

American Society for Reproductive Medicine Menopausal MEDICINE

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FOR CLINICIANS WHO PROVIDE CARE FOR WOMEN

A Personal Memoir

Trudy L. Bush, Ph.D. 1949-2001

I lost a dear friend and colleague on Wednesday, March 14, 2001 – Professor Trudy L. Bush. I had known Trudy over the past 15 years and I always told my colleagues that she was the smartest scientist I had ever known. I worked with Trudy and benefited from her advice as an advisor to our research programs here at Wake Forest University over the past decade.

Trudy's training in sociology and epidemiology at Penn State University and The Johns Hopkins School of Hygiene and Public Health set the stage for a remarkably productive career. In 1983 and again in 1987, she authored two of the most frequently cited papers concerning the relationship between postmenopausal hormone replacement therapy and cardiovascular and all-cause mortality.^{1,2} She went on to serve as a principal investigator of both of the first large clinical trials designed to understand more clearly the effects of hormone replacement therapy on risk for heart disease – the Postmenopausal Estrogen/Progestin Intervention trial (PEPI)³ and the Heart and Estrogen Progestin Replacement Study (HERS).⁴

I believe that Trudy was one of the top five epidemiologists in the world. She was an independent thinker with novel points of view that formed the basis for new directions in research. Her views on estrogens and coronary heart disease as well as estrogens and breast cancer prompted a great deal of research that has proven productive. As an advisor, she challenged us to move forward toward the goal of improved health and longevity for older women. She stimulated our group to think about problems in innovative ways that were of utmost value to our research progress.

Trudy was a warm and caring person who had a special capability of making

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people around her feel comfortable. Her charm and intellectual productivity will be sorely missed.

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FROM THE EDITOR

David F. Archer, M.D.

Trudy Bush in Memoriam 2001



Trudy L. Bush, Ph.D. 1949-2001

This issue of *Menopausal Medicine* is dedicated to a woman who many clinicians do not know. She was quiet and her publications were involved in Epidemiology rather than Clinical Medicine. Despite this, she had an enormous impact on the health care of postmenopausal women.

Meeting Trudy Bush was an honor and you became her friend immediately. Her impact in a room of knowledgeable people was profound. Often she was looked to for the last word or the summation of the discussion. Her views and insight were sought after in these discussions.

Life is indeed short, but we are becoming used to the octogenarian. Dr. Bush left the stage prematurely but will be recalled with fondness and high regard by those who knew her.

Menopausal Medicine

A Newsletter of the
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Surgically Post- Menopausal Monkeys for Assessing Risk and Benefits to the Breast and Endometrium



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INTRODUCTION

The goal of our work over the past several years has been to evaluate the effect of hormonally-active agents on the reproductive tract and breast of monkeys. These animals are used to study the prevention of chronic diseases affecting postmenopausal women, i.e., atherosclerosis, osteoporosis, and cognitive decline, by the use of hormonal treatments. Given the hormonal nature of the interventions used, evaluation of risk or benefit for the reproductive tract and breast is a critical part of our multi-system approach to preclinical studies of women's health.

REPRODUCTIVE FEATURES OF THE MONKEY MODEL

The reproductive physiology and pathobiology of female macaque monkeys is similar to that of women in many aspects, including cyclic hormonal changes, vaginal cytology, endometrial responses to exogenous estrogens, sex steroid receptor expression, expression of estrogen and progesterin regulated genes, and spontaneous reproductive senescence. Much of the basic work demonstrating the function of the hypothalamo-pituitary-gonadal axis and GnRH pulse generator was done in macaques, and with the exception of a few anatomic features, their reproductive biology is quite similar to that of human beings. The presence and distribution of sex-steroid metabolizing enzymes such as aromatase, sulfatase, and 17-beta-hydroxysteroid oxidoreductase has been characterized in male and female macaques. Disruption of reproductive function by aro-

matase inhibitors and antiprogestins has provided important information for further understanding of the mechanisms involved in modulating sex steroid action and metabolism. Serum concentrations of estradiol, progesterone, and gonadotrophins are similar to those seen in women and are useful indicators of ovarian function in macaques. As in women, loss of ovarian function resulting from natural or surgical menopause results in continuous elevation of serum luteinizing hormone and follicle-stimulating hormone, from their normal cycling levels to continuously increased levels. Normal values are well-defined in the literature, and the reproductive features of the model have been reviewed recently.¹

The monkey species most commonly used for reproductive research are macaques, either the cynomolgus monkey (*Macaca fascicularis*) or the rhesus monkey (*Macaca mulatta*). These animals have a monthly menstrual cycle with menarche occurring at about four years of age and menopause occurring naturally at around 20 to 25 years of age. The lifespan of macaques is approximately 30 years. During the reproductive years, histologic changes in the endometrium during the normal menstrual cycle are quite similar to those seen in women. The cycle-staging criteria used for the human endometrium² are applicable to the macaque endometrium, although some criteria differ slightly; for example, epithelial subnuclear vacuolation is considered a post-ovulatory change in women but may occur just before ovulation in macaques.

Beginning with the last day of menstruation, the follicular phase of the cycle is distinguished by progressive glandular and stromal proliferation leading to glandular pseudostratification, glandular lengthening and coiling, and stromal expansion. Superficial stromal edema also occurs. Glandular and stromal proliferation are maximal just before ovulation. Near the time of ovulation, subnuclear vacuoles appear in glandular epithelial cells, and proliferation declines sharply. The glandular epithelium shifts to simple columnar, and throughout the luteal phase of the cycle glandular profiles become more complex, resulting in a "saw-toothed" appearance to the glandular profile. In the mid-luteal phase stromal edema develops throughout the endometrium, and in the late luteal phase prominent clusters of spiral arteries devel-

op. If pregnancy does not occur, the menstrual phase occurs, first manifesting as superficial intrastromal hemorrhage and marked apoptosis of basal glandular epithelium, followed by diffuse sloughing of the adluminal half of the endometrium.

Vaginal bleeding can be observed daily to monitor cyclicity and can also be used as an indirect indicator of endometrial stimulation by observing “withdrawal bleeding,” the latter being observed upon cessation of estrogen or estrogen+progestogen treatment only if endometrial growth was induced.³ Vaginal cytologic examination is a sensitive indicator of estrogenic effect that can be easily measured to assess cyclic or continuous estrogenic effects and can be expressed as continuous data by calculation of the Maturation Value from the relative percentages of parabasal, intermediate, and superficial cells ($MV = 0.2PB + 0.6I + S$).⁴ Endometrial biopsies usually are not possible via the vaginal route in rhesus and cynomolgus macaques because of the tortuous cervical channel.⁵ Ultrasound (Figure 1) and progestin-withdrawal bleeding are alternative methods for assessing responses of the uterus; endometrial biopsies can be taken by laparotomy. The characteristic perineal swellings indicating periods of sexual receptivity in female macaques (“sex skin”) are not useful external indicators of estrogenic effects in the postmenopausal monkey. Cynomolgus monkeys have varying degrees of sex skin prominence, but in general it is a less reliable indicator of cycle stage than in rhesus monkeys. Although sex skin responses are maximal in the estrogen-dominated phases of the premenopausal menstrual cycle, the response may be absent in the face of ele-

Table 1. Histologic Characteristics of Estrogenic and Progestogenic Effects on the Uterus

Estrogens	Progestogens
Glandular coiling	Glandular secretion
Epithelial mitoses	Glandular complexity
Epithelial proliferation (e.g., Ki-67 or PCNA labeling)	Glandular epithelial subnuclear vacuolation
Lowered epithelial nucleus:cytoplasm ratio	Hemorrhage
Epithelial pseudostratification	Vascularization
Epithelial height	Stromal decidualization
Glandular hyperplasia (simple, irregular, or adenomatous)	Decreased ER and PR expression
Squamous metaplasia	Stromal edema and hyperplasia
Epithelial atypia	
Stromal mitoses	
Increased ER, PR expression	

(ER = estrogen receptor/PR = progesterone receptor)

vated serum estradiol during the non-breeding season in rhesus macaques;¹ therefore, the response appears to be mediated by other factors in addition to estradiol.

The mammary glands of macaques are spread over a wide area of the ventral thorax but occupy about 5% of the subcutaneous tissue in the region,⁶ a proportion similar to that seen in women. Mammary cancers are common in aged macaques with an incidence similar to that seen in women.⁷ Mammary ductal hyperplasias and atypias occur in approximately 3% to 7% of clinically normal macaque breasts evaluated histologically at our institution (unpublished data).

ENDOMETRIAL HYPERPLASIA INDUCED BY UNOPPOSED ESTROGEN THERAPY IN MONKEYS

Estrogenic and Progestogenic Effects: Endometrial histologic features typical of estrogenic or progestogenic effects may be scored semiquantitatively to produce an index of overall estrogenic effect (Table 1). This technique has been used to

characterize responses to a variety of agents with potential for hormonal activity. In general, “estrogenic” indicators in the endometrium are physiologic and simply reflect those features that are seen in the follicular phase of the cycle (e.g., glandular proliferation), and “progestogenic” features are those that occur in the

luteal phase of the cycle (e.g., glandular secretion). Other features, such as metaplasias and hyperplasias, are pathologic rather than physiologic.

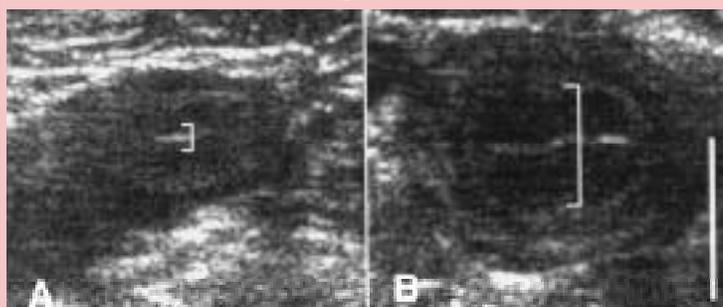
Endometrial Hyperplasia: Endometrial hyperplastic lesions in women are classified as simple or complex (adenomatous) and further subclassified by the presence or absence of cellular atypia. This approach corresponds well to the clinical behavior of the lesions, with the presence of atypia being a strong indicator of the potential for the lesion to progress to malignancy. Among estrogen-treated macaques, nearly all endometrial hyperplastic lesions are of the simple type, although focal adenomatous change and atypia are seen occasionally. As in women, evaluation of endometrial hyperplastic changes is greatly facilitated by the use of markers of proliferation (e.g., Ki-67 or PCNA) and markers of estrogen-responsive gene expression (e.g., PR expression).¹

Uterine Neoplasms: The incidence of reproductive tract neoplasms has been reported to be low in female macaques,⁸ but a variety of reproductive tract neoplasms have been reported, most commonly leiomyomas and endometrial polyps.⁹ We have identified endometrial polyps in tamoxifen-treated animals, but otherwise neoplasms have not been induced in the relatively short treatment periods used in our studies.¹

EFFECTS OF PROGESTINS – DIVERGENT EFFECTS ON THE BREAST AND ENDOMETRIUM

Three years of continuous treatment with oral conjugated equine estrogen (CEE) (0.625 mg/woman/day equivalent) induced marked endometrial glandular proliferation with thickening of the

Figure 1



Ultrasonographic measurement of endometrial thickening in the endometrium of monkeys (*Macaca fascicularis*). Panel A: surgically postmenopausal animal. Panel B: surgically postmenopausal animal treated with estradiol (0.5 mg/ woman/day equivalent). Brackets indicate the approximate thickness of the endometrium. Bar = 1 cm.

agents with potential for hormonal activity. In general, “estrogenic” indicators in the endometrium are physiologic and simply reflect those features that are seen in the follicular phase of the cycle (e.g., glandular proliferation), and “progestogenic” features are those that occur in the

endometrium and markedly increased expression of the proliferation marker Ki-67 in glandular epithelial cells of monkey endometrium, from 10+/-4.2% in control animals to 27.9+/-3.8% in CEE-treated animals (Figure 2, uterine and breast Ki-67). MPA treatment alone did not produce any measurable morphologic effect on the endometrium or breast compared to ovariectomized controls, but did induce a marked decrease in PR expression in both the endometrium and the breast.^{1,6} However, the addition of medroxyprogesterone acetate (MPA) (2.5 mg/woman/day equivalent) to CEE antagonized the endometrial epithelial proliferation induced by CEE, reducing Ki-67 expression back down to 10.7+/-3.8% of glandular epithelial cells. In contrast to the protective effects on endometrium, MPA increased the proportion of glandular tissue in the breast and increased Ki-67 expression in the breast epithelial tissue (13.8+/-3.0% in untreated surgically postmenopausal animals, 17.5+/-2.6% in CEE-treated animals, and 23.4+/-2.6% in animals given CEE+MPA). These adverse findings in the breasts of these animals are of particular importance because they were consistent with a subsequent epidemiological finding in women, indicating that progestins may increase the risk of breast cancer when given as part of hormone replacement therapy (HRT).¹⁰ More recently, a review by Bush *et al.* of human epidemiologic studies states that any association between estrogen replacement therapy (ERT) or HRT and breast cancer risk is absent or too small to be adequately characterized by observational studies, despite the strong evidence from many previous studies that lifetime reproductive history is predictive of risk.¹¹ This conclusion, in the face of continued concern by postmenopausal women regarding breast cancer risk, points out the need for further mechanistic studies on hormonal effects on the breast.

EFFECTS OF TAMOXIFEN ON THE BREAST AND ENDOMETRIUM OF MONKEYS

Three years of continuous treatment with tamoxifen (20 mg/

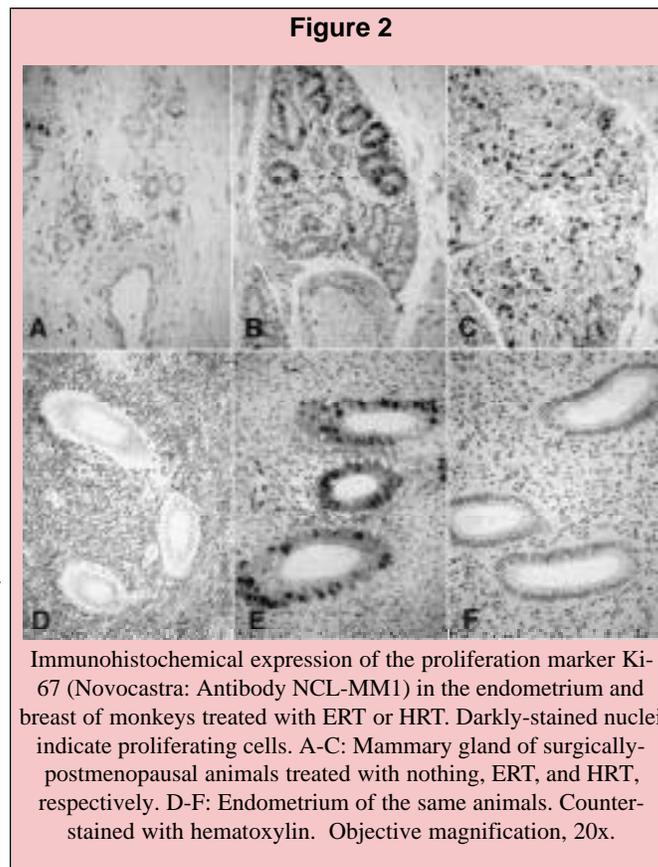
woman/day equivalent) induced cystic endometrial change and endometrial fibrosis in nearly all animals treated.¹ Endometrial polyps were also induced in three of 20 animals. These lesions are similar to those reported in the endometrium of women treated chronically with tamoxifen.¹² Tamoxifen also increased PR expression within endometrial glandular epithelium. PR induction has been reported in the endometrium of women treated with tamoxifen.¹³ Despite the increase in endometrial thickness, tamoxifen did not produce a significant increase in Ki-67 expression in endometrial glands (mean Ki-67 expression was 5.8+/-3.9%, compared to 10+/-4.2% in controls). This dissociation between the morphologic increase in endometrial thickness and the expression of proliferation may indicate either a transient uterotrophic effect, or an anti-apoptotic effect of tamoxifen on the endometrium. Tamoxifen is the archetypal selective estrogen receptor modulator (SERM), and screening for tamoxifen-like effects on the endometrium is an ongoing process in the evaluation of novel SERMs. For some SERMs such as raloxifene, these tamoxifen-like effects have not been seen (unpublished data). For other SERMs (e.g., idoxifene), tamoxifen-like effects have occurred transiently.¹⁴

EFFECTS OF SOY PHYTOESTROGENS: POTENTIAL COMPLEMENTARY EFFECT TO HRT

Soy phytoestrogens (SPEs) are widely marketed as alternatives to HRT for postmenopausal women. SPEs have cardiovascular benefits and may protect women from endometrial and breast cancer.¹⁵ We have previously shown that treatment with SPEs for two months did not induce an estrogenic effect at dietary doses,¹⁶ and that concurrent treatment with SPEs and estradiol for six months resulted in decreased estradiol-induced proliferation, as measured by Ki-67 expression in endometrial glandular epithelium.¹⁷ We have also found recently that the antiproliferative effect of SPEs is not accompanied by down-regulation of PR expression. Thus the antiproliferative effect may be independent of estrogen receptor-mediated events.

SPEs clearly are not mammotrophic or uterotrophic in macaques when given at doses similar to those attained in the Asian diet. Recent work has shown that the estrogenic or anti-estrogenic effect of SPEs is dependent on hormonal context. In monkeys given a hormone replacement dose of estrogen, dietary soy supplementation decreased estrogen-induced proliferation and therefore could potentially decrease the cancer-promoting effect of estrogen.¹⁷ Other studies in rats have indicated that the effects of dietary estrogens and estrogen replacement therapy are interactive and dose-dependent, such that SPEs may increase breast proliferation at low doses of estrogens but may decrease breast proliferation when given in combination with high doses of estrogens.¹⁸ Most recently, we have demonstrated that the estrogen-antagonistic effect of SPEs extends to protection against tumor formation in rats, indicating that dietary soy supplementation may be useful in a "co-HRT" approach.¹⁹

An interesting clinically relevant observation regarding phytoestrogen metabolism is the effect of antibiotic treatment on serum concentrations of phytoestrogens. Monkeys eating a phytoestrogen-rich diet attained a total serum isoflavone concentration of 963+/-116 nM. In contrast, animals that



required antibiotic treatment for diarrheal or other bacterial disease had a reduction in serum isoflavone concentration to 597±91 nM, a 38% reduction. This drug/diet interaction is presumably due to the reduction in bacterial numbers or activity in the intestinal tract. Diarrhea *per se* did not alter serum isoflavone concentrations in these animals, and animals receiving antibiotic treatment for non-enteric conditions also had diminished isoflavone concentrations. This diet/drug interaction could potentially decrease the efficacy of dietary soy supplementation in women taking antibiotics.

EFFECTS OF TIBOLONE: EXPLORATION OF NOVEL MECHANISMS

Tibolone (OrgOD14) is a unique single-agent, tissue-selective steroid for postmenopausal women. Tibolone and its metabolites have estrogenic, progestogenic, and androgenic properties. The estrogenic metabolites provide symptom relief and bone-protective effects, and additional progestagen treatment to protect the uterus is not required because of the progestogenic activity of the delta-4 isomer of tibolone. This isomer is produced locally in the endometrium and prevents endometrial stimulation.²⁰ In addition, tibolone and its metabolites may diminish synthesis of active forms of estrogen in breast epithelial cells²¹ and thus could potentially lower the risk of breast cancer, in contrast to conventional HRT.

Basic and clinical studies with tibolone have been recently reviewed.²² In a recent study, we investigated the effects of tibolone as compared with CEE with and without MPA on the cardiovascular system, mammary gland, and uterus of surgically-postmenopausal monkeys. Drugs were given continuously in the diet for two years, at doses designed to mimic those used clinically in women. Endpoints included histology and histomorphometry of the endometrium and mammary gland, and immunohistochemical measurement of the proliferation marker Ki-67 in endometrial glandular tissue. Treatment with CEE produced an increase in endometrial thickness from < 1 mm to > 3 mm and increased the proportions of proliferating cells in endometrial glandular epithelium from 17% to 62%. This effect was slightly diminished by the addition of MPA. High-dose tibolone (Hi Tib) but not low-dose tibolone (Lo Tib) increased endometrial thickness relative to controls,

and this Hi Tib effect was less than half the effect of CEE+MPA. In contrast to CEE and CEE+MPA, neither Lo Tib nor Hi Tib significantly increased endometrial Ki-67 or mammary lobular area. The lack of a proliferative response of the endometrium to tibolone, coupled with the lower incidence of endometrial bleeding, suggest that tibolone may have advantages over CEE and CEE+MPA regarding endometrial safety and efficacy.²³

CONCLUSIONS

Cynomolgus and rhesus macaques are uniquely suited to studies of multiple chronic diseases of relevance to postmenopausal women. In particular, the unique pathophysiologic features of the primate endometrium and breast require that periclinal studies of hormonally-active compounds be done in monkeys. Studies of the multiple effects of HRTs, SERMs, and phytoestrogens are urgently needed. A cooperative multi-systemic approach to the use of nonhuman primates allows evaluation of novel agents and strategies in a controlled setting in a relatively short period of time while minimizing animal numbers used. With respect to the reproductive tract and breast, results from the monkey model have almost invariably predicted or mirrored human clinical outcomes. Further evaluations of HRTs and dietary phytoestrogens are in progress using these uniquely valuable animals to study issues critical to the health of women.

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Is Premenopausal Protection from Coronary Disease a Myth? Studies in Monkeys Tell the Tale



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INTRODUCTION

The relative sparing of premenopausal women in relation to similarly aged men is a prominent feature of the natural history of coronary heart disease (CHD) and its underlying pathologic process, coronary artery atherosclerosis.¹ The various effects of estrogen are believed to account for most of this phenomenon, which is sometimes referred to as “female protection.”¹ We may question, however,

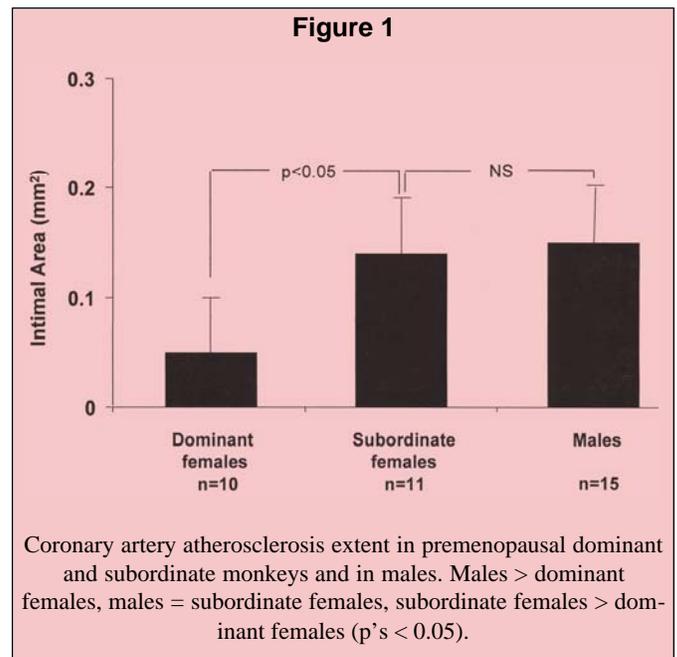
whether such protection truly extends to all premenopausal women. After all, the atherosclerosis that gives rise to CHD progresses over decades. This makes it likely that the clinical events occurring in peri- and postmenopausal women have their beginnings in the premenopausal years. The hypothesis that at least some premenopausal women are at heightened risk for the premature development of atherosclerosis is supported by the most recent report from the Pathobiological

Determinants of Atherosclerosis in Youth (PDAY) study. These data show that one-third of all women under 35 years of age have raised lesions in their coronary arteries.² Another study, using non-invasive imaging, also documents significant focal atherosclerosis in premenopausal women.³

What would put young women at such risk? If estrogen is protective premenopausally, then ovarian abnormalities or failure, by reducing endogenous estrogen, could accelerate atherogenesis in a subset of premenopausal women, thereby accounting for the PDAY observations and predisposing these individuals to CHD in later years. Admittedly, there is currently little direct evidence supporting this suggestion. It is well known, of course, that increased cardiovascular disease risk is observed in relatively young women in association with oophorectomy and early menopause.^{1,4} Furthermore, at least one observational study shows that premenopausal women with angiographically-confirmed CHD have significantly lower plasma estradiol concentrations than do controls.⁵ However, efforts to understand better the influence of endogenous variation in estrogen on the development of atherosclerosis and CHD have been hampered by a paucity of relevant studies in premenopausal women. The reasons for this scarcity of investigations are readily discerned. Ovarian function is relatively difficult to determine in epidemiologic studies. Also, ethical concerns generally preclude the assessment of coronary artery atherosclerosis in asymp-

tomatic individuals. Furthermore, the general assumption that premenopausal women are protected from CHD has led to their exclusion from most investigations in this area.

An alternative research strategy, the one we have employed, involves the use of animal models. In particular, we have focused on female cynomolgus monkeys (*Macaca fascicularis*) – housed in social groups and fed a high-fat diet – to study the initiation and progression of premenopausal atherosclerosis.⁶ These animals are especially useful for such studies because, in addition to having 28-day menstrual cycles that are similar hormonally to those of women, they develop atherosclerotic lesions resembling those seen in people. Furthermore, males of this species typically have more extensive lesions than do premenopausal females. Finally, like humans, these group-living animals are characterized by elaborate patterns of social interaction, including generation-spanning networks of affiliation and well-defined social status relationships and hierarchies. Taken together, the results of our studies tend to discredit the notion that the premenopausal period is pathobiologically benign. Rather, we find that premenopausal females show substantial individual differences in the development of atherosclerosis, with accelerated lesion formation in many animals. The behavioral and hormonal profiles of such “at risk” individuals differ substantially from the characteristics of animals at lower risk for rapid lesion progression. The following paragraphs detail



these findings and explore their relevance for women.

BEHAVIORAL AND HORMONAL INFLUENCES ON ATHEROSCLEROSIS IN PREMENOPAUSAL MONKEYS⁶

In the first of our studies, 30 premenopausal females were housed in social groups of five animals each, with monkeys consuming a moderately atherogenic diet. Repeated behavioral observations were used to determine the outcome of competitive interactions, allowing identification of individuals as ranking higher or lower in their social groups. Averaged over the experiment, animals ranking above their group median in social status were categorized as dominant; the remainder was subordinate. In addition to this behavioral assessment, all females were subjected to daily vaginal swabbing to monitor menses, and across the menstrual cycle we collected blood samples for the determination of plasma estradiol and progesterone concentrations.

Measurement of coronary artery lesions on completion of the study indicated that female protection from atherosclerosis extended only to dominant animals; individual subordinates could not be distinguished from male monkeys housed in social groups and fed the same diet for the same amount of time (Figure 1). Importantly, status-dependent effects on atherosclerosis among females were statistically independent of variation in plasma lipid concentrations and blood pressure. However, we did observe that subordinate females had five times as many anovulatory menstrual cycles and three times as many menstrual cycles characterized by luteal phase progesterone deficiencies (peak plasma progesterone concentrations between 2.0 and 4.0 ng/dl) compared to their dominant counterparts. Not surprisingly, those anovulatory cycles and cycles having luteal phase deficiencies were characterized by lower estradiol concentrations than those recorded during normal cycles. Thus, in comparison to dominant females, subordinate monkeys experienced ovulatory impairment and relative estrogen deficiency.⁷ Subordinate females in the foregoing study also exhibited significant adrenal enlargement and

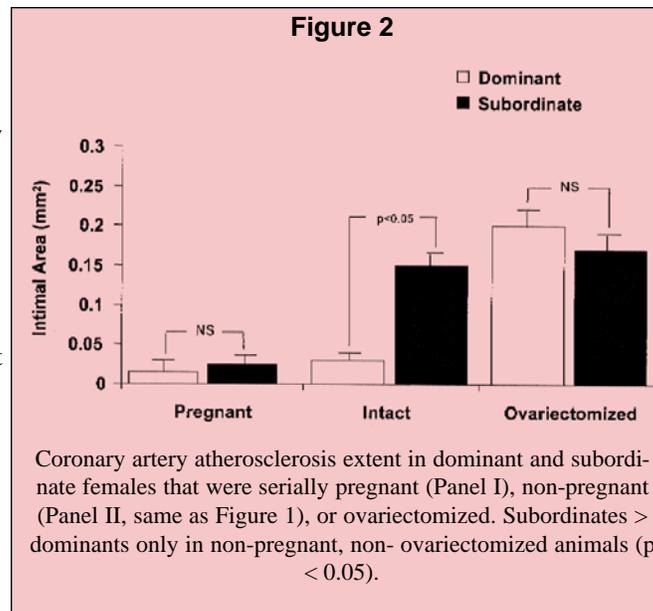
hypercortisolemia in response to a pharmacologic challenge. These data suggest that the animals were under substantial stress. In turn, the association between stress – particularly the stress of social subordination – and ovarian dysfunction is not unique to this species and, in fact, reflects one of the most frequently reported observations in mammalian socioendocrinology.⁸

Based on the ovarian data, we hypothesized that estrogen deficiency was primarily responsible for the worsened atherosclerosis of the subordinate monkeys. Evidence consistent with this hypothesis was provided by two other monkey studies, both conducted in association with the foregoing investigation. In one of these studies, females were housed with reproductively intact males and were allowed to become pregnant serially.⁹ Atherosclerosis was almost completely inhibited among these hyperestrogenic females, irrespective of individual differences in

lowers that treatment with exogenous estrogen should be inhibitory, especially in those animals at highest risk for such deficiency by virtue of their subordinate social status. We tested this hypothesis in 190 premenopausal monkeys, all housed in social groups of five or six animals and consuming a moderately atherogenic diet. Additionally, half of the monkeys were given the triphasic oral contraceptive (OC) TriphasilTM containing ethinyl estradiol and levonorgestrel. At the end of 26 months, we measured atherosclerosis extent in an iliac artery biopsy (as a surrogate for the coronary arteries) from each animal. The animals were then ovariectomized, entered into a three-year “postmenopausal” trial involving treatment with soy plus its isoflavones or conjugated equine estrogens, and were evaluated for extent of coronary artery atherosclerosis.¹²

Animals were again ranked either dominant or subordinate over the course of the premenopausal experiment, based on the outcomes of their competitive interactions. Also, a subset of animals not given OCs was evaluated for ovarian function by vaginal swabbing and blood sampling across the menstrual cycle. The iliac artery atherosclerosis evaluations revealed that untreated subordinates had more extensive atherosclerotic lesions than untreated dominants, as shown in our initial experiment. However, OC treatment inhibited lesion development in subordinates, rendering them indistinguishable from dominants, whether OC-treated or not (Figure 3). Parallel evaluation of plasma lipid concentrations indicated that high density lipoprotein cholesterol

concentrations were suppressed in OC-treated monkeys compared with untreated controls, as well as among subordinates, as compared to dominants. Nonetheless, the protective effect of OC treatment on subordinate monkeys persisted even after statistical adjustment for these differences in plasma lipids. Hence, plaque development was inhibited in OC-treated monkeys despite the reduction in HDLC that accompanied OC exposure. Finally, an evaluation of ovarian function revealed that luteal phase plasma progesterone concentrations were approximately twice as high in dominants as in subordinates; these data are consistent with the conclu-



social status (Figure 2). The other study involved females that were all ovariectomized and thus rendered uniformly estrogen deficient.¹⁰ Here atherosclerosis was extensive in all animals, and again, equivalent in dominant and subordinate monkeys (Figure 2); the practical effect of ovariectomy was to eliminate the usual protection associated with dominant social status.

A subsequent experiment was designed to explore the mechanism mediating the associations among social status, ovarian impairment, and premenopausal atherosclerosis.¹¹ Specifically, if estrogen deficiency potentiates atherosclerosis, it fol-

sion that, as observed in other studies, untreated subordinate animals were ovari-an impaired and estrogen deficient relative to their dominant counterparts.

In summary, this experiment confirmed that the effects of social status on atherosclerosis in premenopausal monkeys are mediated by concomitant ovarian impairment in subordinate animals. Hence, subordinate social status was associated with worsened atherosclerosis among untreated monkeys, whereas treatment with an OC inhibited the development of atherosclerosis in subordinates and, in fact, entirely eliminated the difference in lesion extent between dominant and subordinate monkeys. We may next ask whether these premenopausal effects on iliac atherosclerosis were expressed postmenopausally and whether they extend to the coronary arteries, the site of human clinical interest. The relevant data, briefly reported elsewhere,¹³ show unambiguously that the elevated risk associated with being subordinate, as well as the compensatory protection provided by premenopausal OC exposure, persisted into the postmenopausal period with respect to coronary artery atherosclerosis. Furthermore, the significant persistence of these premenopausal effects occurred irrespective of hormone replacement therapy following ovariectomy. We conclude from these data that premenopausal behavioral and hormonal factors contribute significantly to postmenopausal atherosclerosis.

RELEVANCE TO WOMEN

How do these findings inform our understanding of the natural history of atherosclerosis and CHD in women? Notably, a substantial number of premenopausal women may experience ovarian compromise at some time during their reproductive years.¹⁴ The general term for this compromise is functional hypothalamic hypogonadism (FHH), the manifestations of which range from luteal phase defects with regular menstrual intervals to irregular cycles to amenorrhea. Psychogenic stress is often linked to FHH.¹⁴ We believe that one particular expression of FHH, functional hypothalamic amenorrhea (FHA), offers perhaps the closest parallel between women and the subordi-

nate monkeys described above. A relatively common disorder, FHA in women is associated with abnormal LH pulse generator activity and is accompanied by hypercortisolemia and other neuroendocrine and behavioral indicators of stress.¹⁴

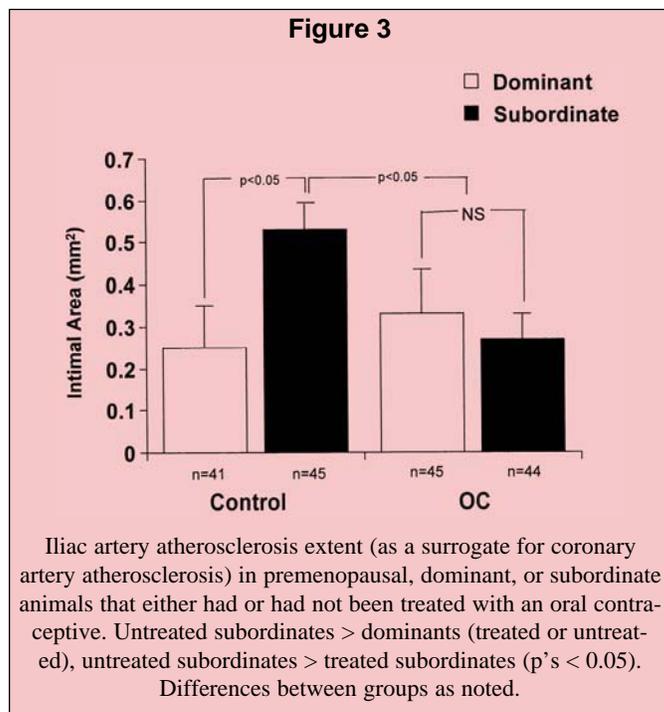
Current data suggest that FHA also may be manifested subclinically. The subclinical expression of FHA may occur frequently and is of particular interest in the current context because it represents a potential marker for pathobiological processes affecting estrogen sensitive tissues, including the skeletal and cardiovascular systems. In one study, for example, disturbed luteal phase function characterized 29% of all menstrual cycles recorded over one year in a sample of 66 premenopausal women thought to be cycling

increased risk of CHD. This is especially true in view of the observation that relatively minor impairment of ovarian function appears sufficient to cause exacerbation of atherosclerosis in monkeys and bone loss in premenopausal women.^{6,15} Ovarian impairment probably has eluded detection as a risk factor for atherosclerosis because it is often occult and because premenopausal women have a low incidence of CHD and thus are generally not recruited for cardiovascular investigations. Nonetheless, surrogate measures for quality of ovarian function (e.g., low circulating concentrations of estradiol) are associated with angiographic evidence of stenotic atherosclerotic lesions and premature CHD.⁵ As a result, we suspect that subclinical FHA is as atherogenic to women as to monkeys. If so, it could be

speculated that the percentage of premenopausal women experiencing accelerated atherosclerosis is much larger than the number clinically diagnosed as amenorrheic or otherwise ovarian impaired, and may contribute substantially to the CHD observed postmenopausally.

The monkey data reviewed here concern the development of atherosclerosis in relation to premenopausal hormonal variation and manipulations. These data suggest that estrogen is antiatherogenic during this period of life, a time during which lesions first begin developing. Such protection may not occur under all circumstances. For example, in the Estrogen Replacement and Atherosclerosis trial, neither unopposed conjugated equine estrogens or equine estrogens plus medroxyprogesterone acetate

slowed the progression of coronary artery atherosclerosis in postmenopausal women with pre-existing disease.¹⁶ These results mirror those of the Heart and Estrogen/progestin Replacement Study, which found that combined hormone replacement therapy (conjugated equine estrogens and medroxyprogesterone acetate) was ineffective in secondary prevention of coronary heart disease.¹⁷ These results are of particular concern because they are derived from controlled, randomized trials. In contrast, results from observational studies largely support the hypothesis that estrogen is protective against CHD.^{18,19} Based largely on the outcome of the controlled trials, the American



normally based on the regular occurrence of menses. Decreases in spinal density at both one and five years were associated significantly with variation in luteal phase function among these otherwise “normally” cycling females.¹⁵ Importantly, 53 of the 66 women in this investigation experienced cycles with abnormal luteal phases at some time during the study year, emphasizing the relatively high incidence of this modest, albeit pathobiologic, degree of impairment.

If ovarian hormones are indeed cardioprotective, women with FHA and subclinical ovarian dysfunction could share with subordinate monkeys a predilection for accelerated atherosclerosis and an

Heart Association recently concluded in a statement for healthcare professionals that estrogen or estrogen/progestin replacement therapy should not be initiated for the sole reason of preventing CHD.²⁰ We believe that the epidemiologic observations on which this recommendation is based in no way obviate the significance of our premenopausal data. Nonetheless, they do provide a strong warning against extrapolating the antiatherogenic effects of estrogen as observed premenopausally to older individuals or those with pre-existing disease.

A SPECULATIVE PRESCRIPTION

We believe that our observations describing the initial development of atherosclerosis, the influence of hormones and behavior on this development, and the predictive relationship between pre- and postmenopausal lesion extent imply a prescription for cardiovascular health in women. First, the data suggest that the community concerned with women's cardiovascular health may want to focus more effort on detecting and regulating premenopausal ovarian dysfunction; that is, a reliance solely on postmenopausal hormonal intervention (at least with respect to cardiovascular disease) may no longer be justified. The data also suggest that the use of OCs to prevent pregnancy and regulate menstrual cycles may have the added benefit of cardioprotection in some women. The final lesson derived from the monkeys is that female protection against coronary heart disease, while perhaps not mythical, is not universal.

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HRT and Coronary Artery Atherosclerosis in Human Beings and Monkeys



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INTRODUCTION

In human populations where coronary heart disease is a major public health problem, its incidence is much lower in premenopausal women than in men of similar age. This sex difference in incidence of coronary events is paralleled by a difference in extent and severity of coronary artery atherosclerosis. It is widely believed that ovarian estrogen is responsible for this relative sparing of women's coronary arteries. However, it remains uncertain whether coronary heart disease risk or atherosclerosis in human beings is influenced by conditions, e.g., such as menopause, that affect endogenous estrogen concentrations. While coronary heart disease risk increases after the menopause, it continues to be controversial whether this represents a direct effect of ovarian senescence and the associated estrogen deficiency or simply a continuation of a linear increase in risk associated with advancing age.

We summarize here the evidence regarding the effects of menopause and estrogen deficiency on atherosclerosis progression and risk of coronary heart disease and describe the usefulness of a monkey model of atherosclerosis in studying the relationship between endoge-

nous estrogen and atherosclerosis progression. We conclude that estrogen deficiency results in an acceleration in the atherosclerotic process and an increase in risk of coronary heart disease, while endogenous or exogenous estrogen inhibits atherosclerosis progression and may reduce risk of coronary heart disease in women without preexisting coronary heart disease.

ESTROGEN AND ATHEROSCLEROSIS IN WOMEN

Few studies have addressed the relationships between endogenous or exogenous estrogen and atherosclerosis in human subjects. There have been three autopsy studies that specifically related age at surgical menopause to degree of coronary artery atherosclerosis. All revealed a trend to worsened atherosclerosis in women who underwent oophorectomy when compared to intact controls. While this difference reached statistical significance in only one of these studies,¹ these data are consistent with the observation of Oliver and Boyd² who showed that women under the age of 35 who underwent bilateral oophorectomy had an eight-fold greater incidence of coronary heart disease before the age of 50 than women who had unilateral oophorectomy.

Three studies related the degree of coronary artery lumen stenosis at coronary angiography to postmenopausal estrogen replacement.³⁻⁵ A 56% to 63% reduction in

risk for developing severe coronary artery stenosis in women who used hormone replacement was observed. Sullivan *et al*⁶ assessed the 10-year survival rate of 2,268 women who had varying degrees of angiographically-defined coronary artery atherosclerosis with respect to whether they used estrogen replacement. In women without demonstrable coronary artery atherosclerosis at the initial angiogram, no statistically-significant difference was found in the 10-year survival rate of women who never used (survival rate of 85%) versus those who ever used (survival rate of 95.6%) estrogen postmenopausally. However, in women with advanced coronary artery atherosclerosis, i.e., more than 70% lumen stenosis at the

initial angiogram, the 10-year survival rate of “never users” dropped to 60% and was significantly less than the 97% survival rate among “ever users.”

These results are consistent with some of the epidemiologic evidence regarding hormone replacement and coronary heart disease risk. Numerous long-term observational studies done in the last 30 years (reviewed in Barrett-Connor and Bush⁷) led many scientists to the conclusion that postmenopausal women who use hormone replacement therapy reduce their risk of myocardial infarction by approximately 50%. However, the recent results of a randomized clinical trial, The Heart and Estrogen/progestin Replacement Study (HERS),⁸ addressing this subject, have

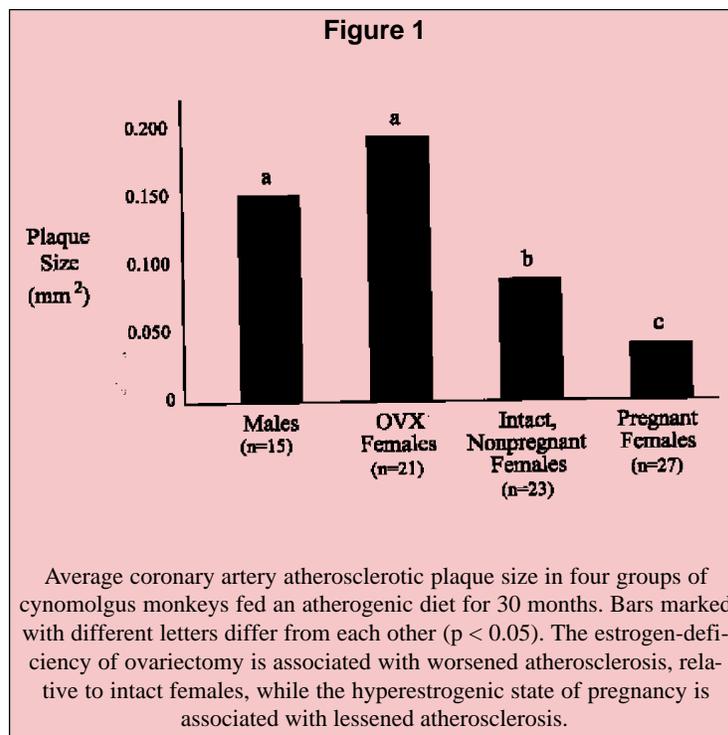
that the results may be specific to combined hormone replacement and to older women with preexisting advanced atherosclerosis.

In agreement with the HERS results are the findings of Herrington *et al*⁹ who found that postmenopausal women with preexisting advanced coronary artery atherosclerosis randomized to treatment with estrogen-only or combined hormone replacement therapy did not differ from a placebo group in the amount of angiographically-defined atherosclerosis present after three years. The results of these two studies have led to widespread speculation that hormone replacement may not be useful for prevention of coronary heart disease. However, these prospective studies

have addressed only the effectiveness of hormone replacement therapy in the secondary prevention of atherosclerosis or coronary heart disease, i.e., the subjects were all women with preexisting coronary heart disease or atherosclerosis. Furthermore, in the case of HERS, only combined hormone replacement was studied. There have been no clinical trials addressing primary prevention of coronary heart disease and atherosclerosis or effects of exogenous hormones in younger and/or healthy older women. Supporting the idea that estrogen may be useful in primary prevention of atherosclerosis and arterial occlusion are the findings of Griewing *et al*¹⁰ who showed that hormone replacement initiated early in

the postmenopausal period is associated with significantly less carotid artery atherosclerosis in later years. This finding is supported by the results of three observational studies.¹¹⁻¹³

Taken together, the autopsy, clinical, and epidemiologic data are consistent with the likelihood that estrogen, either endogenous or exogenous, may be effective in inhibiting the initiation and development of atherosclerosis and preventing coronary heart disease in women without preexisting disease. Restated, it seems likely that endogenous estrogen may suppress atherosclerosis initiation and progression in premenopausal women, and exogenous estrogen may suppress atherosclerosis progression postmenopausally in



cast doubts on the interpretation of the numerous observational studies. This study compared the effects of placebo with those of combined (conjugated equine estrogen plus medroxyprogesterone acetate) hormone replacement on the incidence of myocardial infarction in older postmenopausal women with preexisting coronary heart disease. At the end of four years, there was no overall difference in incidence between the groups. However, when the data were examined year by year, there was evidence of an increase in incidence among the active treatment group in the first year and decreases in subsequent years. Unfortunately, there was no estrogen-only group in this study. Also, it should be considered

women without substantial atherosclerosis. This possibility is further supported by the body of experimental evidence on this subject obtained using animal models.

STUDIES OF ANIMAL MODELS

Avian Species: The initial evidence that endogenous estrogen inhibits atherosclerosis came from a series of studies done in 1950s. Among the important findings from these studies was the resistance of hens to atherosclerosis relative to roosters.¹⁴ Furthermore, ligation of the hen oviduct, which results in a marked hypercholesterolemia, had no effect on this relative resistance of the hen to atherosclerosis while ovariectomy resulted in a marked exacerbation of atherosclerosis.¹⁵ In addition, replacement therapy with physiologic doses of estradiol was found to inhibit progression of atherosclerosis¹⁵ and promote regression of atherosclerosis in this species.¹⁶ Subsequent studies of White Carneau pigeons resulted in similar conclusions regarding inhibitory effects of physiologic doses of estradiol.¹⁷

Cynomolgus Macaque:

In our laboratory, we have employed the cynomolgus macaque to study the effects of endogenous and exogenous estrogen on the initiation and early development of coronary artery atherosclerosis. It is important to note that these subjects are free of advanced atherosclerosis at the initiation of the treatment phase of study. In addition to the fact that this species shares approximately 90% of its genome with human beings, there are several characteristics of this species that render it particularly useful. First, macaques share with human beings a susceptibility to diet-induced atherosclerosis of the main branch coronary arteries. In addition, macaque reproductive physiology is very similar to that of human beings. Female macaques have a 28- to 30-day menstrual cycle and circulating sex hormone patterns which are similar to those of women. Also, a natural menopause occurs in aged macaques.

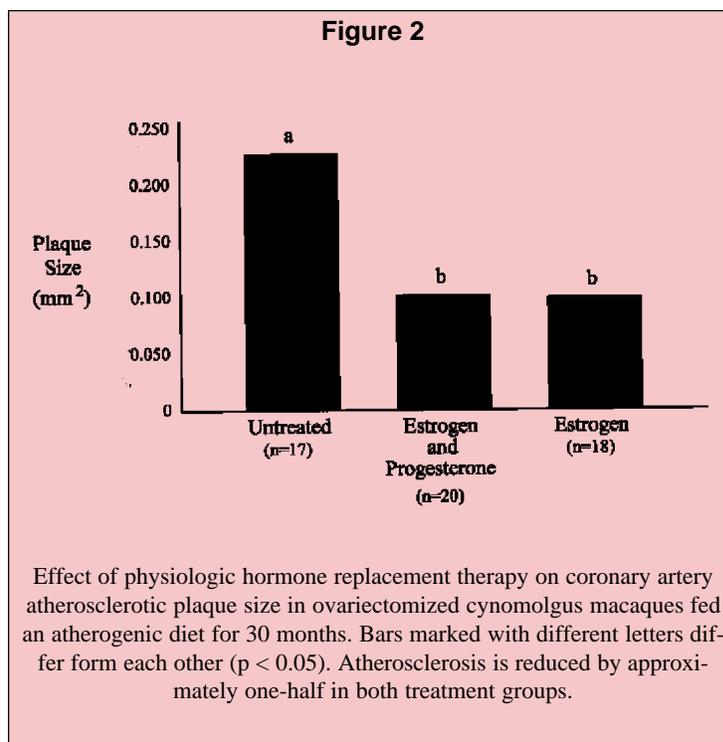
In an initial study of the relationship between endogenous sex steroids and ath-

erosclerosis,¹⁸⁻²⁰ we studied cynomolgus macaques fed an atherogenic diet containing 40% of calories as fat and 0.2% cholesterol for 30 months. There were four experimental groups: males; intact, non-pregnant females; ovariectomized females; and pregnant females. Total plasma cholesterol and high-density lipoprotein (HDL) cholesterol were measured periodically. At the end of 30 months, atherosclerosis extent (cross-sectional plaque area) was determined.

The findings of this study are summarized in Figure 1. As in a previous study, males were found to have more extensive atherosclerosis than females. Males also had significantly lower plasma HDL cholesterol concentrations and higher systolic

tently low plasma estradiol concentrations of less than 20 pg/ml. Atherosclerosis extent was reduced by approximately one-half in intact non-pregnant females, which had much greater plasma estradiol concentrations that fluctuate between 60 and 300 pg/ml depending on the time of the menstrual cycle. Relative to these intact, nonpregnant females, atherosclerosis extent was further reduced by approximately 50% in pregnant females, which experience dramatic elevations in plasma estradiol concentrations in the range of 300 to 1000 pg/ml.

Direct evidence for an inhibitory effect of endogenous estradiol on atherosclerosis is provided by a subsequent study²¹ in which ovariectomized monkeys were randomized to three treatment groups: 1) no hormone replacement, 2) continually-administered estradiol, and 3) continuously-administered estradiol plus cyclically-administered progesterone. Physiologic patterns of plasma estradiol and progesterone concentrations were maintained by administering the hormones in sustained-release subcutaneous Silastic implants. The study lasted 30 months followed by assessment of coronary artery atherosclerosis as in previous studies. The results are summarized in Figure 2. Coronary artery atherosclerosis was inhibited similarly (reduced by approximately one-half) in both hormone replacement groups relative to the untreated control group. Antiatherosclerosis effects of treatment were inde-



pendent of variation in total plasma cholesterol, LDL and HDL cholesterol. Similarly, effects of physiologic hormone replacement on atherosclerosis could not be accounted for by other risk factors (i.e., blood pressure or carbohydrate tolerance). Our findings indicate that physiologic estrogen concentrations inhibit the initiation and early development of atherosclerosis in monkeys. Furthermore, this effect is probably mediated, at least in part, by risk variables other than lipoproteins, blood pressure, or carbohydrate tolerance not assessed in these studies or direct effects on cellular or biochemical effects occurring in arterial intima.

The results of this study provided indirect evidence regarding the effects of endogenous sex hormones on atherosclerosis. Males and ovariectomized females did not differ in extent of coronary artery atherosclerosis and both also had consis-

CONCLUSION

Taken together, the currently available clinical, epidemiologic, and experimental data are consistent with the thesis that endogenous estrogen, or exogenous estrogen in physiologic or pharmacologic concentrations, inhibit the initiation and early progression of atherosclerotic lesions and that the estrogen deficiency of natural or surgically-induced menopause results in accelerated atherosclerosis. This is consistent with the notion that, while hormone replacement therapy may not reduce the risk of coronary heart disease in older postmenopausal women with pre-existing coronary disease, endogenous or exogenous estrogen may inhibit the initiation and progression of atherosclerosis and reduce risk of coronary heart disease in healthy premenopausal or postmenopausal women with limited atherosclerosis.

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Effects of ERT and HRT on Cardiovascular Risk Factors and Coronary Artery Atherosclerosis



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INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in women, particularly those over the age of 50. The increased incidence of CHD observed in women at the fifth decade of life coincides with the onset of menopause, which is associated with significant reductions in estrogen, progesterone, and androgen levels. Preliminary evidence of a role for estrogen in preventing CHD comes from more than 30 observational studies that have found that estrogen replacement therapy (ERT) reduces the risk of CHD in postmenopausal women by 30% to 50%¹⁻⁴ and similarly reduces the extent of atherosclerosis in animal models.³⁻⁷

Although there are numerous potential mechanisms that have been identified supporting these observational and experimental studies, recently published studies from randomized clinical trials have suggested that the relationship between estrogen and progestin use in postmenopausal women and CHD is more complex and that risk or protection may vary depending on clinical circumstances. The Heart and Estrogen/progestin Replacement Study (HERS) Research Group determined in a randomized clinical trial that, among postmenopausal women with an average age of 67 years and preexisting CHD, use of a continuous regimen of conjugated equine estrogen (CEE) and medroxyprogesterone acetate (MPA) compared to placebo was ineffective overall in preventing secondary events, e.g., myocardial infarction or death.⁸ There was an increased rate of CHD and thromboembolism among HRT users compared to placebo in the first year

of follow-up. However, by the fourth year the rate of CHD events in the HRT group was below that of the placebo group. Further analysis of the HERS data indicated that the increased CHD risk was seen primarily in women with low lipoprotein (a) [Lp(a)] levels at baseline, while women with initially high Lp(a) levels seemed to benefit most from HRT as the study progressed.⁹

A second randomized clinical trial, The Estrogen Replacement and Atherosclerosis (ERA) trial, evaluated progression of coronary artery changes in postmenopausal women with angiographically verified CHD at baseline.¹⁰ After about three years of follow-up, neither CEE alone nor CEE in combination with MPA slowed the progression of coronary atherosclerosis as determined angiographically in these women with preexisting disease.

There are many unanswered questions as to why the randomized clinical trials have found results divergent from the observational and experimental studies. The higher rate of events in women in the HERS was limited to the first months of the trial. As discussed by Trudy Bush, Ph.D., M.H.S.,¹¹ a remaining question is whether there was actually an increased rate of events in the treatment group or a decreased rate of events in the control group. However, the initial increased rate in the treated individuals may be real and could go undetected in cohort or observational studies. A complication of clinical trials is that as people develop risk factors, they require intervention. For example, the lipid levels were improved with HRT treatment compared to placebo.⁸ As such, significantly more women in the placebo group were started on statins than women receiving HRT, thus preventing

the ability to have a true placebo group.¹¹ As statins have been shown to be potent drugs for treatment of CHD, this likely affected the power and outcome of the study.

Due to the complexity and cost of the large randomized clinical trials, and perhaps inability to have truly untreated controls, animal studies have many advantages. The cynomolgus monkey has been well characterized as a useful model to study the effects of sex hormones on both cardiovascular risk factors and atherosclerosis.³ Studies in monkeys have found that estrogens are potent inhibitors of primary atherosclerosis progression. Estrogen treatments given parenterally (17 β -estradiol) or orally as conjugated equine estrogen (CEE, 0.625 mg/day human equivalent) inhibit progression of atherosclerosis by 50% to 70%.^{5,6,12} The addition of a progestogen to the HRT therapy has been varied. No effect of intermittent parenteral progesterone was found,⁵ whereas the addition of MPA to the CEE treatment had an intermediate effect in one study⁶ but not in a subsequent study.¹² In this more recent study,¹² CEE and CEE+MPA equally reduced coronary artery atherosclerosis extent by 62%. The major difference between the two studies was the delivery of the hormones. In the second study, the HRT dose was given as a divided dose twice daily. Thus, concentrations of hormones reaching the liver at any one time were essentially halved compared to once daily delivery, but the hormone levels were likely maintained for a longer duration.

Studies from both human and nonhuman primates suggest that the mechanisms of how estrogen provides cardioprotection are complex and likely multifactorial.^{3,4,13} Plasma lipid-dependent mechanisms

account for about 25% of the effect and include increased high density lipoprotein cholesterol (HDL) concentrations, and decreased low density lipoprotein cholesterol (LDL) and Lp(a) concentrations (Figure 1). Estrogen also lowers fibrinogen and improves insulin sensitivity. It has antioxidant activity and suppresses LDL degradation and accumulation in the artery wall. Estrogen also increases vasodilation through endothelium-dependent mechanisms and through effects on the renin-angiotensin system.¹⁴ These lipid-independent mechanisms are thought to account for the remaining 75% of the cardioprotective effects of estrogens.³

ERT/HRT EFFECTS ON LIPIDS AND LIPOPROTEINS

Although there is variability depending on dose and route of administration, estrogens generally cause a decrease in total and LDLC and an increase in HDLC and triglycerides.^{3,4,15} The addition of a progestational steroid may or may not affect lipoprotein concentrations. Results from the Postmenopausal Estrogen-Progestin Interventions (PEPI) Trial indicate that the most favorable effect on HDLC concentrations was in women taking unopposed estrogens.¹⁵ The addition of micronized progesterone to the ERT still resulted in beneficial changes in HDLC; however, the addition of MPA blunted the ERT benefit. Lp(a) levels, on the other hand, are decreased with both ERT and HRT and have been shown to be an independent risk factor for recurrent CHD.⁹

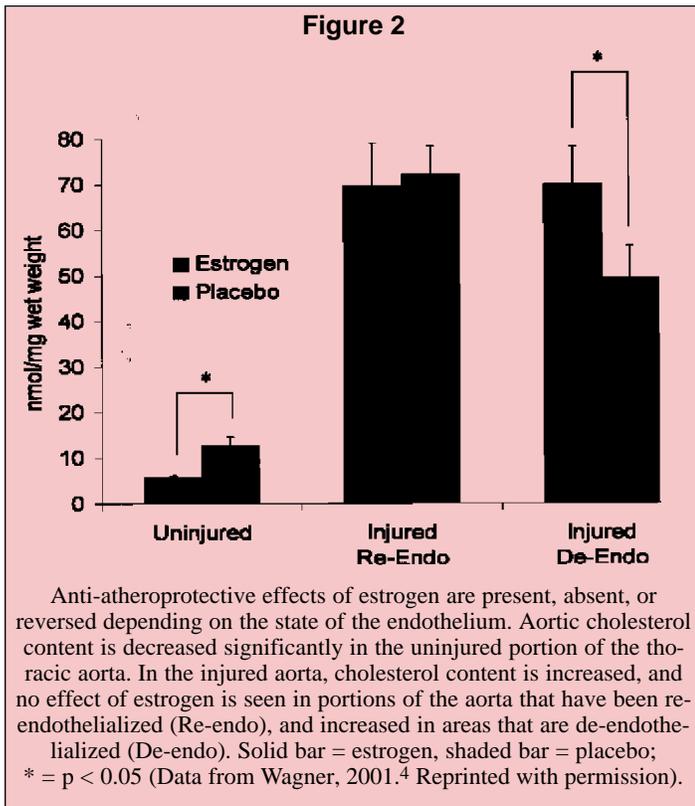
ERT/HRT AND EFFECTS ON THE ARTERY WALL

Estrogens also decrease atherosclerosis by direct effects on the artery wall. One potential site for estrogen effects on atherogenesis is the uptake and metabolism of LDL by cells of the artery. Arterial LDL metabolism can be assessed *in vivo*. LDL particles are radiolabeled and injected into monkeys after relatively short periods of treatment to allow investigation of the hormonal effects prior to treatment effects on atherosclerotic lesions. Using such an approach, it was found that both parenteral and oral estrogens, as well as oral contraceptives, reduce arterial LDL accumulation about 70%.^{3,4} With parenteral HRT, plasma lipid, lipoprotein, or apoprotein concentrations were not affected, suggesting the decrease in arterial LDL metabolism was a direct effect on

Figure 1

Lipid-Independent Mechanisms	Lipid-Dependent Mechanisms
● ↑ Endothelial effects	● ↑ HDL
● ↑ Insulin sensitivity	● ↓ LDL
● ↑ Vascular dilatation	● ↓ Lp (a)
● ↓ Coagulation factors	● ↓ LDL oxidation
● ↓ Coronary artery LDL uptake	

Potential mechanisms of cardioprotection.
Modified from Wagner (2000).³



the artery wall. In a subsequent study using the same techniques, oral esterified estrogens with and without methyltestosterone also decreased coronary artery LDL accumulation. In this study, aortic lipid peroxidation products were decreased, suggesting estrogen's antioxidant activity may be involved with decreased metabolism of modified LDL particles by intimal macrophages.

As LDL, and especially modified LDL, accumulate in the artery wall, monocyte adhesion and chemotaxis are stimulated, resulting in further formation of macrophage foam cells. Estrogen may inhibit atherogenesis by decreasing monocyte adhesion and inhibiting expression of number of adhesion molecules, including vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecules-1 (ICAM-1), and E-selectin.^{4,13} Estrogen may also decrease monocyte chemotaxis, as it was found to suppress monocyte chemoattractant protein-1 (MCP-1). In addition to decreasing monocyte adhesion and migration into the artery, estrogen may also directly affect the metabolism of LDL by macrophages decreasing LDL accumulation and foam cell formation. These effects do not appear to be affected by the addition of progestogens, as combined CEE and MPA as well as CEE and micronized progesterone treatments also decreased E-selectin, ICAM-1, VCAM-1,

increased with both ERT and HRT.¹⁷ It is unclear if the increase in CRP is associated with the early increase in CHD risk described in the prospective trials.

Endothelial damage may increase LDL uptake and atherogenesis. A number of studies have looked at effects of endothelial damage induced by balloon catheter injury in rabbits and how estrogen affects progression of atherosclerosis under these conditions.⁷ As in other studies in rabbits, estradiol significantly inhibited aortic cholesterol in non-injured areas of the aorta; this was independent of plasma cholesterol levels as rabbits were "clamped" at similar plasma cholesterol levels. Areas with injury to the endothelium had increased cholesterol content with no effect of estradiol (Figure 2). Further, in de-endothelialized areas of the injured aorta, estradiol treatment actu-

ally increased cholesterol content.⁷ Thus, the antiatherogenic effect of estradiol was present, absent, or reversed depending on the state of the arterial endothelium. With endothelial damage, there may also be changes in estrogen receptor number or type, which could affect atherogenesis as well as vascular dilation. These effects may be important clues to differences between primary and secondary prevention trials with ERT/HRT.

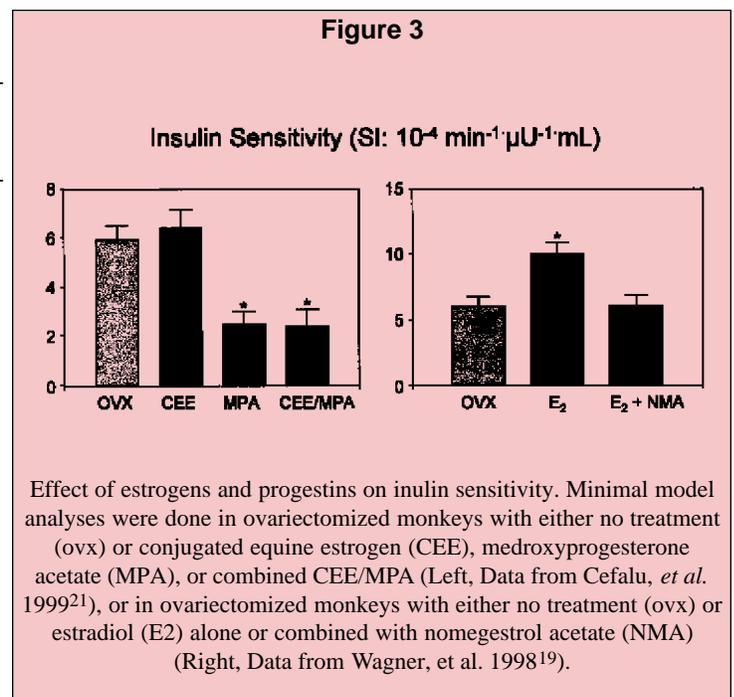
MCP-1, tissue factor antigen, and plasminogen activator inhibitor-1.¹⁶ In addition, ERT improves vasodilation through increases in nitric oxide production and decreases in angiotensin-converting enzyme activity.^{13,14} The effects on markers of inflammation, hemostasis and fibrinolysis inhibition, and vascular relaxation may provide additional non-lipid-mediated mechanisms for cardioprotection. While a number of markers of inflammation are decreased with ERT/HRT, C-reactive protein (CRP) is

ally increased cholesterol content.⁷ Thus, the antiatherogenic effect of estradiol was present, absent, or reversed depending on the state of the arterial endothelium. With endothelial damage, there may also be changes in estrogen receptor number or type, which could affect atherogenesis as well as vascular dilation. These effects may be important clues to differences between primary and secondary prevention trials with ERT/HRT.

ERT/HRT EFFECTS ON CARBOHYDRATE METABOLISM AND BODY COMPOSITION

Estrogen and progestogens affect insulin resistance, which may affect risk of both CHD and diabetes. Changes in body weight and body fat may contribute to the increased insulin resistance after menopause. ERT tends to prevent the weight gain, in both women^{15,18} and monkeys,⁴ primarily by reducing abdominal fat. Changes in body fat may explain, in part, some of the changes in insulin and glucose metabolism with ERT.

Some studies of ERT have reported lower fasting glucose and insulin concentrations¹⁵ or improvement in insulin sensitivity,^{19,20} whereas others have not.²¹ The PEPI Trial¹⁵ reported small but significant decreases in fasting glucose and insulin concentrations in women taking primarily CEE with or without a progestin. However, post-challenge glucose levels tended to increase with treatment and were greatest in those treated with MPA. Likewise,



we²¹ and others²⁰ have reported decreased insulin sensitivity with MPA (Figure 3). However, even nomegestrol acetate, which lacks androgenic activity, diminished the beneficial effects of estradiol on insulin sensitivity (Figure 3).¹⁹ This is not unexpected, as increased progesterone concentrations during the luteal phase of the menstrual cycle also decrease insulin sensitivity compared to the follicular phase.²²

Unlike most chronic diseases, the incidence of diabetes mellitus has been steadily increasing, particularly in older women. This is especially important for women, since diabetes appears to be a greater risk factor for CHD in women than men. Although many ERT studies have excluded diabetic women from the study population, retrospective reports from the Nurse's Health Study have found that ERT users appear to have a reduced relative risk (0.80) of becoming diabetic compared to non-estrogen users.²³ Importantly, CHD risk in diabetic women is also reduced with ERT.²⁴

SUMMARY

The bulk of the experimental and observational data suggest beneficial effects of ERT/HRT on CHD risk factors. Further, these beneficial effects on risk factors result in reduced atherosclerosis in nonhuman primates. The early increase in CHD events in the HERS study has not been noted in animal studies. It is possible that older women who have not been exposed to estrogens for many years may be more sensitive to some estrogen effects, and lower doses of ERT/HRT may be needed. Recent reports suggest that lower hormone doses maintain beneficial effects on lipoproteins and coagulation factors comparable to standard doses.²⁵ These beneficial findings are very promising in light of the improvements in CHD risk and decreased stroke risk reported with low dose estrogens (0.3 mg CEE) compared to doses of 0.65 mg and 1.25 mg CEE.² There are many questions left unanswered at this point. Hopefully some of the answers may come from the Women's Health Initiative which is a large, prospective trial assessing both ERT and HRT. The age range is also relatively large and may be able to determine if older women respond differently to ERT/HRT than younger women. Further research is also needed to determine if lower hormone doses may be equally

atheroprotective without increasing risk of thrombotic and inflammatory complications.

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Effects of Estrogens, Progestogens, and Phytoestrogens on Vascular Reactivity



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INTRODUCTION

Estrogens and progestogens regulate the function of organs and tissues throughout the body. Relatively recently, it has been discovered that sex hormones regulate the function of non-reproductive tissues such as bones and arteries. Effects of sex hormones on these tissues have implications for the pathophysiology of osteoporosis and coronary heart disease (CHD). Besides well-known effects of sex hormones on risk factors of CHD (plasma lipids and lipoproteins), sex hormones may have direct effects on the artery wall that affect atherogenesis and vasodilation and vasoconstriction (vascular reactivity). The subject of this report is to review the action of mammalian sex hormones (estrogens and progestogens) and phytoestrogens (genistein) on vascular reactivity and to relate these effects to coronary artery disease.

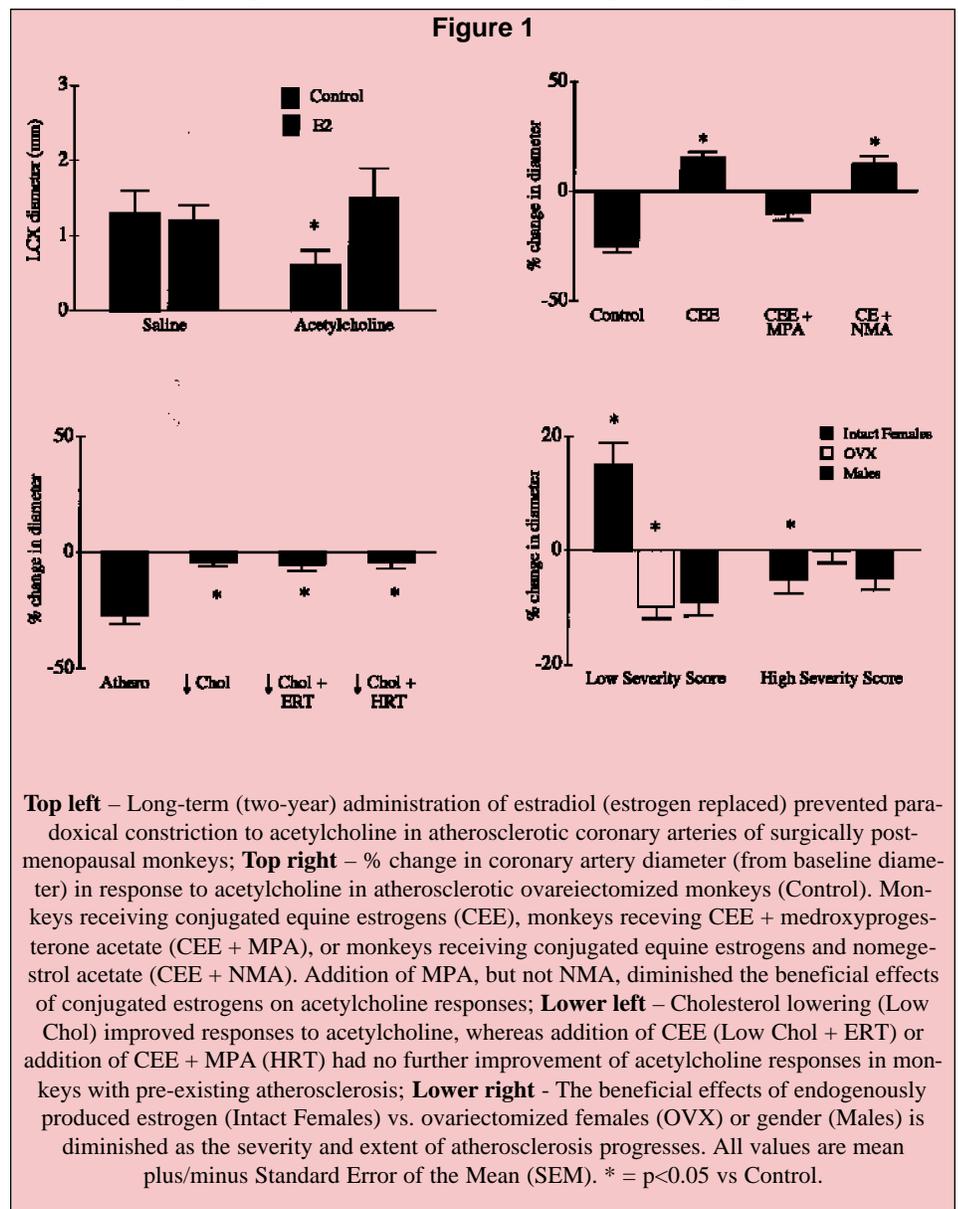
ESTROGENS AND VASCULAR REACTIVITY

Over a century ago, MacKenzie (reviewed in Williams *et al.*¹) reported that menstrual cycle and pregnancy could cause changes in the degree of hyperemia and vascularity of mucous membranes in women. In the late 1980s, Gisclard *et al* and Miller *et al* reported that estrogens

might affect vascular reactivity through actions on vascular endothelial cells (possibly through effects on nitric oxide metabolism).¹ Since publication of these initial studies, many different investigators have examined potential mechanisms by which estrogens affect vascular reactivity. High concentrations of estrogens may promote vasodilation through endothelium-independent ion channels. Studies by Miller *et al* and Jiang *et al* suggest that estrogens inhibit endothelin-1 mediated constriction (reviewed in Williams *et al*¹). Studies by Hayashi *et al*² have examined further the potential role of nitric oxide as a central mediator by which estrogens promote vasodilation. When evaluated in whole, it would appear that estrogens can affect both smooth muscle-mediated and endothelium-mediated dilation depending on dose.

We reported that long-term (two-year)¹ and short-term (20-minute) administration of physiologic doses of estradiol promotes endothelium-mediated dilation of atherosclerotic coronary arteries in nonhuman primates (Figure 1, top left). Both long-term and short-term effects of estradiol were confirmed in studies using postmenopausal women.^{3,4} Results of these studies suggest that one possible mechanism by which estrogens may reduce coronary events is by direct vasodilatory effects on atherosclerotic coronary arteries.

Interaction with Progestogens: A progestogen is usually added to estrogen replacement therapy (ERT) to reduce the increased risk of endometrial cancer associated with unopposed estrogen. However, added progestins may have harmful effects on plasma lipoprotein concentra-



tions and have been speculated to diminish the beneficial effects of estrogens on coronary heart disease risk. We have reported that medroxyprogesterone acetate diminishes the beneficial effects of conjugated equine estrogens on coronary artery reactivity¹ (Figure 1, top right). Miyagawa *et al*⁵ have reported that naturally occurring progesterone does not diminish estrogenic effects on coronary artery reactivity. Additionally, we have reported that not all progestins (e.g., nomogesterol acetate) diminish estrogenic effects on coronary artery reactivity (Figure 1, top right).¹ However, these animal studies must be balanced with results of studies such as that by Arrowood *et al*⁶ indicating that medroxyprogesterone acetate does not diminish estrogen-induced flow-mediated dilation in postmenopausal women. Thus, the question of whether progestogens affect vascular reactivity or work to diminish the effects of estrogen on vascular reactivity remains unanswered.

Effects of Atherosclerosis Progression:

While it seems clear that estrogens and progestogens affect vascular reactivity of both nonatherosclerotic arteries and arteries with moderate amounts of atherosclerosis, results of the HERS trial⁷ suggest that sex hormones may not be as effective in women with established, advanced atherosclerotic disease. In fact, two studies from our laboratories support this concept. In the first study, neither ERT nor hormone replacement therapy (HRT) improved endothelium-mediated dilation of coronary arteries of monkeys with established atherosclerosis⁸ (Figure 1, lower left). In a second study, endothelium-mediated dilation of coronary arteries was progressively diminished in premenopausal female monkeys and ovariectomized monkeys

as atherosclerosis became increasingly severe (increased atherosclerosis extent, necrosis, and mineralization)⁹ (Figure 1, lower right).

Results of these studies call into question the effectiveness of sex hormones to have an effect on the risk of coronary heart disease in women with advanced atherosclerotic disease. Alpasan *et al* recently reported that impaired endothelium-mediated dilation of arteries was associated with later coronary events.¹⁰ Thus, it remains a possibility that sex hormone effects on vascular reactivity in patients with early atherosclerotic disease may help predict event outcome.

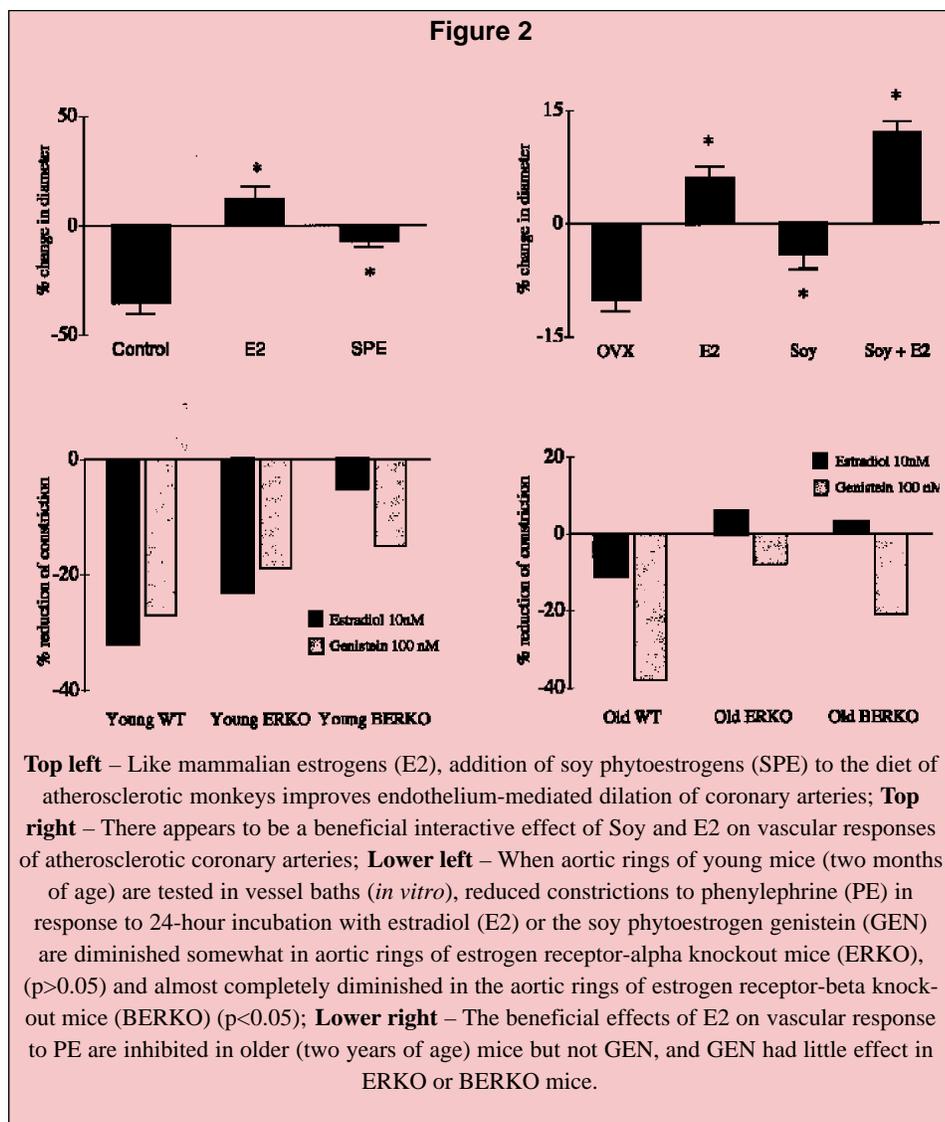
Phytoestrogens and Vascular Reactivity: Some legumes, such as soybeans, produce compounds that bind with estrogen receptors. These compounds are isoflavones that are referred to as phytoestrogens. Similar to studies done with estradiol, both short-term administration of genistein (a phytoestrogen) and long-term con-

sumption of soy protein improved impaired endothelium-mediated dilation of atherosclerotic coronary arteries¹¹ (Figure 2, top left). Interestingly, consumption of soy phytoestrogens seem to interact with administration of estradiol to promote endothelium-mediated dilation of atherosclerotic coronary arteries,¹² suggesting complimentary mechanisms of action (Figure 2, top right).

Estrogen Receptors and Vascular Reactivity: Estrogens diffuse in and out of cells but are retained with high affinity and specificity in target cells by an intranuclear binding protein termed the estrogen receptor (ER).¹³ To date, two ERs have been identified (ER- α [ER- α] and ER- β [ER- β]). Studies of the receptor tissue distribution and expression pattern indicate that ER- α has a broad expression pattern, whereas ER- β has a more focused pattern with high levels in the ovary, prostate, lung, and hypothalamus.¹⁴ Both ER-subtypes are found in coronary arteries.

However, it remains unclear if estrogen receptors modulate vasodilation and, if so, which of the estrogen receptor subtypes predominate. Results of studies support the hypothesis that ER- α mediates, in part, nitric oxide-mediated vascular reactivity.¹⁵ Results of other studies indicate that ER- β may modulate vascular reactivity through Maxi-K channel activity.¹⁶ Still other studies report a potential role of both ER subtypes in mediating vascular physiology, depending on ER relative density and activity.^{17,18}

One of the problems with interpreting these seemingly con-



flicting studies is that no side-by-side comparisons have been made examining the independent effects of ER subtypes using similar methodology and similar doses of estrogens. Thus, side-by-side studies of mice with targeted disruption of either ER- α or ER- β were used to compare the effects of ER subtypes on vascular reactivity.

Age, Estrogens, and Vascular Reactivity: The incidence of myocardial infarction is higher in older individuals even with the absence of cardiovascular risk. With normal aging, several functional and structural changes in the vasculature, including an age-associated decline in vasodilation,¹⁹ occur. Of question is whether and to what extent mammalian and plant estrogens may preserve an age-associated decline in vascular reactivity.

Preliminary studies in our laboratories with mice indicate that in young mice (one to two months), E2 reduced constriction to phenylephrine (PE) by 32% in wild type (WT) ($p < 0.05$ vs. control), 23% in estrogen receptor-alpha knockout (ERKO) ($p < 0.05$), and 5% in estrogen receptor-beta knockout (BERKO) mice ($p > 0.05$) (Figure 2, Lower left). In old mice of the wild type (one to two years old), E2 did not affect vascular responses to PE (all p -values > 0.5). In young and old mice, genistein (GEN) reduced constriction to PE but had little effect in ERKO or BERKO mice (Figure 2, lower right). So in this study, it is concluded that aging inhibits the beneficial effects of E2 to a greater extent than GEN on vascular response to PE. Both ER- α and ER- β play roles in modulating these responses, but it also showed that there are ER-independent mechanisms modulating the effects of GEN on vascular reactivity other than through ER. Figtree *et al* indicated that genistein shifted the calcium concentration curve to the right, which suggests it is acting via a calcium antagonistic mechanism to cause relaxation of coronary arteries.²⁰

Estrogens and Myocardial Ischemia: While it is fairly well established that estrogens affect vascular reactivity, it is less clear if effects of estrogens on coronary artery reactivity affect myocardial ischemia. As reported earlier in this review, estrogens promote acetylcholine-induced vasoreactivity and flow-dependent dilation of both large epicardial and smaller resistance size coronary arteries by approximately 30%.¹ This amount of

“improvement” in coronary vasodilation is associated with reduced risk of cardiovascular events.²¹ Rosano *et al*^{22,23} and Albertsson *et al*²⁴ have reported that acute administration of estradiol lessens symptoms (increases time to angina, time to ST-segment depression, and exercise time) of exercise-induced myocardial ischemia in women with syndrome X. Therefore, it is possible that estrogens may affect the incidence of coronary events.

Recently, we reported that treatment of atherosclerotic ovariectomized monkeys with conjugated equine estrogens promotes both endothelium-mediated dilation of coronary arteries and reduces the incidence of dobutamine-induced ischemia.¹² What was not determined was if the effect of estrogen on myocardial ischemia was due to a direct effect on the myocardium or mediated indirectly through its effects on vascular reactivity. Either way, it seems that estrogen may reduce the incidence of heart-rate-induced myocardial ischemia (angina). Whether or not estrogen can affect the incidence of unstable angina is unknown.

CONCLUSIONS

A preponderance of experimental evidence (both in animal models and in women) indicates that estrogens (both mammalian and plant) promote dilation and inhibit constriction of coronary arteries. There is also evidence of estrogens reducing exercise-induced myocardial ischemia. Thus, estrogens appear to have a biological role on the pathophysiology of coronary artery disease. What remains is the question of whether estrogens affect the incidence and prevalence of coronary artery disease in women. While it would appear that estrogens may not affect coronary artery pathophysiology in older women with pre-existing coronary artery disease, it remains unclear if and to what extent estrogens affect coronary arteries (vascular reactivity, atherogenesis) in primary prevention. Such research may help define the degree to which ERT or HRT may affect the incidence of coronary heart disease in postmenopausal women.

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Effects of SERMs, Tibolone, and Soy Isoflavones on Cardiovascular Risk Factors and Coronary Artery Atherosclerosis of Surgically Post-Menopausal Monkeys



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INTRODUCTION

Less than a century ago, women rarely lived beyond the menopause. Today, women live 35 or more years beyond the menopause and expect a satisfactory quality of life during those years. There has evolved a reasonable consensus that some form of estrogen replacement postmenopausally is important for many women in order to maintain normal cognitive function, inhibit progression of coronary artery atherosclerosis, reduce risk of vertebral and hip fractures, and sustain normal genito-urinary function. Despite these needs for estrogen replacement (ERT), most (80%) postmenopausal women do not take ERT, primarily because of fear of breast cancer and, to a lesser extent, side-effects of the progestin required to prevent endometrial cancer.

Consequently, there has been a major effort to identify therapeutic alternatives that would have the benefits of traditional ERT without its adverse effects on breast and endometrium. Currently, there are three classes of interventions that may meet some of those objectives. One of these is the synthetic selective estrogen

receptor modulators (SERMs) of which raloxifene is the only one now in clinical use. Another class is the naturally occurring SERMs, with soy phytoestrogens being best studied, and the third class is target tissue-selective steroids that differ from SERMs in that their multiple tissue effects result from distinct metabolites of a primary molecule, tibolone being an example. We review here the cardiovascular effects of these three interventions based on our studies of the cynomolgus monkey model.

RALOXIFENE

Raloxifene lowers the total plasma cholesterol concentrations of rats, and when given to postmenopausal women, decreases low-density lipoprotein cholesterol (LDLC) concentrations modestly (12% to 15%).¹ Raloxifene also lowers plasma homocysteine concentrations but had no effect on high-density lipoprotein cholesterol (HDLC) concentrations. The modest reductions in LDLC concentrations prompted clinical investigators to speculate that raloxifene would be cardioprotective.

During the period from 1995 to 1997, we were fortunate to have had an opportunity to conduct a randomized trial, using surgically postmenopausal cynomolgus monkeys (n=84), designed to determine whether raloxifene had the same effects as traditional estrogen replacement therapy in inhibiting coronary artery atherogenesis.² The effects of raloxifene on the plasma lipid concentrations of those monkeys were generally comparable to those reported in postmenopausal women treated with raloxifene (i.e., reductions in LDLC and no significant effect on HDLC) (Figure 1 A). There was no evidence that raloxifene inhibited the progression of coronary artery atherosclerosis as compared to the placebo group (Figure 1 B). In that same trial, conjugated equine estrogen (CEE) treatment was used as the comparator group. Treatment with CEE resulted in about a 70% reduction in coronary artery plaque size relative to that in the placebo group. The results of that study suggested to us that it was unlikely that raloxifene would be cardioprotective.

At a recent meeting of the American College of Cardiology, a presentation was made on behalf of the investigators in the Multiple Outcomes of Raloxifene Evaluation (MORE Trial) concerning the cardiovascular events noted in that trial. The

MORE Trial enrolled 7,705 osteoporotic postmenopausal women with a mean age of 67 years. They were randomized to placebo or raloxifene at a daily dose of 60 mg or 120 mg each day. The interim report is based on three years of treatment.

There was no effect of raloxifene treatment on cardiovascular events. There were 2,557 women treated with the 60 mg dose of raloxifene with a relative risk for any cardiovascular event of 0.89 (confidence interval 0.64, 1.25). There were 2,572 women treated with the 120 mg raloxifene dose with a relative risk of any cardiovascular event of 1.00 (confidence interval 0.72, 1.39). The relative risk for coronary events was 0.88 (confidence interval 0.56, 1.39) at the 60 mg dose and 1.10 (confidence interval 0.72, 1.70) at the 120 mg dose. We view the three-year result as being consistent with our finding of a lack of an effect of raloxifene on coronary artery atherosclerosis in the cynomolgus monkey model.

SOY AND SOY PHYTOESTROGENS

Some plants, primarily the legumes, contain compounds referred to as phytoestrogens (also called isoflavones). They are so named

because they bind to the estrogen receptor, very weakly to ER- α but with an affinity that is 87% of that of estradiol for ER- β . Soy protein is a particularly rich source of phytoestrogens containing 2 to 4 mg/gram of protein. The phytoestrogens of soybeans are genistein, daidzein, and glycitin. The phytoestrogens have both estrogen agonist and antagonist properties and are thus considered natural SERMs.

For more than a decade we have researched the effect of soy with and without its phytoestrogens on the brain,

cardiovascular system, bones, breast, and uterus of surgically menopausal cynomolgus monkeys. We have found the soy phytoestrogens to be estrogen agonists for brain in studies with rats³ and have evidence for antagonistic effects for breast and uterus in monkeys.⁴ This brief review does not permit us to elaborate on the non-cardiovascular tissues.

It has been known for more than 50 years that feeding soy protein in place of animal protein lowers the plasma lipid concentrations of both human beings and experimental animals. Our studies with

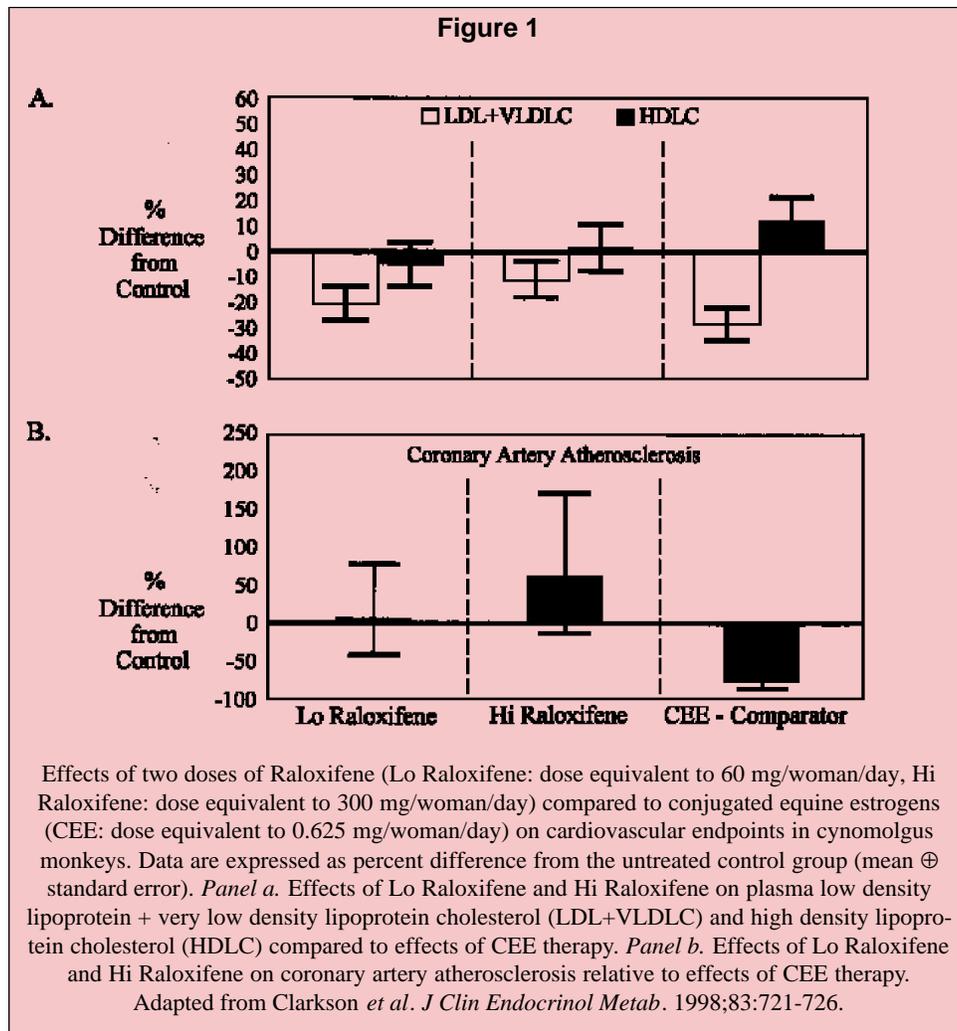
Soy (+) with Soy (-), the high-density lipoprotein cholesterol (HDL) concentrations of the monkeys fed Soy (+) are higher than those fed Soy (-). Similarly, the low-density lipoprotein cholesterol (LDL) concentrations are much lower in monkeys fed Soy (+) compared with Soy (-).^{5,6} We were inclined to interpret those observations as evidence that the lipid lowering property of soy protein is related in a major way to the presence of the soy phytoestrogens or isoflavones. We recently completed a study that contradicts that theory, since we have been unable to

restore the plasma lipid lowering effect of Soy (-) by adding back its isoflavones. Further evidence against our initial interpretation are several clinical reports suggesting that plasma lipid concentrations of women are consistently lowered by feeding isolated soy protein with isoflavones but not by feeding pills of isolated soy isoflavones.⁷ The cardiovascular beneficial components of soy protein remain an enigma and will be the subject of intensive research over the next few years not only by our own group but others.

Coronary artery vascular reactivity refers to the ability of the coronary

cynomolgus monkeys have attempted to determine the extent to which the lipid lowering properties of soy protein relate to the presence of the phytoestrogens or isoflavones and how much relates to effects of the soy peptides. Our general approach has been to compare intact soy protein with soy protein that has been alcohol washed to remove the phytoestrogens. In our research we have referred to the intact soy protein as Soy (+) and to the alcohol washed soy protein as Soy (-). Consistently, when we have compared

arteries to dilate when necessary. This dilation is largely endothelium-mediated via the production and release of nitric oxide. Abnormal coronary artery vascular reactivity can be associated with anginal pain and increase the likelihood of coronary events. Soy (+) but not Soy (-) has been shown to prompt coronary artery dilation following acetylcholine perfusion, the extent of dilation which is generally equivalent to that produced by exogenous estrogen treatment. Interestingly, favorable vascular reactivity responses to soy



are dependent on the presence of some amount of estrogen. There seems to be an additive and/or interactive effect between the presence of estrogen and soy phytoestrogens in prompting coronary artery dilation.⁸

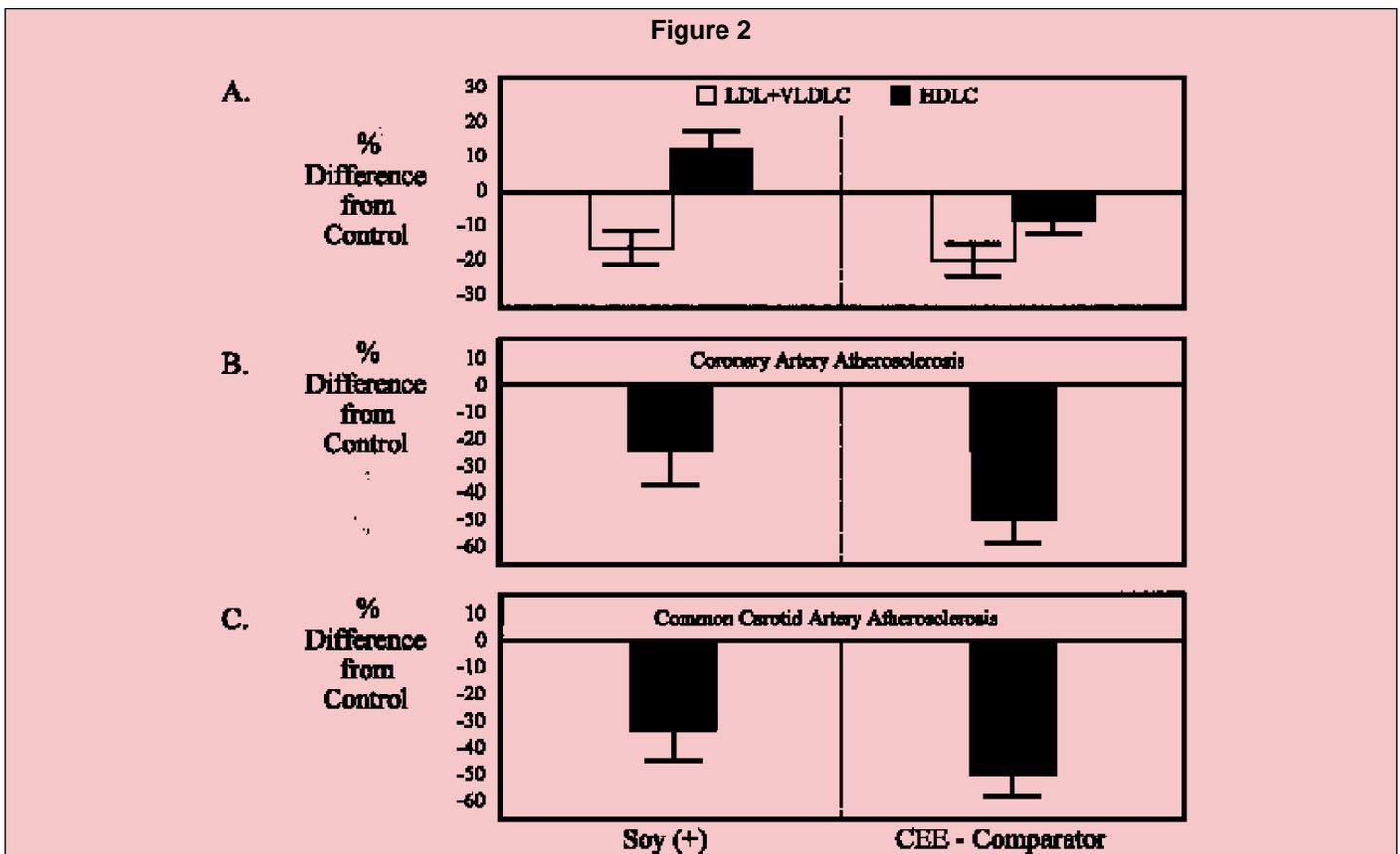
We have also studied the effect of soy on coronary artery blood flow following intracoronary artery platelet aggregation. Soy protein inhibits platelet activation, and it has been reported that the phytoestrogens of soy protein have the capacity to inhibit serotonin uptake by platelets. Since we were well aware that atherosclerosis augments the constrictor response of coronary arteries to serotonin, we undertook a study to determine if feeding diets containing Soy (+) would inhibit platelet-mediated constriction of atherosclerotic arteries following intracoronary artery aggregation of platelets by collagen infusion. The Soy (+) treatment inhibited the reduction in coronary artery blood flow

following the collagen-induced platelet aggregation and serotonin release.⁹ Those studies probably have high clinical relevance for human subjects with pre-existing and symptomatic coronary heart disease.

Finally, we have completed and reported on a randomized, prospective trial using our cynomolgus macaque surgical postmenopausal model and with pathologic endpoints that involved quantification of atherosclerosis.¹⁰ We compared the effect of alcohol washed soy [Soy (-), n=56] as the control group with non-alcohol washed soy [Soy (+), n=59]. We used as a comparator group monkeys treated with conjugated equine estrogens (CEE, n=62) at a dose for monkeys that would be comparable to a dose of 0.625 mg/day for women. Over the three-year trial the monkeys treated with Soy (+) had a more favorable lipid profile than did those in the Soy (-) or the CEE group.

The more favorable lipid profile relative to Soy (-) included higher plasma concentrations of high-density lipoprotein cholesterol and lower plasma concentrations of very low-density lipoprotein plus low-density lipoprotein cholesterol (LDL+VLDLC) (Figure 2 A).

Treatment with CEE, the comparator in this study, resulted in a marked inhibition in the development of coronary artery atherosclerosis. The effect of the diet containing the soy phytoestrogens on coronary artery atherosclerosis was intermediate between the effect of CEE and the control group (Figure 2 B). Interestingly, the effect of the diet containing soy phytoestrogen on common and internal carotid artery atherosclerosis was as large as the inhibitory effect of the CEE treatment (Figure 2 C – common carotid artery data shown). It would have been helpful if the study had included a second type of control, specifically one contain-



Effects of soy with phytoestrogens [Soy(+), phytoestrogen dose equivalent to 129 mg/woman/day] compared to conjugated equine estrogens (CEE: dose equivalent to 0.625 mg/woman/day) on cardiovascular endpoints in cynomolgus monkeys. Data are expressed as percent difference from the control group fed alcohol-washed, phytoestrogen-devoid soy protein (mean ± standard error). *Panel a.* Effects of Soy(+) on plasma low density lipoprotein + very low density lipoprotein cholesterol (LDL+VLDLC) and high density lipoprotein cholesterol (HDLC) compared to effects of CEE therapy. *Panel b.* Effects of Soy(+) on coronary artery atherosclerosis relative to effects of CEE therapy. *Panel c.* Effects of Soy(+) on common carotid artery atherosclerosis relative to effects of CEE therapy.

Adapted from Clarkson *et al. J Clin Endocrinol Metab.* 2001;186:41-47.

ing animal protein such as casein/lactalbumin. Based on several other studies, it is our impression that the amount of coronary artery atherosclerosis that occurred in the Soy (-) group was less than that we would have expected to have seen in a group fed an animal protein diet such as casein/lactalbumin. Given that the Soy (+) effect was much less robust than that of the CEE treatment, one might speculate that the most effective primary prevention regimen for women might be both low-dose estrogen and soy phytoestrogens.

Much remains to be done relative to the effect of soy and its various components on cardiovascular disease of aging females. In particular, we must have a better understanding of the role of the various soy peptides and a better understanding of the relationship between the

dose of soy phytoestrogens and coronary artery atherosclerosis.

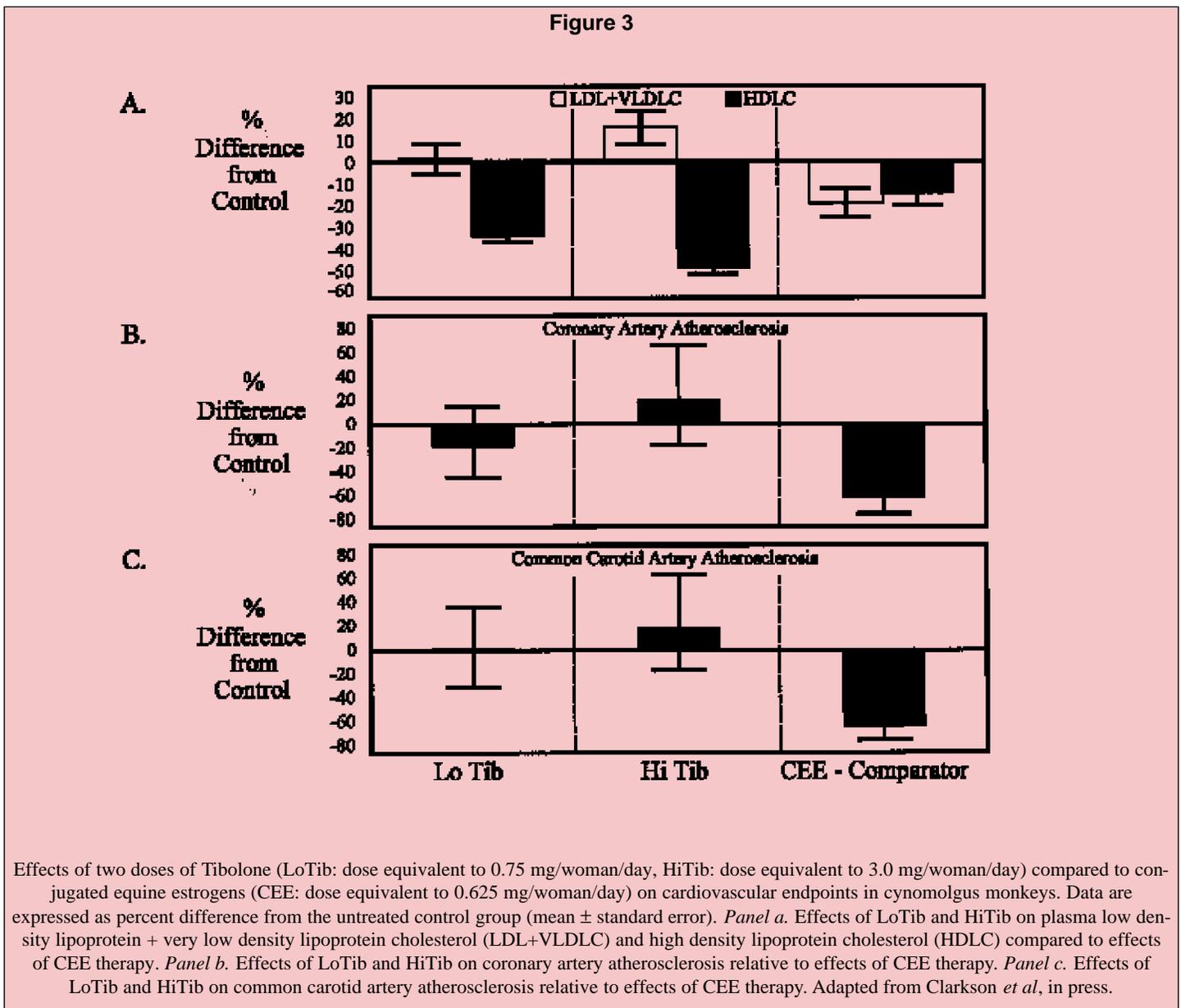
TIBOLONE

Tibolone has been used widely in several countries for the treatment of menopausal symptoms and for the prevention of postmenopausal osteoporosis. Among human and nonhuman primates, tibolone is metabolized into three biologically active metabolites; the 3-beta hydroxy metabolite and the 3-alpha hydroxy metabolite having estrogen agonist properties, while the delta-4 keto isomer has progestogenic and androgenic effects. The delta-4 isomer, produced primarily within the endometrium, protects the endometrium from the agonist effects of the two estrogenic metabolites.

Although tibolone treatment of post-

menopausal women has some beneficial effects on plasma lipid/lipoprotein concentrations (reductions in plasma triglyceride (TG) and lipoprotein(a) [Lp(a)] concentrations), concern has arisen about its "cardiovascular safety" because of reductions in plasma concentrations of high density lipoprotein cholesterol concentrations (HDLC) following both short- and long-term treatment.¹¹ Crona *et al*¹² reported that tibolone treatment reduced HDLC by about 30%. The same magnitude of HDLC reduction following tibolone treatment was confirmed in a more recent study by Bjarnason *et al*.¹¹

We determined that postmenopausal cynomolgus monkeys (*Macaca fascicularis*) shared with postmenopausal women decreases in HDLC following tibolone treatment (40% to 50% decreases were



observed with monkeys). We then conducted a long-term study, with surgically postmenopausal cynomolgus monkeys designed to evaluate the effects of tibolone on coronary artery atherosclerosis and to use treatment with CEE as a comparator group.¹³ The control group received no hormones (Control, n=31). The treatments used were either tibolone (Org OD 14) at a dose for the monkeys comparable to a dose of 3.0 mg for women (HiTib, n=31), tibolone at one-fourth that dose (comparable to a woman's dose of 0.75 mg) (LoTib, n=30) or CEE (Premarin®) (CEE, n=28) at a dose for the monkeys comparable to a dose of 0.625 mg/day for women.

The primary objective of this study was to determine whether reductions in plasma concentrations of HDLC resulting from tibolone treatment were associated with any exacerbation of diet-induced coronary artery atherosclerosis in the cynomolgus monkey model. Despite the finding that tibolone treatment increased plasma concentrations of LDL+VLDL (in HiTib group) and markedly reduced plasma concentrations of HDLC in both LoTib and HiTib groups, there was no exacerbation of coronary artery atherosclerosis nor common carotid artery atherosclerosis as compared to the control group (Figure 3 A, B, & C). This finding may be an underestimate of tibolone's potential for inhibiting coronary artery atherosclerosis in human subjects, both because the HDLC decreases were greater (in the HiTib group) than those seen in human subjects, and because human subjects do not increase their LDL in response to tibolone treatment (as seen in the HiTib group). Moreover, the monkeys show an increase in TG levels after tibolone treatment, whereas a decrease is found in human primates. CEE treatment was associated with a reduced amount of coronary artery atherosclerosis in this study consistent with what we have observed in other studies.^{2,10}

While LoTib and HiTib treatment resulted in lower plasma HDLCs by 34% and 49% respectively, coronary artery atherosclerosis extent was not significantly different from the control group. To better understand the finding, we plotted each monkey's coronary artery atherosclerosis cross-sectional area against its average HDLC during treatment and compared that with the same plot for the control group. These plots showed that at the

same HDLC concentration, the tibolone groups had, on average, less atherosclerosis than the control group. We explored a potential metabolic explanation for the observation by measuring the cholesterol efflux potential of each monkey's serum using 3H-labeled cholesterol and Fu5AH cells.¹⁴ With a 30% reduction in HDLC, there was no reduction in efflux potential. Even with a 50% reduction in HDLC, there was only a 14% reduction in efflux. The studies suggested to us that even though HDLC is reduced by tibolone, reverse cholesterol transport is not compromised and thus atherosclerosis is not exacerbated.

SUMMARY

The studies in nonhuman primates suggest that the SERMs and target tissue-selective steroids currently evaluated have effects that are less robust for the primary prevention of atherosclerosis than is estrogen replacement therapy (i.e., CEE). However, with effective cardiovascular drugs such as statins available, the cardiovascular benefits of postmenopausal therapy might be less important, as long as they have no adverse effects on the cardiovascular system. The nonhuman primate model has provided evidence about pharmaceutical and natural SERMs and target tissue selective steroids that is consistent with data in women. Generally the data in nonhuman primates are available years before there is evidence from clinical trials. Another advantage of this animal model is that pathologic and mechanistic studies can be done that are not feasible in women. While there is currently no ideal postmenopausal therapy (i.e., one with benefits for bone, cognitive function, and cardiovascular system and no adverse effects on cancer risk), there are new compounds being developed, and combined therapies can be tested that might provide this ideal profile. The nonhuman primate model seems useful for evaluating these new therapies and providing direction for clinical trials.

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FROM THE EDITOR

David F. Archer, M.D.

In memory of Trudy Bush, Ph.D., M.H.S., we present six essays on cardiovascular effects of hormones, phytoestrogen, selective estrogen receptor modulators, and tissue selective steroid, all from the Comparative Medicine Clinical Research Center at Wake Forest University School of Medicine.

Coronary artery disease means atherosclerotic involvement of the vessel, with resultant decrease in diameter, loss of vessel reactivity, and propensity for clot formation at the atherosclerosis plaque. These authors report research using the cynomolgus macaque as a model for human disease. The authors are well-known to followers of the publications of this group.

Jay Kaplan, Ph.D., uses his unique observation of the extent of atherosclerosis in dominant and submissive female cynomolgus monkeys to present a

provocative hypothesis relative to premenopausal interventions to prevent atherosclerotic coronary artery disease in postmenopausal women.

Michael Adams, D.V.M., integrates human epidemiologic and observational data with his experience on the retardation of atherosclerosis by estrogen in the cynomolgus macaque. He emphasizes early treatment intervention for prevention.

Janice Wagner, D.V.M., Ph.D., places into perspective the data from the cynomolgus macaque regarding estrogen, progesterin, and the development of atherosclerosis plaque. She develops this information in relationship to human observational data on CAD. Of interest is her interpretation of hormonal effects on carbohydrate metabolism.

J. Koudy Williams, D.V.M., presents the evidence that estrogens and phytoestrogens enhance vascular reactivity

(vasodilation). Age and capillary endothelium prevent this effect of estrogen. This example stresses early preventive intervention before development of atherosclerosis.

Thomas Clarkson, D.V.M., gives us a nice review of estrogen, soy, serum (Raloxifene) and tissue selective steroids (Tibolone) on coronary atherosclerosis. This is a useful essay for the clinician.

J. Mark Cline, D.V.M., Ph.D., presents the data regarding the breast and endometrial changes found in the normal and experimentally treated surgically postmenopausal cynomolgous macaque. The results of the use of conjugated estrogens, estradiol, medroxyprogesterone acetate, tamoxifen, and tibolone all appear to mirror those seen in humans. Their studies with soy phytoestrogen indicate that there is no stimulation of breast or endometrium with this intervention.

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