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Effects of phytoestrogen-coumestrol on lipid and carbohydrate metabolism in young ovariectomized rats may be independent of its estrogenicity

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Two groups of young ovariectomized female rats received one of two treatments. The first group was fed coumestrol in lab chow (200 μg of coumestrol per day) for 14 days; the second group received coumestrol (40 mg/L) via perfusion medium. There was a significant increase (78% compared with the control group) in the uterine weight after coumestrol treatment, which supports the estrogen-like activity of coumestrol. Phytoestrogen diminished the liver and skeletal muscle glycogen contents by 18% and 29%, respectively, and increased the blood glucose level by 24%. Glycogenolytic activity of coumestrol was observed when it acted directly on the liver areas. Although phytoestrogen did not influence insulin and glucagon blood level, liver and to some degree muscle susceptibility to insulin (measured as hormone binding by insulin receptors) was decreased. Coumestrol increased the content of triglycerides in muscle by 113% and enhanced the liver lipid synthesis from glucose by 179%. Liver cholesterol concentration was increased both after coumestrol feeding (by 12%) and when it acted directly on the liver (by 16%). These observations suggest that coumestrol is in general anabolic with regard to lipid and catabolic within-carbohydrate metabolism of young ovariectomized female rats. Based on the results of this study, it is concluded that influence of coumestrol on lipid and carbohydrate metabolism of ovariectomized rats is in part not related to its estrogenic action. (J. Nutr. Biochem. 10:664–669, 1999) *© Elsevier Science Inc. 1999. All rights reserved.*

Keywords: coumestrol; lipids; carbohydrate; young ovariectomized rats

Introduction

The presence in plants of naturally occurring nonsteroidal substances with estrogenic activity has been recognized for over 50 years. These compounds, known as phytoestrogens, are divided into two main groups: isoflavonoids and coumestans.[1] Coumestrol, which was first isolated and characterized by Bickoff et al.,[2] belongs to the latter class. A study of the relative potency of various phytoestrogens showed coumestrol to be the most potent of the estrogen-like plant compounds, as measured by the mouse uterine weight bioassay,[3] although coumestrol is many times less potent than animal estrogens. Coumestrol occurs in plants that are commonly used in animal nutrition. Lookhart[4] reported that 30 mg coumestrol/kg dry matter alfalfa adversely affects cattle, and LeBars and Hurard[5] reported that 80 mg coumestrol/kg decreased fertility in bulls. In addition, products of plants known to be rich in coumestrol are often used as a component of many "health" foods for humans. The coumestrol content of 16 selected human food products of plant origin has been measured.[6] Alfalfa sprouts and soybean sprouts in particular appeared to be rich in coumestrol, containing 0.5 mg/100 g dry weight and 7.11 mg/100 g dry weight, respectively.[6]

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Received April 15, 1999; accepted June 28, 1999.

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655 Avenue of the Americas, New York, NY 10010
0955-2863/99/$–see front matter
PII S0955-2863(99)00047-9

That coumestrol can interfere with normal reproductive processes of domestic and laboratory animals is well documented in the literature.[7–10] The influence of phytoestrogens on reproduction processes is often compared with hyperestrogenization. Animal estrogens have been described for several decades to have extragenital metabolic action as well. Our earlier experiments revealed that coumestrol also could affect energy metabolism in rats.[11,12] The purpose of the present study was to elucidate the mechanism of coumestrol action on carbohydrate and lipid metabolism in young ovariectomized rats by studying (1) the prolonged, 14 days' effects of dietary coumestrol on liver and muscle carbohydrates and lipids and (2) the short-term effects (perfusion) of coumestrol on hepatic metabolism of lipids and carbohydrates. The results are discussed in comparison to estrogen's action.

Methods and materials

Experiment in vivo

Sixteen female Wistar rats weighing 130 ± 5 g (at the start of experiment) were used for the experiments. The rats were young (2 months old) and showed intensive body weight gain. They were housed at 23°C and fed Murigran (Poland) commercial rat chow (ground wheat, 20%; ground corn, 17.5%; ground oat, 15%; wheat bran, 10%; fish meal, 8%; skim milk powder, 12%; casein, 5.5%; yeast powder, 5%; dried green forage, 5%; mineral and vitamin additives, 2%; metabolizable energy, 12.8 MJ/kg of dry matter; crude protein, 24%; crude fat, 3.7%) and tap water. The food was free of coumestrol, which was checked by high performance liquid chromatography (HPLC). Ovariectomy was performed by dorsal incision under light diethyl ether anesthesia. Experiments were performed after 10 days when the animals had recovered and re-entered their original weight-gaining cycle. The animals in the experimental group were fed with food previously supplemented with coumestrol (Eastman Kodak, Rochester, NY USA). Coumestrol was dissolved in ethanol and added to chow, which was then pelleted and evaporated at 50°C. Food for the control animals was prepared in the same way without the addition of coumestrol. Food was given to the rats in amounts that had been estimated during the adaptation period, so that they received 200 μg of coumestrol per day. After 14 days on the experimental diet, the rats were decapitated and the blood, uterus, liver, and sample of thigh muscle (m. biceps femoris) were taken. The uterus was weighted immediately after sacrifice. Liver and muscle were frozen in liquid nitrogen and kept in −20°C until analysis. Serum glucose was measured enzymatically using glucose oxidase and peroxidase (according to Sigma Diagnostic kit, Sigma Chemical Co., St. Louis, MO USA). Blood levels of insulin and glucagon were analyzed radioimmunologically with RIA-Ins (IBJ, Swierk, Poland) and Serano-Diagnostics (Biodata-Diagnostics, Serano, Italy) kits, respectively. Liver and muscle glycogen was extracted with 30% KOH and ethanol, hydrolyzed with amyloglucosidase (Sigma), and estimated as glucose. Blood triglyceride levels were determined by the method of Foster and Dunn,[13] total cholesterol by the method of Richmond,[14] and free fatty acids by the method of Duncombe.[15] Tissues were analyzed in the same way as serum except that the lipids were extracted first.[16]

Liver membranes were prepared according to Havrankova et al.[17] and dissolved in incubation buffer [40 mmol/L TRIS-HCl, pH 7.4, 1% bovine serum albumin (BSA)] to a final concentration of 0.5 mg of membrane protein per 1 mL of incubation mixture. The liver membranes were incubated in triplicate at 4°C for 16 hours in the presence of 0.03 nmol/L of [^{125}I] porcine insulin (IBJ) with increasing amounts of unlabeled porcine insulin (Novo) up to 200 nmol/L. Nonspecific binding was measured with 10 μmol/L of porcine insulin (Novo) and did not exceed 5%. After incubation, the tubes were centrifuged at 15,000 × g, and the pellets were counted in a gamma counter. The binding capacity and dissociation constant were estimated using the computer program LIGAND.[18]

Table 1 Effect of coumestrol in diet on body gain and uterine weight of young ovariectomized female rats

	Control group	Coumestrol group	*P*-value*
Body gain (g/day)	3.0 ± 0.1	3.2 ± 0.2	NS
Uterine weight (mg/100 g BW)	33.0 ± 6.8	58.9 ± 8.8	<0.001

Values are expressed as mean ± SEM. $n = 8$ in each group.
*Estimated by Student's *t*-test.
NS–not significant. BW–body weight.

Liver perfusion

The liver perfusion procedure was adapted from Waynforth.[19] Ten rats were anesthetized with an intraperitoneal injection of urethane prior to opening the abdomen. Any small venous off-shoots from the portal vein were ligated. The portal vein was then canulated, and perfusion was started slowly. The thoracic cavity was opened quickly, and the posterior vena cava was catheterized. The catheter was connected to flasks in which the perfusion medium was collected after passing through the liver. The perfusions were carried out in a nonrecirculating manner.

Eagle's minimum essential medium (MEM; Lublin, Poland) supplemented with 2% of BSA with 0.2 mmol/fatty acids, and gassed with 95% oxygen (O_2)/5% carbon dioxide (CO_2) was used as a perfusate; the temperature was 37°C, pH 7.4. Coumestrol was dissolved in ethanol; subsequently solution was put into flat-bottomed flask and evaporated under nitrogen at 50°C. Medium was added to the flask and mixed with a magnetic stirrer until coumestrol was dissolved. The concentration of coumestrol was 40 mg/L. The flow rate was 2 mL/min. Perfusions were performed as follows: 20 minutes of equilibration with the medium itself and then 30 minutes with coumestrol ($n = 5$). The control perfusions ($n = 5$) were performed in the same way without coumestrol in the medium. To estimate glycogen and lipid synthesis, 2.5 μL/mL of ^{14}C-glucose (NEN Research Products), with radioactivity of 0.1 mCi/mL (specific activity, 9.80 GBq/mmol) were added to the medium.

Samples of liver were taken at the end of the experimental period. The lipid and carbohydrate parameters in the perfusate and liver were determined according to the procedures described for the in vivo experiment.

Statistics

Data from in vitro and perfusion experiments are given as means ± SEM. Measured parameters from control and experimental groups were compared statistically by Student's *t*-test to unpaired data.

Results

Coumestrol fed to ovariectomized female rats did not change their daily body weight gain (*Table 1*). A statistically significant increase in uterine weight was observed

Table 2 Effect of coumestrol in diet on the blood glucose and liver and muscle glycogen levels in young ovariectomized rats

	Control group	Coumestrol group	*P*-value*
Blood glucose (mmol/L)	6.11 ± 0.26	7.56 ± 0.48	<0.02
Liver glycogen (g/kg)	62.0 ± 3.0	51.1 ± 2.9	<0.02
Muscle glycogen† (g/kg)	3.66 ± 0.36	2.60 ± 0.26	<0.02

Values are expressed as mean ± SEM. $n = 8$ in each group.
*Estimated by Student's *t*-test.
†m. biceps femoris.

after phytoestrogen treatment (*Table 1*), which provides evidence for its estrogen-like activity.

As shown in *Table 2* liver and muscle glycogen content decreased markedly as a result of coumestrol feeding. Simultaneously, the serum glucose level was enhanced. Insulin and glucagon do not seem to be responsible for the changes in carbohydrate metabolism parameters because their blood levels were not altered after coumestrol treatment (*Table 3*). However, the decreased binding capacity of high affinity insulin receptors points to the decreased responsiveness of liver to insulin (*Table 4*).

When coumestrol-containing medium was perfused through the liver, there was increased glucose output into the perfusate and diminution of liver glycogen content (*Table 5*). This indicated that coumestrol was glycogenolytic. Coumestrol did not appear to alter glycogen synthesis because it did not change incorporation of ^{14}C-glucose into this carbohydrate (*Table 5*).

The effects of coumestrol on some lipid parameters are shown in *Table 6*. The data show that 14 days' exposure of ovariectomized rats to phytoestrogen resulted in a statistically significant increase in muscle triglyceride content with diminution of serum free fatty acid concentration. The total cholesterol level was enhanced both in the liver and blood serum of coumestrol treated rats (*Table 6*). As shown in *Table 7* perfusion of the liver with coumestrol increased lipid synthesis as measured by incorporation of ^{14}C-glucose. This resulted in higher triglyceride output into the perfusate in the coumestrol group than in the control group. In addition, liver cholesterol concentration was enhanced following the perfusion of the liver with coumestrol (*Table 7*).

Table 3 Effect of coumestrol in diet on the pancreas hormones' blood level in young ovariectomized rats

	Control group	Coumestrol group	*P*-value
Insulin (μU/mL)	40 ± 4	39 ± 5	NS
Glucagon (pg/mL)	320 ± 33	308 ± 34	NS
Insulin/glucagon molar ratio	3.41 ± 0.40	3.44 ± 0.48	NS

Values are expressed as mean ± SEM. $n = 8$ in each group.
*Estimated by Student's *t*-test.
NS–not significant.

Table 4 Effect of coumestrol in diet on characteristics of insulin binding to liver and muscle membrane receptor of young ovariectomized rats

	Control group	Coumestrol group	*P*-value*
Liver			
HAIR			
R_O (fmol/mg of protein)	124 ± 13	69 ± 18	0.05
K_D (nmol/L)	0.59 ± 0.07	0.35 ± 0.09	NS
LAIR			
R_O (pmol/mg of protein)	11.2 ± 5.0	13.1 ± 7.1	NS
K_D (nmol/L)	230 ± 110	98 ± 52	NS
Thigh muscle			
HAIR			
R_O (fmol/mg of protein)	5.36 ± 2.13	2.52 ± 1.12	NS
K_D (nmol/L)	0.06 ± 0.01	0.05 ± 0.01	NS
LAIR			
R_O (pmol/mg of protein)	11.4 ± 4.8	5.1 ± 2.2	NS
K_D (nmol/L)	405 ± 119	249 ± 133	NS

Values are expressed as mean ± SEM. $n = 8$ in each group.
*Estimated by Student's *t*-test.
HAIR–high affinity insulin receptors. LAIR–low affinity insulin receptors. R_O–binding capacity. K_D–dissociation constant. NS–not significant.

Discussion

The estrogenic effect of phytoestrogens is well recognized, because it increases uterine growth in immature or ovariectomized animals.[1] In the present experiment, dietary coumestrol also caused a statistically significant increase in uterine weight (*Table 1*). Coumestrol acts directly on the uterus because it mimics estrogen activity by binding to the mammalian estrogen-receptor sites.[20,21] However, animal estrogens not only affect organs of the reproductive system, but can influence general biochemical pathways as well, mainly those involving carbohydrate and lipid metabolism. The influence of coumestrol on these processes will be discussed.

Ingestion of coumestrol had demonstrable effects on blood glucose levels and on glycogen concentration in the liver and skeletal muscle, which are the main areas of glycogen deposition in the body. Specifically, dietary coumestrol diminished both liver and skeletal muscle glycogen, while increasing the blood glucose level in the young ovariectomized rats (*Table 2*). Similar results have been

Table 5 Effect of coumestrol on carbohydrate metabolism in perfused liver of young ovariectomized rats

	Control group	Coumestrol group	*P*-value*
Glucose output (μmol/30 min)	198 ± 14	358 ± 10	0.001
Liver glycogen† (g/kg)	39.6 ± 8.4	12.9 ± 3.5	0.02
Incorporation of C^{14} glucose into glycogen (cpm * 10^5/g of liver)	5.92 ± 0.88	3.44 ± 0.48	NS

Values are expressed as mean ± SEM. $n = 5$ in each group.
*Estimated by Student's *t*-test.
†Determined after perfusion.
NS–not significant.

Table 6 Effect of coumestrol in diet on some lipid metabolism indices in young ovariectomized rats

	Control group	Coumestrol group	*P*-value*
Blood serum			
Triglycerides (mmol/L)	1.25 ± 0.04	1.38 ± 0.05	NS
Free fatty acids (mmol/L)	0.36 ± 0.03	0.26 ± 0.01	0.01
Total cholesterol (mmol/L)	1.66 ± 0.09	2.00 ± 0.09	0.02
Liver			
Triglycerides (mmol/kg)	25.5 ± 0.7	24.7 ± 1.5	NS
Total cholesterol (mmol/kg)	7.79 ± 0.28	8.75 ± 0.29	0.05
Thigh muscle			
Triglycerides (mmol/kg)	26.4 ± 4.4	56.3 ± 7.0	0.005
Total cholesterol (mmol/kg)	3.38 ± 0.24	3.57 ± 0.38	NS

Values are expressed as mean ± SEM. $n = 8$ in each group.
*Estimated by Student's *t* test.
NS–not significant.

Table 7 Effect of coumestrol on lipid metabolism in perfused liver of young ovariectomized rats

	Control group	Coumestrol group	*P*-value*
Triglycerides output (μmol/30 min)	48.4 ± 4.9	108.0 ± 4.0	0.001
Free fatty acids output (μmol/30 min)	5.02 ± 1.10	3.48 ± 0.37	NS
Liver triglycerides† (mmol/kg)	14.9 ± 2.6	21.2 ± 2.3	NS
Liver cholesterol† (mmol/kg)	6.28 ± 0.31	7.31 ± 0.18	0.02
Incorporation of C^{14} glucose into lipids (cpm/g of liver)	307 ± 63	857 ± 128	0.005

Values are expressed as mean ± SEM. $n = 5$ in each group.
*Estimated by Student's *t*-test.
†Determined after perfusion.

obtained in immature female and adult ovariectomized rats.[11] However, in intact adult rats, glycogen was depleted and uterine weight was unaffected after coumestrol feeding.[11] Thus, the data presented in this article and results of our previous experiments suggest that the decrease of muscle and liver glycogen after coumestrol feeding to female rats may be independent of this phytoestrogen estrogenicity. This was particularly so because the actions of coumestrol differed from those of steroidal estrogens, which had no effect[22] on increased tissue glycogen levels,[23] primarily through stimulation of glucose transport into muscle[24,25] and liver cells.[26] Some authors suggest that this activity of estrogens can be mediated by elevated insulin secretion[27,28] or by enhanced cell receptivity to this hormone.[25,29] The present study did not reveal significant changes in either insulin or glucagon blood levels after coumestrol treatment (*Table 3*). However, the eventual metabolic effect of insulin is the result not only of hormone secretion and its blood concentration but also its interaction with specific cell membrane receptor, internalizations, and postreceptor events cascade.[30–32] Coumestrol in the diet of ovariectomized rats caused a statistically significant decrease in the number of high affinity insulin receptors (HAIR) in the liver (*Table 4*). A similar tendency was observed in skeletal muscle. Decreased HAIR numbers in the liver were also found in male rats[33] and in ovariectomized female rats when coumestrol was administered subcutaneously.[34] Thus, the decreased tissue glycogen content in ovariectomized female rats fed with coumestrol could be partly explained by decreased cellular susceptibility to insulin. This activity of coumestrol differs from estrogens' influence, which are known to play an important role in the maintenance of the normal insulin sensitivity in oophorectomized rats.[25]

However, based on the results from experiments with liver perfusion, another important possibility should be considered. Short-term perfusion of high amounts of coumestrol caused a statistically significant increase in glucose output into the perfusate with concomitant diminution of liver glycogen content (*Table 5*). Such a phenomenon may reflect the glycogenolytic activity of coumestrol when it acts directly on the liver. The unchanged incorporation of ^{14}C-glucose into glycogen in liver perfused with coumestrol suggests that the phytoestrogen acts by promoting liver glycogen decomposition rather than by inhibiting glycogen liver synthesis. Whether this is similar to the direct effect of coumestrol in skeletal muscle remains to be determined.

Because coumestrol changed glycogen metabolism in ovariectomized rats and because carbohydrate biochemical pathways directly linked with lipid metabolism, we investigated the effect of dietary phytoestrogen on the level of triglycerides and cholesterol in blood, liver, and skeletal muscle. In addition, the direct influence of coumestrol on these indices of lipid metabolism in perfused liver was studied. The effect of coumestrol on triglyceride metabolism in perfused liver of ovariectomized female rats seems to be similar to that of animal estrogens, which are known to enhance triglyceride synthesis and secretion in the liver.[35–37] Likewise, coumestrol, acting directly in the liver, promoted lipid synthesis, which was measured as incorporation of ^{14}C-glucose, and it also increased triacylglycerol secretion into the perfusate (*Table 7*). However, the effect of phytoestrogen on blood triglyceride levels seems to be different from that of animal estrogens. Estrogens increase the triglyceride blood levels, not only by stimulation of their liver synthesis, but also through inhibition of this lipid uptake by adipose and muscle tissues secondary to decreased lipoprotein lipase activity.[38–40] Coumestrol may have the opposite effect because it markedly increased the triglyceride accumulation in skeletal muscle of young ovariectomized rats (*Table 6*). This observation is supported by our earlier experiments in which we showed higher muscle triglyceride deposition in immature female and old, ovariectomized before sexual maturity, rats feeding 10 days with coumestrol.[12] This would be in agreement with the work of Oldfield et al.[41] who observed an upward tendency in

fattening of growing lambs fed with coumestrol. On the other hand, the elevated levels of triglycerides in the muscle tissue of young ovariectomized rats after coumestrol feeding might also be the result of lipolysis inhibition because free fatty acid serum concentration was diminished in these animals (*Table 6*). The deposition of triglycerides in the muscle tissue of rats after coumestrol treatment seems to be independent of sex because a similar finding was observed in male rats when phytoestrogen was incorporated into their diet for 10 days.[33]

The influence of coumestrol on cholesterol level in serum and tissues of rats was inconsistent. We observed increased blood and liver cholesterol levels after phytoestrogen feeding (*Table 6*) and increased liver concentrations of cholesterol after perfusion with coumestrol (*Table 7*). However, in male rats, the phytoestrogen had the opposite effect.[33] The obtained findings are not in agreement with fact that phytoestrogens (among them coumestrol) diminished blood cholesterol level in ovariectomized rats.[42] However, although animal estrogens show in general similar action,[43,44] their influence may be opposite depending on dose, age, sex, species, type of estrogen, and duration of treatment.[45] It is possible that coumestrol may also reveal different effects on cholesterol level.

In conclusion, dietary coumestrol fed to young ovariectomized rats produced an accumulation of triglycerides in skeletal muscle with a simultaneous decrease of glycogen levels in muscle and liver. Such changes seem to be at least in part a result of the direct influence of phytoestrogen on liver metabolism and are, to some degree, independent of its estrogenicity. Although results obtained in rats should not be extrapolated directly to livestock and humans, this study points to desirability of controlling coumestrol level in food suspected to contain this phytoestrogen.

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