Estrogens and Experimental Atherosclerosis in the Baboon (Papio cynocephalus)

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SUMMARY One hundred twenty-six adult female baboons (Papio cynocephalus) were hysterectomized and all except 18 were ovariec- tomized. The animals were fed a moderately atherogenic diet (40% calories from hydrogenated vegetable oil, 1.5 mg cholesterol/kcal) for two years. Ovariectomized-hysterectomized animals received estrone sulfate, ethinyl estradiol, or diethylstilbestrol orally in daily doses similar to those given humans. An ovariectomized-hysterectomized group and a hysterectomized group received no drug. The average total serum cholesterol concentration rose from 136 mg/dl to 223 mg/dl and declined to 186 mg/dl. Concentrations of cholesterol, triglyceride, and phospholipid in whole serum, low density lipoproteins and high density lipoproteins showed no consistent statistically significant differences among the groups. Triglyceride and phospholipid concentrations were higher in the estrogen-treated and intact-ovary groups than in the ovariectomized nonestrogen treated group, but not all pairwise comparisons were statistically significant. There were no consistent statistically significant differences in atherosclerotic lesions among the groups. Neither ovariectomy nor estrogen replacement influence diet-induced experimental atherosclerosis in the baboon within two years.

THERE ARE CONFLICTING OBSERVATIONS on the relationship between estrogens and atherogenesis in both humans and experimental animals. Premenopausal white women consistently have a lower incidence and prevalence of myocardial infarction5-4 and a lessened extent of advanced atherosclerotic lesions in the coronary arteries5-6 than do white men of the same age. However, some studies show that women ovariectomized before the natural meno-pause lose their relative immunity to coronary heart dis-ease,7-9 while others find no effect of ovariectomy.10, 11 One study finds atherosclerosis more severe in ovariec-tomized women,12 but another finds no difference.13 According to observations from The Framingham Study, postmeno-ulosa women have a higher incidence of coronary heart dis-ease than age-matched premenopausal women.14 Despite a prevailing impression that the menopause increases risk of coronary heart disease, age and sex specific mortality rates do not increase after the menopause more than would be expected with age.5, 12, 15 Attempts to reduce the incidence of coronary heart disease in postmenopausal women by estrogen replacement therapy also have yielded conflicting results.16-18

The results of animal experiments on the relationship between estrogens and atherogenesis are as inconsistent as those from human studies. Estrogens induced hyperlipidemia in chickens20 but inhibited coronary artery lipido-sis in cockerels21; reduced the size of plaques in White Carneau pigeons in high doses22-24; produced a variety of changes in arteries of rats25-28; and augmented, inhibited, and failed to affect diet-induced hypercholesterolemia and atherosclerosis in rabbits.29-32

In the hope of resolving some of these conflicting observations, this experiment was planned to examine the effects of ovariec-tomy and estrogen replacement therapy on serum lipids and atherogenesis in the baboon, whose reproductive physiology, estrogen and cholesterol metabolism, greater prevalence of aortic fatty streaks in the feral female compared to the feral male, and response to an atherogenic diet are more human-like than are those of chickens, rats, and rabbits.33-36 In order to induce moderate hyperlipidemia and experimental atherosclerosis, the animals were fed a mildly atherogenic diet for two years. The estrogens tested were estrone sulfate, a biologically active conjugate of estrone and a major circulating estrogen in humans; 17α-ethyl estradiol, a highly potent, orally effective synthetic estrogen structurally similar to estradiol; and diethylstilbestrol, a non-steroidal compound differing in chemical structure from the natural estrogens but having estrogenic activity.

Methods and Materials

Subjects

The subjects were 126 adult female baboons (Papio cynocephalus) ranging in estimated age from 5 to 15 years. The animals were of feral origin, but many had been in the Southwest Foundation for Research and Education (SFRE) breeding colony for several years. During this experiment, they were individually caged. All animals were hysterecto-mized to eliminate the uterine bleeding that occurs with continuous estrogen therapy. All but 18 also were ovariec-tomized at the time of hysterectomy. Eight animals died during the experiment from causes unrelated to the experi-mental procedure. On the average, these did not differ in age, serum lipids, or weight from the remaining 118.

Experimental Design

Although the original principal investigator (LRA) did not use a strict randomization procedure for the assignment of treatments to animals, we analyzed the results as a com-
pletely randomized design with seven treatments. There was no evidence of bias in the assignment process. Table 1 outlines characteristics of the treatment groups. All animals received the atherogenic diet from the beginning of the experiment. Drug treatment for groups 1, 3, and 5 began on day 133 after initiating the diet. Drug treatment for groups 2 and 4 began on day 498 to examine the possibility that estrogens might modify lesions previously established by diet-induced hyperlipidemia.

### Table 1. Selected Characteristics of Experimental Animals, Ovarian Status, and Estrogen Treatment by Experimental Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of baboons</th>
<th>At start of experiment</th>
<th>Days after operation</th>
<th>Ovarian status</th>
<th>Duration, days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td>Estimated age, years</td>
<td>Weight, kg</td>
<td>Serum cholesterol</td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>17</td>
<td>1.6</td>
<td>14.3</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>17</td>
<td>1.6</td>
<td>14.3</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>18</td>
<td>1.6</td>
<td>14.3</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>17</td>
<td>1.6</td>
<td>14.3</td>
<td>1.6</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>15</td>
<td>1.6</td>
<td>14.3</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>16</td>
<td>1.6</td>
<td>14.3</td>
<td>1.6</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>18</td>
<td>1.6</td>
<td>14.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

| Abbreviations: ES = estrone sulfate; EE = 17 a-ethyl estradiol; DES = diethylstilbestrol. |

Estrogen Dosage and Administration

Estrogen doses approximated, on a weight basis, the usual human doses for replacement therapy. Drugs were administered by embedding tablets in fig bars. The rate of failure to take the drug ranged from 0.1% of treatment days in group 2 to 1.5% in group 3. The overall omission rate for all groups was 0.64%, and only one animal had an omission rate greater than 5%.

Baboons in groups 1 and 2 received 0.625 mg/day, or about 45 μg/kg/day, of Ogen (Abbott Laboratories) of pure crystalline estrone sulfate (ES) stabilized with piperazine. This dose in baboons may equal a higher dose in humans since plasma concentrations of estrogenic steroids are lower in baboons than in humans.

Baboons in groups 3 and 4 received 17 μg/day, or about 1.2 μg/kg/day, of Estinyl (Schering Corporation) as the source of 17α-ethyl estradiol (EE).

Baboons in group 5 received 62.5 μg/day, or about 5 μg/kg/day, of diethylstilbestrol, USP (DES) (Eli Lilly and Co.).

Diet

The baboons had been fed SFRE baboon chow (Ralston Purina Co.) from the time of their arrival at SFRE until beginning the atherogenic diet for this experiment. This diet (table 2) was prepared by mixing ground baboon chow, hydrogenated vegetable oil (Crisco, Procter and Gamble Co.), frozen egg yolk, cholesterol, and 6 ml water per 100 g. Its caloric value was about 3.8 kcal/g wet weight. Each baboon received 360 g per day, about 90 kcal/kg/day, for the first 487 days of the experiment. Because many animals gained excess weight, the amount offered each day thereafter was reduced by 20%. The final diet mixture contained 5.62 mg/g (wet weight) cholesterol, 0.12 mg/g campesterol, and 0.40 mg/g β-sitosterol by gas-liquid chromatographic analysis. Thus, the diet contained about 1.5 mg cholesterol per kcal.

### Table 2. Composition and Nutritional Value of the Atherogenic Diet per 100 g Dry Weight

<table>
<thead>
<tr>
<th>Component</th>
<th>Total g</th>
<th>Protein</th>
<th>Fat</th>
<th>Carbohydrate</th>
<th>Fiber</th>
<th>Ash</th>
<th>Cholesterol mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baboon chow</td>
<td>67.1</td>
<td>14.1</td>
<td>3.7</td>
<td>32.9</td>
<td>2.8</td>
<td>5.2</td>
<td>4</td>
</tr>
<tr>
<td>Crisco</td>
<td>11.7</td>
<td>0</td>
<td>11.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Egg yolk, frozen</td>
<td>20.8</td>
<td>3.1</td>
<td>5.7</td>
<td>2.1</td>
<td>0</td>
<td>0</td>
<td>239</td>
</tr>
<tr>
<td>Cholesterol, USP</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>366</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>17.2</td>
<td>21.1</td>
<td>35.0</td>
<td>2.8</td>
<td>5.2</td>
<td>609</td>
</tr>
</tbody>
</table>
Autopsy Procedures

We sedated the animals with an intramuscular injection of Sernylan and performed complete autopsies, including gross and histologic examination of all major viscera. The iliac, femoral, innominate, brachial, carotid, and coronary arteries were opened longitudinally and prepared by methods used in the International Atherosclerosis Project. The aorta from the ligamentum arteriosus to its bifurcation was divided along anterior and posterior midlines. The right half of the aorta was frozen for chemical analysis, and the left half, after being sampled for electron microscopy, was prepared for grading like the other arteries.

Grading of Atherosclerotic Lesions

Two experienced pathologists (one of the authors, H.C. McGill; and a consultant, J.P. Strong) independently graded the stained arterial specimens by the method of the International Atherosclerosis Project. Graders were not aware of treatment group or other data, but all the arteries from each animal (except the coronary arteries) were in the same plastic bag. Product moment correlation coefficients between grades of different arterial segments did not exceed 0.34, a value which indicated that the grades of some segments did not unduly influence the grades of other segments. The intraclass correlation coefficient between estimates by the two pathologists of percent intimal surface area involved with fatty streaks for the aorta ranged from 0.77 to 0.84, values indicating acceptable interobserver variability of the grading. Independent regrading of 20 sets of specimens at a later time indicated that variability within pathologist was similar to variability between pathologist.

We compared aortic fatty streaks among treatment groups on the basis of extent of intimal surface involved. Because grades of fibrous plaques in all arteries and of fatty streaks in some arteries frequently were small or zero, we compared these lesions among treatment groups on the basis of prevalence rather than extent. Specimens were scored as positive only if both pathologists independently classified the specimen as positive.

Aortic Intimal Cholesterol

A technician stripped the intima from the unfixed half of each aorta. Lyophilized and saponified tissue was analyzed by gas-liquid chromatography. A technician measured the total intimal surface area of the unfixed half of the aorta from full size photographs using an electronic digitizer (Wang 662). The coefficient of variation after retreading a randomly selected sample of 20 photographs was 2.0%. From the area and weight of the intima and the concentration of cholesterol, we estimated cholesterol per unit area of intimal surface.

Statistical Methods

Responses were analysed in separate univariate analyses, usually by analysis of variance (ANOVA). For ANOVAs that resulted in a statistically significant overall F test, all pairwise contrasts between treatment group means were computed and evaluated with Tukey's t-test. Analyses by the Kruskal-Wallis ANOVA for ranks confirmed the results of the parametric ANOVAs. In some instances, as noted, analysis of covariance (ANCOVA) was used. For data such as presence of lesions, we tested the null hypothesis of independence between treatment groups and presence of lesions by a Chi-square test.

Evidence of Estrogen Stimulation

All baboons in the group with intact ovaries (7) continued normal menstrual cycles throughout the experiment as indicated by periodic turgescence of the sex skin. The ovariotomized group (7) showed no turgescence of the sex skin. Baboons in groups which received estrogens (1-5) showed moderate sustained turgescence of the sex skin, an observation indicating that they were receiving estrogenic stimulation.

Body and Organ Weights

The group receiving EE from the start of the experiment (3) gained less weight than other groups, and the group receiving EE in the second year (4) also gained less weight after hormone treatment began. The DES treated group (5) lost weight steadily after about day 300 of the experiment; the weight loss began before the daily diet ration was reduced. Mean weights of all groups fluctuated over time in a manner apparently unrelated to treatment. Mean body weights at autopsy of the two EE treated groups (3 and 4) were lower than those of all other groups, and pairwise comparisons by ANCOVA with initial body weight as the concomitant variable were statistically significant for these two groups as compared to the group treated with ES for two years. Organ weights at autopsy, analyzed by ANCOVA with total body weight at autopsy as the concomitant variable, showed no real differences.

Serum Lipids and Lipoproteins

The mean total serum cholesterol concentration of all baboons rose in the first three months of the experimental period from 136 mg/dl to 223 mg/dl, declined during the following 18 months to 186 mg/dl, and increased slightly near the end of the experiment. We compared serum lipid and lipoprotein concentrations among groups by ANOVA, using values from two blood samples drawn between 653 and 814 days after the start of the experiment. We reasoned that long term effects of the drugs should be apparent during this period, and that they would not be masked by the large changes associated with beginning the atherogenic diet. Table 3 shows results for cholesterol, triglyceride, and phospholipid in whole serum, LDL, and HDL. Chyomicrons and VLDL (not included in table 3) contained only a minor fraction of total lipids and showed no consistent differences among groups.

Neither cholesterol nor phospholipid concentrations in lipoprotein fractions or in whole serum differed between the ovarietomized group (6) and the group with intact ovaries (7). Total triglyceride, LDL triglyceride, and HDL triglyceride concentrations were higher in all estrogen treated groups (1-5) and in the group with intact ovaries (7) than in the ovarietomized group (6). However, statistically significant pairwise comparisons were limited to groups 1 and 3.

Phospholipid concentrations were slightly higher in the
groups treated with ES and EE for 2 years (1 and 3) than in controls or other treatment groups, and several of the differences were statistically significant.

The decrease in caloric intake after day 487 on the atherogenic diet showed no relationship to changes in cholesterol, triglyceride, or phospholipid concentrations in whole serum or in lipoprotein fractions.

Characteristics of Arterial Lesions

Table 4 shows overall measures of fatty streaks and fibrous plaques by artery. Grossly, the lesions resembled those previously described in the baboon. Although some coronary artery lesions were elevated and resembled fibrous plaques, none produced significant stenosis.

Microscopically, all lesions showed intimal thickening with smooth muscle cells. Lesions classified grossly as fibrous plaques were principally smooth muscle cells with varying amounts of lipid in the subintimal layer, and occasionally a core of necrotic debris and extracellular lipid. Electron micrographs from the thoracic and abdominal aorta and coronary arteries of several animals from each experimental group showed that most intimal lipid was within smooth muscle cells, but some was extracellular and some was in macrophages. We detected no qualitative differences among the experimental groups in histologic sections or electron micrographs.

Aortic Fatty Streaks

Table 5 shows treatment group means of percent surface involvement with fatty streaks by aortic segments. The component of variance within animal (due to interobserver difference in grading) was less than the component of variance between animals. Only two pairwise comparisons were statistically significant — in the abdominal aorta, DES treated (5) and ovariectomized untreated (6) groups were more extensively involved by fatty streaks than the untreated group with intact ovaries (7).

In five of the other arteries, the null hypothesis of independence between treatments and presence of fatty streaks was rejected ($P < 0.05$). These five were further examined by partitioning the degrees of freedom. The group treated with EE for two years (3) had a significantly lower prevalence of fatty streaks in the right carotid, left carotid, left brachial, and left circumflex coronary arteries. The group treated with ES in the second year (2) and the ovariectomized group (6) had a significantly higher prevalence of fatty streaks in the left coronary circumflex artery than other groups. The group treated with ES from the beginning (1) and the group with intact ovaries (7) had a significantly lower prevalence of fatty streaks in the left anterior descending coronary artery than did all other groups.

Concentration of Cholesterol in Aortic Intima

The overall means of concentrations of cholesterol in the stripped aortic intima were 25.0 mg/g and 111.9 mg/cm².

### Table 4. Percent of Intimal Surface Involved with Fatty Streaks and Prevalence of Fatty Streaks and Fibrous Plaques by Arterial Segment

<table>
<thead>
<tr>
<th>Arterial segment</th>
<th>Intimal surface involved with fatty streaks, percent</th>
<th>Prevalence, percent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Aortic arch</td>
<td>11.8</td>
<td>0</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>35.1</td>
<td>1</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>18.6</td>
<td>0</td>
</tr>
<tr>
<td>Right carotid</td>
<td>7.1</td>
<td>0</td>
</tr>
<tr>
<td>Left carotid</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>Right brachial</td>
<td>6.1</td>
<td>0</td>
</tr>
<tr>
<td>Innominate</td>
<td>12.3</td>
<td>0</td>
</tr>
<tr>
<td>Left brachial</td>
<td>11.3</td>
<td>0</td>
</tr>
<tr>
<td>Right iliac-femoral</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td>Left iliac-femoral</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>Right coronary</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Left coronary, ant. desc.</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>Left coronary, circumflex</td>
<td>0.7</td>
<td>0</td>
</tr>
</tbody>
</table>

N = 118 baboons.

*Prevalence less than 100% although lowest percent surface involvement is not 0 because range is from the mean of independent estimates and prevalence is based on both observers scoring specimen as positive.

†Mean of independent estimates by 2 observers.
and overall estimated standard deviations were 17.8 and 60.3. No real differences among the treatment groups were apparent by either measure.

Fibrous Plaques

The null hypothesis of independence between treatments and presence of fibrous plaques was rejected ($P < 0.05$) only for the left iliac-femoral artery. The group treated with ES for the second year (2) and the group treated with DES (5) had a significantly higher prevalence of fibrous plaques as indicated by partitioning the degrees of freedom.

Discussion

Serum Lipids and Lipoproteins

The slight and consistent, but not always statistically significant, elevations in serum triglyceride and phospholipid concentrations in the estrogen treated groups and in the group with intact ovaries compared to ovariectomized untreated controls are consistent with the reported effects of estrogenic compounds in humans. The difference we observed, however, is small, and slight elevations of triglyceride and phospholipid concentrations in the absence of increased cholesterol levels would not be expected to augment atherogenesis. Furthermore, no major shifts in distribution of lipids among the lipoproteins appear to result either from ovariectomy or estrogen replacement. The failure to find a higher serum cholesterol concentration in ovariectomized untreated baboons than in those with intact ovaries or in those receiving estrogens is not consistent with the elevation in serum cholesterol associated with surgical or natural menopause in humans. Detailed comparison of these results with observations on other animal species shows some similarities but many differences.

Atherosclerosis

The comparisons of arterial lesions in this experiment indicate no consistent effect of estrogen deficiency or estrogen replacement on extent or quality of atherosclerotic lesions. The results, therefore, are consistent with reports that ovariectomized women have no greater atherosclerosis at autopsy and experience no higher incidence or prevalence of atherosclerotic disease than women with intact ovaries. They are consistent with reports that estrogen replacement therapy does not influence coronary heart disease prevalence in postmenopausal women. They also are consistent with reports that physiological doses of estrogens do not reduce spontaneous atherosclerosis in pigeons or experimental atherosclerosis in rabbits. We have found no reports of the effects of estrogens on diet-induced atherosclerosis in nonhuman primates.

The extent of fatty streaks exceeded that produced in other baboons by similar moderate levels of hyperlipidemia. This difference may be due to age or sex, since most experiments with atherogenic diets in baboons have used young adult males rather than females. The abundant smooth muscle and fibrous tissue in fatty streaks may be typical of the reaction of nonhuman primates to moderate prolonged hyperlipidemia, as observed in rhesus monkeys. These lesions differ from the foam cell lesions typical of experimental animals with higher serum cholesterol levels. Multiple pregnancies also may have caused smooth muscle proliferation, since Wexler has described nonlipid containing intimal thickening in the arteries of breeder female rats. Most of the baboons were multiparous, but because they were feral, accurate assessment of parity was not possible.

Lesions classified grossly as fibrous plaques resembled fibromuscular cushions described in the coronary arteries and aortas of normal rhesus monkeys and feral baboons autopsied immediately after capture. We believe that our baboons probably had developed many of these plaques prior to the experiment. The relatively high frequency of fibrous plaques in the femoral arteries may have been due to trauma to the arteries during venipuncture. Hyperlipidemia may have augmented fibrous plaques, and probably was responsible for lipid deposition within them as reported for hyperlipidemic rhesus monkeys. Whatever their origin, they do not appear to have been affected either by ovariectomy or estrogen replacement.

Conclusions

In the female baboon fed a moderately atherogenic diet for two years, neither ovariectomy nor estrogen replacement after ovariectomy produces consistent and statistically significant differences in serum lipid or lipoprotein concentrations or in atherosclerosis. There is a trend (not statistically significant) for baboons with intact ovaries or ovariectomized baboons receiving exogenous estrogens to have slightly higher serum triglyceride and phospholipid concentrations than ovariectomized baboons. The results are consistent with epidemiologic studies of humans which show no effects of either estrogen deficiency or estrogen replacement therapy on serum lipid or lipoprotein concentrations or on atherosclerosis.

Acknowledgments

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