Contraceptive Steroid Effects on Lipids and Lipoproteins in Cynomolgus Monkeys

John S. Parks, Susan J. Pelkey, John Babiak, and Thomas B. Clarkson

Seventy-three adult female cynomolgus monkeys fed an atherogenic diet were studied to determine the effect of two different combination contraceptive steroid preparations containing equivalent amounts of estrogen but different progestin components on plasma lipids and lipoproteins. Our hypothesis was that any high density lipoprotein (HDL) lowering effect of the contraceptive steroid preparations was proportional to the rise in total serum cholesterol caused by the progestins. For 2 years, one group (Ovral [Wyeth Laboratories], n=23) received 75 \( \mu \text{g} \) norgestrel and 7.5 \( \mu \text{g} \) ethinyl estradiol daily, while another (Demulen [Searle & Co.], n=25) received 150 \( \mu \text{g} \) ethynodiol diacetate and 7.5 \( \mu \text{g} \) ethinyl estradiol daily. The control group (n=24) received no treatment. On average, the two oral contraceptive groups had higher total serum cholesterol and triglyceride concentrations but lower HDL cholesterol concentrations and smaller low density lipoproteins (LDL) compared with the control group. There was an inverse relationship between total serum cholesterol and HDL cholesterol for all three groups, but at any given total serum cholesterol concentration between 250 and 500 mg/dl, the Ovral group had HDL cholesterol concentrations that averaged 37% and 14% lower than the control and Demulen groups, respectively. The decrease in HDL concentrations with oral contraceptive treatment was associated with a sharp decrease in (HDL\(_{2a}\))\(_{\text{apo}}\) protein (82% for Ovral and 59% for Demulen) and a corresponding increase in (HDL\(_{3a}\))\(_{\text{apo}}\) protein as determined by gradient gel electrophoresis. Of 23 animals in the Ovral group, six had LDL subfractions >10 nm diameter (HDL\(_{2a}\))\(_{\text{apo}}\), compared with 22 of 24 animals in the control group. Although LDL size, on average, was smaller and plasma triglycerides were greater with oral contraceptive treatment compared with controls, there was no apparent relationship between LDL size and plasma triglyceride concentrations. We conclude that: 1) the smaller LDL particles of Demulen vs. control female cynomolgus monkeys was not related to the differences in plasma triglyceride concentrations between the two groups, and 2) treatment of female cynomolgus monkeys fed cholesterol with Ovral results in additional HDL\(_{2a}\) lowering compared with that of dietary cholesterol alone or dietary cholesterol with Demulen. (Arteriosclerosis 9:261-268, March/April 1989)

Oural contraceptives usually contain a synthetic estrogen (i.e., ethinyl estradiol) and one of several synthetic progestins. Since estrogen and progestins affect lipoprotein metabolism in unique ways, combined therapy can result in a variety of plasma lipoprotein changes that depend on the relative amounts and kinds of the two steroids. Most estrogens increase plasma high density lipoprotein (HDL) concentrations, while most combination oral contraceptives cause a decrease in plasma HDL due to the progestin component.\(^1\)\(^2\) Many oral contraceptives increase plasma low density lipoprotein (LDL) concentrations.\(^3\) In particular, an increase in the concentration of small, dense LDL particles has been reported, whereas larger, less dense LDL particles may actually decrease in concentration.\(^4\) There has been concern about these effects of oral contraceptives on lipoprotein metabolism because the changes are in the direction of a possible increased risk of coronary heart disease.

For all our studies on contraceptive steroids, we have chosen to study female cynomolgus monkeys (Macaca fascicularis) because, like women, these animals respond to oral contraceptive treatment with marked increases in plasma triglyceride (TG) concentrations and decreases in plasma HDL concentrations, particularly the HDL\(_2\) subfraction.\(^5\)\(^6\)\(^7\) Since an inverse relationship between total serum cholesterol (TSC) and HDL cholesterol (HDL-C) exists for hypercholesterolemic monkeys (TSC>200 mg/dl),\(^8\) we were interested in exploring the interrelationship between TSC and HDL-C in monkeys given progestins of differing structures and pharmacological effects with an equivalent amount of estrogen. We hypothesized that any HDL-lowering effect of the contraceptive steroid preparations would be, as seen with dietary hypercholesterolemia, proportional to the rise in TSC caused by the...
### Table 1. Effects of Oral Contraceptive Treatment on Plasma Lipids and Lipoproteins of Female Cynomolgus Monkeys

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSC (mg/dl)</td>
<td>HDL-C (mg/dl)</td>
</tr>
<tr>
<td>Control (n=24)</td>
<td>316±26</td>
<td>67±6</td>
</tr>
<tr>
<td>Ovral (n=23)</td>
<td>295±27</td>
<td>69±6</td>
</tr>
<tr>
<td>Demulen (n=26)</td>
<td>329±26</td>
<td>72±8</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control vs. Ovral</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Control vs. Demulen</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ovral vs. Demulen</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Posttreatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=24)</td>
<td>270±22</td>
<td>65±7</td>
</tr>
<tr>
<td>Ovral (n=23)</td>
<td>331±28</td>
<td>33±3</td>
</tr>
<tr>
<td>Demulen (n=26)</td>
<td>362±23</td>
<td>41±4</td>
</tr>
<tr>
<td><strong>p value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control vs. Ovral</td>
<td>NS</td>
<td>0.05</td>
</tr>
<tr>
<td>Control vs. Demulen</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Ovral vs. Demulen</td>
<td>NS</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Pre- vs. posttreatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=24)</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Ovral (n=23)</td>
<td>NS</td>
<td>0.01</td>
</tr>
<tr>
<td>Demulen (n=26)</td>
<td>NS</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values were determined on a single blood sample from each animal taken 2 months before oral contraceptive treatment (pretreatment) and between 21 and 23 months after treatment (posttreatment). Values are means±SEM.

*Analysis of variance with Fisher's least significant difference test; NS = not significant at p=0.05.
†Paired t test.

In the experiment reported here, the effects of a norgestrel/ethinyl estradiol containing oral contraceptive on plasma lipoproteins of female monkeys were compared with those of a preparation containing ethynodiol diacetate/ethinyl estradiol. These female monkeys were fed a moderately atherogenic diet for 31 months during which oral contraceptive treatment was given for 2 years.

### Methods

#### Animals

Seventy-three adult female cynomolgus macaques (Macaca fascicularis) were used for the study. The animals were fed for 31 months a moderately atherogenic diet, supplied by Bioserve Inc. (Frenchtown, NJ), that contained 0.39 mg cholesterol/kcal and 38% of calories as saturated fat (lard).

#### Experimental Design

The study was divided into two periods: a 7-month baseline and a 24-month experimental period. During the baseline period, the animals consumed the atherogenic diet, and blood samples were taken for TSC and HDL-C determinations. Using a stratified randomization technique, the animals were assigned to either the control group or to one of two treatment groups based on body weight, TSC, HDL-C, and menstrual cycle history. After assignment of all animals into one of the three groups, the mean and variance of TSC, HDL-C, body weight, and number of menstrual cycles of each of the three groups were essentially the same (Table 1).

During the 24-month experimental period, one group received 75 μg norgestrel and 7.5 μg ethinyl estradiol daily (Ovral, Wyeth Laboratories, Philadelphia, PA), and another group received 150 μg ethynodiol diacetate and 7.5 μg ethinyl estradiol daily (Demulen, Searle & Co., San Juan, Puerto Rico). The contraceptive steroid pills were ground with a mortar and pestle and incorporated into the ground diet with a mixer. The laboratory prepared diets were kept frozen until time for feeding. Oral contraceptive dosages for these animals were equivalent, on a caloric intake basis, to human females consuming 1800 kcal and 1 oral contraceptive pill per day. The control group received no contraceptive steroids.

#### Lipid and Lipoprotein Quantitation

At 2-month intervals throughout the baseline and experimental periods, blood samples were taken from all animals to quantitate TSC, HDL-C, and serum TG concentrations. TSC and TG concentrations were determined by an autoanalyzer method using Lipid Research Clinic methodology.9 HDL-C concentrations were quantitated after precipitation of apoprotein (apo) B containing lipoprotein from serum with heparin-manganese.10

#### Low Density Lipoprotein Molecular Weight Determination

LDL molecular weight measurements were made for all animals during the baseline period (5 months after diet.
initiation; 2 months before treatment started) and after 24 months of oral contraceptive treatment. Blood samples were obtained from the femoral vein of fasted animals after intramuscular administration of ketamine (10 mg/kg). Blood was collected in tubes containing 0.1% EDTA, 0.02% NaN₃, 0.04% DTNB, pH 7.4 (final concentration), and this was immediately placed on ice. Plasma was harvested by low speed centrifugation at 4°C. The lipoproteins were isolated from plasma by ultracentrifugation and agarose column chromatography. With this procedure, LDL size (i.e., LDL molecular weight) was determined for each sample by including a trace amount of an iodinated LDL preparation of known molecular weight.

**Gradient Gel Electrophoresis**

Prepoured polyacrylamide gradient gels (4% to 30%; Pharmacia, Piscataway, NJ) were used to investigate HDL subfraction size heterogeneity. Gels were run as described previously. Briefly, d<1.225 g/ml lipoproteins (four parts) were mixed with one part of a solution consisting of 40% sucrose and 0.01% bromphenol blue, and a 10 to 20 μl aliquot containing 10 μg protein was applied to the gels. Gels were subjected to electrophoresis for 24 hours at 125 volts (10°C). After electrophoresis, gels were stained with Coomassie blue G-250 and were destained in 5% acetic acid. After destaining, the gels were scanned using a laser densitometer, and the percentage HDL subfraction protein distribution was calculated from the area units. Calculation of HDL particle size was based on the mobility of protein standards of known Stokes’ diameters.

**Statistics**

All values are reported as means±standard error. Statistical comparisons were made between control and treatment groups by using analysis of variance and Fisher’s least significant difference test. A paired t test was used for comparisons before and after treatment within a single group.

**Results**

Table 1 contains the TSC, HDL-C, and TG concentrations and the LDL molecular weight values for the three groups of animals during the baseline period (5 months after diet initiation) and after 21 to 23 months of treatment. There was no significant difference in any lipid or lipoprotein measurements among groups during the baseline period. As expected, the lipid and lipoprotein measurements for the control group were similar after the 24-month experimental period compared with the pre-experimental values (Table 1). Compared with the control group at 21 to 23 months of treatment, the Demulen group had significantly higher HDL-C concentrations and significantly lower LDL-C concentrations and smaller LDL. After 21 to 23 months of treatment, the only significant difference between the Ovral versus Demulen groups was high TG concentrations for the Demulen group.

Next, the interrelationship between TSC, TG, and HDL-C concentrations for the three groups of animals were examined. To do this, the average bimonthly TSC or TG values for each group taken throughout the baseline and experimental periods were plotted with the corresponding HDL-C value as shown in Figure 1. Note that each point in Figure 1 represents the mean of 23 to 26 animals in its respective group. There was an inverse relationship between TSC and HDL-C concentrations: as TSC increased, HDL-C decreased. Interestingly, the control and Demulen values appeared to fall on the same regression line, while the Ovral values were uniformly lower. At any given TSC concentration from 375 to ~500 mg/dl, the Ovral group had significantly (p<0.01) lower HDL-C concentrations compared with the control and Demulen groups (32±1, 50±1, and 43±1 mg/dl HDL-C, respectively). A similar relationship was found when plasma TG concentrations were plotted with HDL-C, that is, an inverse relationship was found between TG and HDL-C. The Ovral group had lower HDL-C concentrations at any given TG concentration compared with the control and Demulen groups.

To examine the effect of oral contraceptive treatment on HDL subfraction size distribution, gradient gel electrophoresis was used to further subfractionate HDL. The frequency of occurrence of different HDL size populations for the three groups was examined first. These data are shown in Figure 2. The Rₘ values previously determined for HDL subclasses of human HDL are indicated by the vertical lines. The most striking difference in HDL subfraction distribution with oral contraceptive treatment was found for the Ovral group. Only 6 of 23 animals in the Ovral group had HDL subfractions >100 Å in diameter (HDLₑ₀₀) compared with 22 of 24 animals in the control group. The Demulen group was intermediate between the control and Ovral groups with 15 of 24 animals having detectable (HDLₙₐₙ) subfractions. A similar, but less striking trend was seen for the (HDLₒ₉ₐ) subfraction.

Using the nomenclature of Blanche et al. and the subfraction size ranges established for a human population, we quantitated the percentage protein distribution of HDL subfractions for the three groups of animals by using laser densitometry. The demarcations of each subfraction size range are shown in Figure 2. Table 2 summarizes the data from these analyses. HDL protein was approximately equally distributed among all four HDL subclasses for the control animals. Both contraceptive steroid treatments...
were associated with a statistically significant decrease in the percentage of (HDL_{2a})_{apo} protein (largest subfractions) and a corresponding increase in (HDL_{3a})_{apo} protein (smallest subfractions). However, the difference in (HDL_{2a})_{apo} distribution was not statistically significant between the two oral contraceptive treatment groups. HDL subfractions of intermediate size [(HDL_{2b})_{apo} and (HDL_{3a})_{apo}] were similar for control, Ovral, and Demulen groups.

To examine the effect of total HDL concentrations on the HDL subfraction distribution among the three groups, we plotted HDL-C (a measure reflective of total HDL) versus the percentage HDL subfraction protein distribution. The results are shown in Figure 3. There was an increase in the percentage of (HDL_{2a})_{apo} protein with increasing HDL-C concentrations. Note that many of the Ovral-treated animals had values along the abscissa, indicating that they had no detectable (HDL_{2a})_{apo}. The percentage (HDL_{3a})_{apo} protein showed a marked decrease as HDL-C increased.

Oral contraceptive treatment was associated with an increase in plasma TG and a decrease in LDL size (Table 1). Since we had previously found an inverse relationship between LDL size and plasma TG concentrations, a plot of the individual values of these variables for the three groups of animals was made (Figure 4). There was no apparent relationship between LDL size and plasma TG concentrations over the range of values found in this study.

Discussion

This study was undertaken to determine the effect of two oral contraceptive formulations with the same dose and kind of estrogen but with different progestins on the plasma lipid and lipoprotein concentrations and HDL subfraction distribution of female cynomolgus monkeys. This animal model was chosen because we had previously shown that this species is suitable for studies on the effect of contraceptive steroids on plasma lipoproteins and atherosclerosis. Both contraceptive formulations contained equivalent amounts of ethinyl estradiol (7.5 μg daily) but different progestins; the Demulen group received 150 μg of ethynodiol diacetate daily while the Ovral group received 75 μg of norgestrel daily. The two oral contraceptive groups, on average, had higher TSC and TG concentrations but lower HDL cholesterol concentrations and smaller LDL compared with the control group. The (HDL_{2a})_{apo} subfraction protein was markedly reduced and the (HDL_{3a})_{apo} was increased for the oral contraceptive groups. Similar findings in women taking combination oral contraceptives and in cynomolgus monkeys given Ovral have been reported. In the latter study, Ovral treatment resulted in a significant reduction of HDL concentrations (measured by analytical ultracentrifugation) and LDL size compared with the female control group; total plasma cholesterol concentrations were not significantly different between the two groups. The present study extends our initial findings to investigate the interrelationships between plasma lipids and lipoproteins when two oral contraceptive formulations containing different progestins are used and to test our hypothesis that the HDL lowering effect of the two oral contraceptive preparations was proportional to the increase in TSC caused by the progestins. Despite the HDL lowering induced by both oral contraceptives, coronary artery atherosclerosis was not exacerbated and was reduced among...
Figure 2. Histogram of high density lipoprotein (HDL) subfraction size for female cynomolgus monkeys treated with oral contraceptives. d<1.225 g/ml lipoproteins from each animal were separated on 4% to 30% polyacrylamide gradient gels, and stained gels were scanned with a laser densitometer. Peak Rf values for each HDL subfraction relative to albumin were plotted for each animal. The particle diameter scale was generated by using molecular weight standards of known Stokes' diameters. Vertical dashed lines indicate the HDL subfraction size ranges reported for a human population.

Perhaps the most striking effect of oral contraceptive treatment was its effect on HDL concentrations and subfraction distribution. In general, when nonhuman primates become hypercholesterolemic by consuming dietary cholesterol and saturated fat, there is a decrease in HDL concentrations and a redistribution of HDL subfraction mass; larger, less dense HDL₂b subfractions decrease in amount relative to the smaller, more dense HDL₃ subfractions. The same trend appears to occur for the control and Demulen groups; as TSC increased there was a proportional decrease in HDL-C (Figure 1A). In addition, as HDL-C decreased, the proportion of (HDL₂b)/ₐₗₐ₄₉ decreased.
Table 2. HDL Subtraction Protein Distribution Determined by Gradient Gel Electrophoresis

<table>
<thead>
<tr>
<th>Group</th>
<th>HDL&lt;sub&gt;2b&lt;/sub&gt;</th>
<th>HDL&lt;sub&gt;2a&lt;/sub&gt;</th>
<th>HDL&lt;sub&gt;3a&lt;/sub&gt;</th>
<th>HDL&lt;sub&gt;3b,c&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.4±4.1</td>
<td>23.2±4.0</td>
<td>27.6±4.4</td>
<td>17.6±3.4</td>
</tr>
<tr>
<td>Ovral</td>
<td>5.4±2.5*</td>
<td>23.1±4.7</td>
<td>29.1±4.4</td>
<td>41.4±5.4*</td>
</tr>
<tr>
<td>Demulen</td>
<td>12.0±3.2*</td>
<td>19.6±3.0</td>
<td>33.5±4.1</td>
<td>33.2±3.6*</td>
</tr>
</tbody>
</table>

Values are means±SEM.

*Significantly different from the control group (p<0.05) by analysis of variance and Fisher's least significant difference test.

Figure 3. Plot of high density lipoprotein (HDL) cholesterol concentrations (mg/dl) vs. percentage of HDL<sub>2b</sub> (A) and HDL<sub>3b,c</sub> (B) protein for female cynomolgus monkeys treated with oral contraceptives for 2 years. Percentage HDL protein distribution was determined by gradient gel electrophoresis. Each point represents data from an individual animal.

Figure 4. Plot of serum triglycerides (mg/dl) vs. low density lipoprotein (LDL) molecular weight (MW) for female cynomolgus monkeys treated with oral contraceptives for 2 years. LDL molecular weight (g/μmol) was measured by agarose column chromatography. Each point represents the value for an individual animal.

Decreased and (HDL<sub>3b,c</sub>)<sub>gge</sub> increased (Figure 3). However, these relationships were not observed for the Ovral-treated group. HDL-C concentrations were uniformly lower than the control or Demulen groups regardless of TSC concentrations (Figure 1, Top) and there were few HDL particles in the (HDL<sub>2b</sub>)<sub>gge</sub> size range for the Ovral group (Figure 2). Thus, even though Demulen treatment resulted in a higher average degree of hypercholesterolemia than Ovral treatment, the HDL cholesterol concentrations were 26% lower for the latter group when TSC concentrations were greater than 375 mg/dl (Figure 1). These results suggest: 1) that for an equivalent degree of hypercholesterolemia, Ovral treatment results in additional HDL lowering compared with that of dietary cholesterol alone or dietary cholesterol with a different progestin (i.e., ethynodiol diacetate), and 2) that this lowering occurs in the HDL<sub>2b</sub> size range (Table 2). The decrease in HDL concentrations with Ovral treatment may be secondary to an increased catabolism of apo A-1 as suggested for anabolic steroid therapy or may result from a decreased production of HDL precursor particles by the liver.

Progestins alone given to women have reportedly decreased plasma concentrations of TG and HDL<sub>2</sub> and increased hepatic lipase activity, while estrogenic agents have the opposite effect. When combination contraceptive steroids are given, the plasma lipoprotein response depends on the dosage and biological effects of the progestin and the dosage of the estrogen component. Several studies have examined the effect of Ovral and Demulen on lipo-
protein concentrations of women. Ovral treatment was associated with an increase in plasma TG, 3,14,16,17,18 an increase3,17,18 or no change14 in total plasma and LDL cholesterol, and a decrease in HDL-C. 3,14,16,17,18 Demulen treatment was associated with an increase in plasma TG, 3,14,16,17,18 an increase3,16,17,18 or no change14 in total plasma cholesterol, an increase17 or no change in LDL cholesterol,14,16,18 and no change in HDL-C concentrations.3,14 Many of the same responses were seen in the Ovral and Demulen treated animals of this study. It has been suggested that the decrease in HDL-C concentrations accompanying progesterin administration is related to an increase in hepatic lipase activity.1 However, in studies of a subset of our animals, hepatic TG lipase was reduced 20% to 35% with oral contraceptive treatment compared with controls, but lipoprotein lipase activity was similar for all three groups.21 Thus, the ethinyl estradiol component of these two contraceptive formulations appeared to have a predominant effect on plasma TG concentrations and hepatic lipase activity. However, HDL concentrations and subfraction distribution appear to be influenced more by the progesterin by mechanisms that presumably include synthesis and catabolism of HDL or other intravascular metabolic events that modify HDL (i.e., lecithin: cholesterol acyltransferase) and not by hepatic lipase activity.

LDL size measured as LDL molecular weight was significantly smaller for the Demulen-treated group compared with control animals. LDL molecular weight is a strong positive predictor of the extent and severity of coronary artery atherosclerosis in nonhuman primates.22 Diet-induced hypercholesterolemia in male cynomolgous monkeys is associated with an increase in LDL size, but for female cynomolgus monkeys it is associated with an increase in the number, not size, of LDL particles in plasma.29 However, factors that control LDL particle size are poorly understood. One hypothetical mechanism involves TG for cholesterol ester (CE) exchange. In this scheme, as plasma TG concentrations increase, there is TG for LDL CE exchange. Subsequent hydrolysis of the exchanged TG results in a reduction of LDL size. If this proposed mechanism was responsible for the smaller LDL of the Demulen group, which had higher TG concentrations, then an inverse relationship between LDL size and plasma TG concentrations would be expected. However, such a relationship was not apparent over the range of plasma TG values in this study (Figure 4). This is in contrast to hypertriglyceridemic humans and to ethinyl-induced hypertriglyceridemic monkeys (reference 24 and LL Rudel, personal communication), both of which demonstrated an inverse relationship between LDL size and plasma TG concentrations. Therefore, oral contraceptive treatment apparently affects LDL size by different mechanisms. In at least one other species of nonhuman primates, the African green monkey, a strong correlation between LDL size and hepatic CE content, as well as hepatic very low density lipoprotein cholesterol secretion, has been found (JS Parks, FL Johnson, and LL Rudel, unpublished observations). Whether such a relationship exists in the animals of this study is unknown.

Acknowledgments
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