

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Aortic Plaque Size and Endometrial Response in Cholesterol-Fed Rabbits Treated With Estrogen Plus Continuous or Sequential Progestin

Ute Brehme, Birgit Bruck, Natalia Gugel, Manfred Wehrmann, Sybille Hanke, Gerald Finking, Friedrich W. Schmahl and Hartmut Hanke

Arterioscler Thromb Vasc Biol 1999;19:1930-1937

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association,
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 1999 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online
ISSN: 1524-4636

The online version of this article, along with updated information and services, is
located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/19/8/1930>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular
Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters
Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax:
410-528-8550. E-mail:

journalpermissions@lww.com

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

Aortic Plaque Size and Endometrial Response in Cholesterol-Fed Rabbits Treated With Estrogen Plus Continuous or Sequential Progestin

Ute Brehme, Birgit Bruck, Natalia Gugel, Manfred Wehrmann, Sybille Hanke, Gerald Finking, Friedrich W. Schmahl, Hartmut Hanke

Abstract—ERT is associated with a reduced incidence of coronary risk and cardiac events in postmenopausal women, but increases the risk of endometrial hyperplasia and carcinoma. Combined estrogen and progestin therapy protects the endometrium; however, its effects on heart disease risk factors are not completely known. In our study, 56 ovariectomized New Zealand White rabbits in 7 groups received a 0.5% cholesterol diet for 12 weeks. Controls were not treated with hormones. All other animals received (per kilogram body weight per week) intramuscular injections of either 0.3 mg estrogen (estradiol valerate) alone, 8.3 mg progestin (hydroxyprogesterone caproate) alone, estrogen and progestin continuously in 3 different dosages (0.3 and 8.3 mg; 1 and 8.3 mg; or 1 and 2.8 mg; estrogen and progestin, respectively), or 1 mg estrogen with 25 mg progestin sequentially in 2-week cycles. Eight non-ovariectomized animals served as further controls for endometrial analysis. Morphometric analysis of plaque size in the aortic arch showed that estrogen monotherapy, and the 3 combined therapies with 1 mg estrogen, significantly reduced intimal thickening ($P<0.05$). The application of progestin alone had no effect on plaque size. The endometrium was enlarged by 3-fold after estrogen treatment, and was decreased by half after progestin treatment, compared with control uteri ($P<0.05$). In all groups with combined hormone regimens, endometrial size was not significantly different from control uteri. However, these uteri showed more inflammatory reactions, especially when higher doses of hormones were given. In this animal model, doses of progestin that are able to successfully reduce the proliferative effect of estrogen on endometrium do not diminish the desirable antiatherosclerotic properties of estrogen. (*Arterioscler Thromb Vasc Biol.* 1999;19:1930-1937.)

Key Words: atherosclerosis ■ estrogen ■ progestin ■ endometrium ■ rabbits

Natural menopause is associated with an increased incidence of coronary risk and cardiac events.¹ Numerous studies have evaluated the relationship between estrogen replacement therapy (ERT) and the risk of coronary heart disease (CHD) in postmenopausal women. ERT is thought to reduce morbidity and mortality from CHD \approx 50% in women using unopposed oral estrogen.^{2,3} Proposed mechanisms involve favorable changes in lipid and lipoprotein levels,⁴⁻⁶ as well as less well-understood nonlipid effects, including direct actions on the vascular endothelium and vascular smooth muscle cells.^{7,8} Because unopposed long-term estrogen therapy considerably increases the risk of developing endometrial hyperplasia and endometrial cancer, the concomitant use of progestogens has been recommended for women with an intact uterus.⁹⁻¹¹ Combined estrogen-progestin regimens effectively relieve menopausal symptoms,^{12,13} and protect the endometrium from the carcinogenic effects of estrogen.¹⁰ However, possible adverse effects of progestins on the atheroprotective properties of estrogen are a matter of de-

bate.¹⁴ Compared with unopposed estrogen treatment, both favorable¹⁵ and unfavorable¹⁶⁻¹⁸ effects of combined hormone replacement therapy (HRT) on HDL cholesterol have been reported. Recently published data from the Nurses' Health Study, however, suggest that the beneficial effects of estrogen therapy for the prevention of CHD are not attenuated by addition of progestin.¹⁹

Animal studies involving combined estrogen-progestin therapy have focussed predominantly on hormone effects on coronary vessels or the aorta. Depending on the type and dose of the progestational agents added, in different experimental studies using mostly monkeys and rabbits, the beneficial lipid or cardiovascular effects of estrogen were found to be maintained as well as reduced. In studies using natural progesterone, no attenuation of the estrogenic effect was found,^{20,21} except for a study by McKinney et al,²² where progesterone impeded the positive influence of estrogen on LDL oxidation. When medroxyprogesterone acetate or other progestins were used, the cardioprotective effects of estrogen

Received September 1, 1998; revision accepted December 29, 1998.

From the Department of Occupational and Social Medicine (U.B., B.B., N.G., F.W.S.), and the Department of Pathology (M.W.), University of Tübingen; and the Department of Internal Medicine, Division of Cardiology, University of Ulm (S.H., G.F., H.H.), Germany.

Correspondence to Ute Brehme, PhD, Department of Occupational and Social Medicine, Wilhelmstr. 27, 72074 Tübingen, Germany. E-mail ute.brehme@uni-tuebingen.de

© 1999 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

TABLE 1. Dosage of Estradiol Valerate and Hydroxyprogesterone Caproate Administered Intramuscularly in the Different Treatment Groups, Either Continuously Once a Week, or Sequentially in 2-Week Cycles

Group	Treatment	Estradiol Valerate (per kg body weight)	Hydroxyprogesterone Caproate (per kg body weight)
1	Control
2	E	0.3 mg/week	...
3	P	...	8.3 mg/week
4	E and P	0.3 mg/week	8.3 mg/week
5	3× E and P	1 mg/week	8.3 mg/week
6	3× E and 1/3 P	1 mg/week	2.8 mg/week
7	3× E and 3× P _{sequ}	1 mg/week	25 mg in 2-week cycles

E indicates estradiol valerate; P, hydroxyprogesterone caproate; and P_{sequ}, sequential administration of hydroxyprogesterone caproate.

were reduced in most,^{23–27} but not all,²⁸ studies. The uterus was rarely analyzed in these studies.

The present study was designed to investigate the effects of estradiol valerate plus hydroxyprogesterone caproate on intimal plaque development in the aortic arch, and on the endometrium, of rabbits. Three regimens with continuous-combined, and one regimen with sequential-combined, progestin application were tested. Progestational agents are added to ERT to counteract the risk of increased endometrial hyperplasia and carcinoma. Because the endometrium is the main target organ of steroid hormones, it is important to analyze in what way hormone doses used in animal models for evaluating atherogenesis affect the endometrium.

Methods

Adult female New Zealand White rabbits (n=56) with body weights between 2.5 and 3.0 kg were supplied by Charles River (Kiblegg, Germany). They were allowed to acclimatize in the laboratory environment for 3 weeks. Animals were housed individually with 12-hour light periods and had free access to food and water throughout the study period. At the beginning of the study, they were ovariectomized bilaterally under anesthesia (ketamine hydrochloride, xylazine hydrochloride, and atropine sulfate). They were then randomized into 7 groups of 8 rabbits each and were fed a standard diet supplemented with 0.5% cholesterol (Altromin) for the study period of 12 weeks. Once a week, estrogen and/or progestin were administered intramuscularly. Estrogen was given as estradiol valerate (Progynon-Depot-10), and progestin as hydroxyprogesterone caproate (Proluton Depot), both from Schering AG. Group 1, the controls, received no hormones; group 2 received estrogen; group 3 received progestin; and groups 4 to 7 received both hormones. In groups 4, 5, and 6, hormones were given continuously, once a week. In group 7, a sequential regimen was followed, with estrogen given continuously, and progestin given sequentially, in 2-week cycles during weeks 3 and 4, weeks 7 and 8, and weeks 11 and 12 (Table 1).

Blood was collected at the beginning of the study, and on days 28, 56, and 84 (1 week after the last hormone injection). After an overnight fast of 18 hours, animals were anesthetized and blood was collected from the central ear vein into EDTA-containing tubes, and was centrifuged at 4°C for 10 minutes at 3000 rpm. All experimental procedures were approved by the Animal Research Committee of the regional governmental authorities and conformed to the American Heart Association *Guidelines for Use of Animals in Research*.

Plasma cholesterol concentrations were determined enzymatically (CHOD-PAP-Method, Boehringer Mannheim). The area under the curve (AUC) was calculated using the 4 different measured concentrations of total plasma cholesterol. AUC represents the total plasma cholesterol to which the arteries were exposed over a duration of

the study period. For determination of plasma triglyceride concentrations, an enzymatic method (Peridochrom Triglyceride GPO-PAP-Method, Boehringer Mannheim) was also used.

Plasma levels of 17 β -estradiol and 17 α -hydroxyprogesterone were determined by radioimmunoassay (DPC Biermann GmbH). The 4 measured values of 17 β -estradiol and 17 α -hydroxyprogesterone were also calculated as AUC.

On day 84, animals were euthanized with an intravenous injection of 2 mL of the barbiturate T61 (Hoechst Roussel Vet GmbH). The aortic arch and uterus were excised and immersion-fixed in 0.1 M cacodylate-buffered 2% paraformaldehyde solution for at least 24 hours.

The aortic arch was divided into 3 sections: the first section was \approx 0.5 cm proximal to the ostium of the innominate artery; the second section was located between the left carotid artery and the left subclavian artery; and the third section was a descending segment of the aortic arch \approx 0.7 cm beneath the subclavian artery. Paraffin-embedded tissue samples were sliced 4 μ m thick and stained with Elastica-van-Gieson (EvG). The extent of the neointimal plaque was measured in the 3 sections using a digital image analyzer (software from Bilany Consultants GmbH). The arithmetic mean was calculated and used for further statistical analyses of the plaque size in the aortic arch.

The bicornis uterus was cut transversally at its broadest part. After paraffin embedding, 4- μ m-thick slices of tissue were cut and hematoxylin-eosin (HE) stained. Both endometrial areas were measured in as many slices as necessary to determine the maximum area. A blinded histopathologic analysis of the HE-stained uterus sections was performed by one of the authors (M.W.), evaluating inflammation, necrosis, branching of mucosal folds, edema, and vascularization (0, not present; +, <25%; ++, 25% to 50%; +++, >50%). Uteri with major pathological changes were stained with a mouse monoclonal antibody against rabbit macrophages (RAM 11, DAKO). Immunohistological detection of macrophages was performed with the avidin-biotin method combined with hemalaune staining. The uteri of the control animals were not included in the analysis, but the 8 uteri from non-ovariectomized rabbits of similar age fed a normal standard diet without cholesterol supplementation were included, representing normal organs from untreated animals.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed on the data. AUCs of 17 α -hydroxyprogesterone and final triglyceride concentrations were analyzed by Welch ANOVA, allowing unequal variances. Data representing intimal plaque size, endometrium area, and AUC of 17 β -estradiol were used after log₁₀ transformation because of the nonhomogenous variance between groups. Equality of group variances was analyzed by Bartlett's test. Although statistical analyses were performed with transformed data, original data are shown in the figures and cited in the text. For multiple comparisons between the different treatment groups, the Tukey-Kramer test was used when ANOVA showed an F-value <0.05. Intimal plaque size was also analyzed in an analysis of covariance (ANCOVA) by adding AUC of total plasma cholesterol as a covariate of the model.

Log₁₀ transformed data of intimal plaque size were correlated with the parameter AUC of total plasma cholesterol using Pearson's correlation coefficient.

Statistical analyses were carried out with JMP (Version 3.1.6.2, SAS). Data are presented as mean \pm SD, unless stated otherwise. P<0.05 was considered to indicate statistical significance for all tests.

Results

Body Weight

At the beginning of the study, animals had a mean body weight of 2.75 \pm 0.16 kg with no significant differences between the single groups. At the end of the study, animals with estrogen monotherapy (group 2) had the highest body weight, whereas animals with progestin monotherapy (group 3) had the lowest body weight. Compared with controls, body weights in groups 2 and 4 were significantly higher (Table 2).

TABLE 2. Initial and Final Values of Body Weight, 17β-Estradiol, 17α-Hydroxyprogesterone, Total Plasma Cholesterol, and Triglyceride Concentrations in the Groups Treated With Estrogen and/or Progestin

Treatment Group	Body Weight (kg)		17β-Estradiol (pmol/L)		17α-Hydroxyprogesterone (nmol/L)		Total Plasma Cholesterol (mmol/L)		Triglycerides (mmol/L)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
1 Control	2.7±0.1	3.4±0.3 ^{2,4}	ND	ND	ND	ND	1.3±0.4	47.3±10.5	0.8±0.2	0.9±0.5
2 E	2.8±0.3	4.0±0.3 ^{1,3,5}	ND	414±184 ^{5,6}	ND	ND	1.5±0.5	61.4±10.2 ³⁻⁷	0.6±0.2	0.6±0.4
3 P	2.7±0.1	3.1±0.3 ^{2,4,6,7}	ND	ND	ND	ND	1.4±0.4	40.4±7.5 ²	0.6±0.2	0.8±0.3
4 E and P	2.7±0.1	3.9±0.2 ^{1,3}	ND	185±81 ^{5,6}	ND	ND	1.8±0.4	44.8±11.0 ²	0.7±0.2	0.5±0.2
5 3× E and P	2.8±0.2	3.5±0.3 ²	ND	1420±723 ^{2,4}	ND	1.5±0.6	1.9±0.3	39.8±12.5 ²	0.6±0.2	0.4±0.2
6 3× E and 1/3 P	2.8±0.1	3.6±0.3 ³	ND	1397±803 ^{2,4}	ND	ND	1.7±0.4	45.2±7.4 ²	0.5±0.1	0.7±0.5
7 3× E and 3× P _{sequ}	2.7±0.1	3.8±0.3 ³	ND	726±338	ND	0.4±0.1	1.6±0.6	35.2±12.3 ²	0.7±0.3	0.9±0.6
ANOVA P value	=0.35	<0.0001	...	<0.0001	...	=0.001	=0.15	=0.0003	=0.13	=0.06

ND indicates not detectable. Superscripts indicate group numbers that are significantly ($P<0.05$) different from each other (Tukey-Kramer test). Exposure to 17β-estradiol and total plasma cholesterol was calculated additionally as AUC (see Figures 1 and 2).

Plasma Hormone Concentrations

Initial values of 17β-estradiol and 17α-hydroxyprogesterone were below detectable limits in all animals.

In animals of group 1 and group 3, which were not treated with estrogen, levels of plasma 17β-estradiol remained below the detection limit throughout the study period. After 12 weeks, final 17β-estradiol levels in the groups receiving the high estrogen dosage and continuous progestin treatment reached values of 1420±723 pmol/L (group 5) and 1397±803 pmol/L (group 6). The concentration of 17β-estradiol was lower in animals with sequential progestin treatment, but not significantly different from groups 5 and 6. Animals in groups 2 and 4, receiving the lower estrogen dosage, showed final 17β-estradiol concentrations of 414±184 pmol/L and 185±81 pmol/L, respectively, which were significantly lower than those of groups 5 and 6 (Table 2). AUC of 17β-estradiol was significantly lower in groups 2 and 4, compared with groups 5, 6, and 7 (Figure 1).

In group 1, group 2, and 3 of the 5 progestin-treated groups (3, 4, and 6), 17α-hydroxyprogesterone levels were not detectable one week after the last hormone injection. An increase in 17α-hydroxyprogesterone concentrations was observed in groups 5 and 7, with final values of 1.5±0.6 nmol/L and 0.4±0.1 nmol/L, respectively ($P=0.001$, Table 2). AUC of 17α-hydroxyprogesterone was also significantly higher in group 5 compared with group 7 (16.2±7.2 versus 7.1±2.3 nmol/L · 12 weeks; $P=0.009$).

Total Plasma Cholesterol

Before cholesterol feeding, animals had an average total plasma cholesterol concentration of 1.6±0.5 mmol/L. No significant differences (ANOVA, $P=0.15$) were found between the groups (Table 2).

Final total cholesterol concentrations, which ranged from 61.4±10.2 mmol/L (group 2) to 35.2±12.3 mmol/L (group 7), were significantly different by ANOVA ($P=0.0003$). With the exception of the control group, all other groups had significantly lower final cholesterol values compared with group 2, which received estrogen monotherapy ($P<0.05$). When the whole period of cholesterol exposure was considered, AUC of total plasma cholesterol also showed significant differences (ANOVA, $P<0.0001$) between the groups.

administered 1 mg/kg body weight per week of estrogen (groups 5, 6, and 7) compared with controls and animals administered 0.3 mg/kg body weight per week of estrogen ($P<0.05$); progestin-treated animals and group 4 animals (administered progestin combined with the lower estrogen dosage) were not significantly different from the other groups. There were no significant differences in AUC of total plasma cholesterol between the 4 groups administered the combined treatment (Figure 2).

Plasma Triglycerides

Initial plasma triglyceride levels, after an overnight fast, were between 0.5±0.1 mmol/L (group 6) and 0.8±0.2 mmol/L (group 1), and were not significantly different (ANOVA, $P=0.13$). Final values were in the same range as initial values, and did not differ significantly between the groups (Welch ANOVA, $P=0.06$; Table 2).

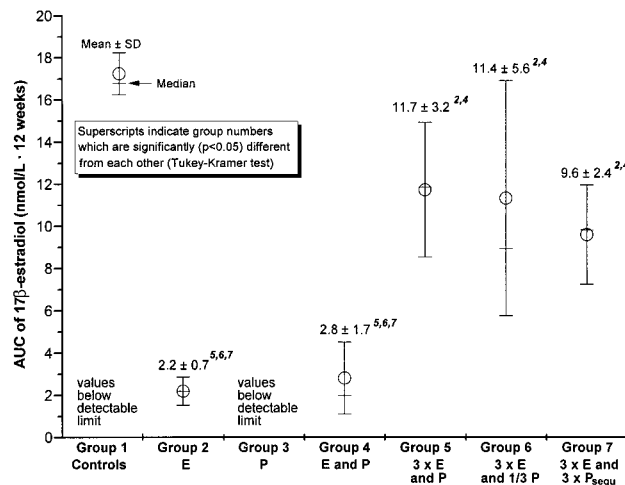


Figure 1. Exposure to 17β-estradiol (calculated as AUC for the 12-week study period), after administration of estrogen and/or progestin to rabbits. Animals without estrogen treatment had no measurable plasma concentrations of 17β-estradiol one week after the last hormone injection. In animals who had received a dosage of 1 mg/kg per week of estrogen (groups 5, 6, and 7), 17β-estradiol concentrations were ≈4- to 5-fold higher than in animals treated with one-third of the dose (groups 2 and 4).

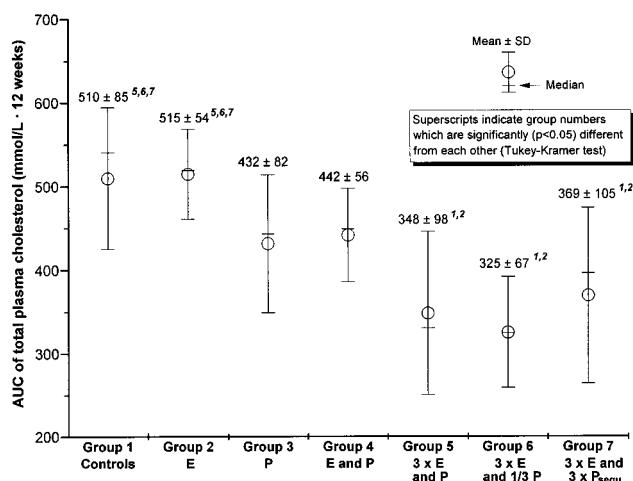


Figure 2. Exposure to total plasma cholesterol (calculated as AUC for the 12-week study period), after administration of estrogen and/or progestin to rabbits. AUC of total plasma cholesterol was significantly higher in controls and estrogen treated animals than in the 3 groups treated with 1 mg/kg per week of estrogen plus progestin. No significant differences were observed between the 4 groups with combined treatment.

Intimal Plaques in the Aortic Arch

Plaque development in the aortic arch after 12 weeks of cholesterol feeding was significantly different between the groups (ANOVA, $P < 0.0001$). Animals treated with estrogen alone (group 2) showed a significantly smaller plaque development ($0.9 \pm 0.7 \text{ mm}^2$) compared with controls ($3.5 \pm 1.7 \text{ mm}^2$). The largest plaques ($3.9 \pm 1.6 \text{ mm}^2$) were observed in the group treated with progestin alone, but they were not significantly different from controls. Compared with controls, plaque development was not significantly reduced in group 4, administered estrogen at the lower dosage of 0.3 mg/kg per week combined with 8.3 mg/kg per week of progestin ($1.7 \pm 1.2 \text{ mm}^2$). However, the extent of atherosclerotic lesions after administration of continuous (group 5, $0.8 \pm 0.7 \text{ mm}^2$; and group 6, $0.7 \pm 0.4 \text{ mm}^2$) or sequential (group 7, $0.5 \pm 0.4 \text{ mm}^2$) progestin application in combination with the higher dosage of estrogen (1 mg/kg per week), was significantly smaller than in controls and progestin-alone treated animals. No differences in plaque development were seen between continuous and sequential hormone regimens with identical estrogen dosages (Figure 3).

As mentioned above, AUC of total plasma cholesterol differed widely between the groups. Because there was a correlation between AUC of total cholesterol and intimal plaque size of $r = 0.51$ ($P = 0.0001$, $n = 56$), we analyzed the intimal plaque size, taking into consideration the differences in AUC of plasma cholesterol as a covariate in an ANCOVA model. All statistically significant differences in plaque size from ANOVA remained significant in the ANCOVA model.

Endometrium

The endometrial area of untreated animals had an extension of $8.7 \pm 2.7 \text{ mm}^2$. Compared with these normal controls, a significant increase was observed after single estrogen treatment ($23.7 \pm 3.7 \text{ mm}^2$) and a significant decrease was observed after single progestin treatment ($3.9 \pm 0.7 \text{ mm}^2$). In the 4 groups with combined hormone treatment, the extent of endometrium was not significantly different from those of control animals ($P > 0.05$). When progestin was administered in 2-week cycles,

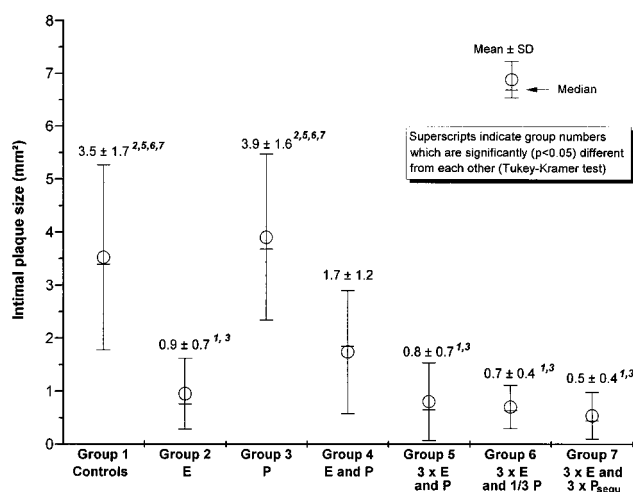


Figure 3. Morphometrically analyzed area of neointimal plaques in cross-sections of the aortic arch of rabbits treated with estrogen and/or progestin. Because AUC of total plasma cholesterol was different between the groups, the intimal plaque size was additionally analyzed in an ANCOVA with the confounder AUC of total plasma cholesterol. All statistically significant differences in plaque size observed in the ANOVA were maintained in the ANCOVA.

significantly larger endometria were seen versus groups 5 and 6 (continuous progestin treatment; Figure 4).

Inflammatory, necrotic, and other changes of endometria were not observed in control animals (group 1) and rarely observed in animals treated with one hormone alone (groups 2 and 3). Moderate changes were seen in group 4, which was administered both hormones in the same dosages as in groups 2 and 3; and in group 6, where the lowest progestin dose was used. In the 2 other groups with combined treatment (groups 5 and 7), inflammatory and necrotic alterations were more frequent. A comparison of groups 5 and 6, which had received the same estrogen dose but different progestin dosages, showed that uteri were in better histopathologic

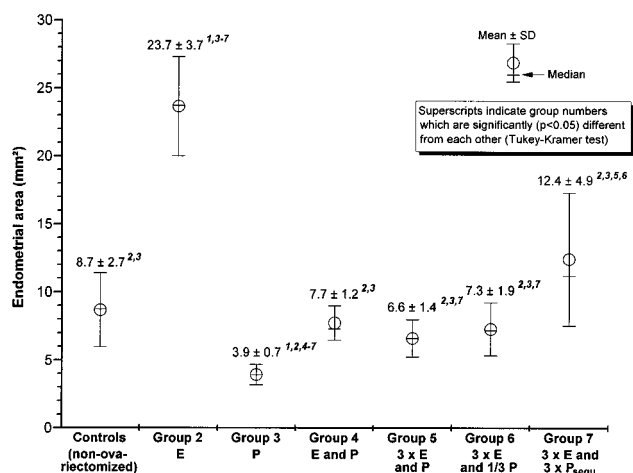


Figure 4. Morphometrically analyzed area of the endometrium of rabbits treated with estrogen and/or progestin. Estrogen monotherapy increased endometrium size ≈ 3 -fold compared with untreated controls. Progesterone monotherapy decreased endometrium size $> 50\%$. In the 4 combined-therapy groups, the size of the endometrium corresponded to that of untreated controls. However, sequential progestin application resulted in significantly larger endometria than continuous treatment with a corresponding estrogen dose (groups 5 and 6).

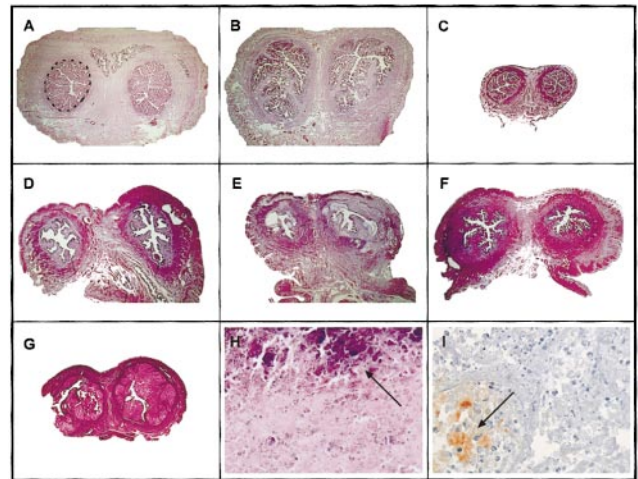
TABLE 3. Frequency of Pathological Findings in Uteri From Each Treatment Group

Treatment Group	No.	Histopathologic Criteria	Frequency of Criteria (No. of animals)			
			0	+	++	+++
1 Controls	8	Inflammation	8
		Necrosis	8
		Branching	8	...
		Edema	8
		Vascularization	...	8
2 E	8	Inflammation	8
		Necrosis	8
		Branching	1	7
		Edema	8
		Vascularization	...	7	1	...
3 P	8	Inflammation	8
		Necrosis	8
		Branching	...	8
		Edema	8
		Vascularization	...	8
4 E and P	8	Inflammation	5	3
		Necrosis	6	...	1	1
		Branching	...	8
		Edema	5	1	2	...
		Vascularization	...	2	5	1
5 3× E and P	7*	Inflammation	4	2	1	...
		Necrosis	...	4	1	2
		Branching	...	6	1	...
		Edema	3	3	...	1
		Vascularization	...	2	3	2
6 3× E and 1/3 P	8	Inflammation	6	2
		Necrosis	4	3	1	...
		Branching	...	4	4	...
		Edema	7	1
		Vascularization	...	8
7 3× E and 3× P _{sequ}	7*	Inflammation	2	3	1	1
		Necrosis	1	2	...	4
		Branching	...	4	3	...
		Edema	1	4	2	...
		Vascularization	...	1	3	3

*Missing values: differentiation of uterus structures was not possible.

condition after exposure to the lower progestin dosage. Comparing groups 4 and 5, which had received the same progestin treatment but different estrogen dosages, uteri of animals administered the lower estrogen dosage exhibited fewer pathological transformations (Table 3).

The typical structure of rabbit endometrium, showing 6 major extensively branched mucosal folds, persisted under hormone monotherapy. With combined treatment, this morphology vanished completely in groups 5 and 7, and to a lesser extent in groups 4 and 6. In groups 5 and 7, large necrotic and inflammatory areas with calcification and expression of macrophages were detected (Figure 5).



A-G: --- = 2.5 mm (HE-staining)

H-I: --- = 50 μm (H = HE-staining; I = RAM 11-staining)

(--- example for measured area)

Figure 5. Photomicrographs of typical uterus structures in the different treatment groups: (A) control uterus of untreated animal; (B) estrogen monotherapy (group 2); (C) progestin monotherapy (group 3); (D) 0.3 mg/kg estrogen+8.3 mg/kg progestin (group 4); (E) 1 mg/kg estrogen+8.3 mg/kg progestin (group 5); (F) 1 mg/kg estrogen+2.8 mg/kg progestin (group 6); (G) 1 mg/kg estrogen+25 mg/kg progestin in 2-week cycles (group 7); (H) group 5, large necrotic area in the endometrium with calcification (---); (I) group 7, necrosis of the endometrium with inflammatory reaction of macrophages (---). The bicornus uteri of control animals (A) and animals with monotherapy (B and C) showed the typical structure of 6 primary mucosal folds bulging into the lumen in the form of broadly based mounds. Superimposed minor folds showed extensive branching. Combined treatment decreased the degree of branching in a hormone dose-dependent manner. In uteri of animals treated with higher estrogen and progesterone doses (E and G), the number of major mucosal folds was reduced and the uterus had lost its typical structures. Structures of uteri of groups 4 and 6 (D and F) were altered to a lesser degree. Frequency of inflammation, necrosis, edema, and vascularization was also higher in groups 5 and 7 (H and I) compared with groups 4 and 6.

Discussion

In this animal study, estrogen monotherapy, progestin monotherapy, and 4 different combined estrogen-progestin treatment regimens (3 with continuous and one with sequential progestin application), were investigated. All 4 combined regimens were effective at reducing the action of estrogen on endometrium proliferation; three of them also resulted in a significantly reduced neointimal plaque size in the aortic arch compared with controls.

In agreement with previous animal studies,²⁹⁻³¹ estrogen monotherapy reduced plaque development, whereas progesterone alone had no significant effect. Haarbo et al²⁸ found no inhibiting effect of norethisterone acetate and levonorgestrel on estrogen action when orally applied to rabbits in a 3- and 6-fold lower dosage than 17 β -estradiol, respectively. In group 4 animals, which were treated with the lower estrogen (0.3 mg/kg per week) and the higher progestin dose (8.3 mg/kg per week), progestin attenuated the beneficial estrogen effect on plaque growth. The same progestin dose, however, showed no attenuating effect in group 5 when combined with 1 mg/kg per week of estrogen. In a similar study, which used

higher hormone dosages of 1 mg/kg per week of estrogen and 25 mg/kg per week of progestin, the protective estrogen effect was diminished.²⁵ In the present study, a 25-mg/kg dose of progestin was also used, but applied in 2-week cycles. This 50% lower dosage did not cause inhibition of the atheroprotective estrogen action. These results show that the absolute estrogen dose, as well as the relation between estrogen and progestin dosages, is important for the maintenance of the atheroprotective estrogen action.

The hormone doses used in the present study resulted in hormone concentrations quite higher than physiological levels usually observed in rabbits. Batra and Källstrand³² showed that 17 β -estradiol levels of adult rabbits are between 40 and 228 pmol/L. In postmenopausal women, 17 β -estradiol concentrations are \approx 300 pmol/L with ERT and \approx 50 pmol/L without ERT.³³ After intramuscular injection of 5 mg estradiol valerate, a high estradiol peak level of 2200 pmol/L was observed within 2 days. After 6 days, estradiol levels were down to \approx 350 pmol/L.³⁴ In rabbits treated with 0.3 mg estrogen valerate, we observed similar 17 β -estradiol levels of \approx 200 to 400 pmol/L seven days after injection. The estrogen dosage of 1 mg/kg per week, however, resulted in concentrations 2- to 8-fold higher.

In addition to hormone dose and timing of administration, different types of estrogen and progestational agents can have different effects on the cardiovascular system. Oral estrogens are usually given as conjugated equine estrogen. Parenteral preparations include conjugated estrogens and estradiol esters, such as estradiol valerate.³⁵ Oral estrogen monotherapy, in the form of conjugated estrogens or estradiol, is known to reduce LDL cholesterol and increase HDL cholesterol.⁵ Progestational agents used in HRT are derivatives of either progesterone, eg, medroxyprogesterone acetate, or testosterone, eg, 19-nortestosterone derivatives such as norethisterone acetate or levonorgestrel.³⁴ Most 19-nortestosterone derivatives have androgenic activity, and, in combination with estrogen, LDL cholesterol levels are reduced to a lesser extent than with unopposed estrogen. Derivatives of progesterone do not seem to have adverse effects on lipid metabolism. The combination of natural estrogens, such as estradiol valerate, and cyclical progesterone, seem to have the most favorable impact on lipids and lipoproteins in women.³⁵ In rabbits, orally administered estrogen or progestin did not produce significant differences in total serum cholesterol.³¹ In our study, intramuscular monotherapy with these hormones also showed no effect on the AUC of total cholesterol compared with controls. However, animals administered high dose estrogen in combined treatment showed a decrease in AUC of total cholesterol. Because animals had free access to food, and thus cholesterol intake was not controlled, it is difficult to exactly determine to what extent hormone treatment was responsible for the decrease in plasma cholesterol concentrations. Because there was a significant reduction in AUC of total cholesterol in group 5 (which had received the same progestin dose but the 3-fold higher estrogen dose compared with animals in group 4), but not in group 4, it can be hypothesized that estrogen affected plasma cholesterol concentrations.

Three studies^{29,36,37} provided information on the lipid effects of intramuscular injection of estrogens to cholesterol-fed rabbits. In the studies by Hagen and Swain,³⁶ and Fischer and Swain,³⁶ estrogen given as 0.5 mg/kg per week of 17 β -estradiol cypionate and 50 μ g per week of estradiol, respectively, did not affect lipid metabolism. In contrast, Kushwaha and Hazzard³⁷ found that a dosage of estradiol cypionate between 0.2 and 0.5 mg/kg per week considerably decreased the diet-induced rise in total plasma cholesterol, compared with cholesterol-fed rabbits not treated with hormones. In women, nonoral estrogen therapies produce less-pronounced favorable effects on LDL and HDL cholesterol than their oral counterparts.³⁸ However, triglyceride concentrations, known to be increased by oral HRT therapy,¹¹ are not altered by transdermal hormone application.^{39,40} In rabbits, no change in triglycerides was observed with intramuscular hormone treatment. Although our results of total plasma cholesterol and triglycerides were comparable to the situation in humans, one disadvantage of the rabbit model has to be considered. The lipid and lipoprotein profile of rabbits is different from that of humans. VLDL cholesterol predominates in rabbits, LDL cholesterol in humans.⁴¹ Therefore, results of hormone treatment on changes in lipid metabolism obtained in rabbits should be cautiously extrapolated to humans.

A number of nonlipid effects of estrogen for protection against atherosclerosis have been discussed, ie, endothelium dependent and independent effects, hemostasis and direct antiatherosclerotic effects.⁴² In the rabbit model used here, the majority of atheroprotective estrogen effects must be lipid-independent because the neointima was significantly smaller in groups with combined treatment compared with controls, even when AUC of total cholesterol was added as a covariate to the statistical analyses.

Prolonged use of unopposed estrogen was found to be associated with a 10-fold increase in the risk of endometrial cancer.⁴³ Therefore, the concomitant use of sequential or continuous progestins is considered mandatory for women who have an intact uterus.⁴⁴ A number of studies showed that endometrial hyperplasia, caused by treatment with unopposed estrogen, can be effectively prevented when estrogen is combined with a progestin.⁴⁵⁻⁴⁷ In the endometrium of the rabbits studied, the proliferative activity of estrogen was seen in group 3 animals, who underwent estrogen monotherapy. Compared with untreated intact controls, endometrium size was increased 2.7-fold. This result is in agreement with a study examining the uteri of ovariectomized or sham-operated cholesterol-fed rabbits after a 45-week study period. Uteri were found to be \approx 2.5-fold bigger in rabbits fed a diet supplemented with estrogen, and $>$ 50% smaller in ovariectomized animals without hormone treatment, compared with controls.⁴⁸ In the group treated with progestin alone, we observed a 55% reduction in endometrial size. Thus, progestin did not seem to have a stimulating effect on uterine tissue proliferation. Batra and Källstrand,³² who observed a tendency for a cyclic pattern in 17 β -estradiol levels of rabbits, supposed that a minimal estrogen concentration is necessary for optimal response of endometrium to progesterone.

When applied in combination with estrogen, progestin doses were clearly able to confer protection against the undesired proliferative action of estrogen. In all 4 groups with combined treatment, uteri showed neither an increase, nor a reduction, in endometrial size, compared with controls. This result is in agreement with a study by Hagen and Swain³⁶ who found that progestin without measurable plasma proges-

Downloaded from <http://ajph.org/> by guest on November 9, 2009

terone concentrations in groups 4 and 6. Considered over the whole study period, animals treated with 1 mg/kg per week of estrogen were found to have \approx 4- to 5-fold higher plasma levels of 17β -estradiol than animals treated with 0.3 mg/kg of estrogen. Nevertheless, progestin was able to counteract estrogenic actions on the uterus at both plasma concentrations. The lowest dosage of continuously administered progestin, 2.8 mg/kg per week in group 6, was as effective as the 3-fold higher continuous dosage in group 5. Because the lowest dose of progestin used in this study was fully capable of attenuating proliferative estrogen action on the uterus, even lower doses might be adequate. Although no statistically significant difference in endometrium size was observed between controls and sequentially treated rabbits, the continuous regimens were advantageous over sequential therapy with respect to endometrial response. Despite the fact that the progestin dose was highest in the sequential therapy, the endometrium was significantly enlarged compared with the 2 analogous continuous-application groups.

Furthermore, histopathologic evaluation showed that uteri of rabbits treated with combined therapy exhibited inflammatory or necrotic changes less frequently when the lowest progestin dose was administered. However, uteri of animals treated with one hormone alone showed no signs of inflammation or necrosis. Thus, in the rabbit uterus, combined hormone treatment has more side effects than the identical doses given as monotherapy.

The dosage of 25 mg/kg of progestin applied in 2-week cycles (group 7) caused scores of pathological transformations higher than those of the other 3 groups with combined treatment. Although the dosage of 8.3 mg/kg per week of progestin (group 5) added up to a 33% lower dose over the entire study period, AUC of 17α -hydroxyprogesterone was significantly lower in group 5 than in group 7. It could be speculated that progesterone concentrations after the injection of 25 mg/kg of hydroxyprogesterone caproate reached higher peaks, which might have caused the damage. From our results, it could not be determined with certainty whether the sequential application form, or the high dose of progestin, was responsible for the undesired changes seen in group 7. Generally, continuous treatment seems to be superior to sequential treatment with respect to endometrial reactions in rabbit uterus. Considering results of all groups, however, hormone dose seems to be more important than the timing of administration.

The parallel examination of hormone effects on plaque development in aortic vessels and on the uterus of rabbits revealed that there could be a different hormone threshold for cardiovascular and endometrial effects. Results in group 4 showed that the estrogen dose required for protective effects in the aortic arch was substantially higher than the dose able to produce effects on the endometrium. On the other hand, progestin doses able to attenuate negative estrogen effects on the endometrium did not impede beneficial estrogen actions at the vessel wall (groups 5, 6, and 7). A study in apoE-deficient mice also showed that the atheroprotective effects are achieved at higher 17β -estradiol levels than those required by other estradiol target tissues, such as uterus.⁴⁹

The rabbit model provided information about atheroprotective estrogen properties in relation to different progestin doses, and about continuous versus sequential progestin

application regimens. Doses applied for the investigation of estrogen and progesterone action in cholesterol-fed animals are reasonable because they are in ranges not only appropriate for cardiovascular factors, but also for the uterus.

In this animal model, doses of progestin were able to successfully reduce the proliferative effect of estrogen on endometrium without diminishing the desirable antiatherosclerotic properties of estrogen. The rabbit model could therefore also be valuable for testing new antiestrogenic substances as well as HRT regimens involving different types of estrogens and progestogens with respect to cardiovascular and endometrial effects.

Acknowledgments

The authors wish to thank H. Bacher, A. Glückman, M. Heilig, M. Holz, G. Kaletta, C. Lenz, and J. Schatz for technical assistance; Mag. A. Rosenberger, Institute of Medical Information Processing, University of Tübingen, for his help with the statistical analysis; and Dr P.F. Kahle for his help with the English manuscript.

References

- White CR, Darley-USmar V, Oparil S. Gender and cardiovascular disease: Recent insights. *Trends Cardiovasc Med.* 1997;7:94-100.
- Barrett-Connor E, Bush TL. Estrogen and coronary heart disease in women. *JAMA.* 1991;265:1861-1867.
- Sullivan JM, Fowlkes LP. The clinical aspects of estrogen and the cardiovascular system. *Obstet Gynecol.* 1996;87(suppl 2):36S-43S.
- Bush TL, Barrett-Connor E, Cowan LD, Criqui MH, Wallace RB, Suchindran CM, Tyroler HA, Rifkind BM. Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. *Circulation.* 1987;75:1102-1109.
- Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnikar V, Sacks FM. Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *N Engl J Med.* 1991;325:1196-1204.
- Karjalainen A, Heikinen J, Savolainen MJ, Bäckström A-C, Salinto M, Kesäniemi YA. Metabolic changes induced by peroral oestrogen and transdermal oestradiol gel therapy. *Br J Obstet Gynaecol.* 1997;104(suppl 16):38-43.
- Gilligan DM, Badar DM, Panza JA, Quyyumi AA, Cannon RO. Acute vascular effects of estrogen in postmenopausal women. *Circulation.* 1994;90:786-791.
- McCrohon JA, Adams MR, McCredie RJ, Robinson J, Pike A, Abbey M, Keech AC, Celermajer DS. Hormone replacement therapy is associated with improved arterial physiology in healthy post-menopausal women. *Clin Endocrinol.* 1996;45:435-441.
- Voigt LF, Weiss NS, Chu J, Daling JR, McKnight B, van Belle G. Progestagen supplementation of exogenous oestrogens and risk of endometrial cancer. *Lancet.* 1991;338:274-277.
- Creasy GW, Kafrisen ME, Upmalis D. Review of the endometrial effects of estrogens and progestins. *Obstet Gynecol Surv.* 1992;47:654-678.
- The Writing Group for the PEPI Trial. Effects of estrogen or estrogen/progestin regimens on heart disease risk factors in postmenopausal women: the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. *JAMA.* 1995;273:199-208.
- Prough SG, Aksel S, Wiebe RH, Shepherd J. Continuous estrogen/progestin therapy in menopause. *Am J Obstet Gynecol.* 1987;157:1449-1453.
- Luciano AA, Turksoy RN, Carleo J, Hendrix JW. Clinical and metabolic responses of menopausal women to sequential versus continuous estrogen and progestin replacement therapy. *Obstet Gynecol.* 1988;71:39-43.
- Soma MR, Baetta R, Crosignani PG. The menopause and lipid metabolism: Strategies for cardiovascular disease prevention. *Curr Opin Lipidol.* 1997;8:229-235.
- Darling GM, Johns JA, McCloud PI, Davis SR. Estrogen and progestin compared with simvastatin for hypercholesterolemia in postmenopausal women. *N Engl J Med.* 1997;337:595-601.
- Kim CJ, Min YK, Ryu WS, Kwak JW, Ryoo UH. Effect of hormone replacement therapy on lipoprotein(a) and lipid levels in postmenopausal women: Influence of various progestogens and duration of therapy. *Arch*

17. Lahdenperä S, Puolakka J, Pyörälä T, Luotola H, Taskinen M-R. Effects of postmenopausal estrogen/progestin replacement therapy on LDL particles: Comparison of transdermal and oral treatment regimens. *Atherosclerosis*. 1996;122:153–162.
18. Medical Research Council's General Practice Research Framework. Randomised comparison of oestrogen versus oestrogen plus progestogen hormone replacement therapy in women with hysterectomy. *BMJ*. 1996;312:473–478.
19. Grodstein F, Stampfer MJ, Manson JE, Colditz GA, Willett WC, Rosner B, Speizer FE, Hennekens CH. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. *N Engl J Med*. 1996;335:453–461.
20. Adams MR, Kaplan JR, Manuck SB, Koritnik DR, Parks JS, Wolfe MS, Clarkson TB. Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys: lack of an effect of added progesterone. *Arteriosclerosis*. 1990;10:1051–1057.
21. Wagner JD, Clarkson TB, St. Clair RW, Schwenke DC, Shively CA, Adams MR. Estrogen and progesterone replacement therapy reduces low density lipoprotein accumulation in the coronary arteries of surgically postmenopausal cynomolgus monkeys. *J Clin Invest*. 1991;88:1995–2002.
22. McKinney KA, Duell PB, Wheaton DL, Hess DL, Patton PE, Spies HG, Burry KA. Differential effects of subcutaneous estrogen and progesterone on low-density lipoprotein size and susceptibility to oxidation in postmenopausal rhesus monkeys. *Fertil Steril*. 1997;68:525–530.
23. Clarkson TB. Estrogens, progestins, and coronary heart disease in cynomolgus monkeys. *Fertil Steril*. 1994;62:147S–151S.
24. Williams JK, Honoré EK, Washburn SA, Clarkson TB. Effects of hormone replacement therapy on reactivity of atherosclerotic coronary arteries in cynomolgus monkeys. *J Am Coll Cardiol*. 1994;24:1757–1761.
25. Hanke H, Hanke S, Finking G, Muhic-Lohrer A, Mück AO, Schmahl FW, Haasis R, Hombach V. Different effects of estrogen and progesterone on experimental atherosclerosis in female versus male rabbits: quantification of cellular proliferation by bromodeoxyuridine. *Circulation*. 1996;94:175–181.
26. Manning JM, Campos G, Edwards IJ, Wagner WD, Wagner JD, Adams MR, Parks JS. Effects of hormone replacement modalities on low density lipoprotein composition and distribution in ovariectomized cynomolgus monkeys. *Atherosclerosis*. 1996;121:217–229.
27. Adams MR, Register TC, Golden DL, Wagner JD, Williams JK. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arterioscler Thromb Vasc Biol*. 1997;17:217–221.
28. Haarbo J, Leth-Espensen P, Stender S, Christiansen C. Estrogen monotherapy and combined estrogen-progestogen replacement therapy attenuate aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. *J Clin Invest*. 1991;87:1274–1279.
29. Hough JL, Zilversmit DB. Effect of 17 beta estradiol on aortic cholesterol content and metabolism in cholesterol-fed rabbits. *Arteriosclerosis*. 1986;6:57–63.
30. Adams MR, Williams JK, Clarkson TB, Jayo MJ. Effects of oestrogens and progestogens on coronary atherosclerosis and osteoporosis of monkeys. *Baillières Clin Obstet Gynaecol*. 1991;5:915–934.
31. Haarbo J, Svendsen OL, Christiansen C. Progestogens do not affect aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. *Circ Res*. 1992;70:1198–1202.
32. Batra S, Källstrand K. Are there cyclic variations in estradiol secretion in the non-pregnant rabbit? *Experientia*. 1979;35:699–701.
33. Ginsburg ES, Mello NK, Mendelson JH, Barbieri RL, Teoh SK, Rothman M, Gao X, Sholar JW. Effects of alcohol ingestion on estrogens in postmenopausal women. *JAMA*. 1996;276:1747–1751.
34. Kuhl H. Pharmacokinetics of oestrogens and progestogens. *Maturitas*. 1990;12:171–197.
35. Rijpkema AHM, van der Sanden AA, Ruijs AHC. Effects of postmenopausal oestrogen-progestogen replacement therapy on serum lipids and lipoproteins: a review. *Maturitas*. 1990;12:259–285.
36. Fischer GM, Swain ML. Effects of estradiol and progesterone on the increased synthesis of collagen in atherosclerotic rabbit aortas. *Atherosclerosis*. 1985;54:177–185.
37. Kushwaha RS, Hazzard WR. Exogenous estrogens attenuate dietary hypercholesterolemia and atherosclerosis in the rabbit. *Metabolism*. 1981;30:359–366.
38. Crook D. The metabolic consequences of treating postmenopausal women with non-oral hormone replacement therapy. *Br J Obstet Gynaecol*. 1997;104(suppl 16):4–13.
39. Crook D, Cust MP, Gangar KF, Worthington M, Hillard TC, Stevenson JC, Whitehead MI, Wynn V. Comparison of transdermal and oral estrogen-progestin replacement therapy: effects on serum lipids and lipoproteins. *Am J Obstet Gynecol*. 1992;166:950–955.
40. Tikkanen MJ. The menopause and hormone replacement therapy: lipids, lipoproteins, coagulation and fibrinolytic factors. *Maturitas*. 1996;23:209–216.
41. Haarbo J. Hormone replacement therapy and cardiovascular disease: the rabbit model. *Br J Obstet Gynaecol*. 1996;103(suppl 13):49–52.
42. Prelevic GM, Jacobs HS. Menopause and post-menopause. *Baillières Clin Endocrinol Metab*. 1997;11:311–340.
43. Grady D, Gebretsadik T, Kerlikowske K, Ernster V, Petitti D. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. *Obstet Gynecol*. 1995;85:304–313.
44. Hirvonen E. Progestins. *Maturitas*. 1996;23(suppl):S13–S18.
45. Speroff L, Rowan J, Symons J, Genant H, Wilborn W. The comparative effect on bone density, endometrium, and lipids of continuous hormones as replacement therapy (CHART Study): a randomized controlled trial. *JAMA*. 1996;276:1397–1403.
46. The Writing Group for the PEPI Trial. Effects of hormone replacement therapy on endometrial histology in postmenopausal women: the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. *JAMA*. 1996;275:370–375.
47. Ferenczy A, Gelfand MM. Endometrial histology and bleeding patterns in post-menopausal women taking sequential, combined estradiol and dydrogesterone. *Maturitas*. 1997;26:219–226.
48. Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Christiansen C. Raloxifene inhibits aortic accumulation of cholesterol in ovariectomized, cholesterol-fed rabbits. *Circulation*. 1997;96:1964–1969.
49. Elhage R, Arnal J-F, Pieraggi M-T, Duverger N, Fiévet C, Faye J-C, Bayard F. 17 β -estradiol prevents fatty streak formation in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 1997;17:2679–2684.