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**—Original—**

# Coxsackievirus B3 infection reduces female mouse fertility

Hye Min SHIM[1)], Ji Young HWANG[1)], Kyung Min LEE[1)], Yunhwa KIM[1)], Daewon JEONG[1)], Jaesook ROH[2)], Hyeonhae CHOI[2)], Jung Hye HWANG[3)], and Hosun PARK[1)]

[1)]*Department of Microbiology, College of Medicine, Yeungnam University, 170, Hyeonchung-ro, Namgu, Daegu 705-703, Republic of Korea*
[2)]*Department of Anatomy and Cell Biology, College of Medicine, Hanyang University, 222, Wangsimni-ro, Seongdong-gu, Seoul 133-791, Republic of Korea*
[3)]*Department of Obstetrics and Gynecology, College of Medicine, Hanyang University Hospital, 222, Wangsimni-ro, Seongdong-gu, Seoul 133-791, Republic of Korea*

**Abstract:** Previously we demonstrated coxsackievirus B3 (CVB3) infection during early gestation as a cause of pregnancy loss. Here, we investigated the impacts of CVB3 infection on female mouse fertility. Coxsackievirus-adenovirus receptor (CAR) expression and CVB3 replication in the ovary were evaluated by immunohistochemistry or reverse transcription-polymerase chain reaction (RT-PCR). CAR was highly expressed in granulosa cells (GCs) and CVB3 replicated in the ovary. Histological analysis showed a significant increase in the number of atretic follicles in the ovaries of CVB3-infected mice (CVBM). Estrous cycle evaluation demonstrated that a higher number of CVBM were in proestrus compared to mock mice (CVBM vs. mock; 61.5%, 28.5%, respectively). Estradiol concentration in GC culture supernatant and serum were measured by an enzyme-linked immunosorbent assay. Baseline and stimulated levels of estradiol in GC were decreased in CVBM, consistent with significantly reduced serum levels in these animals. In addition, aromatase transcript levels in GCs from CVBM were also decreased by 40% relative to the mock. Bone mineral density evaluated by micro-computed tomography was significantly decreased in the CVBM. Moreover, the fertility rate was also significantly decreased for the CVBM compared to the mock (CVBM vs. mock; 20%, 94.7%, respectively). This study suggests that CVB3 infection could interfere with reproduction by disturbing ovarian function and cyclic changes of the uterus.

**Key words:** coxsackievirus-adenovirus reseptor (CAR), coxsackievirus B3 (CVB3), estradiol, granulosa cell, infertility

## Introduction

According to a 2012 national survey on fertility in the Republic of Korea, the infertility rate of 15- to 49-y-old couples is 34.9%. The etiologies are unknown for 46.3% of infertile females and 72.9% infertile males [21]. Therefore, it is very important to elucidate the underlying mechanisms to prevent and treat infertility. The etiologies of infertility are diverse and include endocrinological, anatomical, genetic, infectious, behavioral, environmental, or unexplained factors [5, 25]. Among these, *Chlamydia trachomatis*, *Neisseria gonorrhea*, *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*, and *Mycoplasma genitalium* are well-

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*Address corresponding: H. Park, Department of Microbiology, College of Medicine, Yeungnam University, 170, Hyeonchung-ro, Namgu, Daegu, 705-703, Republic of Korea*
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known bacterial infectious agents that cause both male and female infertility [33]. Furthermore, hepatitis B (HBV) and C (HCV) viruses, human immunodeficiency virus (HIV), human papillomavirus (HPV), herpes simplex virus (HSV), and cytomegalovirus (CMV) are considered risk factors for male infertility [5, 13]. However, viral agents associated with human female infertility are still unknown.

Coxsackievirus is a member of the family *Picornaviridae* and is a positive-sense, single-strand RNA virus. There are several serotypes of coxsackievirus; six of them (CVB1-6) belong to CVB. It is widely known that the coxsackievirus-adenovirus receptor (CAR) is crucial for the entry of CVB into host cells and essential for embryonic heart development [1, 2, 10]. Previously, we found that CAR is highly expressed in uterus and CVB3 infection induces abortion during early pregnancy [16]. CAR is also abundantly expressed in the ovary; a main organ of female fertility. In the present study, we investigated the influence of CVB3 infection on fertility and the underlying mechanism in female imprinting control region (ICR) mice.

## Materials and Methods

*Cells and virus*

The Nancy strain (American Type Culture Collection No. VR-30) of CVB3 was propagated in HeLa cells with Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza) and 1% antibiotic-antimycotic solution (Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% $CO_2$. After 3 days post viral infection, the culture supernatants of the HeLa cells were harvested and centrifuged. Supernatants were transferred to 1.5 ml tube for CVB3 Nancy strain stock and stored at −80°C until use.

*Animals*

This investigation was approved by the Institutional Animal Care and Use Committee of the College of Medicine, Yeungnam University (protocol no. YUMC-AEC2010-002). Six-week-old ICR mice were obtained from Hyochang Science (Daegu, Republic of Korea) and Samtako Biokorea Co. (Kyunggi-do, Republic of Korea). The mice were allowed to acclimate for 1–2 week prior to the experiments. The animals were anesthetized with enflurane inhalation and sacrificed by cervical dislocation while under deep anesthesia at the end of each experiment.

*Histological evaluation of female reproductive organs*

Virgin female ICR mice 8 to 10 week old were used for histological examination of the uterus and ovary. The mice were infected with 0.25 ml of CVB3 ($5 \times 10^4$ plaque forming units [PFUs]/animal) or DMEM culture medium (mock) intraperitoneally and were sacrificed at 7 days post-infection (dpi). Tissue samples were immediately fixed with 4% formalin and embedded in paraffin.

*1. CAR expression in the ovary*

To evaluate constitutive CAR expression, an ovary was obtained from a 10-week-old uninfected mouse. Sections (4-$\mu$m thick) of ovary were placed on glass slides and deparaffinized at 60°C. Immunohistochemistry (IHC) specific for CAR was performed as previously described [16]. Rabbit anti-CAR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody.

*2. Histological analysis of the ovary and follicle counting*

Serial 4-$\mu$m paraffin-embedded sections of the entire ovary were stained with hematoxylin and eosin (H&E). Follicles were classified as secondary, tertiary, or large antral follicles according to the layers of GCs and presence of a visible antrum. Secondary follicles were defined as ones with a larger oocyte, more than one layer of GCs, and containing theca cells. Tertiary (small antral) follicles were similar in size to secondary follicles but included an antrum. Antral follicles were the largest follicles and contained large antral spaces [19]. Only follicles in which the nucleus of the oocyte was visible were counted on the largest cross section from each ovary using a light microscope (Leica, Wetzlar, Germany). Morphologically healthy and atretic follicles were evaluated as previously described [6]. The percentages of healthy growing follicles were calculated for each follicle population.

*3. Evaluation of the estrous cycle and uterine width*

To determine the effect of CVB3 infection on the uterus, the estrous cycle and uterine width were assessed using H&E-stained uteri (n=14 mock, n=13 CVBM) taken at 7 dpi. Uteri sections containing all structures including the lumen, luminal epithelium, gland, and myometrium were captured with Aperio ImageScope

(Aperio Technologies, Vista, CA, USA). The estrous cycle was evaluated based on histology and divided into four phases: diestrus, proestrus, estrus, and metestrus [36]. The mean uterine width was calculated by measuring four different regions including the thinnest and thickest parts of the organ [36].

*CVB3 RNA detection*

Total RNA was extracted from the ovary with an Easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. cDNA synthesis and PCR was performed using a Maxime PCR PreMix kit (iNtRON Biotechnology). Primer sequences for CVB3 (GenBank no. M16572) were as follows: sense 5′-ccc cgg act gag tat caa ta-3′(position 180–199) and antisense 5′-gca gtt agg att agc cgc at-3′ (postion 460–479). Primers for β-actin (GenBank no. BC138611) were as follows: sense 5′-act ctt cca gcc ttc ctt c-3′ (position 830–844) and antisense 5′-atc tcc ttc tgc atc ctg tc −3′ (postion 977–996). After amplification, the PCR products were separated on a 1.5% agarose gel containing RedSafe™ (iNtRON Biotechnology).

*GC preparation and culture*

Ovaries collected from the mock (n=3) and CVBM (n=3) groups at 7 dpi were separately punctured in L-15 Leibovitz medium (Life Technologies, Inc., Santa Clara, CA) under a dissecting microscope. All visible antral follicles were punctured and ovarian debris was removed. The remaining medium containing GCs was collected by low-speed centrifugation at 500 × *g* for 10 min. GCs were dispersed by repeated washing and suspended in McCoy's 5a culture medium (Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (BioWhittaker, Inc., Wakersville, MD). GCs ($5 \times 10^4$ viable cells/well) were cultured in 24-well plates with or without follicle-stimulating hormone (FSH; NV Organon Oss, the Netherlands) in a 5% $CO_2$–balanced nitrogen atmosphere for 24 h at 37°C. At the end of culture period, the media or GCs were harvested and stored at −70°C for the estradiol assay or RNA extraction.

*Measurement of estradiol production*

The amount of estradiol was quantified using a specific EIA kit (Cayman chemical company, Ann Arbor, MI) according to the manufacturer's protocol. This assay was used to analyze both sera (mock, n=8; CVBM, n=9) at 7 dpi and GC culture supernatant (mock, n=3; CVBM, n=3). Under our test conditions, the limit of detection was 6.3 pg/ml.

*Real-time reverse transcription-polymerase chain reaction (RT-PCR) to assess aromatase mRNA expression*

Total RNA of GC was isolated using an RNeasy extraction kit (Qiagen Inc., Valencia, CA). After RNA quantification and verification of integrity, 0.5 $\mu$g of total RNA was annealed (5 min at 70°C) to oligo (dT) 18 primers and reverse transcribed with an Advantage RT for PCR kit (BD Biosciences; Clontech, Palo Alto, CA, USA). Primers specific for aromatase (*Cyp19a1*; GenBank no. NM_007810) were sense 5′- cgg gct acg tgg atg tgt t-3′ (position 586–604) and antisense 5′- gag ctt gcc agg cgt taa ag-3′ (position 701–720). Amplification of 18S ribosomal RNA (rRNA; GenBank no. NR_003278.3, sense 5′-cat ggc cgt tct tag ttg gt-3′ [position 1331 to 1350] and antisense 5′- atg cca gag tct cgt tcg tt-3 [position 1378 to 1397]) was performed for normalization. Real-time PCR reactions were carried out in a total volume of 20 $\mu$l with SYBR Green I Master (Roche Applied Science, Indianapolis, IN, USA) using a LightCycler 480 II System (Roche Applied Science). Samples were run in triplicate in 96-well multiwell plates (Roche Applied Science), and the mean values were compared with those of the untreated control value to calculate the relative amount of transcript.

*Micro-computed tomography*

To monitor osteoporosis development, bone mineral density (BMD) was measured in formalin-fixed tibias from mice (n=6 for the mock group and n=13 for the CVBM group) at 28 dpi. Trabecular morphometry of the proximal tibia was performed by high-resolution Micro-Computed Tomography (MCT) (Skyscan, Aartselaar, Belgium).

*Fertility rate determination*

To exclude cases of natural infertility, the fecundity of female and male ICR mice was confirmed by mating 7- to 8-week-old virgin female mice with male animals prior to CVB3 infection. Four weeks after giving birth, 14- to 15-week-old primipara mice were infected with CVB3 (CVBM group, n=15) or culture medium (mock group, n=19) and then mated with fertile male mice after 7 dpi. For the CVBM group, CVB3 (0.25 ml of $5 \times 10^4$ PFUs/animal) was administered intraperitoneally. The

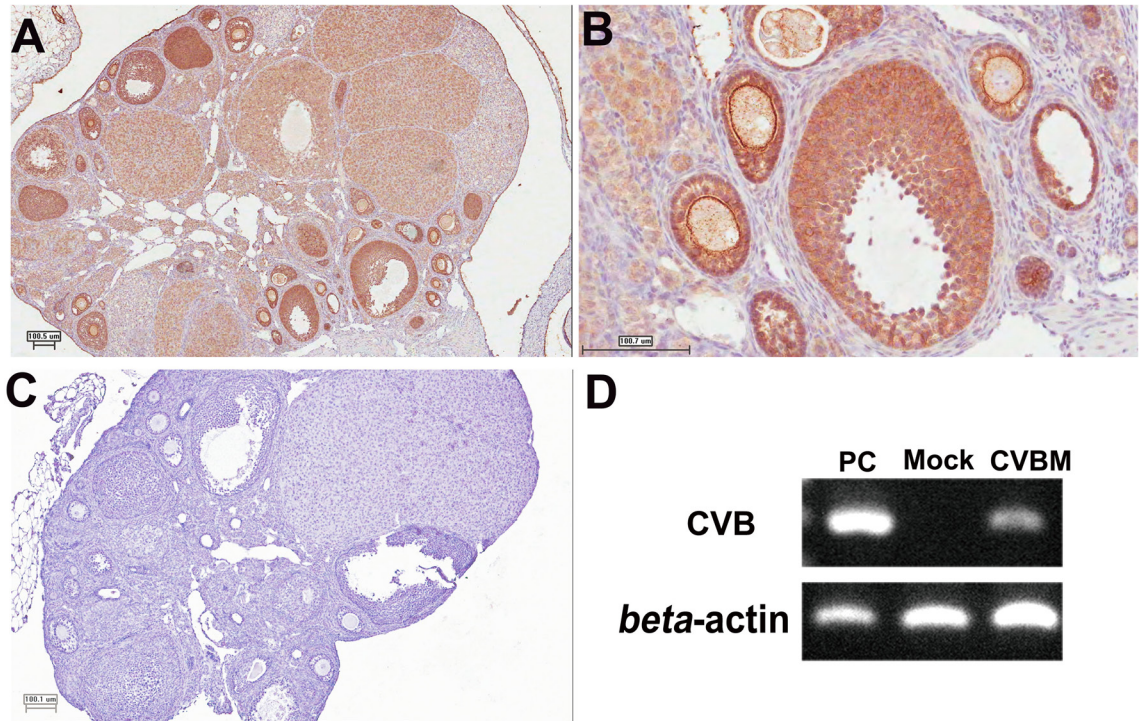


**Fig. 1.** CAR expression and CVB3 replication in the ovary. CAR expression and localization was confirmed in normal 10-week-old mouse ovary by IHC. CAR immunoreactivity was strong in the ovarian epithelium, oocyte membrane, GCs, and corpus luteum but not in theca cells (A, low magnification (scale bar=100.5 $\mu$m); B, high magnification (scale bar=100.7 $\mu$m)). (C) No immunoreactivity was observed in the negative antibody control (scale bar=100.1 $\mu$m). (D) CVB3 replication in the ovary was confirmed by RT-PCR. CVB3-infected HeLa cells (PC), uninfected ovary (mock), and ovary from a CVB3-infected female mouse at 3 dpi (CVBM).

mock group received 0.25 ml of DMEM cell culture medium. Pregnancy and delivery were observed until 30 days after mating.

*Statistical analysis*

All data were analyzed using SPSS (version 18.0 SPSS Inc.). Student's *t*-test, chi-square test, Kruskal-Wallis test, and Mann-Whitney *U* test were performed. *P*-values<0.05 were considered significant.

## Results

*Abundant CAR expression and CVB3 replication in the ovary*

CAR expression in the ovary was detected by IHC (Figs. 1A–C). CAR immunoreactivity was strong in the ovarian epithelium, oocyte membrane, GCs, and corpus luteum. In contrast, CAR expression was not found in theca cells. CAR-specific signals were localized in the cell membrane and cytoplasm but not in the nucleus. CVB3 replication in the ovary was confirmed by RT-PCR at 3 dpi (Fig. 1D). These results suggest that the ovary is an important target of CVB3 and infection with this virus might impact female fertility.

*Effects of CVB3 infection on ovarian histology*

CVB3 replicated in ovary as shown above, we analyzed the impact of CVB3 on follicles in ovaries obtained from seven different mice per group. Each section of ovary was examined to determine the characteristics and quantity of follicles in different stages of development. The data are summarized in Fig. 2. The total numbers of secondary or tertiary follicles per ovary in both groups were similar (12.6 ± 6.0 and 3.9 ± 1.8, respectively, for the mock, and 11.4 ± 3.6 and 2.6 ± 1.0, respectively, for the CVBM). However, the number of healthy follicles for the CVB3-infected mice significantly decreased compared to the mock animals for each follicle population (for secondary follicles, 3.6 ± 2.6 versus 8.5 ± 5.1 [$P$<0.02]; for tertiary follicles, 0.4 ± 0.5 versus 1.6 ± 1.2 [$P$<0.02]). Furthermore, the number of secondary atretic follicles was significantly increased for the CVBM

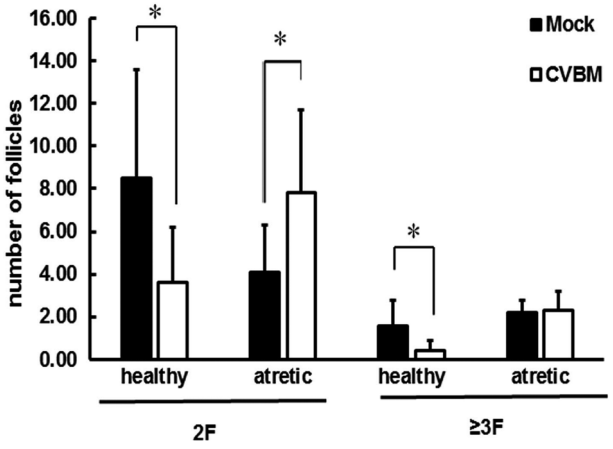


**Fig. 2.** Analysis of follicles in the ovary of control and CVB3-infected mice. The numbers of healthy and atretic follicles were counted in secondary and tertiary follicular population. Mock, control mice; CVBM, CVB3-infected mice; 2F, secondary follicle; 3F, tertiary follicle including large antral follicles. **P*<0.02 versus the mock group.

group compared to the mock group (7.8 ± 3.9 versus 4.1 ± 2.2, *P*<0.02) but this was not observed for the tertiary follicles. Considering the relatively low number of total tertiary follicles for the CVBM group, the proportion of atretic follicles for each follicle population was significantly increased in mice infected with CVB3. These results suggest that CVB3 infection significantly reduces the number of healthy follicles and causes an increase in atretic follicles.

*Effects of CVB3 infection on estradiol production and BMD*

Because estradiol is a hormone that influences fertility, estradiol concentrations in GC culture supernatant and serum were measured. As shown in Fig. 3A, basal estradiol production of GCs obtained from mice infected with CVB3 was decreased compared to that of the mock mice (14.7 ± 8.1 versus 25.6 ± 13.3 pg/ml, respectively; *P*>0.05). To test the effect of CVB3 on stimulated estradiol production, GCs were treated with FSH (50 ng/ml). Administration of FSH increased estradiol production by 10-fold compared to basal levels in both groups (mock versus CVBM, 264 ± 42 versus 110.2 ± 29.4 pg/ml, respectively; *P*<0.05). Similar to the basal estradiol levels, FSH-stimulated estradiol concentrations

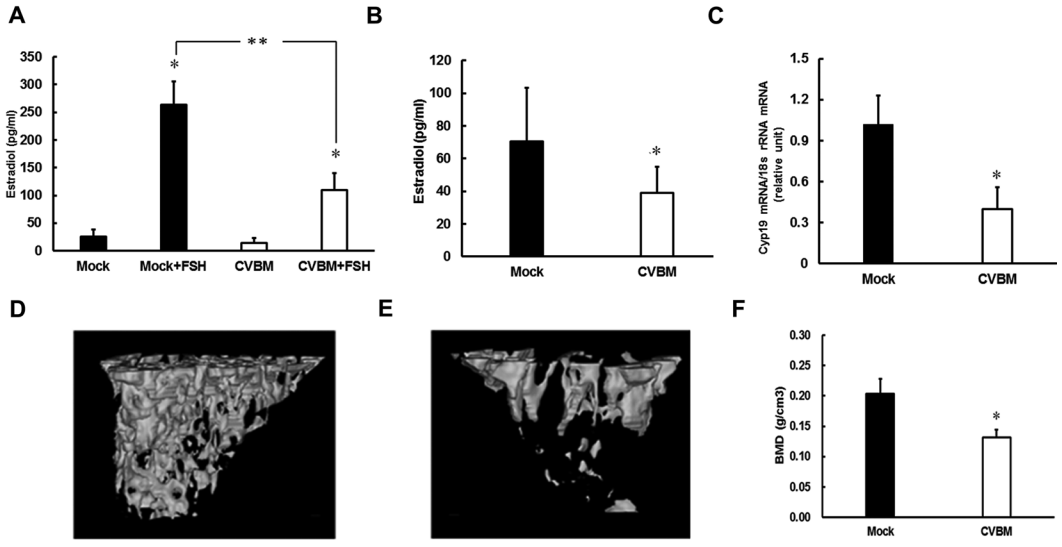


**Fig. 3.** Effects of CVB3 infection on estradiol production and BMD. (A) Basal and FSH-stimulated estradiol production by cultured GCs. The cells were obtained from mock- or CVB3-infected mice at 7 dpi and cultured for 24 h in serum-free McCoy's 5a medium with or without FSH (50 ng/ml) and androstenedione. Data are presented as the mean ± SD of two independent experiments with each condition tested in triplicate. **P*<0.05 mock versus mock + FSH; CVBM versus CVBM + FSH; ***P*<0.05 mock + FSH versus CVBM + FSH. (B) Estradiol concentration in sera. Serum samples were obtained from mock- or CVB3-infected mice at 7 dpi. Data are presented as the mean ± SD of each value for sera tested in duplicate. (C) Analysis of aromatase (*Cyp*19) mRNA expression in GCs by real-time RT–PCR. Each bar indicates the fold-change relative to 18S rRNA, and represents the mean ± SD for two independent experiments with each condition tested in triplicate. Mock, control; CVBM, CVB3-infected group. *Significantly different from the mock (*P*<0.05). MCT images of the tibia of mock (D) and CVB3-infected (E) mice at 28 dpi. (F) Mean BMD values measured in the tibia of the mock (n=6) and CVBM (n=13) groups at 28 dpi were significantly different (**P*<0.05).

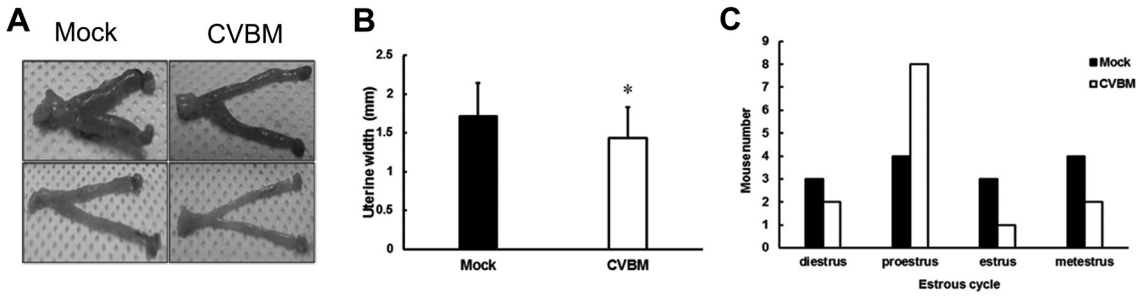


**Fig. 4.** Changes of the uterus. (A) Gross morphology and width of the uterus. Uteri during the estrus phase (left panel) and proestrus phase (right panel). (B) Mean uterine widths of the mock (n=14) and CVBM (n=13) groups were significantly different (*$P$<0.01). Values are presented as the mean ± SD. (C) Distribution of the estrous cycle. The estrus cycle was evaluated based on uterine histology of the mock (n=14) and CVB3-infected (n=13) mice. Images of the H&E-stained uteri sections were captured with Aperio ImageScope and then analyzed. Four estrous phases (diestrus, proestrus, estrus, and metestrus) were evenly distributed for the mock group ($P$>0.005) while the proestrus phase was significantly prolonged for the CVBM group ($P$<0.05).

were significantly decreased by 50% in the CVBM group compared to the mock group ($P$<0.05). Serum estradiol levels of the CVBM group (39.9 ± 15.8 pg/ml) were also significantly decreased compared to those of the mock group (70.7 ± 32.5 pg/ml) at 7 dpi ($P$<0.05; Fig. 3B).

To determine whether CVB3 infection affected aromatase expression in GCs, transcript levels were analyzed. Consistent with estradiol concentration, aromatase transcript levels were also decreased in the GCs of CVB3-infected mice compared to those of the mock animals (Fig. 3C). In the GCs of CVB3-infected mice, the level of aromatase mRNA normalized relative to 18S rRNA was approximately 40% of the control value. These results demonstrated that CVB3 infection decreased estradiol production by inhibiting aromatase gene expression.

The influence of decreased estradiol production on the bone was also analyzed. BMD of the tibia was measured using MCT at 28 dpi (Figs. 3D and E). BMD was significantly decreased ($P$<0.05) in the CVBM group compared to the mock group (mock versus CVBM, 0.20 ± 0.02 versus 0.13 ± 0.01 g/cm$^3$, respectively; Fig. 3F). Therefore, we confirmed that not only the concentration of estradiol but also the function of estradiol were reduced in CVBM group.

*Effects of CVB3 infection on uterine histology*

Since the uterine cycle is dependent on estradiol, we evaluated the effect of CVB3 infection on the uterus. Uterine histology was assessed based on the estrous cycle. CVB3-infected uteri were long and thin compared to ones from the mock group (Fig. 4A). Indeed, the mean uterine width was significantly thinner ($P$<0.05) for the CVBM group (1.44 ± 0.39 mm) than the mock group (1.71 ± 0.44 mm; Fig. 4B). The percentage of mice in diestrus, proestrus, estrus, and metestrus was 21.4%, 28.5%, 21.4%, and 28.5%, respectively, in the mock group (n=14), and 15.3%, 61.5%, 7.6%, and 15.4% in the CVBM group (n=13), respectively (Fig. 4C). In contrast to the mock group in which the four estrous phases were evenly distributed ($P$>0.005), the proestrus phase was significantly increased in the CVBM group ($P$<0.05). This finding suggested that the estrous cycle did not progress to the estrus phase and may have affected fertility.

**Table 1.** Fertility rates of mock and CVB3-infected mice

| Group | Pregnancy (%) | | Total No. |
|---|---|---|---|
| | Yes | No | |
| Mock | 18 (94.7) | 1 (5.3) | 19 |
| CVBM | 3 (20) * | 12 (80) | 15 |

Mock, control mice; CVBM, CVB3-infected mice. *$P$<0.05 versus the mock group.

*Reduced fertility rate in female mice caused by CVB3*

Since CVB3 infection reduced estradiol production in the ovary and halted progression of the estrous cycle, the fertility rate of CVB3-infected female mice was evaluated. Cell culture media (mock group) or CVB3 (CVBM group) were administrated to primipara female mice that were then mated at 7 dpi with fertile male mice. The fertility rate of the mock mice was 94.7% and that of the CVBM group was 20%, illustrating that CVB3

infection significantly reduced fertility (Table 1, $P<0.01$).

## Discussion

CVB infection causes severe diseases such as meningitis and myocarditis in children. However, most cases of CVB3 infection in adults are asymptomatic or produce flu-like symptoms. Rubella, one of the most important pathogens that affect pregnancy, is easily diagnosed by characteristic clinical symptoms and serologic tests whereas CVB infection is usually not recognized nor diagnosed in pregnant women. Recently, we found that CVB3 infection during early pregnancy causes pregnancy loss [15, 16]. This effect is related with abundant expression of CAR (a major CVB receptor) in the uterus and embryos. CAR is also highly expressed in the ovary and CVB3 replicates in this organ (Fig. 1). Therefore, we examined the effects of CVB3 on the ovary and female fertility in ICR mice.

The number of healthy follicles in the ovary was significantly decreased while the number of atretic follicles was significantly increased in mice infected with CVB3. Therefore, CVB3 infection during the reproductive age could have a negative effect on the growth or development of ovarian follicles and be linked to atresia in mice. Additionally, benign cysts were incidentally found in the ovaries of four animals from the CVBM group. These cysts were not observed in the mock group. Two of them were cystic changes of degenerating follicles and the others were inclusion cyst or ovarian endosalpingiosis (Supplementary Fig. 1). Cystic changes of degenerating follicles were possibly associated with increased incidence of atresia in the ovaries of the CVBM group. Benign inclusion cysts, possibly resulting from invagination of the ovarian surface epithelium into the ovarian stroma [12], are thought to be precancerous lesions in humans [27]. Formation of inclusion cyst might be due to weakened adhesion of ovarian epithelial cells caused by translocation of the CAR-CVB3 complex in tight junctions to the cytoplasm during CVB3 infection.

The role of CAR in tumor development and progression has been reported although there are controversies depending on tissue type [28–32, 37]. CAR mRNA expression is significantly decreased in invasive bladder cancer specimens and the adhesive properties of CAR inhibit the growth of bladder cancer cells [28, 32]. Therefore, CAR has been found to possess tumor suppressive activities in bladder. On the other hand, expression of soluble isoforms but not the transmembrane form of CAR is increased in cases of epithelial ovarian cancer [31]. However, further studies are necessary to determine whether CVB3 infection is related to the development of precancerous lesions in the ovary.

Endosalpingiosis is characterized by the presence of extra-tubal cyst lined with a tubal-type epithelium. Although the origin and transformative potential of these ectopic cells are unknown, many cases of endosalpingiosis are accompanied by additional pelvic pathologies and associated with ovarian surface papillary tumors, adenocarcinoma of the fallopian tubes, and endometriosis [8, 14]. The tubal-type epithelium lining the cysts is thought to originate from the Mullerian epithelium [4] and these cells can be influenced by steroid background [24]. As shown in Fig. 3A, altered secretory activity of GCs obtained from CVB3-infected mice could be partly implicated in occurring cysts. In mice, significantly more cysts have been observed with age. For instance, the frequency of benign cysts was more than 90% for 8-month-old mice but less than 10% for 3-month-old animals [35]. Considering that 2-month-old mice were used in the present study, finding benign cysts in four out of seven animals infected with CVB3 represented a relatively high frequency of cyst formation.

Because morphologic changes were observed in the CVB3-infected ovary, estradiol production was evaluated. The generation of estradiol by GCs and serum levels of this hormone were significantly decreased in the CVBM group in which severe osteoporosis also developed. Decreased aromatase mRNA expression explained the reduced estradiol synthesis by GCs. Taken together, our findings demonstrated that CVB3 infection hindered the production of estradiol through the suppression of aromatase synthesis in GCs. However, further studies are necessary to elucidate the detailed mechanism underlying the inhibition of aromatase mRNA expression by CVB3 infection.

Morphological changes in the uterus of CVB3-infected mice were noted. Uteri of the CVBM group were long and thin compared to those of the mock group. Indeed, the mean uterine width was significantly smaller for the CVBM group than the mock group. Because uterine width changes during the estrous cycle, we evaluated the estrous cycle of both groups. The estrous cycle of mice is divided into four phases (i.e., diestrus, proestrus, estrus, and metestrus) that were evenly distributed for the mock group. In contrast, the proestrus phase was sig-

nificantly prolonged compared to the other phases for the CVBM group. The proestrus phase corresponds to the proliferative phase of the human menstrual cycle, and the uterus during the proestrus phase is long and thin. In contrast, the uterus during the estrus phase, which corresponds to the secretory phase in humans, is short, thick, and ready for implantation [7, 36]. Our results suggested that CVB3 infection led to a halt in the progression of proestrus to estrus and an onset of infertility. Not surprisingly, the fertility rate of the CVB3-infected mice was significantly reduced compared to the mock-infected mice.

Some viral infections are known to cause male infertility. Mumps and HIV hinder spermatogensis in testes, and HSV has been found in semen of infertile men [3, 9, 22]. HBV infection was also found to promote male infertility by reducing sperm motility or inducing sperm necrosis and apoptosis [26, 38]. Recently, a case-control study indicated that HBV antigen seropositive female *in vitro* fertilization (IVF) patients have reduced optimal embryo quality, decreased fertilization rates, and lower ovarian responses compared to seronegative women [34]. HCV also significantly reduces ovarian responses of IVF female patients [11]. However, neither HBV nor HCV infection was found to influence the implantation and delivery rates after IVF [11, 34].

Enterovirus infection is prevalent during childhood, especially before 10 years old. However, enteroviruses have diverse serotypes and children are not exposed to all of the serotypes. According to survey for the prevalence of enterovirus infection in Korea performed by Korean Center for Disease Control, EV71 was the most prevalent type during 1999–2013 [17, 23]. E30 was also usually prevalent; however the prevalence of other types have been changed annually [18, 20]. CVB3 was not very prevalent in Korea except the year 2012 when the CVB3 was the fifth prevalent enterovirus [23]. Therefore, we can expect the immunity against CVB3 might be not very high in children in Korea. In our previous study, the prevalence of CVB3 infection was high in missed abortion compared to full-term or pre-term deliveries [15]. In that study, we enrolled patients from Oct. 2010, however the missed abortion patients were mainly enrolled during Dec. 2011 to May 2012. That result was simultaneous with the high prevalence of CVB3 infection in Korea. Therefore, the serotype changes during enterovirus epidemic might be one of the factors to affect female fertility. And also, there might be some other factors such as different tropism of CVB3 between children and childbearing-aged women. We do not know yet the exact factors to influence the CVB3 tropism, however, hormones and CVB3 receptor (CAR) expression in reproductive organs during childbearing-aged women might be related with the CVB3 tropism.

To the best of our knowledge, ours is the first report to confirm that disturbed female reproductive function is related to CVB3 infection. An underlying mechanism of this phenomenon is decreased estradiol production by GCs due to reduced aromatase synthesis. Further studies will be necessary to evaluate the prevalence of CVB3 infection among infertile women.

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