Effects of Coumestrol Administration to Pregnant and Lactating Mice on Intestinal Alkaline Phosphatase Activity

Yuka Kirihata, Yoshihiro Horiguchi, Michihisa Ueda, Miki Sugimoto, Shuntaro Ikeda, and Shinichi Kume*

Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Correspondence author: Shinichi Kume,
Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
Tel) +81-75-753-6325, Fax) +81-75-753-6345,
E-mail: kume@kais.kyoto-u.ac.jp

* Corresponding author to: Prof. Shinichi Kume, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
e-mail: kume@kais.kyoto-u.ac.jp

The present study was conducted to clarify the effects of coumestrol administration during pregnancy on Ca metabolism during pregnancy and in lactating mice. From 6.5 to 16.5 days post coitus (dpc), pregnant mice were administered at 200 μg/kg body weight/day of coumestrol. The duodenum, jejunum and blood samples were obtained at 17.5 dpc or 10 days after parturition (dap). Coumestrol administration decreased alkaline phosphatase (ALP) activity and mRNA expression of *IAP* and estrogen responsive genes, *c-fos* and vascular endothelial growth factor (*VEGF*), in the duodenum and jejunum of pre-delivery mice. In lactating mice, the ALP activity and mRNA expression of *IAP* were not changed, although coumestrol administration decreased mRNA expression of *c-fos* in the duodeum and *VEGF* in the jejunum. Coumestrol did not affect serum Ca and the expression of vitamin D receptor protein in the duodenum and jejunum. Thus, coumestrol administration during pregnancy may decrease the mRNA expression of *IAP* and the ALP activity in the intestine of the pre-delivery mice through ERα, but coumestrol had little effect on intestinal ALP activity at 10 days after parturition.

Keywords; coumestrol: alkaline phosphatase: Ca metabolism; pre-delivery mice

INTRODUCTION

Phytoestrogens are plant-derived compounds that have similar chemical structures to endogenous estrogens and the potential to mimic estrogen activity (Ososki and Kennelly, 2003). 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. Because phytoestrogens may help to prevent carcinomas, heart diseases, osteoporosis and menopausal disorders (Ososki and Kennelly, 2003), it is expected that the phytoestrogens can be used for estrogen replacement therapy to improve the health status of humans and animals.

Coumestrol, a phytoestrogen and the most prevalent coumestan derivatives, interacts with ERs most strongly in phytoestrogens and has various physiological effects through ERs *in vivo* and *in vitro* (Morito *et al.*, 2002). Ca plays a crucial role in some targeted organs, and the demands for fetal accretion of Ca and output of milk are met by physiological changes in Ca metabolism during pregnancy and lactation, such as renal Ca conservation, increased Ca resorption from the skeleton or enhanced Ca absorption (Kovacs and Kronenberg, 1997). Estrogen plays some physiological role in Ca homeostasis through intestine, kidney and bone (Farhan and Sundeep, 2005). Coumestrol stimulates bone cells and exerts effective prevention against bone resorption in estrogen deficient rats (Ye *et al.*, 2003).

Phytoestrogens can act as both estrogen agonists and antagonists (Ososki and Kennelly, 2003). The effects of oral administration of coumestrol at doses of 0.1 to 30 mg/kg body weight have been examined in ovariectomized rats; ≥ 1 mg/kg body weight of coumestrol increased uterine weight, ≥ 10 mg/kg body weight increased bone mineral density of the tibia, and ≥ 100 μg/kg body weight lowered cholesterol in sera

significantly (Dodge *et al.*, 1996). 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 isoflavone. This corresponds to about 458-570 μg/kg body weight/day on the average body weight of Japanese adults. Based on less than half this limit for isoflavone intake, we previously examined the effects of coumestrol on Ca metabolism in the post-delivery mice given 200 μg/kg body weight/day during pregnancy and showed that coumestrol decreased activity of intestinal alkaline phosphatase (IALP) in both duodenum and jejunum at 1 day after birth (Kirihata *et al.*, 2008). These results suggest that coumestrol at a dose of 200 μg/kg body weight antagonizes the effects of ER on expression of *IAP* mRNA, resulting in decreased activity of *IALP* in duodenum (Kirihata *et al.*, 2008), but it is not clear whether coumestrol administration during pregnancy affects expression of *IAP* mRNA and activity of *IALP* in maternal mice before delivery and lactating mice.

The objective of this study was conducted to clarify the effect of coumestrol administration during pregnancy on Ca metabolism including IALP activity and expression of vitamin D Receptor (VDR) protein in pre-delivery and lactating mice in order to evaluate the role of phytoestrogen for Ca metabolism during periparturient period.

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 h-1900 h)/ 10-h dark cycle. All animals were given free access to tap water and MF rodent feed (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) and “Regulation on Animal Experimentation at Kyoto University”.

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 h-1300 h. As a normal control (NC group), pregnant females were kept under the same conditions without administration of either vehicle or coumestrol solution. The day on which pups were found in the morning was assigned as 1 day after parturition (dap), and the numbers of pups for each dam were reduced to 10 at 2 dap. At 17.5 dpc or 10 dap, blood samples were obtained for biochemical analysis by cardiac puncture under anesthesia by diethyl ether or avertin, and then the duodenum and jejunum 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).

Enzymehistochemical and immunohistochemical analyses. After formalin fixation, the duodenum and jejunum samples were dehydrated in a graded series of ethanol and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). ALP activity and VDR protein expression in the duodenum and jejunum were examined using

enzymehistoshemical and immunohistochemical analyses, respectively. These analyses were conducted as previously described (Kirihata *et al.*, 2008).

Semi-quantitative RT-PCR. Total RNA was extracted from homogenized duodenum and jejunum 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 semiquantified using ImageJ software (National Institute of Health, Bethesda, MD, USA). The relative abundance of specific mRNA was normalized by the abundance of glyceraldehydes 3-phophate dehydrogenase (*GAPDH)* mRNA. The primer pairs and PCR conditions used for *IAP*, *c-fos*, *VEGF*, *VDR*, epithelial Ca channel 1 (*ECaC*1), *ECaC*2 and *GAPDH* are same as those in the previous study (Kirihata *et al.*, 2008).

Biochemical analysis. Each blood sample was stabilized at room temperature for 1 h and then centrifuged at 3000 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.

Statistical analysis. Statistical analysis was carried out by analysis of variance. 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

Intestinal ALP activity

ALP activity was detected on the surface of the epithelia of villi and upper crypts. ALP activity weakened in the apical region of villi. ALP activity was not detected in the Brunner's glands in the duodenums. At 17.5 dpc, the duodenums of the CM group (Fig. 1c) showed weaker activity than those of the NC (Fig. 1a) and VC groups (Fig. 1b). In the jejunum, coumestrol also decreased ALP activity (Fig. 1d-f). In contrast, no remarkable differences were detected among the NC, VC and CM groups in both of the duodenum and the jejunum at 10 dap.

Intestinal VDR expression

No remarkable differences were detected in intestinal VDR expression in the duodenum and jejunum among the NC, VC and CM groups at 17.5 dpc and at 10 dap (not shown). In the duodenums, VDR was expressed strongly in the entire epithelium of villi compared with in the epithelium of the jejunum and scattered in the Brunner's glands.

In the jejunums, VDR expression was detected in the entire epithelia of villi and crypts and declined from the villi top to the villi stem.

Intestinal mRNA expression

IAP, *c-fos* and *VEGF* mRNA was expressed both in the duodenum and jejunum. At 17.5 dpc, coumestrol administration decreased the mRNA expression of *IAP*, *c-fos* and *VEGF* compared with the NC and VC groups in the duodenum and jejunum ($P<0.05$; jejunum, $P<0.01$; duodenum; Table 1). At 10 dap, there were no significant differences in *IAP* mRNA expression in the duodenum and jejunum among the NC, VC and CM groups, but expression of *c-fos* in the duodeum and *VEGF* in the jejunum were decreased in CM group. *VDR* and *ECaC2* mRNA was expressed in the duodenum and jejunum, but *ECaC1* mRNA was not expressed. No significant differences were detected in *VDR* and *ECaC2* mRNA expression among the NC, VC and CM groups at 17.5 dpc and at 10 dap (data not shown).

Serum Ca and Pi

No significant differences were detected in the serum Ca and Pi levels among the groups at 17.5 dpc and at 10 dap (Table 2).

DISCUSSION

Estrogen regulates expression of some genes related to Ca metabolism, such as ALP and VDR. Intestinal ALP, which is encoded by *IAP* gene, increases Ca absorption in the jejunum and duodenum (Dulpuis *et al.*, 1991; Halloran and De luca, 1981). Estrogen regulates activity and mRNA expression of IALP in rats (Matsumoto *et al.*, 1997) and

179 estrogen responsive genes, *c-fos* and *VEGF*, in mice (Hyder *et al.*, 1991; Wang et al.,
180 2009). Coumestrol has been shown to be an oestrogen agonist (Dodge *et al.*, 1996;
181 Jefferson *et al.*, 2002; Kanno *et al.*, 2004; Ye *et al.*, 2003), and phytoestrogens increase
182 intestinal Ca uptake *via* enhancement of ALP activity in ovariectomized mice
183 (Mukherjee *et al.*, 2006).

184 In this study, however, coumestrol at a dose of 200 μg/kg body weigh during
185 pregnancy caused a decrease in IALP activity and mRNA expression of *IAP, c-fos and*
186 *VEGF* in duodenum and jejunum of pre-delivery mice, although serum Ca and
187 expression of VDR protein in the small intestine were not affected. Because
188 phytoestrogens (at 100-1000 times of estradiol) compete effectively with endogenous
189 estrogens, bind to the ER and prevent estrogen-stimulated growth in mammals (Kurzer
190 and Xu, 1997), the effects of phytoestrogens on ALP activity may be related to the level
191 of endogenous estrogen. In general, pregnancy induces a dramatic rise in estrogen, and
192 serum estrogen increases with gestation but decreases after parturition. According to the
193 previous (Kirihata *et al.*, 2008) and present studies, coumestrol administration during
194 pregnancy decreased the expression of *IAP, c-fos* and *VEGF* mRNA in both duodenum
195 and jejunum at 17.5 dpc and only in duodenum at 1 dap, resulting in the decreased
196 activity of IALP in both duodenum and jejunum at 17.5 dpc and 1 dap. Thus, in the
197 presence of high level of endogenous estrogen in mice before parturition, coumestrol
198 administration may interrupt the ER-derived increase of ALP activity in mice around
199 parturition. However, the antagonistic effects of coumestrol may not be maintained for
200 lactating mice, because coumestrol administration had little effect on IALP activity in
201 mice at 10 days after parturition.

202 Vitamin D and VDR also play important roles in Ca metabolism *via* regulation of

ECaCs and *IAP* transcriptionally and the expression of VDR is regulated by estrogen (Bouillon *et al.*, 2003). In this study, the VDR protein expression and the expressions of *VDR* and *ECaC2* mRNA in mice were not affected by the administration of coumestrol, which agreed with the previous study (Kirihata *et al.*, 2008). The expression of *VDR* mRNA is mediated by ERβ in human and rats, while the expression of murine *c-fos* and *VEGF* are induced by ERα dependent pathway (Jelinsky *et al.*, 2003; Wang *et al.*, 2009). It has been reported that the estrogen-antagonistic action of coumestrol observed in mouse brain is mediated by ERα (Jacob *et al.*, 2001) and ERα mRNA expression is much more abundant in murine duodenum than ERβ (Duan *et al.*, 2006; Kanno *et al.*, 2004; Smith *et al.*, 2008). Our results suggest that coumestrol regulates the expression of *IAP* mRNA and the activity of IALP through ERα in mice.

Recent studies in IALP deficient mice reported that lack of IALP does not result in Ca mal-absorption (Narisawa *et al.*, 2003) but impairs intestinal barrier function (Goldberg *et al.*, 2008). Because serum levels of Ca in mice were unchanged at 17.5 dpc, 1dap and 10 dap by coumestrol administration in both a previous (Kirihata *et al.*, 2008) and the present study, it can be concluded that coumestrol at a dose of 200 μg/kg body weight during pregnancy does not have a significant effect on Ca metabolism in periparturient mice. However, laboratory studies in developmental animals have demonstrated the potential for adverse effects following exposure to high levels of soy isoflavones, although perimenopausal and early menopausal women may be more receptive to the therapeutic effects of isoflavones on bone loss prior to the diminution of ERs (Reinward and Weaver, 2006). Thus, feeding large amounts of phytoestrogen may be not recommended for pregnant animals, especially in pre-delivery periods, to prevent adverse effects on Ca metabolism.

In summary, coumestrol administration during pregnancy may decrease the expression of *IAP* mRNA and the activity of IALP in the intestine of the pre-delivery mice through ERα, but coumestrol had little effect on IALP activity at 10 days after parturition.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 21580328) from Japan Society for the Promotion of Science.

References

Bouillon R, Van Cromphaut S, Carmeliet G. 2003. Intestinal Calcium absorption: Molecular vitamin D mediated mechanisms. *J Cell Biochem* 88: 332-339.

Dodge JA, Glasebrook AL, Magee DE, Phillips DL, Sato M, Short LL, Bryant HU. 1996. Environmental estrogens: Effects on cholesterol lowering and bone in the ovariectomized rat. *J Steroid Biochem Mol Biol* 59: 155-161.

Duan LP, Wang HH, Ohashi A, Wang DQ. 2006. Role of intestinal sterol transporters abcg5, abcg8, and npc1l1 in cholesterol absorption in mice: Gender and age effects. *Am J physiol* 290: G269-276.

Dupuis Y, Tardivel S, Porembska Z, Fournier P. 1991. Effect of some alkaline phosphatase inhibitors on intestinal calcium transfer. *Int J Biochem* 23: 175-180.

Farhan S, Sundeep K. 2005. Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun* 328: 688-696.

Goldberg RF, Austen WG, Jr., Zhang X, Munene G, Mostafa G, Biswas S, McCormack M, Eberlin KR, Nguyen JT, Tatlidede HS, Warren HS, Narisawa S, Millan JL, Hodin

RA. 2008. Intestinal alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. *Proc Natl Acad Sci USA* 105: 3551-3556.

Halloran BP, De Luca HF. 1981. Intestinal calcium transport: Evidence for two distinct mechanisms of action of 1,25-dihydroxyvitamin d3. *Arch Biochem Biophys* 208: 477-486.

Hyder SM, Stancel GM, Loose-Mitchell DS. 1991. Presence of an estradiol response region in the mouse c-fos oncogene. *Steroids* 56: 498-504.

Jacob DA, Temple JL, Patisaul HB, Young LJ, Rissman EF. 2001. Coumestrol antagonizes neuroendocrine actions of estrogen via the estrogen receptor alpha. *Exp Biol Med (Maywood)* 226: 301-306.

Jefferson WN, Padilla-Banks E, Clark G, Newbold RR. 2002. Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses. *J Chromatogr B Analyt Technol Biomed Life Sci* 777: 179-189.

Jelinsky SA, Harris HA, Brown EL, Flanagan K, Zhang X, Tunkey C, Lai K, Lane MV, Simcoe DK, Evans MJ. 2003. Global transcription profiling of estrogen activity: Estrogen receptor alpha regulates gene expression in the kidney. *Endocrinology* 144: 701-710.

Kanno S, Hirano S, Kayama F. 2004. Effects of the phytoestrogen coumestrol on rank-ligand-induced differentiation of osteoclasts. *Toxicology* 203: 211-220.

Kirihata Y, Kawarabayashi T, Imanishi S, Sugimoto M, Kume S. 2008. 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. *J Reprod Dev* 54: 35-41.

Kovacs CS, Kronenberg HM. 1997. Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. *Endocrine reviews* 18: 832-872.

Kurzer MS, Xu X. 1997. Dietary phytoestrogens. *Annu Rev Nutr* 17:353-381.

Matsumoto HN, Yamamoto A, Iimura T, Oida S, Ezawa I, Sasaki S, Goseki-Sone M. 1997. Effects of ovariectomy on intestinal alkaline phosphatase expression in rats. *J Nutr Sci Vitaminol (Tokyo)* 43: 529-539.

Morito K, Aomori T, Hirose T, Kinjo J, Hasegawa J, Ogawa S, Inoue S, Muramatsu M, Masamune Y. 2002. Interaction of phytoestrogens with estrogen receptors alpha and beta (ii). *Biol Pharm Bull* 25: 48-52.

Mukherjee M, Das AS, Das D, Mukherjee S, Mitra S, Mitra C. 2006. Role of oil extract of garlic (Allium sativum Linn.) on intestinal transference of calcium and its possible correlation with preservation of skeletal health in an ovariectomized rat model of osteoporosis. *Phytother Res* 20: 408-415.

Narisawa S, Huang L, Iwasaki A, Hasegawa H, Alpers DH, Millan JL. 2003. Accelerated fat absorption in intestinal alkaline phosphatase knockout mice. *Molecular and cellular biology* 23: 7525-7530.

Ososki AL, Kennelly EJ. 2003. Phytoestrogens: A review of the present state of research. *Phytother Res* 17: 845-869.

Reinwald S, Weaver CM. 2006. Soy isoflavones and bone health: a double-edged sword? *J Nat Prod* 69:450-459.

Smith A, Contreras C, Ko KH, Chow J, Dong X, Tuo B, Zhang HH, Chen DB, Dong H. 2008. Gender-specific protection of estrogen against gastric acid-induced duodenal injury: Stimulation of duodenal mucosal bicarbonate secretion. *Endocrinology* 149: 4554-4566.

Wang M, Tan J, Coffey A, Fehrenbacher J, Weil BR, Meldrum DR. 2009. Signal transducer and activator of transcription 3-stimulated hypoxia inducible factor-1alpha mediates estrogen receptor-alpha-induced mesenchymal stem cell vascular endothelial growth factor production. *J Thorac Cardiovasc Surg* 138: 163-171,

Ye SF, Saga I, Ichimura K, Nagai T, Shinoda M, Matsuzaki S. 2003. Coumestrol as well as isoflavones in soybean extract prevent bone resorption in ovariectomized rats. *Endocr Regul* 37: 145-152.

Figure legend

Figure 1. Alkaline phosphatase activity in the duodenums (a-c) and jejunums (d-f) of mice at 17.5 dpc in 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.

317

Table 1. The *IAP*/*GAPDH*, *c-fos*/*GAPDH* and *VEGF*/*GAPDH* ratio among three groups in the duodenum and jejunum at 17.5 dpc and 10 dap.

Genes	NC	VC	CM
IAP			
at 17.5 dpc			
Duodenum	0.60 ± 0.14	0.63 ± 0.22	0.32 ± 0.10**
Jejunum	0.94 ± 0.19	0.98 ± 0.20	0.69 ± 0.23*
at 10 dap			
Duodenum	1.59 ± 0.19	1.61 ± 0.18	1.67 ± 0.30
Jejunum	0.95 ± 0.12	1.19 ± 0.13	1.10 ± 0.15
c-fos			
at 17.5 dpc			
Duodenum	0.75 ± 0.14	0.68 ± 0.17	0.40 ± 0.12**
Jejunum	0.64 ± 0.29	0.61 ± 0.21	0.39 ± 0.11*
at 10 dap			
Duodenum	1.25 ± 0.27	1.31 ± 0.24	0.46 ± 0.12*
Jejunum	0.80 ± 0.14	1.03 ± 0.11	0.50 ± 0.15
VEGF			
at 17.5 dpc			
Duodenum	0.41 ± 0.09	0.34 ± 0.07	0.22 ± 0.04**
Jejunum	0.61 ± 0.20	0.64 ± 0.22	0.30 ± 0.09*
at 10 dap			
Duodenum	1.30 ± 0.21	1.09 ± 0.17	1.23 ± 0.32
Jejunum	0.86 ± 0.14	0.86 ± 0.10	0.42 ± 0.12*

Each value represents the mean value±SE (n=9 per group at 17.5 dpc and n=11 per group at 10 dap).
*: P<0.05 vs. NC, **: P<0.01 vs. NC.
NC, normal control; VC, vehicle control; CM, coumestrol

318
319

320
321
322

Table 2. Serum Ca and Pi among the three groups at 17.5 dpc and 10dap.

Parameters	NC	VC	CM
at 17.5 dpc			
Ca(mg/dl)	10.79 ± 0.49	10.62 ± 0.80	10.31 ± 0.71
Pi(mg/dl)	9.49 ± 2.24	7.69 ± 1.69	9.18 ± 1.93
at 10 dap			
Ca(mg/dl)	10.27 ± 0.45	10.02 ± 0.47	10.19 ± 0.42
Pi(mg/dl)	9.58 ± 0.68	9.36 ± 0.78	8.70 ± 0.53

Each value represents the mean value ±SE (n=9 per group at 17.5 dpc and n=15 per group at 10 dap).
NC, normal control; VC, vehicle control; CM, coumestrol

323

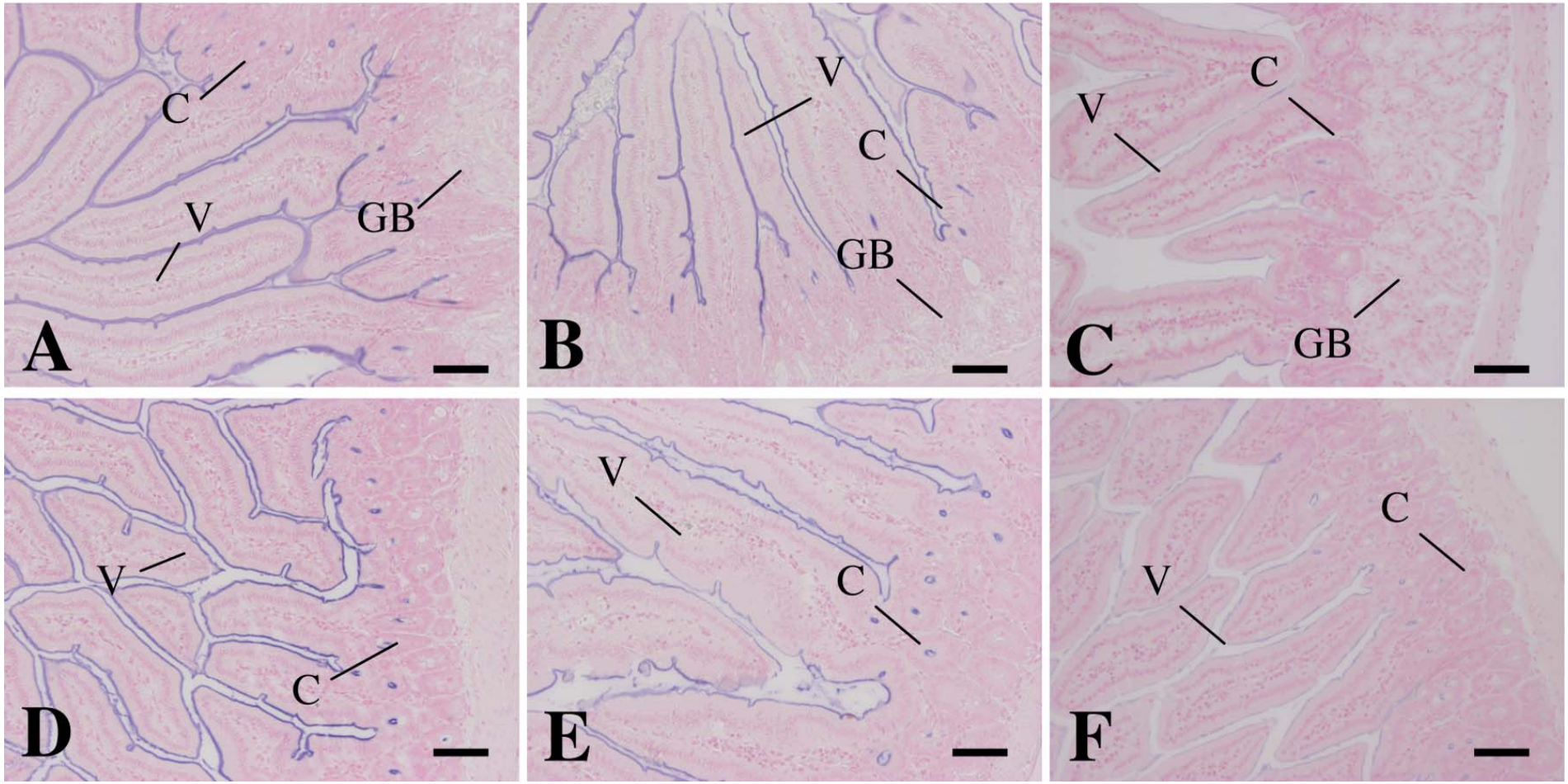

Figure 1