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—Full Paper—

Coumestrol Decreases Intestinal Alkaline Phosphatase Activity in Post-delivery Mice but does not Affect Vitamin D Receptor and Calcium Channels in Post-delivery and Neonatal Mice

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Abstract. In this study, we investigated the effects of administration of coumestrol during pregnancy on calcium (Ca) metabolism in post-delivery maternal and neonatal mice. From 6.5 to 16.5 days post coitus (dpc), pregnant females were administered daily doses of coumestrol (200 μg/kg body weight/day). One day after parturition, blood samples and the kidneys, liver, jejunum and duodenum were obtained from each of maternal mouse, and blood samples and the kidneys and liver were obtained from neonatal mice. Coumestrol did not have any significant effect on the Ca and inorganic phosphorus concentrations in the sera of the maternal and neonatal mice. No notable effects of coumestrol were observed in relation to Vitamin D receptor expression in the maternal and neonatal mice by immunohistochemical analysis. Coumestrol did not affect the Vitamin D receptor and epithelial calcium channel1 and 2 mRNA levels in any of the organs investigated. Enzyme histochemical analysis showed that coumestrol decreased intestinal alkaline phosphatase activity in the maternal jejunum and duodenum. In the duodenum, coumestrol decreased expression of intestinal alkaline phosphatase, *c-fos* and vascular endothelial growth factor at the mRNA level. However, we did not observe any significant effects of coumestrol on the expression of these genes. In conclusion, coumestrol decreased intestinal alkaline phosphatase activity in the small intestines of maternal mice at the level used in the present study, and the mechanisms underlying this effect are different for the jejunum and duodenum.

Key words: Coumestrol, Delivery, Epithelial calcium channels, Intestinal alkaline phosphatase, Mouse, Vitamin D receptor

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Exogenous endocrine disruptors (EDs) are chemicals that exist in our environment and disrupt normal endocrine systems. Phytoestrogens, candidates of EDs, are plant-derived compounds that have a similar chemical structure to endogenous estrogen and the potential to mimic estrogen activity [1]. These chemicals can compete with 17β-estradiol for binding to estrogen receptors (ERs), although their relative affinity to ERs and transcription activity are substantially less than those of endogenous estrogens. Phytoestrogens can act as both estrogen agonists and antagonists [2]. Legumes are rich sources of phytoestrogens, and our foods and forage crops also contain them. There are several health benefits from these compounds, and phytoestrogens may help to prevent carcinomas, including breast, prostate and colon cancer, heart diseases, osteoporosis and menopausal disorders [2]. Thus, it is expected that phytoestrogens can be used to improve the health of humans, to prevent sickness and for estrogen replacement therapy (ERT). However, in the mid-20th century, sheep fed a diet of predominately subterranean clover suffered from a reproductive disorder that reduced their lambing rates and produced abnormalities in the reproductive organs, permanent infertility, prolapsed uterus and maternal dystocia [3]. These symptoms were called "clover disease", and their cause is thought to be the phytoestrogens in the subterranean clover [2].

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Coumestrol, a phytoestrogen and the most prevalent coumestan derivatives, is found in legumes, particularly food plants such as alfalfa sprouts and various clovers and other leguminous plants [2]. Coumestrol shows some reproductive effects, such as a uterotrophic effect, body weight decrease at vaginal opening and early onset of persistent estrus [4, 5]. On the other hand, coumestrol stimulates bone cells. In cultured cells, coumestrol inhibits differentiation of osteoclasts from macrophage-like cell line RAW264.7 cells and bone resorption and increases bone mineralization in osteoblast primary culture or in osteoblast-like cell line ROS 17/2.8 cells [6–8]. Phytoestrogens interact with ERs [2]. Estrogen plays a major role in the regulation of bone turnover (i.e., inhibition of bone remodeling, suppression of bone resorption and stimulation of bone formation). Coumestrol interacts with ERs most strongly in phytoestrogens [9] and has various effects through ERs. Thus, its stimulating effects on bone cells are considered to be dependent on its estrogenic activity.

Calcium (Ca) plays a crucial role in some targeted organs and is maintained at nearly constant levels in the blood and extracellular fluids by many factors. The demands for fetal accretion of Ca and output of milk are met by physiological changes in Ca metabolism that occur independently of Ca intake and presumably in response to hormonal changes during pregnancy and lactation [10]. Estrogen plays some physiological role in Ca homeostasis [11]. For

example, estrogen regulates activity and mRNA expression of intestinal alkaline phosphatase (IALP) in rats [12] and chicks [13]. IALP, which is encoded by *IAP* gene, is a vitamin D-dependent intestinal protein that regulates Ca absorption [14, 15]. Vitamin D and vitamin D receptor (VDR) also play important roles in Ca metabolism *via* regulation of the epithelial Ca channels (ECaCs). The expression of VDR is, in turn, regulated by estrogen [16]. Although VDR is expressed in most organs, strong expression is observed in the organs involved in Ca metabolism, such as the kidneys, intestines and bones [16]. VDR regulates gene expression by forming a heterodimer with retinoid X receptor (RXR) [17]. The target genes of VDR are associated with mineral homeostasis, particularly regulation of Ca metabolism, and bone formation [17]. ECaCs are important proteins involved in transepithelial Ca absorption [18]. Two ECaCs, namely ECaC1 and ECaC2, belong to the superfamily of transient receptor potential (TRP) channel. The channels are primarily expressed in Ca-transporting epithelia and work as gatekeepers of transepithelial Ca transport, regulating Ca entry [18]. ECaC1 is abundantly expressed in the kidney, whereas low expression is observed in the small intestine. Conversely, ECaC2 is mainly localized in the small intestine and is expressed in almost all organs, such as the kidney [18].

Disorder of Ca metabolism during pregnancy and lactation sometimes causes disease. Milk fever is a hypocalcemic disorder caused by the onset of lactation in the dairy cow. Feeding too much alfalfa, legume pasture, increases the incident rate of milk fever. Although the mineral balance of alfalfa is considered to be a cause of milk fever, phytoestrogens, which are contained in alfalfa at high levels, are thought to be another cause of this disease [2]. In the present study, the effects of coumestrol on the changes in Ca metabolism associated with delivery were investigated in mice to obtain basic information concerning the relationship between phytoestrogens and diseases.

Materials and Methods

Animals and coumestrol treatment

Pregnant ICR mice were obtained from Clea Japan (Tokyo, Japan), housed in polycarbonate cages and maintained in a temperature-controlled room (24 ± 2 C) on a 14-h light (0500–1900 h)/10-h dark cycle. All animals were given free access to tap water and MF rodent feed (composed of soybean cake, white fish meal, yeast, a germ, soybean oil, wheat bran, rice bran, alfalfa meal, wheat flour, maize, milo; Oriental yeast, Tokyo, Japan). They received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (Kyoto University Animal Care Committee according to NIH No. 86–23; revised 1999). From 6.5 to 16.5 days post coitus (dpc), pregnant females were administered a daily dose of coumestrol (200 μg/kg body weight/day, oral gavage, 11 times; Toronto Research Chemicals, Tronro, ON, Canada) dissolved in ethanol (Wako Pure Chemicals, Osaka, Japan) and purified olive oil (Wako; CM group) or ethanol dissolved in purified olive oil (5 ml/kg/day) as vehicle control (VC group) at 1200–1300 h. As a normal control (NC group), pregnant females were kept under the same conditions without administration of either vehicle or coumestrol solution. One day after parturition, blood samples were obtained by cardiac puncture under diethyl ether anesthesia for biochemical analysis, and then the kidneys, liver, jejunum and duodenum were rapidly removed. Portions of these samples were immediately fixed in 10% neutral-buffered formalin (Wako) for immunohistochemistry and enzyme histochemistry. The remaining portions of these samples were frozen in liquid nitrogen and stored at –80 C for semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Neonatal mice were euthanized under diethyl ether anesthesia, and then the kidneys and liver were removed. These samples were immediately fixed in 10% neutral-buffered formalin (Wako) for immunohistochemical analysis. Each group consisted of eight dams. Neonatal mice from the same mother were divided into female group and male groups, and blood samples were obtained under diethyl ether anesthesia for biochemical analysis from 3 to 5 neonatal mice in each group by decapitation.

Biochemical analysis

Each blood sample was stabilized at room temperature for 1 h and then centrifuged at 3,000 rpm for 15 min. The serum was fractionated, and the serum Ca and inorganic phosphorus (Pi) levels were measured using an automatic analyzer (Fuji Drychem 3500 V; Fuji Film, Tokyo, Japan) according to manufacturer's instructions.

Immunohistochemical analysis

After formalin fixation, the kidney, liver, jejunum and duodenum specimens from the maternal mice and kidney and liver specimens from the neonatal mice were routinely embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Subsequently 3-μm thick sections were mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Aldrich Chemical, Milwaukee, WI, USA), deparaffinized with xylene and rehydrated in a graded series of ethanol. The sections were then treated with citrate buffer (pH 6.0) for 20 min at 95 C for antigen retrieval. After washing in phosphate-buffered saline (PBS, pH 7.4), endogenous peroxidase was inactivated by 3% hydrogen peroxide in PBS, and the sections were treated with goat normal serum supplemented with 1% polyxyethylene sorbin monolaurate (Tween 20). The sections were then incubated with specific antibodies for VDR (the dilution ratio was 1:1,000; anti-Vitamin D Receptor, Chicken, Rat-Monoclonal Antibody; Chemicon, Temecula, CA, USA) for 1 h at room temperature. Subsequently, the sections were incubated at room temperature for 30 min with anti-rat immunoglobulin antibody labeled by HRP [Histofine Simple Stain Mouse MAX-PO (Rat); Nichirei, Tokyo, Japan]. The sections then were stained with DAB solution (3,3'-diaminobenzidine tetrahydrochloride; Dako Cytomation, Carpinteria, Glostrup, Denmark). After couterstaining with hematoxylin, the sections were mounted with entellan (Merck) and examined under a light microscope.

Enzymehistochemical analysis

After formalin fixation, the jejunum and duodenum samples from the mother mice were dehydrated in a graded series of ethanol and embedded in paraffin (Histosec). Subsequently, 3-μm thick sections were mounted on glass slides, deparaffinized with xylene and rehydrated in a graded series of ethanol. The sections were

Table 1. Primers for semi-quantitative RT-PCR

Gene	Forward primer	Reverse primer	Product size
VDR	5'-GGATCTGTGGAGTGTGTGGAGACC-3'	5'-CTTCATCATGCCAATGTCCACGCAG-3'	209 bp
*ECaC*1	5'-GAAACTTCTCAATTGGTGGGTCAG-3'	5'-TTTGCCGGAAGTCACAGTT-3'	218 bp
*ECaC*2	5'-GCACAGGCCTTGCAGCAACA-3'	5'-GTACTCCCAGCCCTCCCCACTCT-3'	172 bp
IAP	5'-CTCCTCCTCCTATGACTCTGTAGCC-3'	5'-CCAACTGAGGCTCCACTATTACCC-3'	196 bp
c-fos	5'-ATGGGCTCTCCTGTCAACAC-3'	5'-GACACGGTCTTCACCATTCC-3'	229 bp
VEGF	5'-CAGGCTGCTGTAACGATGAA-3'	5'-AATGCTTTCTCCGCTCTGAA-3'	206 bp
GAPDH	5'-GGGTGGAGCC AAACG GGTC-3'	5'-GGAGT TGCTG TTGAAGTCGC-3'	532 bp

then incubated with 1 mg of Fast Blue RR Salt (Muto Pure Chemicals, Tokyo, Japan) dissolved in 1 ml of stock solution (5 mg Naphthol AS-MX phosphate sodium, 2 ml dimethylformamide, 0.8 mg 2-amino-2-methyl-1,3-propanediol, 1 ml hydrochloric acid and 97 ml distilled water) for 3 min at room temperature. After washing the sections with distilled water and counterstaining with Nuclear Fast Red, the sections were mounted in glycerol (Wako), and the stained sections were then observed by optical microscope.

Semi-quantitative RT-PCR

Total RNA was extracted from homogenized kidney, liver, jejunum and duodenum samples from maternal mice using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Complementary DNA (cDNA) was synthesized with oligo- (dT) primer using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Calisbad, CA, USA) and 2 μg RNA from each sample. The cDNA was quick-chilled on ice to denature the RNA-cDNA duplex. PCR was performed using a Platinum Super Mix Kit (Invitrogen). All of the above procedures were performed according to each manufacturer's protocol. The PCR products were electrophoresed in 2% agarose gel and stained with 1 μg/ml ethidium bromide solution. A ready-load 100 bp DNA ladder (Invitrogen) was used as a molecular weight marker for electrophoresis. After electrophoresis, the gels were recorded with a digital recorder, and then the mRNA expression levels were semi-quantified using ImageJ software (National Institute of Health, Bethesda, MO, USA). The relative abundance of specific mRNA was normalized by the abundance of *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* mRNA. The primer pairs used for *VDR*, *ECaC*1, *ECaC*2, *IAP*, *c-fos*, *vascular endothelial growth factor (VEGF)* and *GAPDH* are shown in Table 1. The PCR conditions for *VDR* were 95 C for 5 min to denature the DNA; 35 (kidney, liver and duodenum) or 40 cycles (jejunum) at 95 C for 30 sec, 56 C for 1 min and 72 C for 22 sec; and then 1 cycle at 72 C for 5 min. The PCR conditions for *ECaC*1 were 95 C for 5 min to denature the DNA; 35 cycles at 95 C for 30 sec, 54 C for 1 min and 72 C for 22 sec, and then 1 cycle at 72 C for 5 min. The PCR conditions for *ECaC*2 were 95 C for 5 min to denature the DNA; 35 (kidney, liver and duodenum) or 40 cycles (jejunum) at 95 C for 30 sec, 56 C for 1 min and 72 C for 20 sec; and then 1 cycle at 72 C for 5 min. The PCR conditions for *IAP* were 94 C for 5 min to denature the DNA; 35 cycles (jejunum and duodenum) at 94 C for 30 sec, 62 C for 1 min and 72 C for 20 sec; and then 1 cycle at 72 C for 5 min. The PCR conditions for *c-fos* were 94 C for 5 min to denature the DNA; 30 cycles (jejunum and duodenum) at 94 C for 30 sec, 60 C for 1 min and 72 C for 1 min; and then 1 cycle at 72 C for 5 min. The PCR conditions for *VEGF* were 95 C for 5 min to denature the DNA; 30 cycles (jejunum and duodenum) at 95 C for 1 min, 55 C for 1 min and 60 C for 1 min; and then 1 cycle at 72 C for 7 min. The PCR conditions for *GAPDH* were 95 C for 5 min to denature the DNA; 35 cycles at 94 C for 1 min, 57 C for 1 min and 72 C for 1min; and then 1 cycle at 72 C for 5 min. To ensure that the experiments were performed in the linear range of the amplification cycles, representative samples were subjected to PCR amplification using five different numbers of cycles for each primer pair [28–32 cycles for *c-fos* and *VEGF*; 33–37 cycles for *VDR*, *ECaC*1, *ECaC*2, *IAP* and *GAPDH*; and 38–42 cycles for *VDR* (jejunum) and *ECaC*2 (jejunum), data not shown]. As a negative control for genomic DNA contamination, the PCR was performed using equivalent amounts of total RNA from representative samples without reverse transcription as templates.

Statistical analysis

Statistical analysis was carried out by analysis of variance using Excel 2001 (Microsoft, Tokyo, Japan). This test was used to evaluate the interaction among three groups. When a difference among three groups was shown, the two-independent-sample *t*-test was applied to evaluate whether or not the difference was a treatment effect. Differences of $P<0.05$ were considered significant.

Results

Biochemical analyses

No significant differences were detected between the groups of maternal mice and neonatal mice in relation to the serum Pi and Ca levels (Table 2). Furthermore, no significant differences were detected between the female and male neonatal mice in relation to the serum Pi and Ca levels.

Immunohistochemistry

No remarkable differences were detected in any of the maternal organs investigated among the NC, VC and CM groups. In the kidneys of the mother mice, VDR was expressed in the proximal and distal tubular cells of the renal cortex (Fig. 1a–c), but was not the expressed in glomeruli. In the renal medulla, VDR expression was detected in almost all tubular epithelial cells, but was not detected in interstitial cells. VDR was expressed in some vascular endothelial cells. In the livers of the maternal mice, VDR was expressed at

Table 2. Effect of coumestrol on the serum calcium and phosphorus levels of maternal and neonatal mice

Parameters			NC	VC	CM
Ca (mg/dl)	Maternal mice		11.00 ± 0.82	11.31 ± 1.03	11.54 ± 0.80
	Neonatal mice	Male	9.28 ± 1.06	8.42 ± 0.78	8.06 ± 0.78
		Female	8.75 ± 0.76	9.36 ± 1.36	8.60 ± 0.61
		Average	9.00 ± 0.92	8.89 ± 1.15	8.33 ± 0.71
Pi (mg/dl)	Maternal mice		11.69 ± 2.93	9.81 ± 2.31	10.56 ± 1.63
	Neonatal mice	Male	11.96 ± 2.25	13.98 ± 1.87	13.70 ± 2.61
		Female	13.12 ± 1.74	14.48 ± 1.79	11.86 ± 2.08
		Average	12.54 ± 1.99	14.23 ± 1.74	12.78 ± 2.43

Each value represents the mean value ± SE. Maternal mice: n=8 per group. NC, normal control; VC, vehicle control; CM, coumestrol.

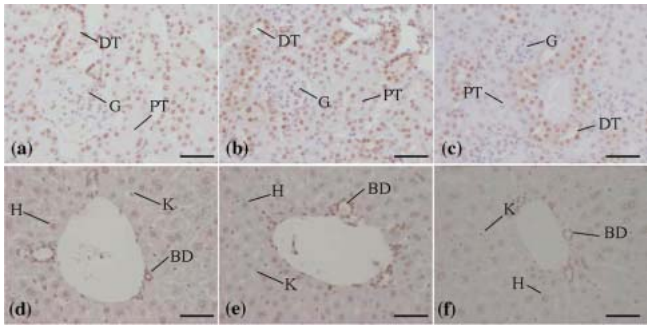

Fig. 1. Vitamin D receptor expression in the kidney cortices of maternal mice in the normal control (a), vehicle control (b) and coumestrol (c) groups and in the livers of maternal mice in the normal control (d), vehicle control (e) and coumestrol (f) groups. Bars= 5 μm. G, glomerulus; PT, proximal tubule; DT, distal tubule; BD, bile duct; H, hepatocyte; K, Kupffer cell.

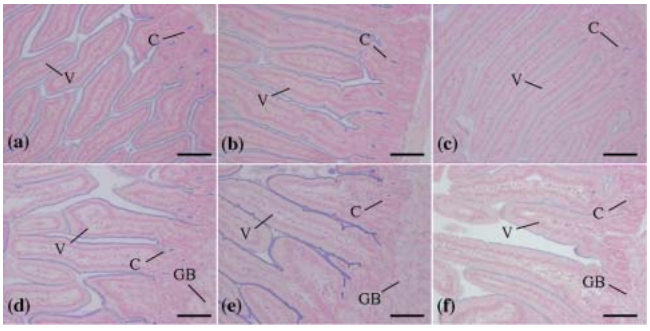

Fig. 3. Alkaline phophatase activity in the jejunums (a–c) and duodenums (d–f) of the normal control (a, d), vehicle control (b, e) and coumestrol (c, f) groups. Blue stains reflect alkaline phosphatase activity. Bars=10 μm. V, villi; C, crypts; GB, Brunner's glands.

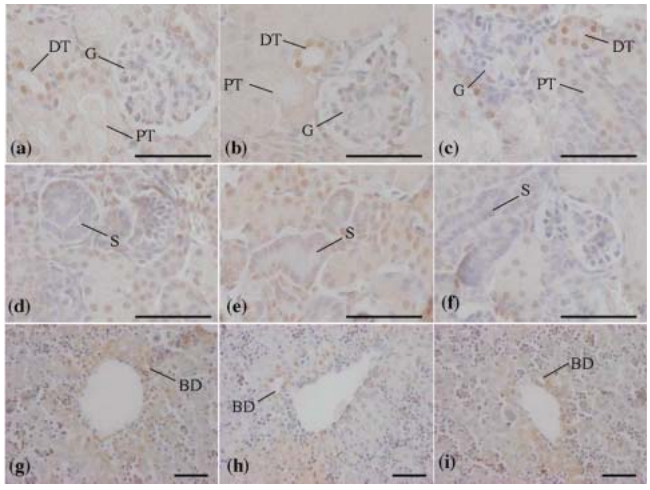

Fig. 2. Vitamin D receptor expression in the kidneys of neonatal males in the normal control (a, d), vehicle control (b, e) and coumestrol (c, f) groups and in the liver from neonatal males in the normal control (g), vehicle control (h) and coumestrol (i) groups. Bars= 5 μm. G, glomerulus; PT, proximal tubule; DT, distal tubule; S, S-shaped body; BD, bile duct.

high level in the bile ductal epithelium and was also weakly expressed in the nuclei of hepatocytes (Fig. 1d–f). However, VDR was not expressed in the nuclei of Kupffer and Ito cells. In the jejunums of the maternal mice, VDR expression was detected in the entire epithelia of villi and crypts and declined from the villi top to the villi stem (not shown). In the duodenums of the maternal mice, VDR was expressed strongly in the entire epithelium of villi compared with in the epithelium of the jejunum. VDR was scattered in the Brunner's glands (not shown).

In the kidneys of the neonatal mice, VDR was expressed in the nuclei of the cells differentiated into glomeruli, proximal tubules and distal tubules (Fig. 2a–c). VDR was not expressed in cells of S-shaped bodies, which have not been wholly differentiated (Fig. 2d–f). In the livers of the neonatal mice, diffusive expression of VDR was detected in the parenchyma (Fig. 2g–i). The bile ducts also showed intense immunoreactions. No remarkable differences were detected in the neonatal mice among the NC, VC and CM groups and between the female and male neonatal mice.

Enzymehistochemistry

In the jejunum, IALP activity was detected in the villous epithelial surface. The jejunums of the CM group (Fig. 3c) showed weaker activity than those of the NC (Fig. 3a) and VC groups (Fig.

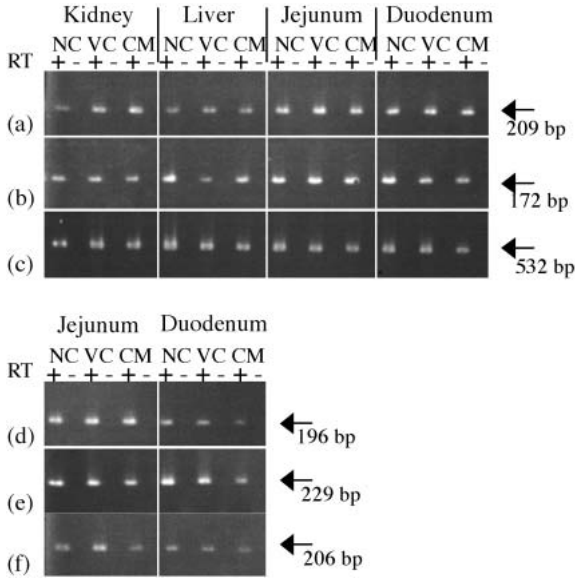

Fig. 4. Expression of mRNA of representative samples for *vitamin D receptor* (*VDR*) (a), *epithelial calcium channel* 2 (*ECaC*2) (b) and *GAPDH* (c) in the kidney, liver, jejunum and duodenum and for *intestinal alkaline phosphatase* (*IAP*) (d), *c-fos* (e) and *vascular endothelial growth factor* (*VEGF*) (f) in the jejunum and duodenum. The lanes marked with "-" contain PCR amplification products from equivalent amounts of total RNA from representative samples without reverse-transcription (RT) as a negative control for genomic DNA contamination.

3b). In the duodenum, IALP activity was detected on the surface of the villous epithelium. IALP activity weakened in the apical region of villi. IALP activity was not detected in the Brunner's glands. Coumestrol decreased IALP activity (Fig. 3d–f).

Semi-quantitative RT-PCR

VDR mRNA was expressed in the kidney, liver, jejunum and duodenum (Fig. 4a). No significant differences were detected among the NC, VC and CM groups for any of the organs investigated (Table 3). *ECaC*1 mRNA was only expressed in the kidney; however, we were able to detect *ECaC*1 mRNA expression in the liver of one of the maternal mice. There were no significant differences in *ECaC*1 mRNA expression in the kidney among the NC, VC and CM groups (not shown). *ECaC*2 mRNA was expressed in the kidney, liver, jejunum and duodenum (Fig. 4b). No significant differences were detected among the three groups in the kidney, jejunum and duodenum. In the liver, administration of vehicle solution and administration of coumestrol decreased the *ECaC*2 at mRNA expression level versus the NC group, respectively ($P<0.01$ for VC and $P<0.05$ for CM; Table 4). *IAP* (Fig. 4d), *c-fos* (Fig. 4e) and *VEGF* (Fig. 4f) mRNA was expressed both in the jejunum and duodenum. No significant difference was detected in the jejunum. In the duodenum, coumestrol administration decreased expression of *IAP*, *c-fos* and *VEGF* compared with the NC and VC groups ($P<0.05$; Table 5).

Discussion

In the present study, we investigated the effects of administration of coumestrol during pregnancy (from 6.5 to 16.5 dpc) on Ca metabolism in post-delivery maternal mice and neonatal mice. As of August 23, 2006, the Ministry of Health, Labour and Welfare of Japan recommends intake of up to 30 mg per day of soybean isofla-

Table 3. Comparion of the *VDR/GAPDH* ratio among the three groups: NC, VC and CM (kidney, liver, jejunum and duodenum)

Organ	NC	VC	CM
Kidney	0.57 ± 0.25	0.70 ± 0.30	0.65 ± 0.28
Liver	0.38 ± 0.26	0.28 ± 0.08	0.45 ± 0.16
Jejunum	1.81 ± 0.84	2.48 ± 1.06	1.68 ± 0.68
Duodenum	1.36 ± 1.04	0.96 ± 0.34	0.86 ± 0.33

Each value represents the mean value ± SE. Kidney and liver: n=5 per group; Jejunum and duodenum: n=8 per group.*VDR*, *Vitamin D receptor*; NC, normal control; VC, vehicle control; CM, coumestrol.

Table 4. Comparion of the *ECaC*2/*GAPDH* ratio among the three groups: NC, VC and CM (kidney, liver, jejunum and duodenum)

Organ	NC	VC	CM
Kidney	1.12 ± 0.19	0.96 ± 0.46	0.80 ± 0.30
Liver	1.06 ± 0.41	0.39 ± 0.11**	0.51 ± 0.10*
Jejunum	2.70 ± 0.91	3.49 ± 1.52	2.55 ± 1.24
Duodenum	1.02 ± 0.39	0.79 ± 0.32	0.89 ± 0.12

Each value represents the mean value ± SE. Kidney and liver: n=5 per group; Jejunum and duodenum: n=8 per group. *: $P<0.05$ *vs*. NC, **: $P<0.01$ *vs*. NC. *ECaC*2, *Epithelial calcium channel* 2; NC, normal control; VC, vehicle control; CM, coumestrol.

Table 5. Comparion of the IAP, c-fos and VEGF/GAPDH ratio among the three groups: NC, VC and CM

Jejunum	NC	VC	CM
IAP/*GAPDH*	1.74 ± 0.61	2.51 ± 1.19	1.91 ± 0.98
c-fos/*GAPDH*	0.87 ± 0.26	1.12 ± 0.50	0.70 ± 0.31
VEGF/*GAPDH*	1.73 ± 0.31	2.22 ± 1.32	1.50 ± 0.71
Duodenum	NC	VC	CM
IAP/*GAPDH*	0.62 ± 0.35	0.44 ± 0.14	0.25 ± 0.13*
c-fos/*GAPDH*	1.23 ± 0.59	0.88 ± 0.25	0.59 ± 0.15*
VEGF/*GAPDH*	0.87 ± 0.50	0.72 ± 0.17	0.44 ± 0.15*

Each value represents the mean value ± SE (n=8 per group). *: P<0.05 *vs*. NC and VC. *IAP*, *intestinal alkaline phosphatase*; *VEGF*, *vascular endothelial growth factor*; NC, normal control; VC, vehicle control; CM, coumestrol.

vone. This corresponds to about 458–570 μg/kg body weight/day based on the average body weight of Japanese adults. We set the dose for oral administration (200 μg/kg body weight/day) based on less than half this limit for isoflavone intake. The effects of oral administration coumestrol at doses of 0.1 to 30 mg/kg body weight have previously been examined in ovariectomized rats; ≥1 mg/kg body weight coumesterol increased uterine weight, ≥10 mg/kg body weight increased the bone mineral density of the tibia and ≥100 μg/kg body weight lowered cholesterol in sera significantly (ED_{50}=0.4 mg/kg body weight) [19]. Based on this data, the dose we used was not expected to have any significant effects on reproductive organs or bone; however, some physiological effects were believed to be possible.

In the pre- and post-delivery maternal body, the circulating Ca level changes remarkably due to the fetal bone mineralization and Ca release into milk [10]. Ca absorption in the intestine and Ca reabsorption in the kidney are essential for maintaining the circulating Ca level. We therefore investigated the effects of coumestrol on VDR, ECaC1, ECaC2 and IALP in association with Ca absorption.

Estrogen increases the expression and activity of VDR in the bones of ovariectomized mice [20]. VDR protein expression is decreased in the liver and increased in the kidney, jejunum and duodenum by estrogen [21–23]. In this study, however, immunohistochemical analysis showed that there were no significant differences in expression of VDR protein among the groups for any of the organs examined (kidneys and livers of maternal and neonatal mice and the maternal jejunum and duodenum). Assessment of the level of *VDR* mRNA expression by semi-quantitative RT-PCR analysis also showed that there were no significant differences. These results suggest that coumestrol at a dose of 200 μg/kg body weight during pregnancy does not have a significant effect on the expression of VDR protein and *VDR* mRNA in maternal and neonatal mice.

Vehicle or coumestrol treatment significantly decreased expression of *ECaC*2 at the mRNA level in the liver, but no significant difference was detected between the VC and CM groups. Thus, this decrease may not have been induced by coumestrol. *ECaC*1 expression is increased by estrogen [24]. However, in the present study, coumestrol had no effect on *ECaC*1 expression in the kidney. Thus, coumestrol may have no effect on the expression of *ECaC*s in post-delivery mice.

IALP regulates Ca absorption in the jejunum and duodenum and its activity and mRNA expression are dependent on vitamin D [14, 15, 25–27]. Absence of estrogen also decreases *IAP* expression and the activity of IALP [12, 13]. In this study, coumestrol decreased the activity of IALP in both the duodenum and jejunum. However, decreased expression of *IAP* mRNA in the CM group was only observed in the duodenum. Futhermore, expression of *c-fos* and *VEGF* mRNA which are increased by estrogen, was decreased in duodenums of the CM group. These results suggest that coumestrol antagonizes the effects of ER on expression of *IAP* mRNA, resulting in decreased *IAP* activity in the duodenum. However, *VDR* expression in the duodenum, which is regulated by estrogen [23], was not affected by coumestrol. An ER-independent mechanism may play a role in *VDR* expression in the duodenum or other factors may regulate expression of *VDR*; however, we do not have any data to support this. In the jejunum, IALP enzyme activity was decreased by coumestrol, but the mRNA expressions of *IAP*, *c-fos* and *VEGF* were unaffected. These results suggest that the decreased IALP activity in the jejunum resulting from administration of coumestrol is caused by a different mechanism from that in the duodenum. Phytoestrogen increases intestinal calcium uptake *via* enhancement of alkaline phosphatase activity in ovariectomized mice [28]. The effects of phytoestrogens on alkaline phosphatase activity may be related to the level of endogenous estrogen. In the presence of high levels of endogenous estrogen, as in the case of the present study, coumestrol may interrupt the ER-derived increase in alkaline phosphatase activity. Although the decrease in IALP activity indicates the possibility that coumestrol decreased Ca absorption from the intestine, we failed to detect a decrease in the serum Ca level.

In conclusion, coumestrol decreased IALP activity in the small intestines of maternal mice at the level used in the present study, and the mechanisms underlying this effect are different in the jejunum and duodenum. In neonatal mice, however, no effects were observed for coumestrol. Further studies are needed to elucidate the effects of coumestrol on calcium metabolism in detail.

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