Effect of Estrogen Dosage upon Plasma, Liver and Bile Lipids in Cholesterol-Fed Cockerels

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ABSTRACT
The effects of four dosages of conjugated equine estrogens (Premarin) on atherogenesis and on plasma, liver and bile lipids were studied in cockerels maintained on a high-fat, high-cholesterol diet. A lowering effect, not dose-dependent, occurred in plasma linoleic and linolenic acid. An augmentative effect, varying with dose, occurred in plasma phospholipids, plasma palmitic, stearic and oleic acids, liver total fat and thoracic atherosclerosis. A lowering, dose-dependent effect occurred in coronary atherosclerosis, plasma cholesterol-phospholipid ratio and plasma arachidic acid. A lowering, dose-dependent effect occurred in plasma cholesterol (decrease at lowest dosage and increase at higher dosages); (2) bile cholesterol (increase at lower dosages and a decrease at higher dosages); (3) liver cholesterol (increase at lowest dose, and decrease at higher dosages); (4) plasma arachidonic acid (increase at lower and decrease at higher dosages). The mechanisms responsible for these changes are not clear but are under investigation.

ADDITIONAL KEY WORDS plasma fatty acids total liver fat
atherosclerosis plasma cholesterol plasma phospholipids fecal sterols
bile acid levels bile cholesterol aorta atherosclerosis

Previous work from this laboratory has shown that cockerels maintained on a high-fat, high-cholesterol diet are protected against coronary atherosclerosis when given estrogens. The exact mechanism of this protective action has not been established to date. Estrogens have a profound effect on lipid metabolism which may be involved in their antiatherogenic action. This does not exclude the possibility of an independent estrogen action on the arterial wall. Estrogens, like diet-induced cholesterol, cause an elevation of the plasma cholesterol level in the chicken. Unlike cholesterol feeding, however, estrogens cause a concurrent and even greater rise in the serum phospholipid level so that the cholesterol-phospholipid ratio (C/P) in the blood declines.1-5

In order to cast further light on this subject, we studied: (1) the effect of four dosages of estrogens; (2) the influence of estrogens on plasma fatty acids, cholesterol, phospholipid and C/P ratio; and (3) the influence of estrogens on bile and liver cholesterol.

Methods
Five groups of approximately 50 Hy-line hybrid strain cockerels, 8 weeks old, were studied. All birds were received on the day of hatching and reared in a battery brooder on commercial chick starter mash until 5 weeks of age. They were then separated and kept in cages until the start of the experiment. All groups received the same diet consisting of commercial chick starter mash (20% protein) supplemented with 1% cholesterol and 5% cottonseed oil. Groups 2 to 5 received conjugated equine estrogens (Premarin) daily in their drinking water. The amount of estrogens added to the water in these groups was 12.5, 25, 50, and 75 mg per
chick per day, respectively. The water containing Premarin was given to each group in the morning before the regular water supply was added in order to ensure total consumption. Precautions were taken to prevent spillage. Obviously, the amount taken by each bird varied. After 5 weeks on these regimens, blood was withdrawn and the animals were killed. Bile samples were obtained by direct puncture of the gall bladder with a syringe, and fecal samples were taken from the terminal colon to avoid contamination with urine. The livers were homogenized in hot ethanol, and an ethanol-ether mixture was added to make the final proportions of these solvents 3:1. These extracts were washed three times with water to remove nonlipid constituents, dried, and the total liver fat was weighed. The plasma, bile and liver cholesterol levels and the digitonin-precipitable sterols in the feces of each animal were determined by a modification of the Sperry-Webb procedure. Plasma phospholipids were determined by the method of Zilversmit et al. after extraction with ethanol-ether (3:1), and the C/E ratio of the plasma was calculated.

Aortic and coronary artery atherosclerosis were determined using our criteria and without knowledge of the group to which the bird belonged. Aortic atherosclerosis was graded grossly from 0 (no lesions) to 4+ (most severe), taking into consideration the area involved and the elevation of plaques. Coronary atherogenesis was determined histologically by taking 2 blocks of tissue from each heart and counting the arteries in one frozen section stained with Sudan IV and hematoxylin from each block; vessels with atheroma were counted and calculated as percent of total vessels. Vessels with intimal and/or medial lipid infiltration without thickening of the intima were counted as normal vessels.

The plasma fatty acid composition was determined by gas-liquid chromatography following methyl transesterification. Analysis was carried out on a Barber Coleman model 10 unit equipped with a 10-foot ethylene glycol succinate column. The column was maintained at 178°C with an argon flow rate of 60 ml per min. National Heart Institute reference compounds were used as standards. Calculations of the area under the curves were made by triangulation, and the results reported here are a percentage of the total fatty acid present.

Some preliminary data were obtained on bile acid levels. Di- and trihydroxycholanic acids were determined by differential spectrophotometry.

Results

Food intake was comparable within each series, and there was no significant effect of estrogens on weight gain.

Plasma fatty acids and fecal sterols were determined only on series 80, liver and bile lipids on series 76 and 80. Other data were obtained from all three series. The data from the three series in these experiments were comparable, and therefore, were combined. Tables 1 to 3 present the mean with the standard error of the data and their statistical significance. Table 4 shows the significance of the values between successively increasing doses of estrogens.

Changes from the control when the various doses of Premarin were given are classed as follows:

1. **No effect.** In the doses used, Premarin had no statistically significant effect on body weight, the severity of abdominal aorta atherosclerosis—although both showed an increase (Table 1), nor on digitonin-precipitable fecal sterols (Table 2), plasma myristic, palmitoleic acids and an unidentified fatty acid (Table 3).

2. **Effect, not dose-dependent.** A significant lowering effect, not dose-dependent, occurred with Premarin on testes weight, comb index (Table 1) and plasma linolenic and linoleic acids (Table 3).

3. **Dose-dependent effect.** An augmentative effect which was dose dependent was obtained with Premarin on the severity of thoracic aorta atherosclerosis (Table 1), plasma phospholipids (Table 2), and plasma palmitic, oleic, and stearic acids (Table 3) and total liver fat (Table 2). The maximum effect on these occurred at different doses. The effect on thoracic aorta atherosclerosis was greatest with 12.5- and 25-mg doses of Premarin, and diminished with higher doses, so that the effect was nil with 75 mg (Table 1). In the case of plasma stearic acid, the effect was limited to 12.5- and 25-mg doses while 50- and 75-mg doses had no effect (Table 3). On the other hand, plasma palmitic and oleic acids had a maximum effect at 50- and 75-mg dose levels (Table 3). In the case of plasma phospholipids, there was a progressive increase with
TABLE 1
Effect of Various Dosages of Premarin on Secondary Sex Characteristics and Aorta and Coronary Atherosclerosis in Cockerels Fed an Atherogenic Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no. of birds</th>
<th>Change in body weight (g)</th>
<th>Testes weight (g)</th>
<th>Comb index</th>
<th>Thoracic aorta lesions % Incidence Average grade</th>
<th>Abdominal aorta lesions % Incidence Average grade</th>
<th>Coronary artery lesions % Birds % Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Premarin (control)</td>
<td>48</td>
<td>+ 560 ± 18†</td>
<td>3.09 ± 0.43</td>
<td>59.1 ± 2.6</td>
<td>98 1.11 ± 0.06</td>
<td>83 0.88 ± 0.10</td>
<td>100 17.0 ± 1.2</td>
</tr>
<tr>
<td>12.5 mg Premarin*</td>
<td>49</td>
<td>+ 614 ± 13</td>
<td>0.47 ± 0.07†</td>
<td>16.5 ± 1.3†</td>
<td>100 1.59 ± 0.11§</td>
<td>94 1.11 ± 0.10</td>
<td>69  8.5 ± 1.5§</td>
</tr>
<tr>
<td>25 mg Premarin*</td>
<td>50</td>
<td>+ 655 ± 18</td>
<td>0.78 ± 0.17†</td>
<td>19.9 ± 1.5†</td>
<td>100 1.70 ± 0.10§</td>
<td>96 1.16 ± 0.11</td>
<td>43  3.9 ± 0.8§</td>
</tr>
<tr>
<td>50 mg Premarin*</td>
<td>48</td>
<td>+ 663 ± 20</td>
<td>0.41 ± 0.02†</td>
<td>18.0 ± 1.2†</td>
<td>100 1.43 ± 0.09§</td>
<td>90 1.08 ± 0.11</td>
<td>27  1.4 ± 0.4§</td>
</tr>
<tr>
<td>75 mg Premarin*</td>
<td>49</td>
<td>+ 642 ± 18</td>
<td>0.39 ± 0.02†</td>
<td>15.5 ± 0.6†</td>
<td>100 1.19 ± 0.08∥</td>
<td>88 0.88 ± 0.09</td>
<td>33  1.7 ± 0.5§</td>
</tr>
</tbody>
</table>

*Conjugated equine estrogens (daily dose).
†Standard error of the mean.
§P < .01.
∥Not significant; calculated by Student's t test.
‡P < .001.

TABLE 2
Effect of Various Dosages of Premarin on Some Plasma Lipids, Liver Lipids, Bile Cholesterol and Fecal Sterols in Cockerels Fed an Atherogenic Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma C/P (mg%: 100 g)</th>
<th>Plasma phospholipids (mg%)</th>
<th>Plasma cholesterol (mg%)</th>
<th>Bile cholesterol (mg%)</th>
<th>Fecal sterols (mg%)</th>
<th>Liver cholesterol (mg%)</th>
<th>Liver total fat (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Premarin (control)</td>
<td>2.43 ± 0.06§</td>
<td>446 ± 16</td>
<td>1120 ± 63</td>
<td>793 ± 64</td>
<td>2111 ± 142</td>
<td>4337 ± 204</td>
<td>12.1 ± 0.5</td>
</tr>
<tr>
<td>12.5 mg Premarin</td>
<td>1.28 ± 0.04§</td>
<td>735 ± 34§</td>
<td>920 ± 38§</td>
<td>1208 ± 69§</td>
<td>1929 ± 84</td>
<td>4593 ± 335§</td>
<td>12.7 ± 0.5</td>
</tr>
<tr>
<td>25 mg Premarin</td>
<td>1.09 ± 0.04§</td>
<td>1328 ± 128§</td>
<td>1245 ± 78§</td>
<td>933 ± 79§</td>
<td>2345 ± 344</td>
<td>3078 ± 252§</td>
<td>13.7 ± 0.7</td>
</tr>
<tr>
<td>50 mg Premarin</td>
<td>0.74 ± 0.04§</td>
<td>2517 ± 196§</td>
<td>1570 ± 73§</td>
<td>846 ± 61§</td>
<td>2219 ± 169</td>
<td>2526 ± 219§</td>
<td>14.3 ± 0.8</td>
</tr>
<tr>
<td>75 mg Premarin</td>
<td>0.60 ± 0.04§</td>
<td>3405 ± 168§</td>
<td>1772 ± 62§</td>
<td>531 ± 24§</td>
<td>2030 ± 236</td>
<td>1877 ± 110§</td>
<td>15.2 ± 0.6</td>
</tr>
</tbody>
</table>

*Series 76 and 80.
†Series 80 only.
§P < .01.
∥Not significant; calculated by Student's t test.
‡Standard error of the mean.
increasing dosage with the maximum effect at 75 mg (Table 2).

A lowering effect which was dose dependent was obtained with Premarin on the severity of coronary atherosclerosis (Table 1), on plasma C/P ratio (Table 2) and on plasma arachidonic acid (Table 3). In the first two, increasing the dose of Premarin increased the lowering effect but in a curvilinear manner, each successive dose caused a smaller decrement than the preceding one. In the case of plasma arachidonic acid, the decline occurred only with 50- and 75-mg doses.

4. Diphasic dose-dependent effect. Plasma cholesterol, bile cholesterol (Table 2), liver cholesterol (Table 2) and plasma arachidonic acid showed a diphasic response (Table 3), large doses producing the reverse effect of small doses. Large doses of Premarin (50 and 75 mg) caused a progressive increase in plasma cholesterol, the 12.5-mg doses caused a decrease, and the 25-mg doses had no significant effect. However, in other experiments a 25-mg dose led to an increase in plasma cholesterol. In bile cholesterol, 12.5 mg produced a rise; 75 mg, a fall; and 25 and 25
mg had no effect. The liver cholesterol level increased with the 12.5-mg dose and was significantly lower than the control at the larger doses of Premarin. The response of plasma arachidonic acid was different; 12.5- and 25-mg doses produced a small rise (which was not statistically significant) while 50 and 75 mg produced a statistically significant fall from the control value.

Discussion

It is apparent from these results that estrogen effects are dose dependent in many instances and may, in some cases, actually be diphasic in that one dosage has an effect opposite to that obtained with another dose. Some of the statistically negative effects noted may be attributable to the limits of the accuracy of measurement used for that parameter or the fact that the number of observations made were not sufficient to give the observed deviation statistical significance. This study also shows that results with any single dose of estrogens may be deceiving if predicting effects over a wide dose range. It would be important to see whether other estrogens have similar dose-dependent effects on cockerels and also what a change in dose level of these estrogens will do in other species. Some published data on estrogens which seem to differ from others may in part be accounted for on the basis of the dose of estrogens used.

We have previously described a dose-dependent effect of estrogens on ulceration of the atheromatous plaque in the chicken. Recently, Horlick found no significant dose-dependent effects of diethylstilbestrol on plasma cholesterol level in the rat in the dose range he employed. We know of no other study on the influence of estrogen dose on lipid metabolism or atherosclerosis.

Atherosclerosis in different vascular beds

The present study shows again the existing difference in behavior of different parts of the systemic arterial tree (to estrogens) which we have previously reported. The differences between the thoracic aorta, abdominal aorta, and the coronary arteries hold over the entire dose range of Premarin used in these experiments. Estrogens have a slight atherogenic effect (not statistically significant), on abdominal aorta atherosclerosis in the dose range employed, whereas it is definitely atherogenic in the thoracic aorta and definitely antiatherogenic in the coronary arteries. Atherogenicity in the thoracic aorta is greatest with the smaller doses used; antiatherogenesis in the coronary arteries is greatest with the larger doses, the maximum being reached at 50 mg. The difference in the effect of Premarin in these three arterial beds is thus shown to exist over a wide range of dosage being greatest at the 25-mg level—the dose we have used most frequently in the past. It would appear that these differences must depend on conditions within the three vascular beds, but exactly what the critical factors are has not yet been established.

When one compares the effect of Premarin on the magnitude of diet-induced atherosclerosis in the coronary vessels with its effect on the plasma lipids studied, parallelism to its effect on the C/P ratio can be clearly seen. The relation to the fatty acids of the plasma are discussed below.

Bile and liver cholesterol

We have previously shown that in bile fistula birds, estrogens had an effect on bile cholesterol opposite to that on plasma cholesterol both with and without added dietary cholesterol. The present study shows that this inverse action of estrogens on bile cholesterol as compared to plasma cholesterol extends over the entire dose range of Premarin used. Both plasma and bile show a diphasic response. With 12.5-mg of Premarin there is a decline in plasma cholesterol and, at the same time, a rise in bile cholesterol. With large doses of Premarin the reverse occurs.

The mode of action of estrogens in leading to these changes is not established. It is possible that an increase in the volume of bile formation occurred with larger doses of Premarin which caused a more dilute bile without any significant alterations in the quantity of cholesterol excreted. However, it is unlikely that this is the sole mechanism because the
opposite effects are seen when bile and plasma cholesterol levels are compared, and similarity of these effects are observed for bile and liver cholesterol levels.

It is more likely that estrogens act through an increased catabolism and excretion of cholesterol metabolites. Changes in the excretion of nondigitation-precipitable neutral sterols and/or bile acids could, at least in part, account for the differences in the bile cholesterol levels of the estrogen-treated birds. However, our results fail to show any effect on fecal neutral sterols. Preliminary results on the bile acid levels in these animals indicate that there is a distinct increase in the level of both the di- and trihydroxycholanic acids in the group receiving 75 mg of the estrogen. There is an initial decrease in the level of the bile acids at the lower dosages as compared to the control group with a gradual decline of the difference as the dosage is increased. The data obtained in the present experiment suggest an effect of Premarin on the handling of cholesterol by the liver in which large doses of Premarin appear to have an effect opposite to the small doses. However, the exact mechanism and importance of estrogens on lipid-bile acid metabolism remains to be found.

**PLASMA LIPIDS**

Small dosages of Premarin lead to a fall in plasma cholesterol while large doses cause a rise. Plasma phospholipids show a dose-dependent progressive increase. The ratio between the two, the plasma C/P ratio, shows a progressive fall because the increase of the phospholipids is greater than that of cholesterol. This C/P ratio fall is curvilinear with increasing dosage of Premarin—the increase becomes progressively less as dosage is increased, primarily because of the biphasic response of plasma cholesterol.

Our results suggest that the actions of estrogens on the distribution of fatty acids in whole plasma are complex. Only the changes in plasma arachidonic acid (and the inverse of the changes in plasma linoleic acid) can be considered to be roughly parallel to the atherogenic action of Premarin on the thoracic aorta at the various doses used. There is some evidence in the literature suggesting that polysaturated fatty acids and especially arachidonic acid play an important role in atherosclerosis. Thus, Sinclair has suggested that dietary deficiency of essential fatty acid is responsible for human atherosclerosis through a replacement of the polysaturated fatty acids in plasma cholesterol esters by saturated and non-essential fatty acids. This is followed by the deposition of the more saturated esters in the arterial wall.

It is possible that further analysis may reveal other, perhaps more complex, interactions among the lipids in ester form in the plasma and the several vascular beds. It would be valuable to know exactly in what form these fatty acids are in the plasma, e.g., their combination with glycerides, cholesterol and phospholipids and their quantitative distribution among the classes of lipoproteins.

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