause Society (EMAS) recommends the use of HRT (estrogen with or without progesterone) in women with climacteric symptoms due to its efficiency/safety profile within the ´window of opportunity´ period (54).

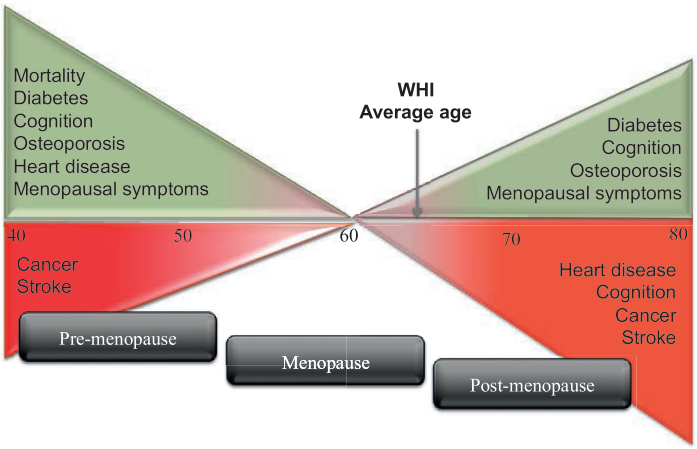


Figure 6. Hormone replacement therapy timing hypothesis. Use of HRT is generally accepted in women close to menopause, without associated risk factors. It is considered that in these women the benefits derived from therapy including osteoporosis and coronary heart disease prevention outweigh the risks. However, in women over 60 years old, HRT should be avoided unless the menopause symptoms affects quality of life.
Modified from Bolton, JL et al (55).

1.3. Endothelium and cell cycle progression

As previously explained, endothelial cell dysfunction is the first hallmark of predisposition to atherosclerosis and could be one of the earliest triggers of CVD events. The phenomenon of atheroprotection is the capacity of endothelial cells to keep a strict barrier with a variety of pathophysiological roles to promote regrowth after external aggression and denudation. To carry it out, the endothelium and the rest of the mammalian cells grow, divide and produce two identical daughter cells through a sequential, ordered and repetitive process over time called the cell cycle. Progression through the cycle has been arbitrarily classified

into five phases, of which replication of chromosomal material (S phase or synthesis) and distribution of the genetic material (M phase or mitosis) are the two essential stages. However, the cells need prior preparation to face the S and M phases, the G1 phase (Gap 1) with proteins and RNA synthesis, and the G2 phase (Gap 2) where the synthesized material is duplicated. Finally, when a cell stops proliferating it leaves the cycle and enters a period of rest or repose called G0 (56).

To ensure the cycle develops correctly, equitable distribution and genomic integrity over the following generations an essential role is played by a group of cyclins and cyclin-dependent kinases (CDKs) that participate in different phases of the cycle (Figure 7).

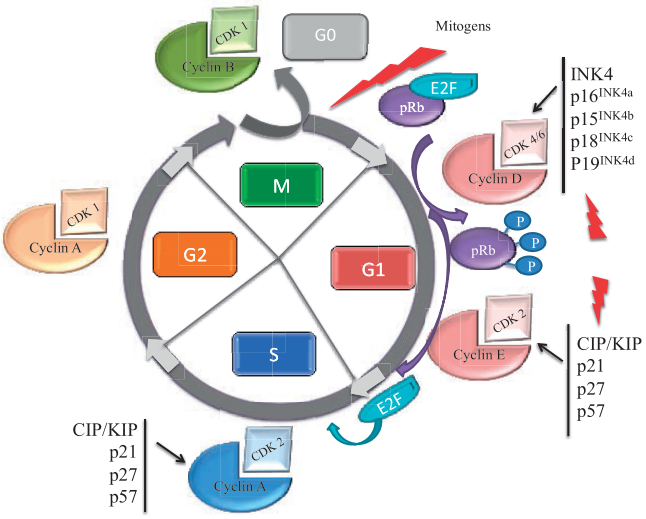


Figure 7. Diagram of cell cycle progression and the proteins involved in this. Stimulation by mitogens activates the sequence through different cell cycle phases controlled by the activating specific CDK/cyclin complexes.
Modified from Fuster, J et al (57).

1.3.1 Role of CDKs, cyclins and CKIs in cell cycle regulation

CDKs are a family of serine/threonine kinases that constitute the nucleus of cell cycle control. The CDKs activation and oscillation of depend on a complex machinery of enzymes and proteins, among which the cyclins represent one of the more critical regulatory catalytic subunits. The levels of the different cyclins, which fluctuate throughout the cell cycle, are controlled transcriptionally and by the ubiquitin-dependent proteolytic machinery. These particular oscillations in the concentration of the cyclins manage to activate each of these key Cdk-

cyclin heterodimeric complexes sequentially for systematized cycle progression (56).

Mammalian G1 cyclins and their associated kinases D-type cyclins D1/D2/D3 which bind to Cdk 4/Cdk 6, and E-type cyclins E1/E2 which link up with Cdk2, are initiators of protein synthesis and RNA/DNA replication. These complexes are responsible for incorporating outside information from mitogen signals and driving cells through the G1 phase of the cycle (58). Both cyclin types (D and E) had long been thought essential for their function of access and advancement through G1 phase; nonetheless, recent work has challenged that view as findings from gene inactivation studies in animal models encoding all G1 cyclins and their Cdks has shown normal animal development in their absence (59).

Cyclins A and B, also called mitotic cyclins, join Cdks during the S, G2 and early M phases, and are necessary to enter into mitosis. Specifically, Cdk1 binds cyclins A and B, and Cdk2 binds cyclins A and E. As we mentioned above, cyclin E is responsible for DNA replication and centrosome duplication, whereas cyclin A supports both of these actions and mitosis, and cyclin B guides mitosis alone (60). Due to cyclin B´s inability to induce replication, and the resulting different biological functions of cyclin A and B, they are located in different parts of the cell and regulated separately, cyclin A being nuclear and cyclin B cytoplasmic.

CKIs (cyclin-dependent kinase inhibitors) are proteins that bind to CDKs by negatively regulating their activity. Depending on their structure and function, CKIs are classified into two families: a) the INK family (p15Ink4b, p16Ink4a, p18Ink4c, and p19Ink4d), characterized by multiple repetitions of ankyrin domains, and by explicitly inhibiting CDK4 and CDK6 kinases; b) the CIP/KIP family (p21Cip1/WAF1, p27Kip1 and p57Kip2), characterized by having a broad spectrum of inhibition, although they are more active against the CKD2 kinase than against the CDK4 and CDK1 kinases. They have a common amino-terminal domain inhibitor of CDK/cyclin, and a structurally and functionally different carboxy-terminal domain (61). $P27^{Kip1}$ is a Cip/Kip family member that plays a significant role in the control of cell sequence in diverse pathophysiological contexts. $p27^{Kip1}$ levels are typically elevated during the G0/G1 phase and rapidly decrease via mitogen action, allowing the cells to enter the S phase. Among the mitogenic factors, certain hormones and growth factors are noteworthy. On the other hand, there are substantial signals that cause cellular quiescence by increasing the levels of $p27^{Kip1}$, such as tumor growth factor β (TGFβ), low serum levels, interleukin-10, interferon-γ, and various cytostatic drugs.

$P27^{kip1}$ also has atheroprotective functions, as has been shown in studies with an animal models. ApoE-null mice with genetic inactivation

of $p27^{Kip1}$ exhibited accelerated development of atherogenesis. In addition to modulating proliferation, $p27^{Kip1}$ may contribute to other cellular mechanisms involved in the atherogenesis, such as apoptosis, migration, and autophagy (57).

Due to their relevance in the experiments conducted in this Doctoral Thesis, the following table summarizes the roles of cyclin A, B, D1, and the CKI $p27^{Kip1}$ (Table 1).

Protein	**Established function**
Cyclin A	Control of S phase of cell cycle in complex with Cdk2 or Cdk1
Cyclin B	Control of M phase of cell cycle in complex with Cdk1
Cyclin D1	Control of G1 phase of cell cycle in complex with Cdk4 or Cdk6
$P27^{Kip1}$	Inhibition of Cdk/cyclin complexes

Table 1. Established functions of cyclins and CKI $p27^{Kip1}$.

1.3.2 Effect of therapies on cell proliferation

Endothelial effects are clinically significant in cardiovascular disease development. The effect of estradiol on the proliferation of endothelial cells has been previously demonstrated, as has its ability to exert a protective influence against apoptosis, in all stages of neo-angiogenesis (62), (63), (64) . During the early atherosclerotic process, damage and removal of luminal endothelium causes arterial lesions to form, whose severity are proportional to the duration of endothelial denudation (65). Cell proliferation is maximal in the initial stages of intimal thickening; animal studies have thus shown cell proliferation and atherosclerotic plaque size to be inversely related.

Although mature endothelial cells exhibit a meagre proliferation rate, during the process of re-endothelization of the denuded arteries, the endothelial cells can re-enter the cell cycle and undergo several proliferation cycles. The balance between action of angiogenic stimulators and inhibitors determines the dynamics of proliferation (66).

Detailed knowledge of the cell cycle is of particular interest. Several studies have focused on raloxifene as a SERM capable of protecting against cardiovascular disease. This conclusion is based on its ability to regulate vital biochemical processes for cellular homeostasis. Raloxifene stimulates eNOS and promotes endothelial health by reducing ischemic damage in experimental conditions (67). Likewise, it supports

non-genomic production of prostacyclin which, together with nitric oxide, inhibits platelet aggregation dependent on vascular endothelium (68). Prostanoids such as PGI2 and thromboxane (TXA2) play an essential role in maintaining vascular homeostasis. In terms of cellular mobility, raloxifene significantly inhibits vascular smooth muscle cell migration (69). One line of research focused on studying whether SERMs promote cell proliferation in the same way as estradiol (70). Using cell cultures of endothelial cells (EC) from the vein of the umbilical cord, the research group demonstrated that the proliferative effect of raloxifene was similar to that induced by E2. This action is also associated with increased expression of cyclins A and B1.

In contrast to the effects of estradiol and raloxifene on endothelial regrowth, the proliferative effects of BZA have not been studied to date.

1.4 Selective estrogen receptor modulators (SERMs)

1.4.1 The SERM family

Interest in the cardiovascular potential of SERMs has emerged out of the discordant behavior of estrogens in observational clinical studies and randomized placebo-controlled trials. Research has identified a wide new range of compounds with heterogeneous chemical structural characteristics capable of binding to ER, and leading to transcriptional activity in some tissues but not in others, depending on the ligand or the target tissue (22), (71), (72). Approximately 70 molecules with a

pharmacological profile of SERM type have been identified, many of them from both *in vitro* cell models and *in vivo* experiments.

The triphenylethylene family represents the most classic members of the SERMs, including compounds such as tamoxifen and clomiphene citrate (first generation SERMs). Tamoxifen, a drug introduced more than 40 years ago for hormone-dependent breast cancer treatment, has been considered an antiestrogen for decades due to its ER blocking action in neoplastic breast cells (73). However, several subsequent studies suggested functional tamoxifen agonist activity in bone, serum lipids, and endometrium (74), (75). This, together with recent insights into how this medicine can modify gene expression according to a particular tissue, has led researchers to develop new compounds with potentially improved safety profiles, such as toremifene, ospemifene, miproxifene, droloxifene, idoxifen and other second and third generation SERMs (76). Toremifene has a similar pharmacological and clinical profile to tamoxifen. It also has antitumor activity in mammary carcinoma, with an estrogenic effect on the uterus and bone. It could probably be prescribed as a second-line agent in cases of resistance to tamoxifen since it has shown cross-resistance with it in resistant mammary tumors (77).

Soon after this initial finding, other antiestrogens were found to exhibit similar mixed functional activities, although not necessarily identical to each other. One example is raloxifene (previously called keoxifene and LY 156758), the SERM that ranks second in accumulating

most clinical information belonging to the benzothiophene family. Raloxifene was initially developed to function as a breast cancer treatment, but its clinical development was subsequently focused on prevention and treatment of postmenopausal osteoporosis, becoming the first SERM approved to prevent and treat this metabolic bone disorder (78). Evidence has accumulated supporting an agonist role for raloxifene in the vascular tree, and it has also been investigated for primary and secondary prevention of cardiovascular disease in postmenopausal women, and prevention of breast cancer in high-risk women, with similar efficacy to tamoxifen (79), (80), (81).

Arzoxifene, a new and more potent raloxifene analog, effectively suppresses the growth of breast cancer cells *in vitro*, with agonist effects on the liver and bone, and antiestrogenic effects on the endometrium (82). As with the other new SERMs, there is little experience of clinical use and it is currently being studied as an anti-osteoporotic drug (83).

The naphthalene family includes lasofoxifene and trioxifene. The first mentioned is of recent development and demonstrates excellent SERM-like properties in animal models *in vivo*, showing a binding affinity to ERα similar to estradiol, and approximately tenfold higher than other SERMs, including tamoxifen and raloxifene (84). The lasofoxifene molecule was investigated for prevention and treatment in two significant areas of menopause, bone and breast, but was not approved by the US Food and Drug Administration owing to a slight increase in endometrial thickness observed (85).

The SERMs belonging to the benzopyran group include several compounds designed primarily as agents to treat hormone-dependent breast cancer and endometrial cancer, some of which are in the early stages of clinical development. The benzopyran ormeloxifene has long been used in India as an oral contraceptive, while levormeloxifene is its active enantiomer whose development has been discontinued due to its association with increased uterine prolapse and urinary incontinence (86).

Finally, pipendioxifene and bazedoxifene are indole derivatives and their main representatives. Pipendioxifene is an interesting option under investigation as an agent with the potential to control proliferation in breast cancer tumor lines resistant to tamoxifen, without stimulating the endometrium (87). Bazedoxifene is a new drug recently approved for the treatment of postmenopausal osteoporosis (88). This new SERM will be discussed in detail in the following sections.

Other SERMs are being developed to prevent and treat osteoporosis, breast cancer and cardiovascular diseases, such as MDL 101,986, SR16234, tetrahydroisoquinoline derivatives, and 2-phenylspiroinden. These new drugs in development should ideally present a neutral profile regarding side effects such as hot flushes or venous thromboembolic disease, the main side effects of raloxifene, tamoxifen and bazedoxifene, also similar to those reported with use of ET/HRT and oral contraceptives. The molecular mechanism that sustains this pro-coagulant activity in the venous territory is still unknown,

seeming to be an estrogen agonist effect which in the SERMs must be minimized or eliminated in the development of new molecules (Figure 8). Finally, an important aspect on which this Doctoral Thesis is based is to study the new therapeutic modalities such as SERM or TSEC, both composed of the bazedoxifene molecule alone or in association with estrogenic derivatives, used in clinical practice in postmenopausal women in various treatments. Given the debate about its impact on atherogenesis, these experiments have great clinical interest.

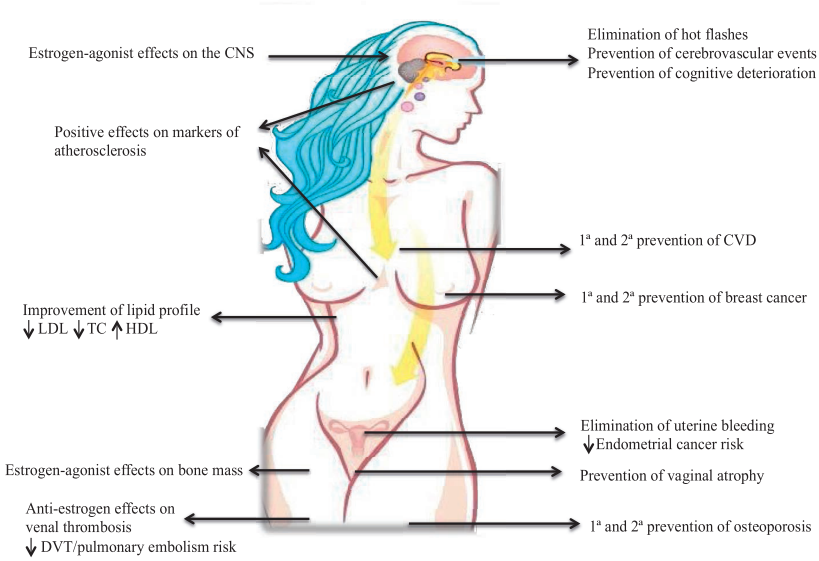


Figure 8. Illustration of the ideal pharmacological profile of SERM as a single drug.
CNS: central nervous system; LDL: low-density lipoproteins; TC: total cholesterol; HDL: high-density lipoprotein; DVT: deep vein thrombosis; CVD: cardiovascular disease.

1.4.2 Molecular actions of SERMs

Estrogens and SERMs constitute a broad arsenal of structurally diverse compounds, but they all share an affinity for estrogen receptors (Figure 9). Several determining factors influence the specific final actions of estrogens and SERMs, of which the chemical structure of the molecule, the isoform of the estrogen receptor to which it binds, and the characteristics of the activated promoter gene are noteworthy. On the other hand, the discovery of protein interaction receptor and the balance between coactivator ligands or corepressors modulates the final transcriptional response, and biological activities could differ between cells (22).

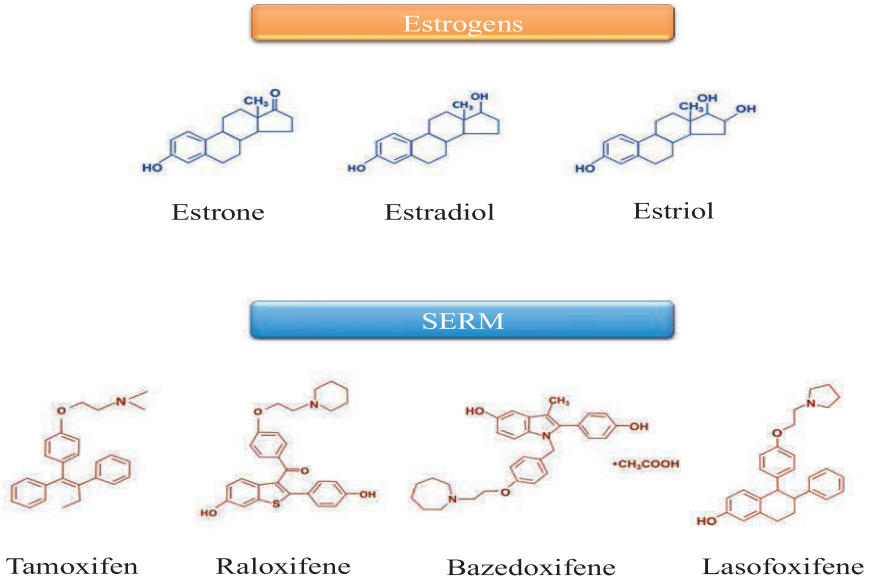


Figure 9. Representation of a group of structurally diverse compounds, both linked to estrogen receptors.

The ability of SERMs to induce their effects reproduce the molecular mechanisms of estradiol, and when they bind to intracellular receptors, there is a recruitment of specific proteins functionally divided into two opposed classes: coactivators, which activate transcriptional events, and in contrast, corepressors which repress them. Several studies have confirmed that the ER does not remain in a stable ´on/off˝ conformation inside the cell, but changes its structure depending on the essence of the bound ligand, becoming a specific three-dimensional conformation that determines a wide range of specific actions (89).

In summary, the estrogen receptor is a crucial point of convergence of multiple signals. The unique characteristics of SERMs are determined by their various synthesis methods and the relative expression of coactivator and corepressor ligands (Figure 10).

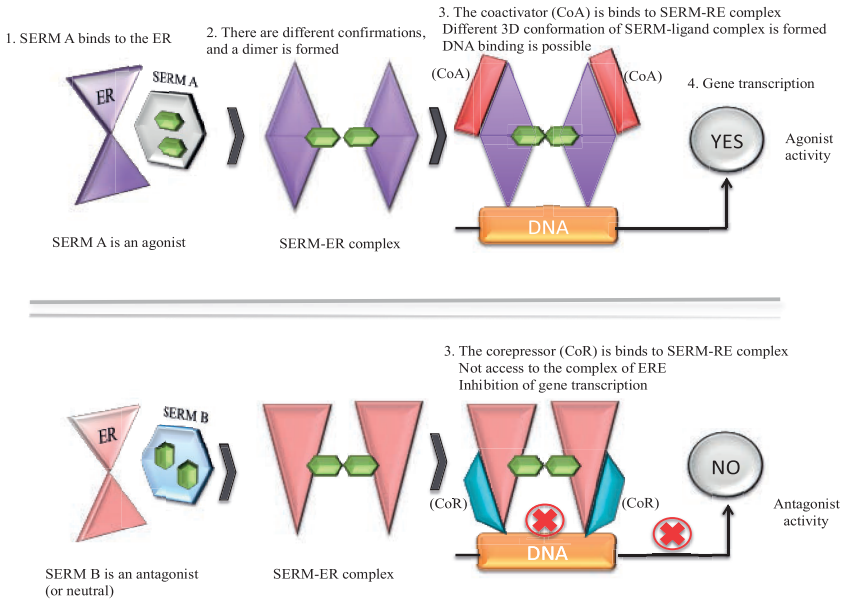


Figure 10. Illustration of an updated model of estrogen receptor action and the capacity of their modulators.
SERM: selective estrogen receptor modulator; ER: estrogen receptor; CoA: coactivator; CoR: correpresor; ERE: estrogen-response element.
Modified from McDonnell, DP et al (89).

1.4.3. Clinical experience of raloxifene

At the level of the vascular wall, SERMs have been investigated in order to check their resemblance to or divergence from estrogen action on atherogenesis. Raloxifene is a SERM that has been analyzed to a greater extent, and was postulated to be cardioprotective. Raloxifene belongs to the benzothiophene chemical class and is characterized by having estrogenic actions in bone and anti-estrogenic actions in breast

tissue (78). In cellular and animal models, raloxifene has shown a protective effect over the endothelium in a similar magnitude to estrogens, avoiding the onset and progression of atherosclerosis by both genomic and non-genomic mechanisms. The studies observed that raloxifene directly and rapidly induces production of vasoactive substances like nitric oxide and nitric oxide synthase, important molecules due to their anti-inflammatory and antiatherogenic effects (67), (90) . Further, *in vitro* experiments have proved that the classical genomic pathways are involved after observing the activation of protein and gene expression of cyclooxygenase-1 and -2, leading to prostacyclin production, which is also antiaggregant and a vasodilatory mediator (68), (91). The capacity of raloxifene to promote proliferation of human endothelial cells in a magnitude similar to estradiol was also demonstrated (70).

Not only in experimental studies, the cardioprotective effect of raloxifene has been corroborated by some animal models, where endothelium-dependent vasodilatation was observed in coronary arteries of ovariectomized rats and ewes (92). Despite these beneficial findings, various adverse effects have been found for raloxifene in postmenopausal women. For example, the Raloxifene Use for the Heart (RUTH) study reported no benefit for coronary heart disease in postmenopausal high-risk women over 60 years, even showing an increased risk of venous thromboembolism and fatal stroke in this group

(80). Raloxifene could also be associated with epithelial proliferation in the endometrium when it is accompanied by estrogens (93).

In order to avoid these deleterious effects, recent research has developed a new third-generation SERM, acetate of bazedoxifene, which can be used to prevent and treat postmenopausal osteoporosis with similar efficacy to raloxifene, but with overwhelmingly positive effects on endometrial thickness (94). Furthermore, bazedoxifene decreases estrogen-dependent proliferation rate in uterus and breast tissues due to its capacity to bind competitively to ER (95).

1.5 New proposal: bazedoxifene and derivatives

1.5.1 Bazedoxifene: SERM with improved selectivity

Bazedoxifene acetate is a third-generation SERM approved for the treatment of postmenopausal osteoporosis in women at increased risk of fracture (96).

This compound is capable of a significant translational impact; its profile has therefore improved compared to its predecessors. Its chemical structure is characterized by having two indoles, each with a phenyl ring, which serve as a specific binding site to the ER. This indole derivative is capable of a significant translational impact. This rigorously selected affinity of the ligand causes the compound to act as an agonist in tissues such as bone and the cardiovascular system and as an antagonistic agent in the uterus and breast, in addition to the particularity of being able to vary the spectrum of action depending on the levels of estradiol. In other

words, in a hyper estrogenic environment the bazedoxifene molecule acts as an estradiol inhibitor and has an antagonistic effect (97).

In the evaluation of any member of the SERM family the primary objective is maintaining bone mass; however, it is essentially important to ensure mammary and endometrial security. In initial studies performed in ovariectomized rat models, it has been shown to bind the estrogen receptor with high affinity, being able to increase bone mineral density and bone strength while reducing raloxifene-induced uterine cell hypertrophy (98).

Its efficacy has been evaluated in randomized studies, demonstrating that it is effective in preventing bone loss and reducing the risk of postmenopausal vertebral fractures compared to placebo. Long-term treatment with bazedoxifene has proven safety, as adverse outcome rates in the endometrium, breast and reproductive tract are low and similar to placebo (99). In the cardiovascular system, bazedoxifene treatment has shown to be safe and well tolerated with no increase in the risk of ischemic cardiac disorders or stroke across 3, 5 and 7 years of use, which signifies an advantage over raloxifene. However, it still presents an increased risk of venous thromboembolic events when compared to placebo in long-term administration for the treatment of osteoporosis, meaning women with established menopause (100), (101). The changes evidenced at the lipid level were beneficial, both a significant decrease in total cholesterol and LDL cholesterol and an increase in HDL cholesterol (102), (103).

Over the past decades, with the aim of improving the profile of SERMs on quality of life of postmenopausal women, different combinations of SERMs with estrogens have been developed, called tissue selective estrogen complex.

1.5.2 Tissue-selective estrogen complex (TSEC)

While conjugated estrogens are agonists of both α and β estrogen receptors, SERMs have tissue-specific estrogenic/anti-estrogenic effects. Laboratory studies have demonstrated distinct gene expression profiles compared with their individual components. The combination maintains the benefits of estrogens without the stimulatory effects on the breast and uterus, improving tolerability.

As no SERM have demonstrated to manage menopausal symptoms such as vasomotor ones, indeed tend to worsen them, and owing to concerns about estrogen/progestin therapy revealed in the Women´s Health Initiative (WHI) study, TSEC has been put forward as a possible solution to this problem. This allows the possibility to omit progestin for endometrial protection and simultaneously offers improvement in vasomotor symptoms and protects against breast cancer and postmenopausal fractures. These effects have been demonstrated in several studies, such as in the Selective estrogen, Menopause and Response to Therapy (SMART) trials.

The first TSEC in clinical development, recently commercialized, combines bazedoxifene with conjugated equine estrogens (CEEs) (104). BZA was selected for its pharmacological profile, particularly its antagonistic capacity at the endometrial and mammary levels, as demonstrated by preclinical studies with other molecules of the SERM class (Figure 11) (105).

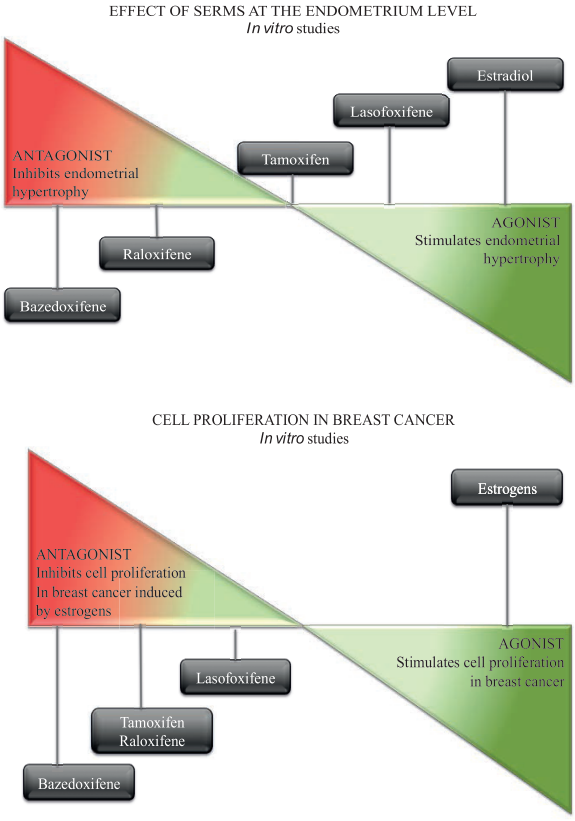


Figure 11. The pharmacological profile of different SERMs at the endometrium and mammary levels.
Modified from Komm, BS et al (106).

On the other hand, CEEs are composed of multiple estrogen agonists of ERα and β. They have established efficacy and a beneficial profile in treatment of vasomotor symptoms and prevention of osteoporosis (107). Considering these properties and working towards an

optimal pharmacological option, preclinical studies have shown that they are suitable for pairing with bazedoxifene (108). Another combination, estradiol with raloxifene, was studied without favorable results due to signs of endometrial stimulation observed in the treated group compared with placebo (109).

To evaluate the efficacy and safety of BZA/CEE, clinical studies were carried out worldwide in more than 7,500 women. A formula combining 20 mg bazedoxifene and 0.45 or 0.625 mg conjugated estrogens has been approved by the FDA and the European Medicines Agency for endometrial safety.

The effects of this therapy on the cardiovascular system have yet to be clearly defined. The SMART trials, which included healthy postmenopausal women aged 40-75 years, reported no increased incidence of adverse cardiovascular events nor venous thromboembolic events (VTE) compared to placebo.

In the SMART-1 trial, performed over two years, incidence of VTEs was similar in both treatment and placebo groups (110). However, the risk is expected to be similar with bazedoxifene alone or low doses of estrogens, with no additive effect when administered together, which means an increased risk of VTEs. These effects may be related to the younger age of participants in the SMART trials. Moreover, this trial showed favorable effects on plasma concentrations of specific coagulation variables and inflammatory markers which have been associated with increased risk of CVD (111).

The SMART-5 trial found an improvement in lipid profiles, with decreased levels of total cholesterol and low-density lipoproteins, and increased levels of high-density lipoproteins. This study also shows stable levels of triglycerides, while the SMART-1 trial found an increase compared to placebo.

Clinical trials involving the use of BZA alone and conjugated estrogens plus BZA have shown cardiovascular safety in healthy postmenopausal women. Nevertheless, a research group working with an atherosclerotic animal model identified the first potential limitations of BZA. Specifically, BZA therapeutic application alone did not affect atherosclerosis, body weight, adiposity, fasting glucose, or plasma lipid profile in surgically postmenopausal cynomolgus monkeys treated with BZA and BZA/CEE over two years. Moreover, BZA completely impeded the antiatherosclerotic effects of estrogen treatment in coronary and common iliac arteries (112). Although this study could represent a setback in the applicability of BZA or TSEC to maintain cardiovascular health in postmenopausal women, further evidence and additional studies are needed before establishing conclusions. Furthermore, TSEC effects have not been tested in women with CV disease, history of previous VTE, renal disease, obesity or age over 75 years, so this therapy should not be used in these groups. Additionally, as its effects remain unclear, the personal risk of cardiovascular events in long-term treatments with TSEC should be evaluated.

We have developed our research amid this passionate controversy, trying to deepen our understanding of the effects of BZA alone or in combination with estrogens on endothelial health through vascular cell proliferation in an *in vitro* model. The zeal of this project comes about because the timing hypothesis has renewed interest in determining the actions of drugs at early postmenopause, when the endothelial impact may be relevant. Moreover, SERMs are being reconsidered as crucial drugs in the treatment of osteoporosis in younger women, due to problems with long-term use of bisphosphonates.

2. HYPOTHESIS AND OBJECTIVES

The current **hypothesis** of our investigation is that new selective estrogen receptor modulator bazedoxifene, alone or in combination with estradiol, promotes the proliferation of endothelial cells in an *in vitro* model, which is associated with increased gene and protein expression in certain regulatory molecules of the cell cycle. We also would like to throw light on bazedoxifene's impact on the cardiovascular system by promoting endothelium anatomical and functional integrity, a pivotal factor in the initiation of the atherosclerosis process.

As the **main objective,** this Doctoral Thesis aims to evaluate the effect of bazedoxifene on human umbilical arterial endothelial cell (HUAEC) proliferation, and to determinate the genetic behavior of the main regulatory molecules of the cell cycle such as cyclin A, B, D1 and the inhibitory protein $p27^{Kip1}$ in both fresh and cryopreserved endothelial cells, seeking to clarify the role of this treatment in the early stages of the atherosclerosis process.

Specific objectives:

1. To determine if bazedoxifene promotes HUAEC proliferation in a magnitude similar to estradiol, raloxifene, and estradiol and bazedoxifene combined.

2. To evaluate whether this action is associated with increased protein and gene expression in some primary cell cycle regulators like cyclins A, B, and D1 or cell cycle inhibitor $p27^{kip1}$.

3. To compare fresh and cryopreserved status of human culture endothelial cells by characterizing their protein and gene expression after drug application.

4. If proliferation is increased, to characterize whether it improves endothelial repair mechanisms that would result in a protective effect against cardiovascular disease.

3. MATERIAL AND METHODS

3.1 Experimental design

Primary HUAECs were isolated from nine umbilical arteries at the time of delivery and subcultured *in vitro* to determine their ability to proliferate and modulate cell cycle genes at the mRNA and protein level when exposed to different concentrations/combinations of the hormonal treatments being assayed.

The nine primary cultures of endothelial cells were subcultured up to three times. A proportion of endothelial cells obtained from the first three cords were resuspended in freezing medium at -80ºC for a 3 to 3.5-month-time span. Fresh, subcultured arterial endothelial cells were used to determinate their ability to proliferate after treatment application. Fresh and cryopreserved subcultured arterial endothelial cells were used to study the protein and gene expression of certain molecules of the cell cycle after treatment application.

The treatments and the dose used for cell proliferation, protein and gene expression measurement were obtained based on binding affinity (EC50) of ligands to their ERα, as described elsewhere (98). Doses of E2 and BZA in combination were half the EC50 of each compound.

- E2 (Sigma, Alcobendas, Spain): 3.2 nM
- BZA (Sigma-Aldrich): 26 nM
- RLX (Sigma-Aldrich): 2.4 nM
- E2 + BZA (Sigma-Aldrich): 1.6 nM + 13 nM

For experimental purposes, cells were made quiescent, and their cell-cycle activities were synchronized at the G0 and G1 phase by the incubation of confluent monolayers (approximately 80%) with steroid-deprived FBS medium for 24 hours previous to testing the experimental conditions of interest.

3.2 Isolation and culture of endothelial cells

3.2.1 Collection and primary culture

The endothelial cells were obtained from human umbilical cord arteries collected from maternity wards at the time of birth, without differentiating between delivery methods. For this, cords were chosen from non-diabetic, non-smoking (or less than 15 cigarettes per day) mothers without cardiovascular problems or infections (hepatitis, HIV, etc.). Exclusion criteria were births with obstetric complication or intrapartum fetal distress. The cord of each new-born was double-clamped immediately after delivery to obtain at least 15-20 cm of sample without knots or punctures, the rest being typically used for gasometry and pH determination. Although the cord is a sterile structure during intrauterine life, it could be contaminated during passage, so exhaustive

lavage was performed on the surgical area. Additionally, cold physiological saline under pressure was injected into the umbilical artery to wash it properly. The ends were surgically sectioned to simplify their cannulation.

The cord was quickly placed in a sterile container and taken to the laboratory, where it can remain refrigerated at 4ºC for up to 24 hours, although our samples all were processed within 8 hours from collection of the material. Once in the laboratory and under sterile conditions (gloves, mask, surgical material and laminar flow chamber), both ends of the cord were cannulated with a fragment of serum system or probe in order to inject solution directly into the artery from the pipette. Holding the cord at its ends, the wall of the vein was carefully cut to expose the arteries and introduce a catheter inside. Once inside, it was first washed abundantly using at least 50 ml of PBS (Phosphate Buffered Saline**,** Gibco®, Thermo Fisher Scientific) to eliminate the remnants of blood that could have remained in the interior, and was checked for perforations. This procedure was repeated 2-3 times. The PBS was then removed with a pipette filled with air and the vessel lumen was filled up with 0.1% collagenase type I adjusted to a pH of 7.4, closing both ends of the cord with ring forceps. The result was incubated at the optimum temperature to separate the endothelial cells from the basement membrane without damaging them, at 37 °C for 15 minutes. Immediately afterwards the contents were collected in 10 ml conical tubes to centrifuge at 800 rpm at room temperature for 5 minutes. After this time,

and once the supernatant was eliminated, the cell pellet was re-deposited in 5 ml of M199 with phenol red (Medium 199, Earles&HEPES, Gibco®, Thermo Fisher Scientific). The prepared volume was seeded in culture flasks and finally, M199 was added as necessary up to a final volume of 5 ml for bottles of 25cm^2 or 13-15 ml for bottles of 75 cm^2 , and was incubated at 37ºC in 5% CO2 atmosphere. The pH of the medium should be maintained optimum for the adequate growth and multiplicity of endothelial cells.

Figure 12. Representation of cannulated cord with serum system inserted directly into both arteries ready for washing with Phosphate Buffered Saline medium.

3.2.2 Subcultures and cell processing

The first renewed culture took place the next day and attempts were made to eliminate the collagenase that might be retained. The successive renovations were carried out every 72 hours until the monolayer of cells reached semiconfluence, occupying approximately 75% of the surface of the dish. When this happened, the surface of the plate was washed 2-3 times with 5 ml of PBS to remove any remains of the medium, then a small amount (1.5 ml) of trypsin was added and the resulting solution was incubated for 3 minutes at 37 °C. Immediately afterwards, 5 ml of M199 was administered to suppress the effect of the enzyme and the contents were transferred into a conical tube which was centrifuged at 900 rpm for 10 minutes at 25 °C to completely remove the trypsin.

The obtained cellular precipitate was re-suspended in new medium 199 with 20% SFB (Fetal Bovine Serum, Gibco®, Thermo Fisher Scientific). The cell count was carried out by the dilution method with Trypan blue over a Neubauer chamber, following the protocol detailed in Appendix 1. Then they were seeded in microanalysis grids and in new Falcons roughly 500.000 to 1.000.000 cells per culture flask. A proportion of endothelial cells obtained from the first three cords were cryopreserved at -80ºC with Dimethyl Sulfoxide-based (DMSO) as described in Appendix 2.

The procedure by which the first subcultures were obtained, once in the culture flasks and reaching the phase of semiconfluence, was also repeated twice, obtaining second and third subcultures. We collected and processed a total of fifteen umbilical cords. Cells from six cords have been eliminated for different reasons: one due to contamination, one due to lack of adhesion and four to weak growth in falcons.

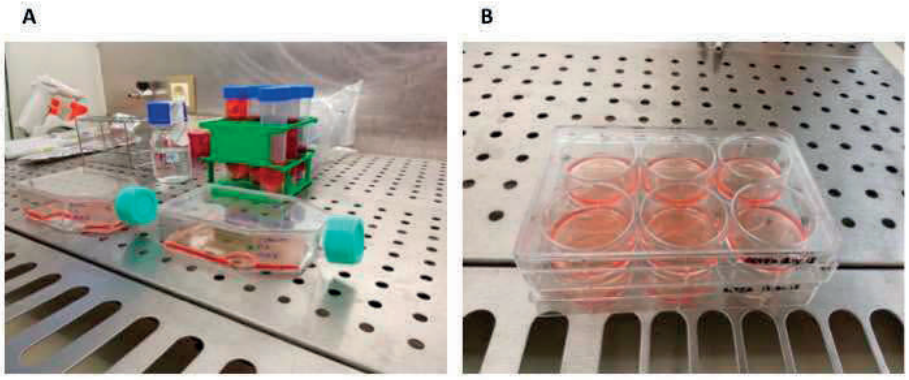


Figure 13. Culture process of human umbilical arterial endothelial cells (HUAEC). Primary culture (A) and subcultures (B) of endothelial cells in medium 199.

3.3 Measurement of cell proliferation (XTT assay)

The freshly prepared cells from passage 3 were seeded onto 96-well plates at 1 x 10^4 cells per well. The relationship between dosage and effect was tested over the pharmacological concentration range of 10-1 to 10+1 of EC50 of each compound (Table 2).

Equivalent comparisons were treatments 1-4 (at equal doses). Procedure 5 resulted from the duplication of treatment 4 doses and pursued the goal of exploring whether two drugs given in combination might have a super-additive (synergistic) effect; specifically, if the action was above what is expected from their individual potencies and efficacies.

Treatment	**Dose 1**	**Dose 2**	**Dose 3**
1. E2	0.32 nM	3.2 nM	32 nM
2. BZA	2.6 nM	26 nM	260 nM
3. RLX	0.24 nM	2.4 nM	24 nM
4. E2+BZA	0.16 nM E2 + 1.3 nM BZA	1.6 nM E2 + 13 nM BZA	16 nM E2 + 130 nM BZA
5. E2+BZA-2	0.32 nM E2 + 2.6 nM BZA	3.2 nM E2 + 26 nM BZA	32 nM E2 + 260nM BZA

Table 2. Doses of different treatments used for HUAEC proliferation in XTT assay.

The cells were exposed to increasing doses of the above-mentioned drugs and were allowed to proliferate at 37ºC with 5% CO_2 for 24 hours. After the incubation period, 50 µl of XTT mixture was added to each well to obtain a final desired concentration of 0.3 mg/ml.

The plate was incubated at 37 °C with 5% CO2 for 24 hours. The enzymes of metabolically active cells reduce the tetrazolium salt XTT to a highly colored water-soluble formazan salt. The extent of XTT reduction was quantified by measuring optical density at 450 nm with reference wavelength of 650 nm using microplate reader Wallac 1420 Victor 2 Multilabel Reader (EG&G, Turku, Finland).

Data are expressed as the index of formazan production by treated cells in comparison to formazan produced by control cells. Six technical replicates were analyzed in cells from three different cultures.

3.4 Protein expression analysis (Western blot)

HUAEC from passage 3 were seeded into 25 cm2 flasks and when 70-80% of confluence was reached, culture medium was replaced with steroid-deprived FBS medium for 24 hours. Then, the active treatment with the above-mentioned concentration (section 3.1) were applied following by incubation period of 24 hours at 37 °C with 5% CO2.

After that time, a 100 µl volume of lysis buffer (50 nM Tris-HCl, 150 nM NaCl, proteinase inhibitor cocktail and phosphatase inhibitor cocktail adjusting to pH 7.4) was added to the samples, reaching a final volume of 50 ml. To achieve cell lysates, the incubation was maintained at 4°C under orbital agitation for 30 min. The scraping method was used to lift and collect the cellular debris from the culture plate surface. To remove the remnants from the membranes, the extracts were centrifuged

at 16100 rpm for 15 min at 4°C. The total protein concentration into lysate supernatant was measured by Pierce Micro BCA Assay (Thermo Fisher Scientific) and samples were frozen at −80°C until assay. The proteins of each sample (20 µg) were separated by 12% of SDS-PAGE gels (SDS-PAGE; Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, Bio-Rad, USA) and the protein was transferred to PVDF membrane (Transblot Turbo Transfer pack, Bio-Rad, USA). To confirm that the transfer was complete, the gel was included in staining solution containing Coomassie Blue with 10% acetic acid and 25% methanol for 2 hours at room temperature, visualizing proteins as blue bands on a clear background. Likewise, to verify that proteins had been transferred to the membrane, we used the aqueous Ponceau red solution, whose red staining could be reversibly visualized in the presence of proteins.

Immunostaining was achieved with specific antibodies against the proteins represented in Table 3 below.

Specific antibodies for immunostaining		
Antibody name and units	**Host**	**Supplier**
1/500 anti-cyclin A (sc-751)	rabbit	Santa Cruz Biotechnology, Santa Cruz, CA
1/200 anti-cyclin B (sc-751)	rabbit	
1/200 anti-$p27^{Kip1}$ (sc-1641)	mouse	
1/25 anti-cyclin D1 (NCL-cyclin D1-GM)	mouse	Novocastra Laboratories, Newcastle, UK

Table 3. List of specific antibodies used for immunostaining.

Blots were blocked in 5 % bovine serum albumin (BSA) and visualization of positive antibody binding was performed with alkaline-phosphatase-linked anti-rabbit antibody (for cyclin A and cyclin B) or anti-mouse antibody (for cyclin D1 and $p27^{Kip1}$), both from Sigma, Alcobendas, Spain. Concurrently, the membranes were re-probed with anti-actin antibody (Sigma, Alcobendas, Spain) to verify equivalent amounts of loaded protein and transfer efficiency. Finally, immunodetection of protein bands was visualized using chemiluminescence films (ImageQuantLAS4000, GE Healthcare Bio-Sciences) and the NIH ImageJ Software performed the densitometric analysis.

Data are expressed as a ratio of each protein band relative to the lane's loading control (β-actin band). Three technical replicates were performed in cells from six cultures with different previous cryopreservation status (cells from three frozen and three fresh cultures).

3.5 Gene expression analysis

To evaluate the effect of drugs (E2, BZA, RLX, E2+BZA) on gene expression of human cell cycle markers (cyclin A, cyclin B, cyclin D1 and inhibitor $p27^{kip1}$) the samples were treated with the same doses as for Western blot analysis, and as mentioned in section 3.1.

3.5.1. RNA extraction

To assess the effect of different drugs on gene expression, total cellular RNA from HUAECs was extracted using the TRIzol reagent (Thermo Fisher Scientific®), a monophasic phenol and guanidine isocyanate solution capable of decomposing the stability of the cell while maintaining the integrity of its RNA. The procedure was carried out according to the manufacturer's instructions of the original March 2013 protocol, with slight modifications as summarized below.

The cells were collected in suspension after centrifugation and elimination of the medium, obtaining samples of 0.25 ml (5-10x106 cells), then adding 0.75 ml of TRIzol Reagent. Once the sample was homogenized, it was transferred to a tube and centrifuged at 12,000 rpm for 10 min at 4°C and the aqueous phase, which contains the RNA, was transferred to a new Eppendorf tube. Samples were stored at -80°C for 24 hours. On the second day, the samples were homogenized at room temperature for 5 min to re-suspend the cells and allow complete dissociation of the nucleoprotein complex. For each ml of extraction solution, 200 µl of chloroform was added, the samples were mixed in Vortex for 15 secs and then incubated 2-3 min at room temperature. After reaching that stage, the result was microcentrifuged again at 12000 rpm for 15 min at 4ºC, and the aqueous phase was transferred to a clean and sterile micro tube.

The nucleic acids were precipitated with 500 µl of isopropanol 100% and incubated for 10 min at -20 °C. The mixture was centrifuged

at maximum power for 10 min, the supernatant was removed, and 1 ml of ethanol 75% was added for every 1 ml of Trizol used in the initial homogenization. Subsequently, it was centrifuged at 7500 rpm for 5 min. The aqueous phase was removed.

The RNA pellet was allowed to dry for 5-10 min at 37°C with the cap of the microtube open, and the pellets were re-suspended in RNase-free water. All phases of the process were executed in RNase-free zones and with RNase-free solution to avoid RNA degradation. When the RNA was not used immediately, it was stored at -80°C until use. RNA was quantified by measuring absorbance spectrophotometrically at 260 nm.

3.5.2. Reverse transcription

To study specific gene expression by real-time quantitative PCR, the first step was RNA transcription into complementary DNA (cDNA) by reverse transcriptase from total RNA, carried out using the SuperScript First-Stand Synthesis System for RT-PCR (Invitrogen, Barcelona, Spain), according to the established protocol.

One µg of RNA was mixed with 0.5 µl of a random combination of primers, 2 µg of an equimolar mixture of 10 mM dNTPs and RNase-free water, reaching a final volume of 9 µg. The RNA was denatured for 5 min at 65°C and immediately cooled to 4°C for a few minutes. Afterwards we added 8 µl of the 5x reaction buffer, 1 µl of 0.1 M DTT, 1 µl of RNase Out and 1 µl of the reverse transcriptase SuperScript IV.

The thermal cycler was programmed with the following incubation times: hybridization process for 60 min at 50°C followed by inactivation reaction for 5 min at 85°C. The obtained cDNA samples were subsequently stored at -20 ° C until further study.

3.5.3. Gene expression using Quantitative Real-Time PCR (QRT-PCR)

PCR reactions were performed in real-time PCR QuantStudio5 (Applied Biosystems, Thermo Fisher Scientific) using an SYBER Green PCR Master Mix (Thermo Fisher Scientific). This mixture includes the required amounts of deoxyribonucleotide triphosphate (dNTPs), AmpliTaq Gold DNA Polymerase, MgCl2, PCR buffer and SYBR Green fluorophore. The latter molecule, for which the product is named, has a distinctive characteristic: when the SYBR green fluorophore intercalates in double DNA helix, its increased fluorescent emission allows detection of the amount of DNA present in the sample without the use of specific probes.

Five genes were amplified in triplicate in each sample, in experiments on two different days. Two microliters of total cDNA were amplified in a 10 μg reaction mix containing 5 μg SYBER Green PCR Master Mix, and 10 μM of forward and reverse primers (Table 4).

Gene name	5´-3´sequence	Concentration
Cyclin A	Forward 5´-TTCCCGCAATCATGTACCCTG-3´ Reverse 5´-TGTAGCCAGCACAACTCCACT-3´	10µM each
Cyclin B	Forward 5´-CATGGTGCACTTTCCTCCTT-3´ Reverse 5´-AGGTAATGTTGTAGAGTTGGTGTCC-3´	10µM each
Cyclin D1	Forward 5´-GAAGATCGTCGCCACCTG-3´ Reverse 5´-GACCTCCTCCTCGCACTTCT-3´	10µM each
P27^{Kip1}	Forward 5´-GGAATAAGGAAGCGACCTGCA-3´ Reverse 5´-CCGTTGACTCCGACCTTCAC-3´	10µM each
GAPDH	Forward 5´-CTGCTCCTCCTGTTCGACAGT-3´ Reverse 5´-CCGTTGACTCCGACCTTCAC-3´	10µM each

Table 4. Forward and reverse primer sequences and concentrations corresponding to QRT-PCR amplification of cyclin A, cyclin B, cyclin D1, p27^{Kip1}, and GAPDH.

Following a 10 min Tag activation step at 95ºC, reactions were subjected to 40 cycles of 15 second denaturation at 95º C and 1 minute extension at 60ºC. In parallel, fivefold serial dilutions of well-known cDNA standard were run as calibration curves. To guarantee that all amplicons were obtained at the same temperature a melting-curve program was applied.

Expression of the genes of interest was normalized to the corresponding value of one of the endogenous genes (housekeeping gene) that show constant expression in different cellular conditions. In our case, we used GADPH as a housekeeping gene, and we also put empty wells as a negative control in each experiment.

The data was collected and analyzed with the QuantStudioTM Design & Analysis Software v.1.3.1 program (Figure 14). The cycle threshold (CT) is obtained when the amplification cycle signal reaches a level above the limit of detection.

Data were expressed as relative quantification of gene expression according to the comparative Ct method (2-ΔΔCt) (113). Three technical replicates were performed in cells from six cultures with different previous cryopreservation status (cells from three frozen and three fresh cultures).

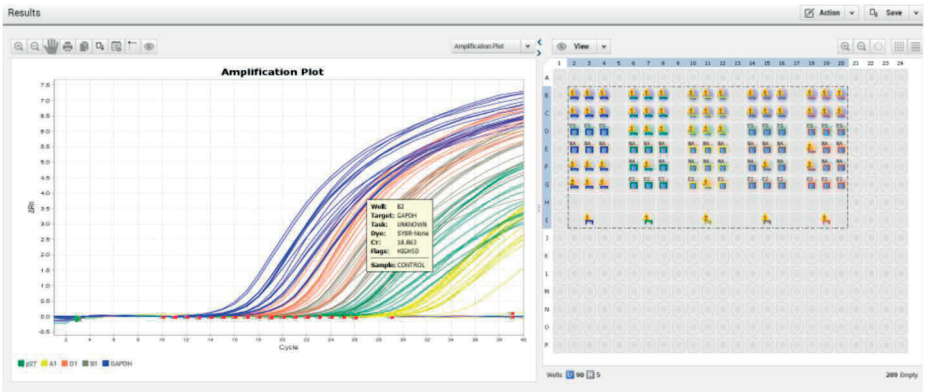


Figure 14. Amplification plot of cyclins and GADPH expressions.

3.6 Statistical analysis

Data analysis was performed using Generalized Estimating Equations (GEE) models. Variables were arranged in a hierarchical three-level structure to adjust standard errors for potential correlation between experiments whose levels of the three variables considered in these hierarchical models were the same (114). To analyze the effect of "cell treatment" on HUAEC proliferation in each one of the four doses, "number of replicates" was clustered within "cell treatment", and this variable within "number of HUAEC cultures". In contrast, to analyze the effect of "dose" on HUAEC proliferation in each one of the five cell treatments, "number of replicates" was clustered within "dose", and this variable within "number of HUAEC cultures". To analyze the effect of cell treatment on gene expression, "time of HUAEC proliferation before gene expression analysis" was clustered within "cell treatment", and this variable within "number of HUAEC cultures". The independent effect of "HUAEC cryopreservation", i.e. whether or not HUAEC were cryopreserved, was also included in this particular GEE model. Finally, to analyze the effect of cell treatment on protein expression, "number of replicates" was clustered within "cell treatment", and this variable within "number of HUAEC cultures". The independent effect of "HUAEC cryopreservation" was included in this hierarchical GEE model. However, as all the cell cultures were exposed to the same "timespan of cell proliferation before protein-expression analysis", this variable was not considered in the GEE model.

A NORMAL response and IDENTITY function were chosen as the MODEL distribution and the LINK function, respectively. The ROBUST variance estimator (a.k.a. the Huber/White/sandwich estimator) was the method used for computing the variance-covariance matrix of the regression parameter coefficients. The sequential Sidak test was used to adjust the significance level for post hoc pairwise multiple comparisons. Finally, the goodness-of-fit Quasi-likelihood under Independence Model Criterion (QIC) was used to choose among several working correlation structures: INDEPENDENT, EXCHANGEABLE and UNSTRUCTURED. The working correlation structure that had the smallest QIC was considered as the matrix providing the best goodness of fit.

Values shown in the text and tables are estimated marginal means ± standard error of the means (SEMs). In the figures, values are exponentiated regression coefficients with 95% confidence interval (CI). Because the dependent variables are continuous and the independent variables are categorical, exponentiated regression coefficients indicate the expected/estimated relative change in value of the dependent variable for a particular category of the independent variable compared to the reference category. When using exponentiated regression coefficients, results are significant when their respective 95% CI does not include 1. A value <1 means that the factor is negatively associated with the outcome. The percentage decrease in the value of the dependent variable is (1 - exponentiated regression coefficient) × 100.

Conversely, a value >1 denotes that the factor is positively associated with the outcome. In this case, the percentage increase in the value of the dependent variable is (exponentiated regression coefficient - 1) × 100 (115). *P*-value≤0.05. All the statistical analysis was carried out using the Statistical Package for Social Sciences (IBM SPSS Statistics, version 24; © Copyright IBM Corporation and its licensors 1989, 2016).

4. RESULTS

4.1 Effect of cell treatment, number of replicates, and dose on HUAEC proliferation.

Table 5 shows that cell treatment had a significant ($P \leq 0.0005$) effect on HUAEC proliferation, irrespective of the doses used in each treatment. The "number of replicates" variable was also a significant ($P \leq 0.0005$) factor.

Table 5. Overall effect of cell treatment and number of replicates on HUAEC proliferation.

Dependent variable	Independent variable	N	*P*-value
HUAEC proliferation	Treatment	52	0.0005
	Number of replicates	52	0.0005

HUAEC were exposed to increasing doses in each of the four treatments (E2, BZA, RLX, E2+BZA) during a 24-hour time span. Table 6 shows the marginal means ± SEMs of data stratified by the different treatments and doses used in these experiments; Figure 15 presents the relative changes in HUAEC proliferation of these marginal means compared to the control untreated group, used as the reference group in the GEE analyses. Figure 15 indicates that at dose 1, E2 ($P \leq 0.0005$), BZA ($P \leq 0.021$), and RLX ($P \leq 0.0005$) treatment displayed significantly higher relative values of HUAEC proliferation than the control group. At dose 2, a positive significant relative change in proliferation was also observed after cell treatment with E2 ($P \leq 0.001$),

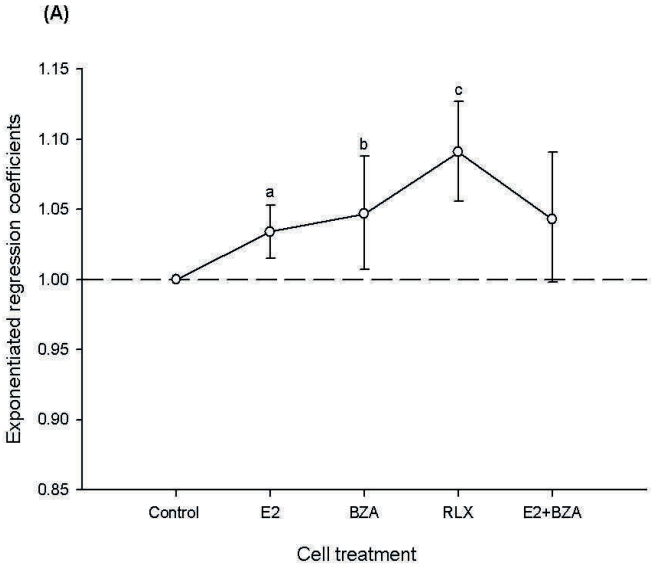
BZA ($P \leq 0.0005$), and RLX ($P \leq 0.001$) versus control values. At dose 3, as in previous doses, increase in HUAEC proliferation was obtained after E2 ($P \leq 0.005$), BZA ($P \leq 0.0005$), and RLX ($P \leq 0.036$) treatment when compared with control.

Table 6. Effect of cell treatment on HUAEC proliferation at doses 1, 2 and 3.

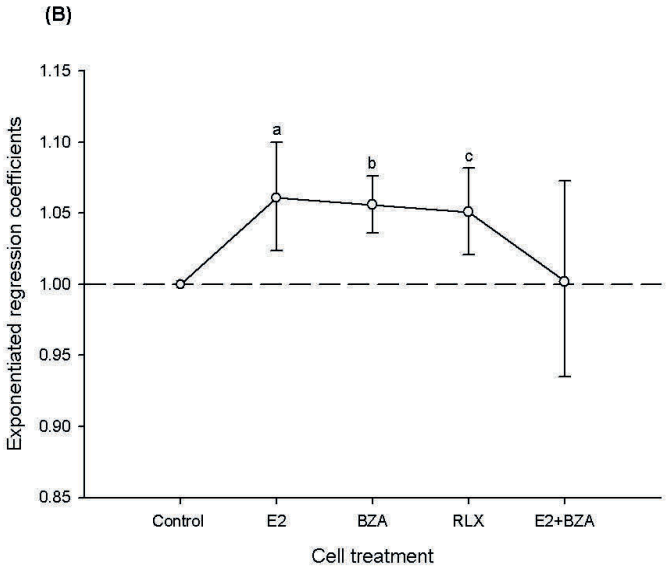
Treatment	**HUAEC proliferation**		
	Dose 1	**Dose 2**	**Dose 3**
Control	0.09 ± 0.016[a]	0.10 ± 0.018 [d]	0.14 ± 0.011[f]
E2	0.12 ± 0.007[b]	0.16 ± 0.001[e]	0.17 ± 0.005
BZA	0.13 ± 0.005	0.15 ± 0.001	0.20 ± 0.009
RLX	0.18 ± 0.001[c]	0.15 ± 0.006	0.18 ± 0.011
E2+BZA	0.13 ± 0.007	0.10 ± 0.016	0.11 ± 0.019[g]

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from the control group ($P \leq 0.002$).
[c] Value significantly different from the control ($P \leq 0.0005$), E2 ($P \leq 0.0005$), BZA ($P \leq 0.0005$), and E2+BZA ($P \leq 0.0005$) group.
[d] Value significantly different from the E2 ($P \leq 0.008$), BZA ($P \leq 0.0005$), and RLX ($P \leq 0.007$) group.
[e] Value significantly different from the E2+BZA group ($P \leq 0.006$).
[f] Value significantly different from the E2 ($P \leq 0.022$) and BZA ($P \leq 0.0005$) group.
[g] Value significantly different from the E2 ($P \leq 0.019$), BZA ($P \leq 0.019$), and RLX ($P \leq 0.0005$) group.

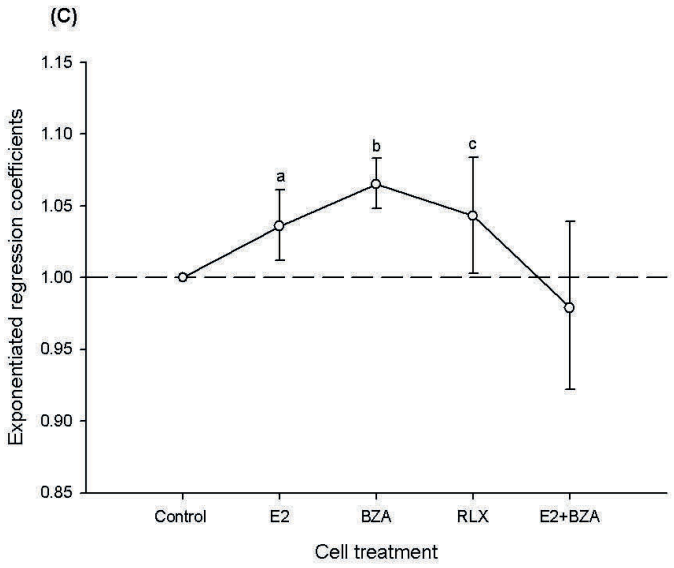
Figure 15. Estimated relative change in HUAEC proliferation after E2, BZA, RLX, and E2+BZA treatment at dose 1(A), dose 2 (B), and dose 3 (C).



Error bars are 95% CIs.
[a,b,c] Values significantly different to the control group ([a]P ≤ 0.0005; [b]P ≤ 0.021; [c]P ≤ 0.0005).



Error bars are 95% CIs.
[a,b,c] Values significantly different to the control group ([a]P ≤ 0.001; [b]P ≤ 0.0005; [c]P ≤ 0.001).



Error bars are 95% CIs.
[a,b,c] Values significantly different to the control group ([a]P ≤ 0.004; [b]P ≤ 0.0005; [c]P ≤ 0.036).

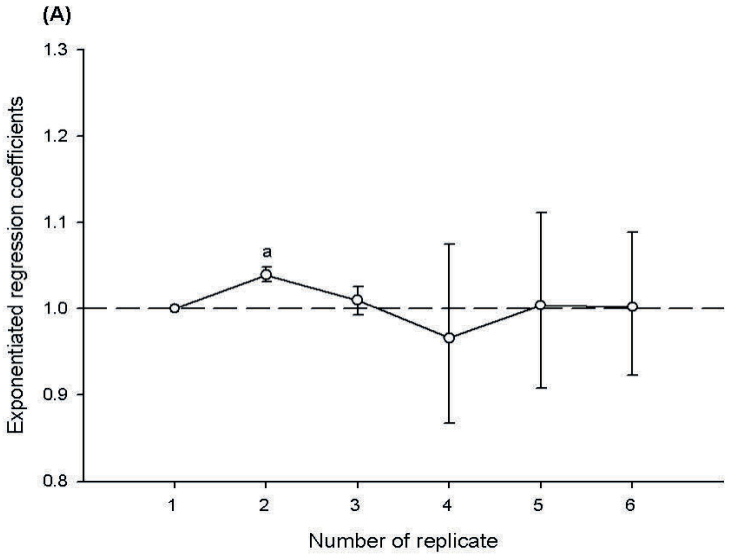
The replicates analysis shown in Table 7 and Figure 16 shows slightly higher HUAEC proliferation with replicate 2 at dose 1 ($P \leq 0.0005$) and at dose 2 ($P \leq 0.009$). Generally, there were no changes in proliferation content among the rest of the replicates at the mentioned doses. Nevertheless, the fluctuation in cell proliferation dependent on the number of replicates was noted at dose 3. In this case, replicate 3 ($P \leq 0.003$), replicate 4 ($P \leq 0.002$), and replicate 5 ($P \leq 0.0005$) we noted significantly higher relative values of HUAEC proliferation than with replicate 1.

Table 7. Effect of number of replicates on HUAEC proliferation at doses 1, 2 and 3.

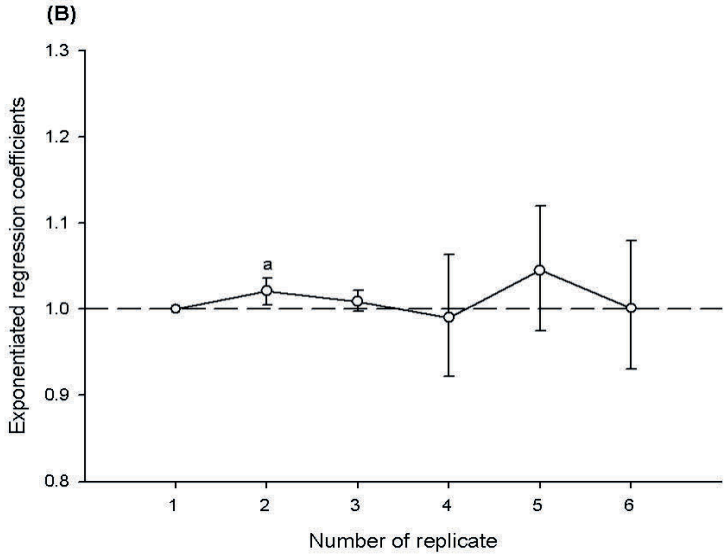
Replicate	HUAEC proliferation		
	Dose 1	**Dose 2**	**Dose 3**
1	0.13 ± 0.028[a]	0.12 ± 0.023	0.11 ± 0.011[e]
2	0.17 ± 0.026[b]	0.14 ± 0.021	0.12 ± 0.019
3	0.14 ± 0.020	0.13 ± 0.018	0.13 ± 0.015
4	0.09 ± 0.026	0.11 ± 0.013	0.22 ± 0.024[f]
5	0.13 ± 0.023[c]	0.16 ± 0.012[d]	0.25 ± 0.022[g]
6	0.13 ± 0.013	0.12 ± 0.015	0.11 ± 0.012

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from replicate 1 ($P \leq 0.0005$) and replicate 3 ($P \leq 0.0005$).
[c] Value significantly different from replicate 4 ($P \leq 0.0005$).
[d] Value significantly different from replicate 4 ($P \leq 0.0005$) and replicate 6 ($P \leq 0.0005$).
[e] Value significantly different from replicate 3 ($P \leq 0.027$), replicate 4 ($P \leq 0.017$), and replicate 5 ($P \leq 0.0005$).
[f] Value significantly different from replicate 6 ($P \leq 0.0005$).
[g] Value significantly different from replicate 2 ($P \leq 0.027$), replicate 3 ($P \leq 0.015$), and replicate 6 ($P \leq 0.0005$).

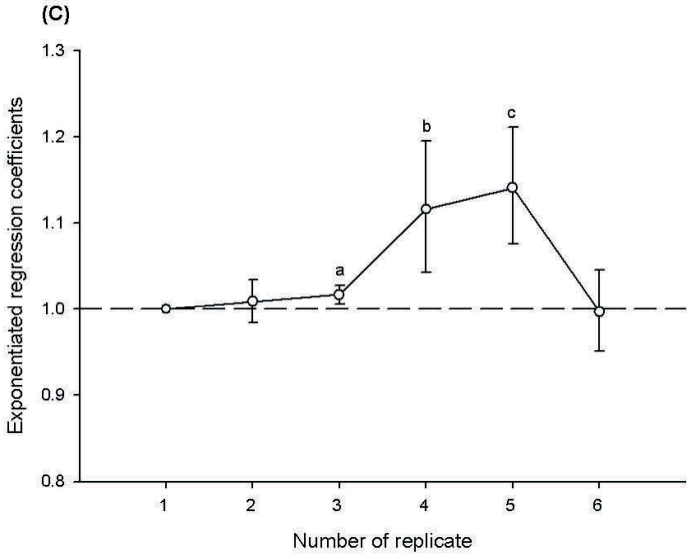
Figure 16. Estimated relative change in HUAEC proliferation depending on replicate number at dose 1 (A), dose 2 (B), and dose 3 (C).



Error bars are 95% CIs.
[a]Values significantly different to the replicate 1 ([a]P ≤ 0.0005).



Error bars are 95% CIs.
[a]Values significantly different to the replicate 1 ([a]P ≤ 0.009).



Error bars are 95% CIs.
[a,b,c] Values significantly different to the replicate 1 ([a]P ≤ 0.003; [b]P ≤ 0.002; [c]P ≤ 0.0005).

Table 8 shows a significant overall effect of dose on HUAEC proliferation, irrespective of the treatment applied. The "number of replicates" variable was also significant ($P \leq 0.0005$) factor.

Table 8. Overall effect of dose and number of replicates on HUAEC proliferation.

Dependent variable	Independent variable	N	*P*-value
Cell proliferation	Dose	43	0.0005
	Number of replicates	43	0.0005

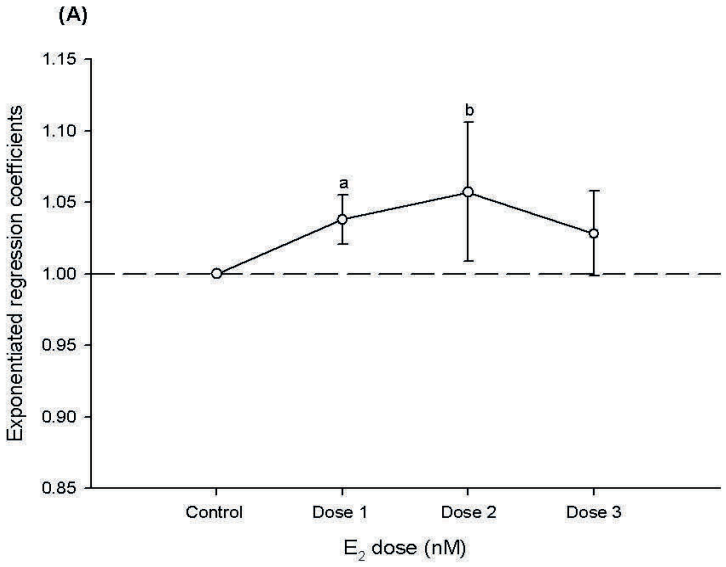
Table 9 shows the marginal means ± SEMs of data stratified by increasing incremental doses of four different drugs. Figure 17 represents their estimated relative variation compared to the control untreated group. Use of increasing doses of either E2 and RLX confirmed that significant changes in proliferation were only observed in response to dose 1 ($P \leq 0.0005$ for E2, $P \leq 0.0005$ for RLX) and dose 2 ($P \leq 0.018$ for E2, $P \leq 0.0010$ for RLX). In case of BZA alone, the three incremental doses produced significant positive effects on HUAEC proliferation after 24 hours ($P \leq 0.001$ at dose 1, $P \leq 0.0005$ at dose 2, $P \leq 0.0005$ at dose 3 vs. control values).

Table 9. Effect of increasing doses of E2, BZA, RLX or E2 + BZA on HUAEC proliferation.

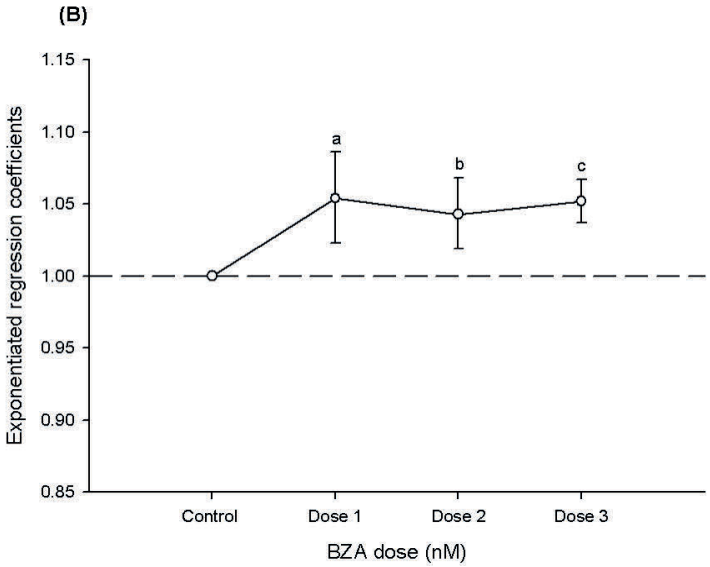
Dose (nM)	HUAEC proliferation			
	E2	**BZA**	**RLX**	**E2+BZA**
Control	0.12 ± 0.028[a]	0.13 ± 0.029 [c]	0.12 ± 0.019[d]	0.10 ± 0.030
Dose 1	0.15 ± 0.019[b]	0.19 ± 0.014	0.20 ± 0.006[e]	0.14 ± 0.002
Dose 2	0.17 ± 0.009	0.18 ± 0.017	0.16 ± 0.005	0.12 ± 0.011
Dose 3	0.14 ± 0.013	0.19 ± 0.02	0.15 ± 0.003	0.08 ± 0.022[f]

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from the control group ($P \leq 0.0005$).
[c] Value significantly different from the dose 1 ($P \leq 0.002$), dose 2 ($P \leq 0.002$), and dose 3 ($P \leq 0.0005$).
[d] Value significantly different from the dose 1 ($P \leq 0.0005$) and dose 2 ($P \leq 0.031$).
[e] Value significantly different from the dose 2 ($P \leq 0.0005$) and dose 3 ($P \leq 0.0005$).
[f] Value significantly different from the dose 1 ($P \leq 0.046$) and dose 2 ($P \leq 0.003$).

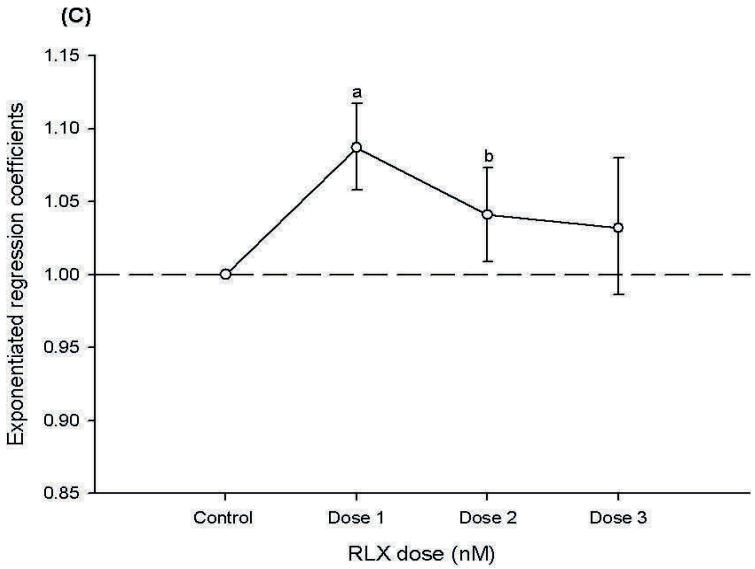
Figure 17. Estimated relative variation in HUAEC proliferation on increasing doses of E2 (A), BZA (B), RLX (C) or E2 + BZA (D).



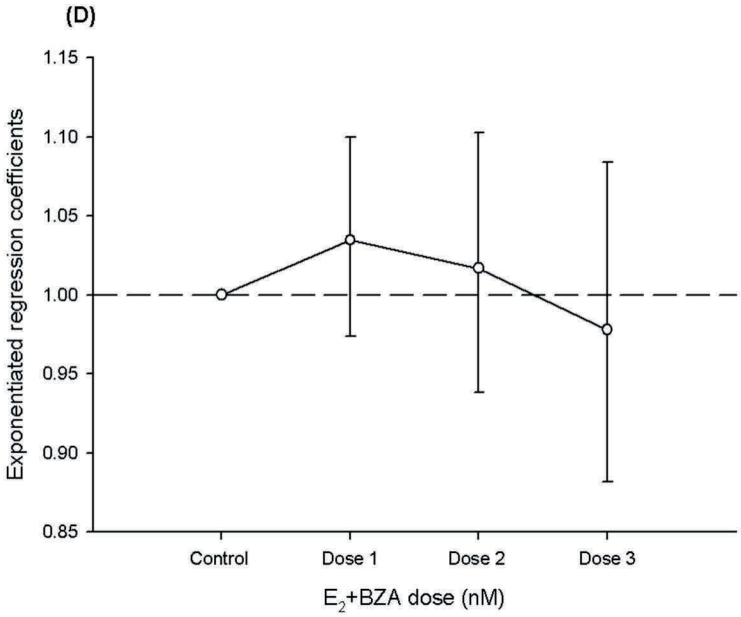
Error bars are 95% CIs.
[a,b] Values significantly different to the control group ([a]P ≤ 0.0005; [b]P ≤ 0.018).



Error bars are 95% CIs.
[a,b,c] Values significantly different to the control group ([a]P ≤ 0.001; [b]P ≤ 0.0005; [c]P ≤ 0.0005).



Error bars are 95% CIs.
[a,b] Values significantly different to the control group ([a]P ≤ 0.0005; [b]P ≤ 0.010).



The effect of number of replicates on cell proliferation after E2, BZA, RLX or E2 + BZA treatment is illustrated in Table 10 as the marginal means ± SEMs and in Figure 18 as their estimated relative change. There is a greater fluctuation in cell proliferation after treatment with BZA, experiencing a significant increase in proliferation after replicate 2 ($P \leq 0.017$), replicate 3 ($P \leq 0.001$), replicate 5 ($P \leq 0.002$), and replicate 6 ($P \leq 0.0005$). E2 treatment produced slightly higher significant values with replicate 2 ($P \leq 0.0005$) and replicate 3 ($P \leq 0.006$), and RLX treatment only with replicate 2 ($P \leq 0.035$). E2+BZA

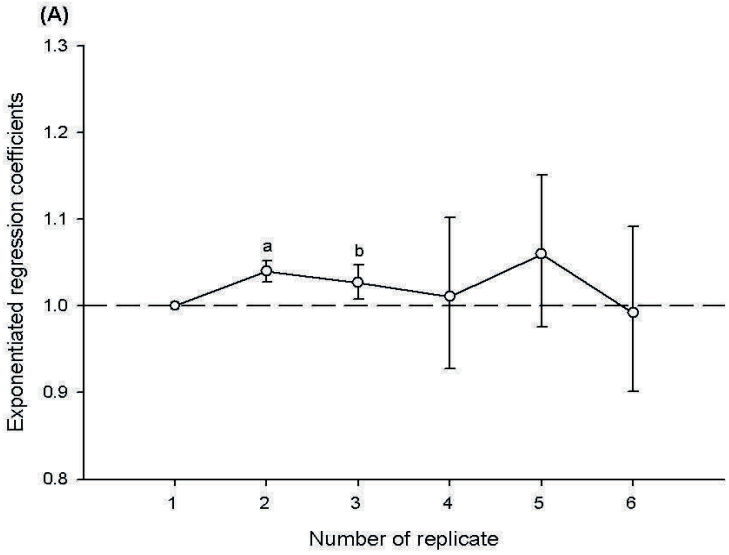
did not significantly alter the HUAEC proliferation dependent on the replicate number.

Table 10. Effect of number of replicates on HUAEC proliferation after E2, BZA, RLX or E2 + BZA treatment.

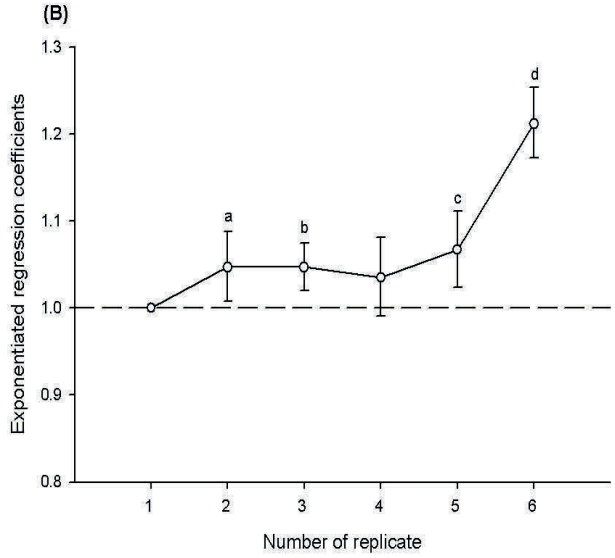
Dose (nM)	HUAEC proliferation			
	E2	BZA	RLX	E2+BZA
1	0.13 ± 0.037[a]	0.11 ± 0.023[c]	0.15 ± 0.031	0.11 ± 0.032
2	0.16 ± 0.041[b]	0.15 ± 0.041	0.16 ± 0.024[d]	0.12 ± 0.016
3	0.15 ± 0.041	0.15 ± 0.033	0.12 ± 0.012	0.08 ± 0.012
4	0.14 ± 0.007	0.14 ± 0.001	0.18 ± 0.006	0.11 ± 0.017
5	0.18 ± 0.008	0.17 ± 0.008	0.20 ± 0.008	0.14 ± 0.019
6	0.12 ± 0.011	0.30 ± 0.033	0.13 ± 0.013	0.10 ± 0.031

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from replicate 1 ($P \leq 0.0005$) and replicate 3 ($P \leq 0.006$).
[c] Value significantly different from replicate 2 ($P \leq 0.017$), replicate 3 ($P \leq 0.01$), replicate 5 ($P \leq 0.002$), and replicate 6 ($P \leq 0.0005$).
[d] Value significantly different from replicate 1 ($P \leq 0.035$).

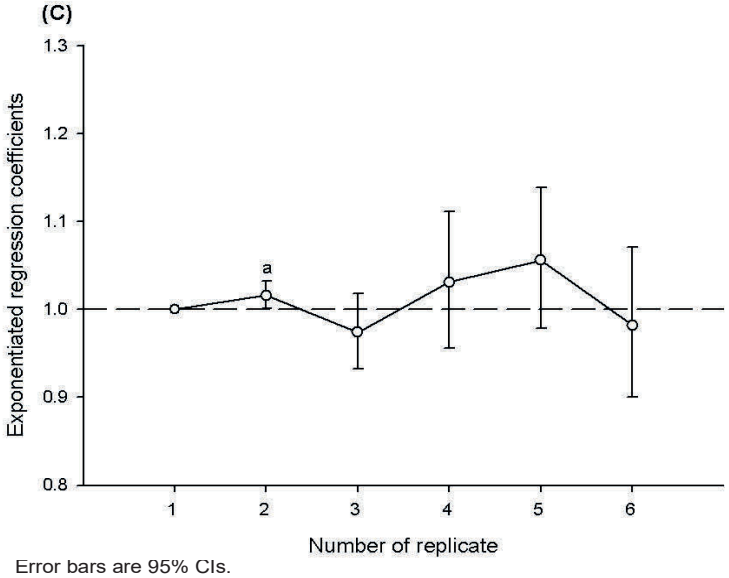
Figure 18. Estimated relative change in HUAEC proliferation depending on replicate numbers after cell treatment with E2 (A), BZA (B), RLX (C), and E2+BZA (D).



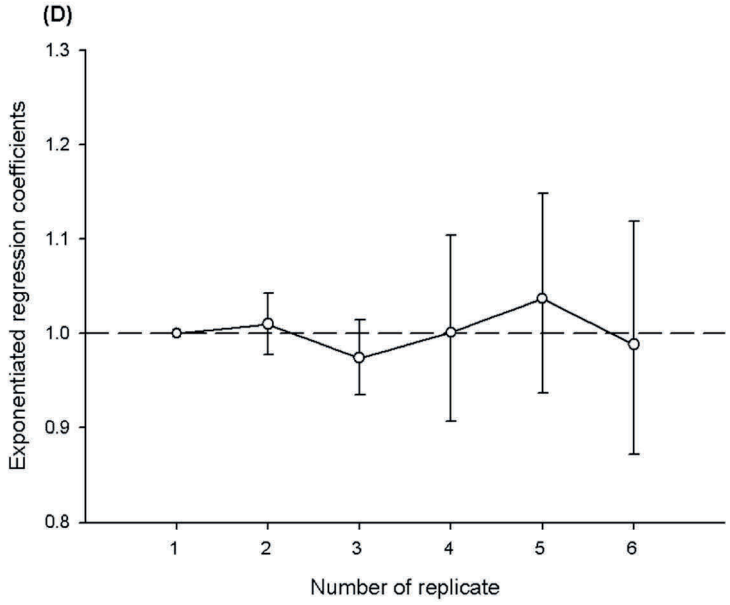
Error bars are 95% CIs.
[a,b]Values significantly different to the replicate 1 ([a]P ≤ 0.0005; [b]P ≤ 0.006).



Error bars are 95% CIs.
[a,b,c,d]Values significantly different to the replicate 1 ([a]P ≤ 0.017; [b]P ≤ 0.001; [c]P ≤ 0.002; [d]P ≤ 0.0005).



Error bars are 95% CIs.
[a]Values significantly different to the replicate 1 ([a]P ≤ 0.035).



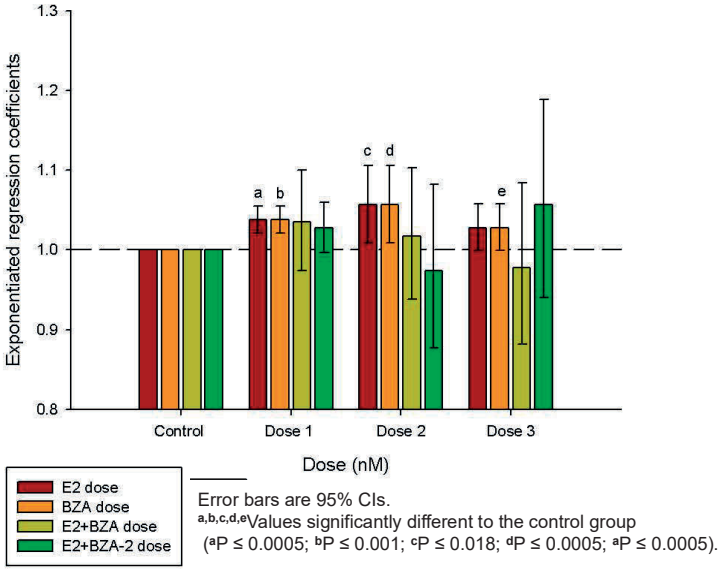
Focusing on our findings of the reduction in cell proliferation after E2+BZA treatment in comparison with individual E2 or BZA efficacy in equivalent doses, we performed the same proliferation XTT assay with a duplicate dose of E2+BZA. We found that high doses of E2+BZA-2 had no significant effect on HUAEC proliferation (Table 11, Figure 19).

Table 11. Effect on HUAEC proliferation of increasing dose of E2 and BZA alone and in combination, in standard dose of E2+BZA and duplicated dose of E2+BZA-2.

	HUAEC proliferation			
	E2	**BZA**	**E2+BZA**	**E2+BZA-2**
Control	0.12 ± 0.028[a]	0.13 ± 0.029 [c]	0.10 ± 0.030	0.11 ± 0.027
Dose 1	0.15 ± 0.019[b]	0.19 ± 0.014	0.14 ± 0.002	0.13 ± 0.018
Dose 2	0.17 ± 0.009	0.18 ± 0.017	0.12 ± 0.011	0.08 ± 0.026[e]
Dose 3	0.14 ± 0.013	0.19 ± 0.02	0.08 ± 0.022[d]	0.16 ± 0.033

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from the control group ($P \leq 0.0005$).
[c] Value significantly different from dose 1 ($P \leq 0.002$), dose 2 ($P \leq 0.002$), and dose 3 ($P \leq 0.0005$).
[d] Value significantly different from dose 1 ($P \leq 0.046$) and dose 2 ($P \leq 0.003$).
[e] Value significantly different from dose 3 ($P \leq 0.0005$).

Figure 19. Relative effect of dose of E2, BZA, E2 + BZA and E2+BZA-2 on HUAEC proliferation.



4.2 Effect of cell treatment on cyclins A, B, D1, and $p27^{Kip1}$ protein expression.

Western blot experiments were performed to investigate whether cell proliferation induced by different treatments was associated with changes in the protein expression of the cell-cycle regulators A, B, D1, and $p27^{Kip1}$. Table 12 shows a significant overall effect of treatment on protein expression of all cyclins except cyclin D1. Number of replicates had no significant effect on A, B, D1, and $p27^{Kip1}$ protein expression. In contrast, previous cryopreservation of HUAEC was a significant factor in A, B, and $p27^{Kip1}$ protein expression only.

Table 12. Overall effect of cell treatment, number of replicates, and previous cryporeservation of HUAEC on cyclin protein expression.

Dependent variable	Independent variable	N	*P*-value
Cyclin A	Cell treatment	72	0.0005
	Number of replicates	72	0.369
	Cryopreservation of HUAEC	72	0.0005
Cyclin B	Cell treatment	72	0.0005
	Number of replicates	72	0.424
	Cryopreservation of HUAEC	72	0.0005
Cyclin D1	Cell treatment	72	0.105
	Number of replicates	72	0.113
	Cryopreservation of HUAEC	72	0.075
$p27^{Kip1}$	Cell treatment	87	0.0005
	Number of replicates	87	0.458
	Cryopreservation of HUAEC	87	0.020

First we focus on the effect of E2, BZA, RLX, and E2+BZA used to study possible changes in the protein content of a group of cyclins. Table 13 shows the marginal means ± SEMs of data stratified by the different treatments; Figure 20 represents the relative changes in protein expression of these marginal means compared to the control untreated group, used as the reference group.

In the case of cyclin A, although there was a tendency to increase protein expression when cells were exposed to different drugs, only E2 treatment exhibited significantly higher relative values ($P \leq 0.002$).

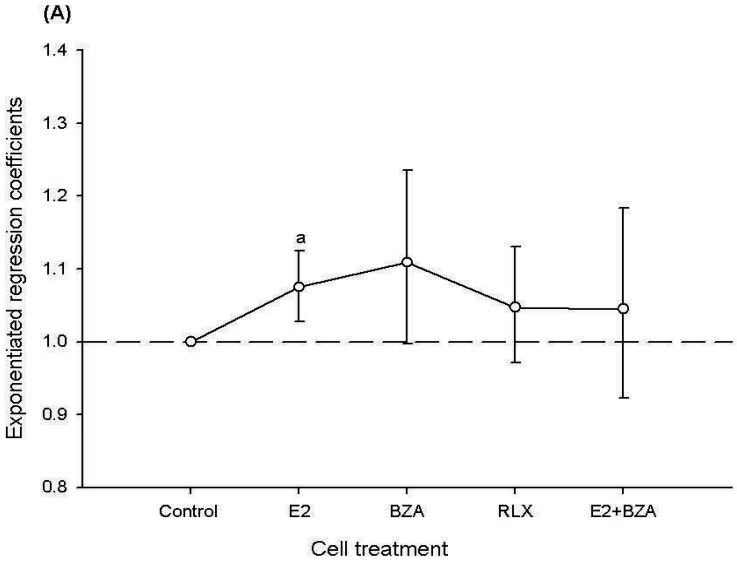
Despite the overall effect of cell treatment to endothelial cells was significant on the cyclin B and cyclin D1 expression, the individual effect of each treatment did not evidence significant changes. Significantly increased $p27^{Kip1}$ concentrations were reached after treatment of cells with E2 ($P \leq 0.006$ vs. control values).

Table 13. Effect of cell treatment on protein expression.

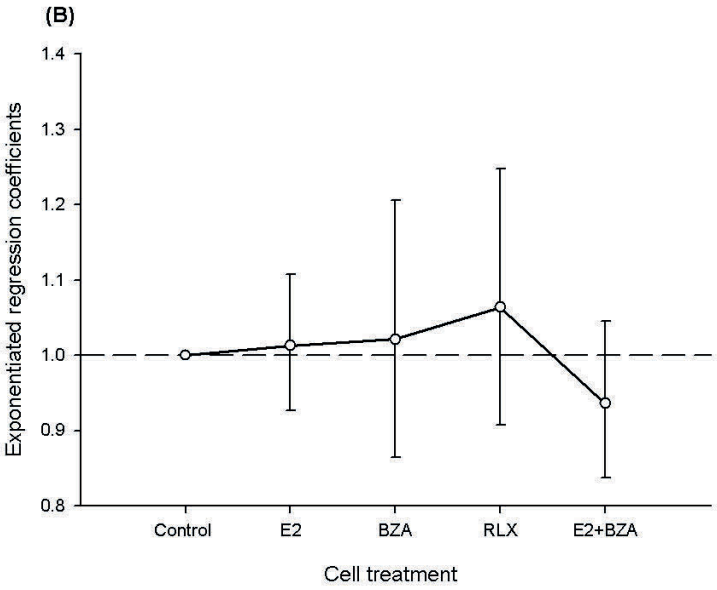
Treatment	Relative protein expression			
	Cyclin A	**Cyclin B**	**Cyclin D1**	**$p27^{Kip1}$**
Control	0.10 ± 0.033[a]	0.21 ± 0.052	0.11 ± 0.027	0.17 ± 0.049
E2	0.17 ± 0.027[b]	0.22 ± 0.053	0.26 ± 0.064	0.31 ± 0.018[c]
BZA	0.20 ± 0.055	0.23 ± 0.113	0.19 ± 0.070	0.22 ± 0.031
RLX	0.15 ± 0.029	0.27 ± 0.103	0.15 ± 0.063	0.21 ± 0.075
E2+BZA	0.14 ± 0.034	0.14 ± 0.030	0.11 ± 0.066	0.21 ± 0.040

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from the control group ($P \leq 0.016$).
[c] Value significantly different from the control group ($P \leq 0.054$).

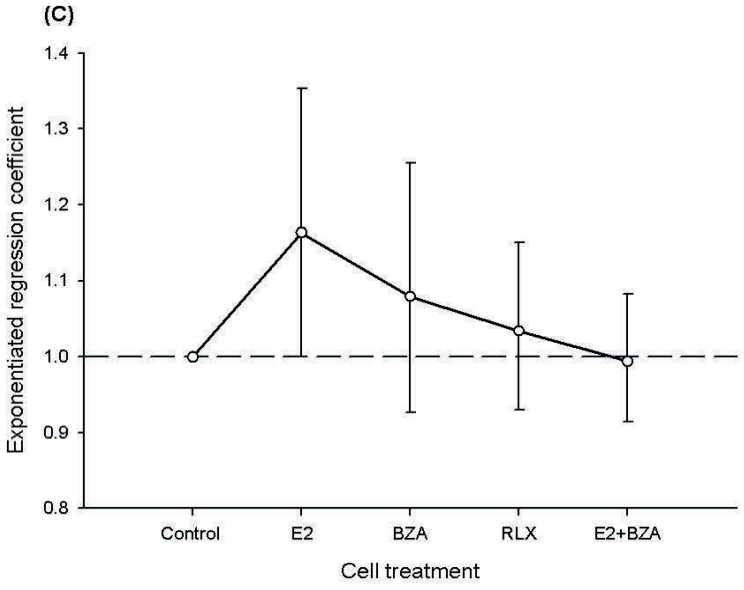
Figure 20. Estimated relative effect of cell treatment with E2, BZA, RLX or E2 + BZA on protein expression of cyclin A (A), cyclin B (B), cyclin D_1 (C) and $p27^{Kip1}$ (D).



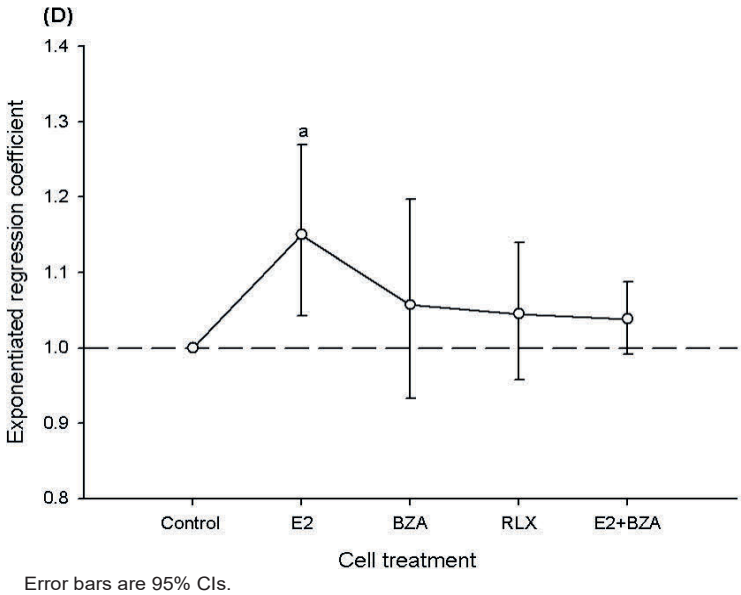
Error bars are 95% CIs.
[a]Values significantly different to the control group ([a]P ≤ 0.002).



Error bars are 95% CIs.



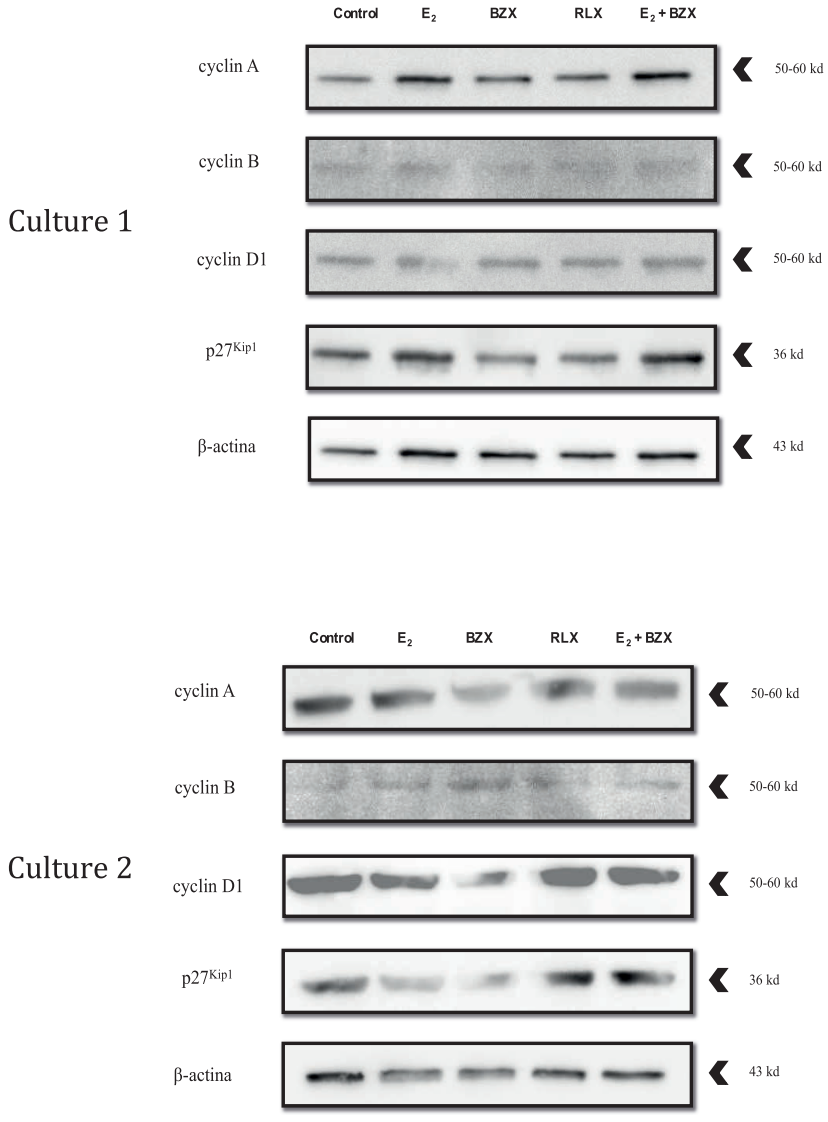
Error bars are 95% CIs.



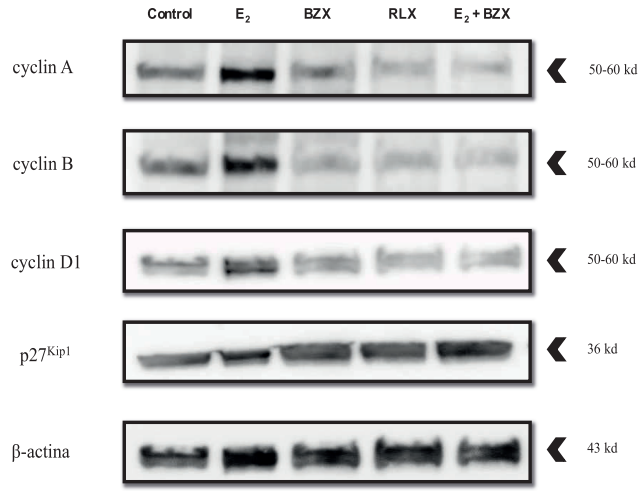
Error bars are 95% CIs.
[a]Values significantly different to the control group ([a]P ≤ 0.006).

Western blots obtained in these experiments are represented in Figure 21.

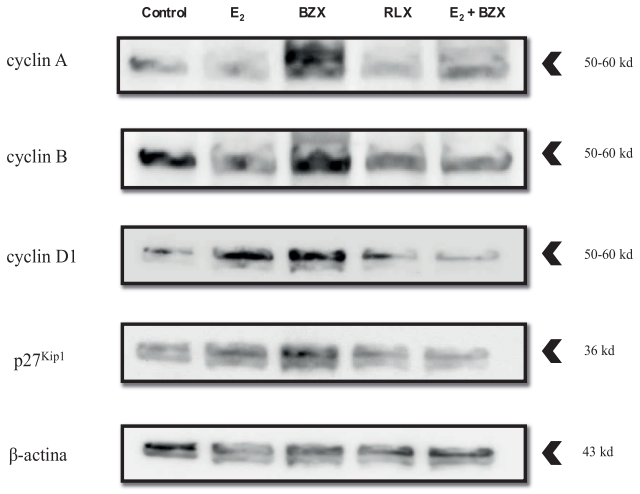
Figure 21. Examples of cyclin protein bands and loading control with their molecular weight from four different cultures.



Culture 3



Culture 4



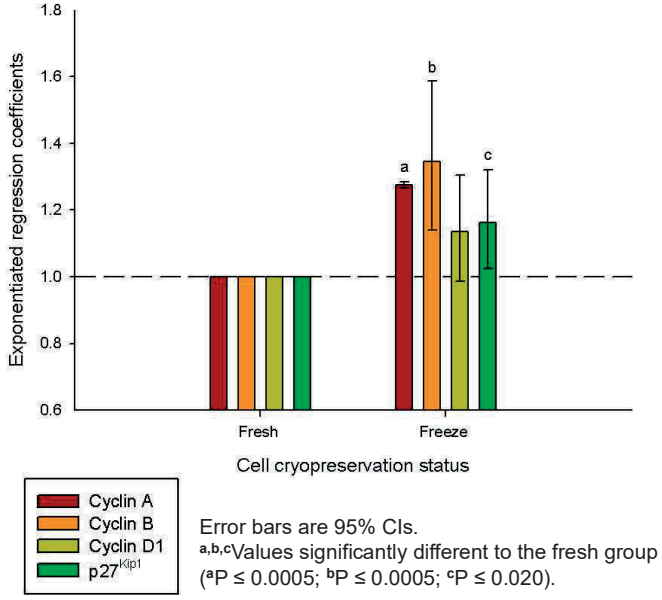
When analyzing data of how previous cryopreservation of HUAEC affects the protein expression after drug application, Table 14 and Figure 22 show a similar pattern of expression, irrespective of cell treatment type. This figure indicates that previously frozen cells displayed significantly higher relative values of protein expression of cyclin A ($P \leq 0.0005$), cyclin B ($P \leq 0.0005$), and $p27^{Kip1}$ ($P \leq 0.020$).

Table 14. Effect of previous cryopreservation of HUAEC on protein expression of cyclin A, cyclin B, cyclin D1 and $p27^{Kip1}$, irrespective of cell treatment.

Cell status	Relative protein expression			
	Cyclin A	**Cyclin B**	**Cyclin D1**	**$p27^{Kip1}$**
Fresh	0.03 ± 0.020[a]	0.07 ± 0.084	0.10 ± 0.075	0.15 ± 0.030
Freeze	0.28 ± 0.020[b]	0.36 ± 0.058[c]	0.23 ± 0.022	0.30 ± 0.055[d]

[a] Data are estimated marginal means ± SEMs.
[b] Value significantly different from the fresh group ($P \leq 0.0005$).
[c] Value significantly different from the fresh group ($P \leq 0.0005$).
[d] Value significantly different from the fresh group ($P \leq 0.020$).

Figure 22. Relative effect of previous cryopreservation status of cells on protein expression of cyclin A, cyclin B, cyclin D_1 and $p27^{Kip1}$.



4.3 Effect of cell treatment on cyclins A, B, D1, and $p27^{Kip\ 1}$ gene expression.

Quantitative Real-Time Polymerase Chain Reaction Assay was performed to investigate whether cell proliferation induced by different treatments was associated with changes in gene expression of the cell-cycle regulators A, B, D1, and $p27^{Kip1}$. The overall output of GEE analysis between independent variables and gene expression of each cyclin is illustrated in Table 15. There were no significant changes in mRNA content after application of different treatment, day of experiment

or basal condition of endothelial cells, aside from the only significant overall effect of cell treatment on $p27^{Kip1}$.

Table 15. Overall effect of cell treatment, day of experiment, and cryporeservation of HUAEC on cyclin gene expression.

Dependent variable	Independent variable	N	*P*-value
Cyclin A	Cell treatment	52	0.208
	Day of experiment	52	0.462
	Cryopreservation of HUAEC	52	0.319
Cyclin B	Cell treatment	55	0.097
	Day of experiment	55	0.682
	Cryopreservation of HUAEC	55	0.934
Cyclin D1	Cell treatment	55	0.154
	Day of experiment	55	0.386
	Cryopreservation of HUAEC	55	0.143
$p27^{Kip1}$	Cell treatment	54	0.0005
	Day of experiment	54	0.081
	Cryopreservation of HUAEC	54	0.960

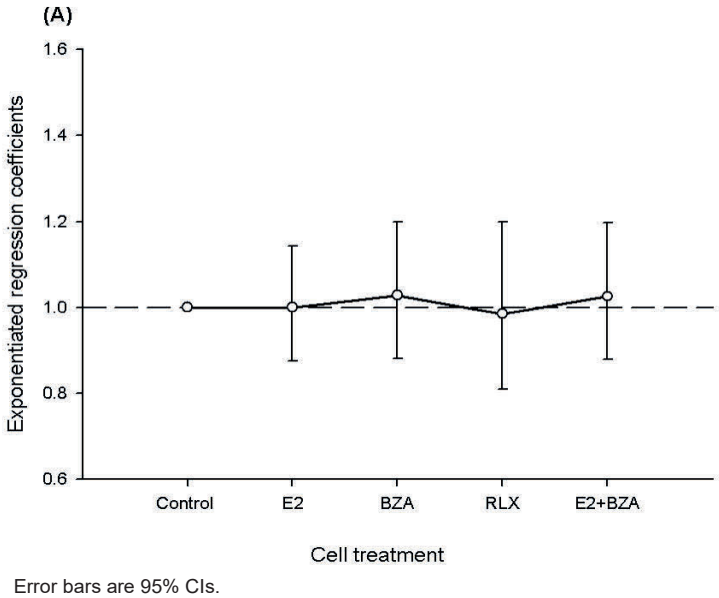
Table 16 and Figure 23 represent the relative changes in gene expression of data stratified by each treatment. No changes in any cyclin gene content were observed for any type of treatment compared to the untreated control cells.

Table 16. Effect of cell treatment on mRNA expression.

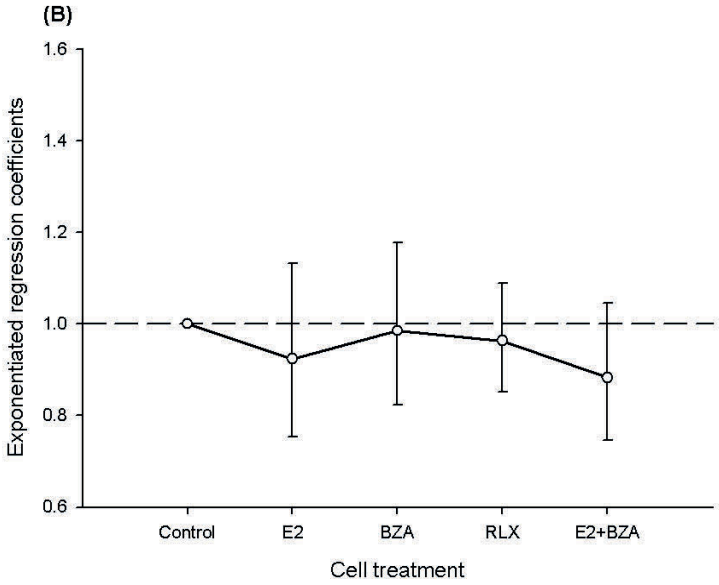
Treatment	Relative mRNA expression			
	Cyclin A	**Cyclin B**	**Cyclin D1**	**p27^{Kip1}**
Control	0.31 ± 0.119[a]	0.95 ± 0.118	1.34 ± 0.168	1.84 ± 0.158
E2	0.31 ± 0.164	0.87 ± 0.105	1.18 ± 0.097	1.78 ± 0.137
BZA	0.34 ± 0.175	0.94 ± 0.113	1.16 ± 0.099	1.81 ± 0.177
RLX	0.29 ± 0.163	0.91 ± 0.072	1.26 ± 0.105	2.03 ± 0.170
E2+BZA	0.33 ± 0.190	0.83 ± 0.105	1.12 ± 0.107	1.93 ± 0.167

[a] Data are estimated marginal means ± SEMs.

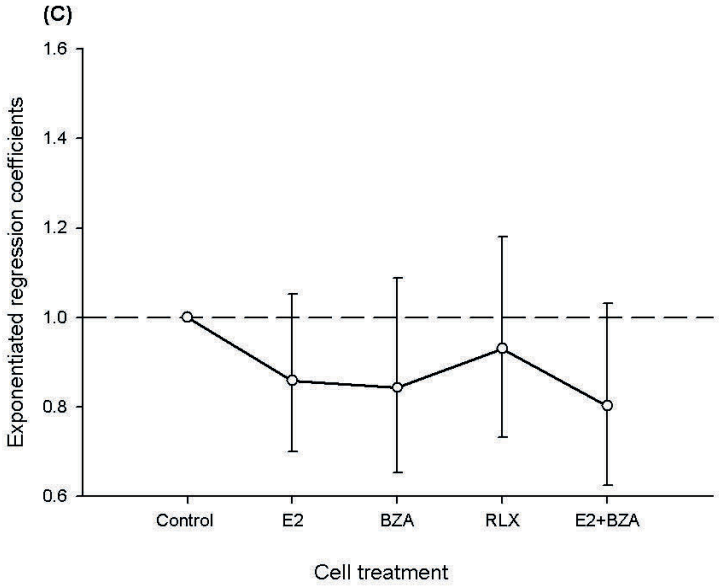
Figure 23. Estimated relative effect of cell treatment type on relative mRNA expression of cyclin A (A), cyclin B (B), cyclin D_1 (C) and $p27^{Kip1}$ (D).



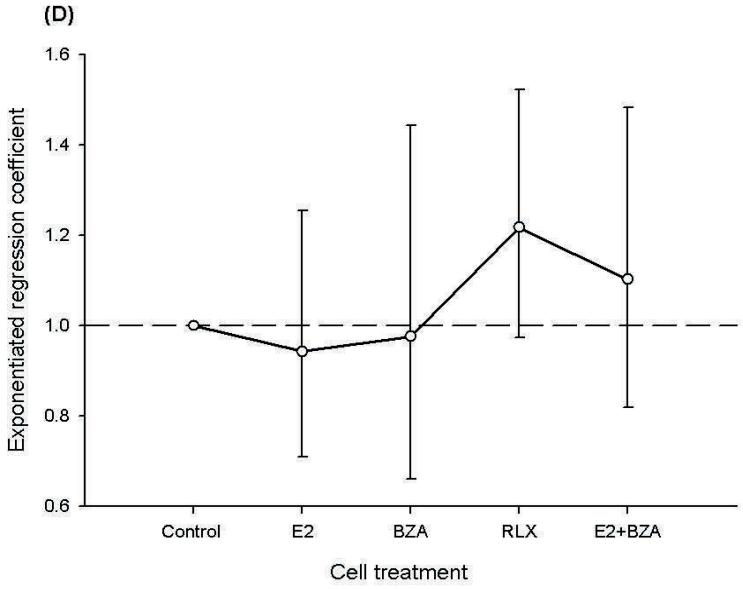
Error bars are 95% CIs.



Error bars are 95% CIs.



Error bars are 95% CIs.



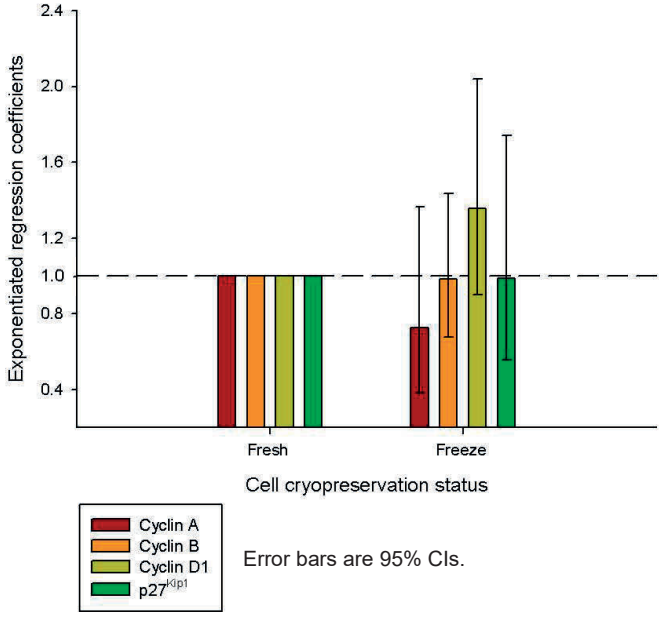
The condition of endothelial cells, whether fresh or frozen, showed no significant variation in gene expression of cyclins either (Table 17, Figure 24).

Table 17. Effect of previous cryopreservation of HUAEC on relative mRNA expression of cyclin A, cyclin B, cyclin D_1 and $p27^{Kip1}$, irrespectively of cell treatment.

HUAEC status	Relative mRNA expression			
	Cyclin A	Cyclin B	Cyclin D1	$p27^{Kip1}$
Fresh	0.48 ± 0.281[a]	0.91 ± 0.167	1.06 ± 0.122	1.89 ± 0.169
Frozen	0.16 ± 0.145	0.89 ± 0.083	1.37 ± 0.163	1.87 ± 0.234

[a] Data are estimated marginal means ± SEMs.

Figure 24. Relative effect of previous cryopreservation status of cells on gene expression of cyclin A, cyclin B, cyclin D_1 and $p27^{Kip1}$.



5. DISCUSSION

Our investigation has focused on the endothelium level, a target organ able to prevent or slow down the pathogenesis of women´s cardiovascular disease. The early stages of menopause is a critical period where atheromatous lesions, which are the substrate of the disease, could develop.

The increased incidence of atherosclerosis in women has been linked to the cessation of gonadal hormone production, although this is not unanimously accepted. When this happens, gynecologists initially lead with the effects of hypoestrogenism that occur after the interruption of ovarian function, and then with cardiovascular pathology after menopause and its hormonal treatment. This treatment initially improves quality of life for many women, but in the longer term promotes health problems such as cardiovascular function, osteoporosis and dementia. Recently, new drugs have been added to menopausal hormone therapy in the treatment spectrum for postmenopausal women, such as SERM and TSEC, of which we still have little information regarding cardiovascular dysfunction.

There are a wide range of research topics focused on the atherosclerosis process. Owing to the slow progression of cardiovascular disease, research in humans is a quite complex process requiring a large number of patients and many years of follow-up to reach conclusions, which in epidemiological studies are often unreliable, yielding insufficient evidence to defend or reject hormone replacement therapy for this pathology.

This has led researchers to develop strategies for assessing changes in vascular endothelium at the preclinical stage. On the one hand, there are experimental animal models in which pathophysiology can be studied over a shorter time span and in a more homogeneous way. And on the other hand, *in vitro* cell culture is useful for understanding endothelial cell behavior, such as interaction with various mediators. The endothelial cells damaged after acute mechanical vessel injury or noxious stimuli represent a physiologic challenge that can further alter endothelial repair, and therefore, proliferation, gene, and protein expression.

In order to gain a clearer understanding of the gap between clinical and basic science, we decided to concentrate on samples and study human endothelial tissue. For this reason, our project has focused on assessing how bazedoxifene, a recently incorporated therapy used in postmenopausal women, is able to induce the proliferation process in healthy human vascular endothelial cells, either alone or in combination with estradiol. Other estrogen-based preparations were also evaluated and compared with bazedoxifene. From this perspective, the method has allowed us to approach the early stages of the atherosclerotic process from an experimental point of view, providing more data pertinent to this treatment in prevention of cardiovascular pathology in postmenopausal women.

It is important to highlight the strengths of this work, which contributes original research on bazedoxifene, and provides data which

elucidates the potential CV impact of bazedoxifene. The area of CV impact of SERMs has been poorly investigated following the neutral effects of raloxifene in the RUTH study. However, limited data exist concerning the impact on the initial stages of atherosclerosis, of value for women entering the postmenopausal period.

5.1 Methodological considerations

Human umbilical vein endothelial cells (HUVECs) and human umbilical artery endothelial cells (HUAECs) derived from human umbilical cord vein and artery, respectively, are the most commonly used cell type for the study of endothelial function and disease adaptation *in vitro*. EC culture has been considered the gold standard in cardiovascular research since the 1980s (116). Although both HUVEC and HUAEC are derived from umbilical cord, they have different functions due to their particular genetic and molecular background (117). Human vascular endothelial substance has had limited availability until recently, and the advances in understanding of vascular physiology have stemmed mainly from experiments performed with cultured ECs. For this reason, this technique could be considered as a helpful and convenient method, complementary to *ex vivo* methods and animal models.

Proliferation and endothelial barrier function have been evaluated *in vitro* in several studies to assess inflammatory status in diabetes and atherosclerosis processes (118). These experiments were basically made

in HUVECs, under a specific design that precipitates the above mentioned pathological conditions, and the main findings were suppressed cell number, delayed replication, disrupted cell cycle, compromised endothelial barrier and accelerated death.

In the study a research group working with hormonal therapy *in vitro* models demonstrated that both estradiol and raloxifene promote proliferation of HUVEC in a similar magnitude. This could suggest a protective response against atherogenesis, as a consequence of the major proliferative effect and therefore better re-endothelization capacity (70). However, the study was conducted in endothelial cells of venous origin, but no data exist on the proliferative effect on arterial endothelial cells after hormonal menopausal treatment. This is a matter of particular interest since atherosclerosis occurs in arteries, and as already discussed, in several genes expression profiling differs in endothelial cells from different blood vessels (117).

The *in vitro* EC culture model has several advantages and some disadvantages. On the one hand, redundant cord tissue can be obtained easily from the umbilical cord after delivery and no further surgical intervention is needed for the patient. The ordinary length of human umbilical cord is approximately 50-55 cm, which provides sufficient amounts of cells to work with (119), cords of at least 15-20 cm of the sample being used in our work. Vascular cells are the potential source for isolation of three main cell types: endothelial cells, smooth muscle cells,

and fibroblasts. Once isolated, cells can be cryopreserved for use when needed. Despite the fact that thawing, reculturing, and expanding techniques are applied after cryopreservation, these cells showed similar properties regarding morphology and proliferation potential, as reported in previous studies (120).

On the other hand, the *in vitro* fabrication process of both HUAEC and HUVEC for research is a complex practice which requires strict quality control to ensure adequate cell isolation, cell cultivation in terms of primary culture, and cell growth leading to the final product prepared for cryopreservation. Several problems may occur during the entire process, such as: 1. absence or few viable cells after thawing (poor quality of culture, microbial contamination, inappropriate freezing rate, etc.); 2. poor cell attachment after thawing from passaging or stock (low room humidity, inadequate mixing of cells, media or other reagents, etc.); 3. slow cell growth (cells over-passaged, lacking CO2 levels, fluorescent light damage, inaccurate cell counting method, etc.); 4. uneven cell growth (inadequate mixing of cells or media, temperature variations within the incubator, uneven evaporation and vibration in the incubator, etc.).

The model used in our study is much more difficult to use than HUVEC, and we worked extraordinarily hard to improve the quality of the results. A total of fifteen umbilical cords were collected and processed, with six losses during the process: one due to contamination,

one due to lack of adhesion, and four due to poor growth in culture flasks. Fortunately, we were able to locate the exact source of the problem in time and correct the multiple potential causes of poor growth, demonstrating the importance of using standardized protocols as a basis for standard operating procedures, to ensure efficiency and reproducibility for future practice.

Looking forward, good manufacturing practice conditions and guidelines have already been established for vascular umbilical cord cells culture, the main advantages of the current cell banks that represent an excellent cell source for cardiovascular tissue engineering (120).

5.2 Effect of cell treatment, number of replicates, and dose on HUAEC proliferation.

Our investigation is novel in that it shows that bazedoxifene alone promotes proliferation of HUAECs in a magnitude similar to estradiol and raloxifene.

Most scientific evidence to date has collected information about estrogens and raloxifene and their ability to accelerate endothelial cell growth in vitro models (121), (62), (70), which is crucial for functional endothelial preservation. Taken together with other actions, this underscores the protective effect of estrogens and raloxifene against atherogenesis. Estradiol has been shown to induce a favorable lipid

profile by decreasing low-density lipoprotein levels and serum Lp (a) lipoprotein concentration, while increasing high-density lipoprotein levels (122). Raloxifene also favorably alters the lipoprotein profile to a similar but less pronounced degree than estrogen (123), both protecting low-density lipoprotein from oxidation (124), (125). The promotion of endothelial health is also suggested by estrogen and raloxifene vasodilatory effects, and more recently by the upregulation of atheroprotective prostacyclin throughout cyclooxygenase 1 and 2, thus reducing some of the adhesion molecules (126). Furthermore, estrogen and raloxifene exert an antiproliferative effect on smooth-muscle cells, contributing directly to vascular long-term protection (127).

Bazedoxifene, the latest selective estrogen to enter the market for osteoporosis treatment in postmenopausal women, was placed top of a long list of SERMs due to its overwhelmingly positive results in relevant clinical trials, notwithstanding the paucity of evidence about efficacy on the cardiovascular system.

Bazedoxifene alone and in combination with CEE was associated with a favorable lipid level profile, obtaining significant reductions in total and LDL cholesterol levels and significant increases in HDL cholesterol levels in women (110), (128) despite producing null effect in monkeys (112).

BZA and BZA/CEE action on extent and severity of atherosclerosis has been investigated by only one research group, which focused their experiments on three arterial territories of surgically postmenopausal monkeys (112), (129). BZA alone had no effect on atherosclerosis of coronary, peripheral and cerebral arteries nor any change in body weight, adiposity, fasting glucose, or plasma lipid profile. Furthermore, they observed BZA interference in the capacity of CEE to prevent atherosclerotic lesions developing in coronary and peripheral vascular sectors, but it did not attenuate CEE's inhibition of plaque complications in the common carotid artery.

The limited current available data on bazedoxifene is via *in vitro* studies at the endothelium level. Only one research group demonstrated that bazedoxifene could act as an anti-inflammatory and anti-oxidative agent against proteins and lipids that become glycated due to exposure to sugars, called advanced glycation end products (AGE), and their receptor RAGE, using HUVECs. There is an accumulating body of evidence that these particles have thrombogenic and inflammatory properties on the endothelial cells, thereby creating a pathological link between complications in diabetes, accelerated atherosclerosis and vascular calcification (130).

There is no information, however, on the effect of BZA alone and in association with estrogen compounds on endothelial cell proliferation. As the integrity and regeneration of endothelium seems relevant for

cardiovascular health, the potential effect of BZA on proliferation is of interest.

Our investigation demonstrated for the first time bazedoxifene's capacity to induce proliferation in endothelial cells, albeit in a modest quantity, but similar to the range reached by estradiol and raloxifene. This seems to be observation congruent since mature ECs exhibit a very low proliferation index (131). Moreover, a comparable effect was observed in the spectrum of concentration range of 10-1 to 10+1 of EC50 of each compound. Both estradiol and raloxifene treatment showed less proliferation at higher concentration. We do not have a clear explanation regarding the decrease in effect with the increase in dose, but this paradoxical result was also observed in previous reports, affirming similar responses to estradiol and raloxifene (62), (70). Nevertheless, these studies were carried out in endothelial cells of human umbilical cord veins, but no arteries, and with different range of dosages.

The relationship between dosage and effect on proliferation after the concurrent cell treatment with bazedoxifene plus estradiol did not yield a significant result either with a standard dose or after performing the same proliferation XTT assay with a duplicated dose. The only experimental work studying analogous correlation showed BZA interference in the capacity of CEE to prevent the development of atherosclerotic lesions in animal models (112).

Our investigation also took into consideration the experimental variation which comes from both the process and the biological source variability. Biological replicates were obtained using umbilical cords from different patients and served to provide information about the population. Supplementary technical replicate analysis was conducted to provide information about process variability and accuracy of laboratory measurements regarding both dose and treatment evaluation. Very slight variability was detected at doses 1 and 2; specifically, one of the six replicates showed significantly different values, whereas a little more fluctuation in proliferation was observed at dose 3. Likewise, the treatment with bazedoxifene showed modest variability, unlike estradiol, raloxifene, and estradiol plus bazedoxifene treatments. This observation may suggest that more accurate measurement is needed to test proliferation at dose 3 and treatment with bazedoxifene, or the need to include a greater number of biological samples in the analysis, since we cannot recognize which six technical replicates came from each biological replicate.

5.3 Effect of cell treatment on cyclins A, B, D1, and $p27^{Kip1}$ protein and gene expression.

In our work the proliferative effect induced by bazedoxifene, estradiol, raloxifene, and combination of estradiol plus bazedoxifene was not associated with increased gene expression of cell cycle key regulators such as cyclin A, cyclin B, cyclin D1 and $p27^{Kip1}$. Despite this, increases in cyclin A and $p27^{Kip1}$ protein content were detected only after treatment with estradiol.

Endothelial cells, under normal conditions and especially when facing physiological and pathological aggression, undergo phenotypic modulation and re-enter the cell cycle, which is a highly complex process due to the larger number of proteins participating at different phases (131). A complex composed of CDK and the regulatory subunit cyclin must be activated to advance through the cell cycle, while CKI modulates it negatively (57). At least twenty different holoenzymes participate in controlling the cell cycle, of which we have studied four, three activators and one inhibitor.

On cell proliferation there is a wide range of evidence regarding the genetic regulation with estrogenic treatment made in experiments with breast cancer cells, which points to the possibility that estrogen may share the same effect in vascular cells.

Most work supports that E2 up-regulates cyclin A2, (70), cyclin B1 (70), cyclin D1 (132), (133), (134). Although it was also observed that long term ethinyl estradiol (EE) treatment in rats decreased levels of

all proteins required for a proper G1/S-transition and S phase progression, such cyclin A, cyclin E and CDK2 (135). This is true even in studies where the cyclin-associated CDK activity didn't seem to depend on an increase in cyclin or CDK protein, or a decrease in CKI protein.

Although only isolated data of significant increases were detected at some point in the curves for protein levels, the confirmation of proliferation draws attention to a propensity towards positive changes in protein content, which could probably be fully achieved by increasing the sample size and procedural rigor, or if other determinants of cell proliferation had been explored.

Our analysis detected no significant changes in the gene expression of cyclins in cells treated with E2, BZA, RLX, and E2+BZA. The relation of these modifications to the cell cycle in endothelial arterial cells treated with these drugs is still unclear, since data are inconclusive. We do not have a clear explanation for the lack of concordance observed between the gene and protein expression of cyclins studied, but post-transcriptional and post-translational modifications could be involved in the process of protein biosynthesis from genetic cell content, and further work should help in resolving this issue.

5.4 Effect of cryopreservation of HUAEC on cyclins protein and gene expression.

Nowadays, the analysis of biological markers (DNA, RNA, and proteins) has become a technique capable of diagnosing disease risk, monitoring and establishing possible responses to potential therapies (136). As not all biological specimens can be processed immediately after collection, and since the process of transportation, storage and verification could require a long time, cryopreservation has become a widely used method over the last few decades. Hence, biobanks, where human biological materials are cryopreserved at -80°C or in liquid nitrogen, are a crucial source in the scientific research. Moreover, the technique of cryopreservation has produced important advances in gynecology, specifically for reproductive medicine, since human oocyte, embryos and ovarian tissue could be cryostored. For this reason, as a matter of interest we investigated changes in RNA and protein expression in arterial endothelial cells after cryopreservation at -80°C and in liquid nitrogen. Naturally, RNAs and proteins are much less unstable and exposed to faster degradation than DNA, and the methodology of sample collection, handling, processing and preservation may easily influence the diagnostic results (137).

We found that freeze arterial endothelial cells displayed significant changes in protein levels for cyclin A, cyclin B and $p27^{Kip1}$ compared to the fresh control sample. However, no obvious significant

trend was observed in RNA profile for any cyclin after freezing and storage. Similar observation was found in previous works showing that the presence of cryoprotective agents, such as DMSO we used, increased the survival rates of cells after cryopreservation, even though there was no consistent trend observed with regard to the RNA expression measurements (136).

The data indicate a need for more accurate strategies for processing biological specimens or new solutions that could protect specific RNA and proteins by eliminating external factors, depending on the methodology.

6. CONCLUSIONS

In the light of the results obtained, the following conclusions can be drawn:

- The use of bazedoxifene at increasing doses is followed by a moderate proliferative effect on HUAEC in an *in vitro* model.
- This action was not associated with major protein and gene expression of key cell cycle regulators such as cyclin A, cyclin B, cyclin D1 and $p27^{Kip1}$. Despite not finding consistency between cell proliferation and greater gene expression of cyclin A, cyclin B, cyclin D1 and $p27^{Kip1}$, we suspect that other cell cycle regulators might be involved in the process.
- Bazedoxifene promotes proliferation of HUAEC in a similar magnitude to estradiol and raloxifene.
- There was no significant relationship between dosage and effect on proliferation after concurrent cell treatment with bazedoxifene plus estradiol.
- The cryopreservation process displayed positive changes in protein content of cyclins compared to fresh endothelial cells. On the other hand, no obvious significant trend was observed in RNA profile for any cyclin after freezing and storage.

7. RESEARCH STRENGTH AND LIMITATIONS

The main strength of this Doctoral Thesis lies in the consistency of the model. Arterial cells from human umbilical cords have been used to assess endothelial function as they allow an excellent approach to the physiological context of the arteriosclerosis process that occurs at the artery level. Until now no data has been published on the proliferative effect on arterial endothelial cells after menopausal hormonal treatment. Most of our knowledge from *in vitro* proliferative studies comes from umbilical vein cell culture since these cells do not experience as much aging in the subcultures. Despite the difficulties experienced in obtaining subcultures of arterial endothelial cells, we refrained from using cell lines.

Another point to highlight in the present Doctoral Thesis is the use of multiple technical replicates in the analysis of proliferation, protein and gene expression, which offered some advantages to our study. Among them the most notable are the evaluation of variation in the experiments, the overall precision of proliferation measurements, and not so suitable in gene and protein expression measurements. The use of proper replicates analysis instead of average values allowed smaller changes to be detected within the sample or the experimental procedure.

Although the present study represents a very limited experimental work, the results obtained allow us to speculate about the approach to understanding of the endothelial proliferative response under specific

hormonal treatment and the beneficial effect of bazedoxifene alone on endothelial disorders like atherosclerosis. Nevertheless, the variability of the data indicates a need for further studies with an increased biological sample size. Also, there is a different mechanism by which the treatment of cells with drugs could enhance proliferation. Apart from the classical ER pathways, the ligand-independent pathway, the ERE-independent pathway and the nongenomic pathway are the potential alternatives, but we did not analyze their possible mechanisms in our work. Nor did we include the entire battery of cell cycle regulators involved in the cell growth and differentiation process.

In this experimental work proliferation exhibited clear conclusions, but the door is open to a further edifying research on mediators and pathways, since relatively few have been studied, yielding a limited number of observations. In contrast, the strength of using HUAEC, and of exploring bazedoxifene or, even more, bazedoxifene in association with estradiol, has intrinsic value. The data regarding possible interference between estradiol and bazedoxifene when acting together are noteworthy and merit further exploration.

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9. APPENDIX

9.1 Cell count

Quantification of endothelial cells was performed using an optical inverted microscope, in which the original substrate was analyzed directly by phase contrast. Cell count was carried out by dilution method over a Neubauer chamber using trypan blue, a dye which contains amino and sulfate groups, allowing us to distinguish between viable and nonviable cells. Notable among its properties are high toxicity and solubility in water. This preparation proved to be of great help in calculating the cells and their viability, as the ions prevent them from passing through the membranes of the intact cells, so they stain only damaged or dead cells.

The Neubauer chamber grid has a depth of 0.1 mm, optimal to adapt to the optical microscope. The grid contains nine large squares, each with a $1mm^2$ area. The large squares are separated by triple lines with a 0.025 mm space between them, and are subdivided into 4x4 groups of 0.2 x 0.2 mm2 squares (Figure 25A).

We took 15 µl of medium with cell suspension and diluted it with 15 µl of 0.4% trypan blue solution, prepared in 0.8% sodium chloride and 0.06% potassium phosphate dibasic (Trypan Blue Solution 0,4%, SIGMA). The mixture was placed in Eppendorf-type bottles and incubated for 5 min at room temperature. With the help of a pipette, 10 µl was injected from one side into the Neubauer chamber. We proceeded

to count the cells using the optical inverted microscope. To avoid counting errors, we started the count at the top left-hand corner and followed the direction shown in Figure 25B. Moreover, only cells completely within the area were taken into account, including cells touching line on top and left, but not those that touch the bottom and the right lines (Figure 25C). We could perceive birefringent or living cells, and other blue colored ones indicating dead or damaged cells. To obtain the final concentration we applied the cell counting formula with Neubauer chamber:

Concentration (cel/ml): number of cells x 10.000 /Number of square x dilution

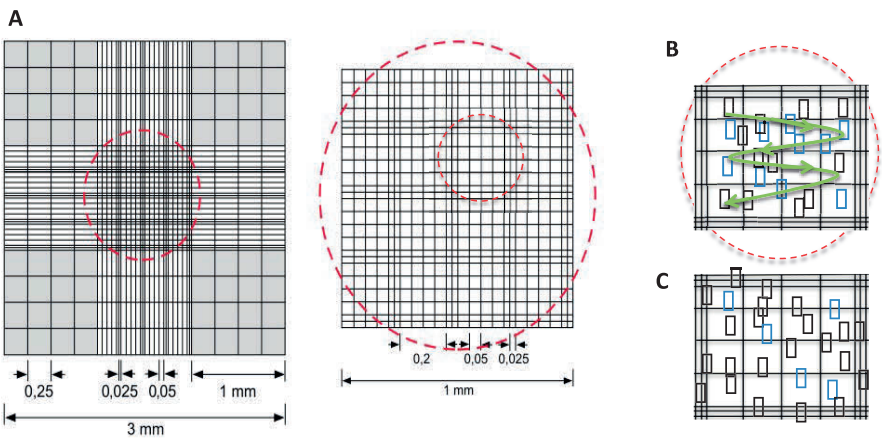

Figure 25. Cell count using Neubauer's chamber. Illustration of Neubauer´s chamber (image taken from (138)). (A) Counting technique in a large square: counting direction (B), and counting system (C).

9.2 Cryopreservation of human endothelial cells

A proportion of the endothelial cells obtained from the first three cords were treated with Dimethyl Sulfoxide-based (DMSO) cryoprotective agent solutions, then resuspended in freezing medium and were slowly frozen overnight in an isopropanol bath freezing container at -80ºC, then transferred to liquid nitrogen to be stored at 195.8ºC. For recovery from cryopreservation, all cells were thawed at room temperature after a slightly variable period of time of cryopreservation (3-3.5 months) and promptly were resuspended drop by drop with prewarmed culture medium. The cells were then centrifuged, washed, and resuspended in culture medium to remove residual dimethyl sulfoxide and plated on pre-coated standard tissue culture plates. The medium was changed the next day, and the cells were passaged when confluence was reached.