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# Effects of Virus-associated Molecular Patterns on the Expression of Cathelicidins in the Hen Vagina

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The aim of this study was to examine the expression profiles of the cathelicidins (*CATH*s) in the oviduct and the effects of Toll-like receptor (TLR) ligands of virus-associated molecular patterns on *CATH*s expression in the vagina of hens. The mRNA expression of cathelicidins (*CATH1*, *-2*, *-3* and *-B1*) in the oviductal mucosa was analyzed by RT-PCR. The effects of viral molecules on the *CATH*s expression in the vagina was examined by incubating the mucosal tissue with virus molecular patterns, including poly I:C (dsRNA virus, TLR3 ligand), R848 (ssRNA virus, TLR7 ligand) and CpG-ODN (DNA virus, TLR21 ligand), followed by real-time PCR analysis. The expression of *CATH1, CATH2* and *CATH3* was identified in all oviductal segments, except for *CATH2* which was lacked in the magnum. The expression of *CATHB1* was not identified at any segments of the oviduct. Poly I:C down-regulated the expression of *CATH1, -2* and *-3*, whereas R848 up-regulated the expression of *CATH1* and *CATH3* but down-regulated the expression of *CATH2*. CpG-ODN did not affect the *CATH*s expression. These results suggest that mucosal tissues of the oviduct express *CATH*s to provide the defense mechanism against microbes, and the expression of *CATH1* and *CATH3* is up-regulated against ssRNA viruses, whereas, dsRNA virus may suppress the expression of *CATH1, -2* and *-3*.

**Key words**: cathelicidins, hen oviduct, Toll-like receptor ligands, virus-associated molecular patterns

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## Introduction

The oviduct of hens is the site where the albumen, egg-shell membranes and egg-shell are secreted surrounding the yolk to complete the egg formation (Nys *et al*., 2004; YU and Marquardt, 1973). If pathogenic microorganisms infect and colonize in the oviduct, they may affect proper functioning of the oviduct associated with egg formation and contaminate the forming eggs (Miyamoto *et al*., 1997; Reiber *et al*., 1995). The mucosal barrier formed by the innate and adaptive immune system is of great importance to maintain the health of the oviduct (Abdel-Mageed *et al*., 2014).

Recognition of specific molecular patterns of the pathogenic microbes, which are known as pathogen-associated molecular patterns (PAMPs), by specific receptors is the first step in the innate immune response. Toll-like receptors (TLRs) are the receptors that recognize PAMPs, and ten TLRs have been identified in chicken to date (Boyd *et al*., 2007; Brownlie and Allan, 2011). TLR2 forming the heterodimer with TLR1 recognizes peptidoglycan and lipoproteins of the Gram-positive bacteria (Mintz *et al*., 2013), whereas TLR3 recognizes the double-stranded RNA (dsRNA) of viruses (Karpala *et al*., 2008). The LPS of the Gram-negative bacteria is recognized by TLR4 (Karnati *et al*., 2014), and bacterial flagellin is recognized by TLR5 (Keestra *et al*., 2008). The single-stranded RNA (ssRNA) of viruses is recognized by TLR7 (Diebold, 2008; Yue *et al*., 2014). Fungal and bacterial proteases are recognized by the chicken specific TLR15 (de Zoete *et al*., 2011; Keestra *et al*., 2013). The oligo-DNA with unmethylated CpG motifs of bacteria and virus is recognized by TLR21, a homologous of mammalian TLR9 (Brownlie *et al*., 2009; Keestra *et al*., 2010). After recognition of different PAMPs by TLRs, the innate immune response is triggered by synthesizing the cytokines and antimicrobial peptides (Yoshimura, 2015).

Cathelicidins are one of the members of host defense peptides with antimicrobial and antiviral activities (Barlow *et al*., 2014). They are widely expressed in the bone marrow, neutrophils and epithelial tissues of animals such as sheep, cattle, goat (Brogden *et al*., 2001; Whelehan *et al*., 2014;

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Zhang *et al*., 2014) as well as in chicken (Xiao *et al*., 2006). In human, rhesus monkey, mice and rats, the structures of cathelicidins, their antimicrobial activities, and other functions including wound repairing and inhibition of tissue injury have been documented (Bals *et al*., 2001; Dürr *et al*., 2006; Nakamichi *et al*., 2014; Ramanathan *et al*., 2002; Zanetti, 2005). In chickens, four gene of cathelicidins (*CATH*s) have been identified; namely, *CATH1, -2, -3* and *CATHB1* in the tissues of the digestive, respiratory, urinary and immune systems (Xiao *et al*., 2006). However, to our knowledge, no reports examined the expression of *CATH*s in the oviduct of chickens.

The viral infection disrupts the proper functioning of the oviduct associated with egg formation. For example, infection by *avian infectious bronchitis virus* causes malformation of eggshell (Nii *et al*., 2014; Nii *et al*., 2015). It is reported that *CATH*s are expressed in the infected tissues and kill the enveloped viruses (Barlow *et al*., 2014). Although it has been reported that stimulation of TLRs by their ligands affected the expression of avian $\beta$-defensins (*AvBD*s) and proinflammatory cytokines in hen oviduct (Abdel-Mageed *et al*., 2014; Mageed *et al*., 2008; Mageed *et al*., 2009; Sonoda *et al*., 2013), the effects of TLR ligands on the *CATH*s expression have not been documented. Many of pathogenic microbes may enter the oviduct through the vagina since it opens to the cloaca. Thus, the aim of this study was to examine the expression profiles of the *CATH*s in different segments of the oviduct and the effects of virus molecular patterns on their expression in the vagina of laying hens.

## Materials and Methods

***Birds and Tissue Preparation***

A total of fifteen White Leghorn laying hens, approximately 250-d-old and regularly laying four or more eggs in a clutch, were used. The hens were reared in individual cages under a lighting regimen of 14 h light: 10 h dark and were provided with feed and water *ad libitum*. The birds were euthanized under anesthesia with sodium pentobarbital (Somnopentyl; Kyoritsu Pharmaceutical Co., Tokyo, Japan) 6 h after oviposition, and the mucosal tissues of the each oviductal segment, namely the infundibulum, magnum, isthmus, uterus and vagina, were collected. This study was carried out in accordance with the Guidelines for Animal Experimentation, Hiroshima University, Japan.

***Tissue Culture of the Vagina Mucosa***

The procedure of tissue culture was described in our previous report in which the effects of TLR ligands on cytokines and AvBDs expression were analyzed (Abdel-Mageed *et al*., 2014). The cultured tissue samples used for dose dependency test in this study were the same ones that had been used in that study. Briefly, the mucosal tissues of the vagina were collected and washed with sterile phosphate-buffered saline (PBS) containing 10 U/mL penicillin and 10 $\mu$g/mL streptomycin (Cosmo Bio Co., Ltd., Tokyo, Japan). The tissue specimens of the mucosa (approximately 2×2×1 mm) were placed in sterile tubes for culture (5 specimens collected from one bird per tube in one analysis; Greiner Bio-One Ltd., Tokyo, Japan) containing 2 mL TCM-199 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with 10 U/mL penicillin and 10 $\mu$g/mL streptomycin. The different TLR ligands, poly I:C (0 to 100 $\mu$g/mL; TLR3 ligand; Imgenex, San Diego, CA, USA), R848 (0 to 5 $\mu$g/mL; TLR7 ligand; Novus Biologicals, Littleton, CO, USA), or CpG-ODN (0 to 10 $\mu$g/mL; oligonucleotide sequence 5 - TCGT-CGTTGTCGTTTTGTCGTT-3; TLR21 ligand; InvivoGen, San Diego, CA, USA) were added to the culture tubes and incubated for 1.5 h or 3 h at 39℃ in 5% $CO_2$ and 95% air. The analysis was repeated five times using five different birds within the examination of the effects of one TLR ligand.

***RNA Extraction and Reverse Transcription (RT)***

Total RNA was extracted from each segment of the oviduct (the infundibulum, magnum, isthmus, uterus and vagina), and from the cultured vaginal tissues using Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan), and dissolved in TE buffer (10 mM Tris, pH 8.0, with 1 mM EDTA). They were mixed with 1 U of RQ1 RNase-free DNase (Promega Co., Madison, WI, USA), and incubated in a PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA, USA) at 37℃ for 45 min and 65℃ for 10 min. Concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). The RNA samples were then reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). The reaction mixture (10 $\mu$L) contained 1 $\mu$g of total RNA, 1×RT buffer, 1 mM dNTP mixture, 20 U RNase inhibitor, 0.5 $\mu$g of oligo(dT) 20 primer, and 50 U ReverTra Ace. Reverse transcription was performed at 42℃ for 30 min, followed by heat inactivation for at 99℃ 5 min using the PTC-100.

***Semi Quantitative PCR***

To examine the presence of mRNA expression of *CATH*s (*CATH1, -2, -3* and *-B1*) in the oviductal mucosa, PCR analysis was performed using Takara Ex Taq (Takara Bio, Inc., Shiga, Japan) according to the protocol of the manufacturer on a PTC-100 Programmable Thermal Controller. The primers for target genes and ribosomal protein S17 (RPS17) used in this study are shown in Table 1. The PCR mixture (25 $\mu l$) contained a 0.5 m*l* aliquot of cDNA, 1×PCR buffer, 0.2 mM dNTP, 1.25 U Takara Ex Taq, and 0.5 mM each primer. The cycle parameters were denaturation at 94℃ for 3 min, 35 cycles for all *CATH*s primers and 30 cycles for *RPS17*; annealing at 60℃ for 30 sec and extension at 72℃ for 30 sec followed by final extension at 72℃ for 6 min. For negative control, the PCR was done by replacing the cDNA by RNA of the same sample. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing 0.025% (w/v) ethidium bromide. The analysis was repeated three times.

***Quantitative Real-time PCR***

Real-time PCR was performed using a Roche Light Cycler Nano System (Roche Applied Science, Indianapolis, IN, USA). The reaction mixture (20 $\mu$L) contained 1 $\mu$L of the cDNA, 1×Thunderbird SYBR qPCR mix (Toyobo), 1×

Table 1. **Primer sequences for cathelicidins and RPS17**

| Target gene | Sequences 5′–3′ | Accession no. (Reference) |
|---|---|---|
| *CATH1* | F: GCTGTGGACTCCTACAACCAAC | NM_001001605.3 |
| | R: GGAGTCCACGCAGGTGACATC | (Achanta *et al*., 2012) |
| *CATH2* | F: CAAGGAGAATGGGGTCATCAG | NM_001024830.2 |
| | R: CGTGGCCCCATTTATTCATTCA | (Achanta *et al*., 2012) |
| *CATH3* | F: GCTGTGGACTCCTACAACCAAC | NM_001311177.1 |
| | R: TGGCTTTGTAGAGGTTGATGC | (Achanta *et al*., 2012) |
| *CATHB1* | F: CCGTGTCCATAGAGCAGCAG | NM_001271172.1 |
| | R: AGTGCTGGTGACGTTCAGATG | (Achanta *et al*., 2012) |
| *RPS17* | F: AAGCTGCAGGAGGAGGAGAGG | NM_204217 |
| | R: GGTTGGACAGGCTGCCGAAGT | (Nii *et al*., 2011) |

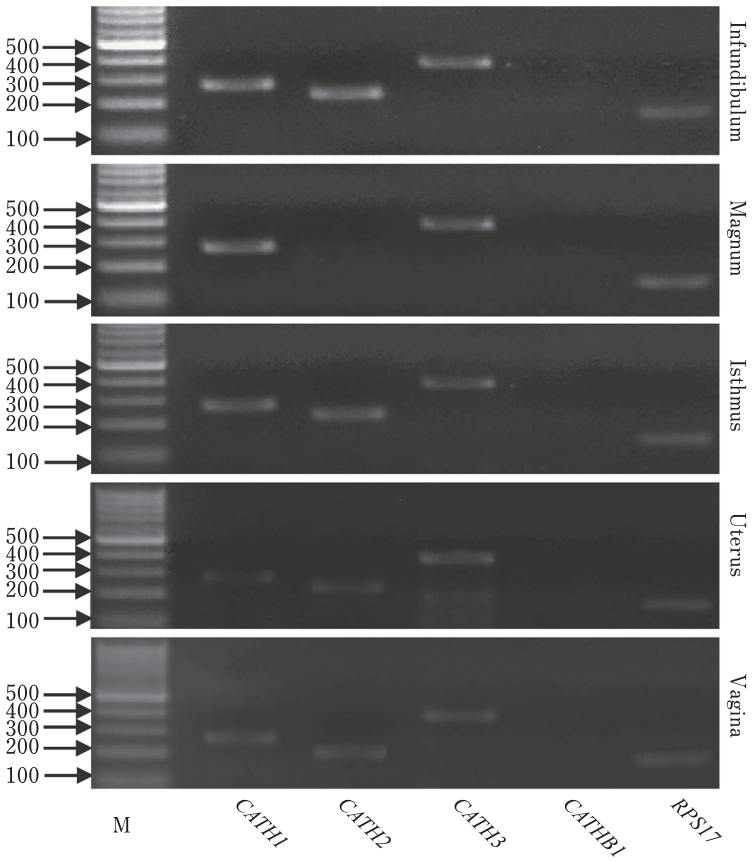


Fig. 1. **Pattern of reverse transcription-PCR products for cathelicidins (*CATH*s) in the mucosal tissues of hen oviduct.** The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. M=marker.

ROX reference dye and 0.5 $\mu$M each primer. The amplification by PCR was performed with 55 cycles for all *CATH*s, and with 45 cycles for *RPS*17. The cycle parameters were 95℃ for 10 sec followed by 57℃ for 10 sec and 72℃ for 30 sec for *CATH1* and 15 sec for *CATH2* and *CATH3*. The cycle parameter for *RPS*17 was 95℃ for 5 sec and 60℃ for 30 sec. Real-time PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of each gene in each sample using *RPS17* as the housekeeping gene (Livak and Schmittgen, 2001). The tissue sample incubated without TLR ligand was used for standardization of values to calculate relative expression level (standard sample). The results were expressed as fold change obtained from the ratio of the expression levels of the samples treated with TLR

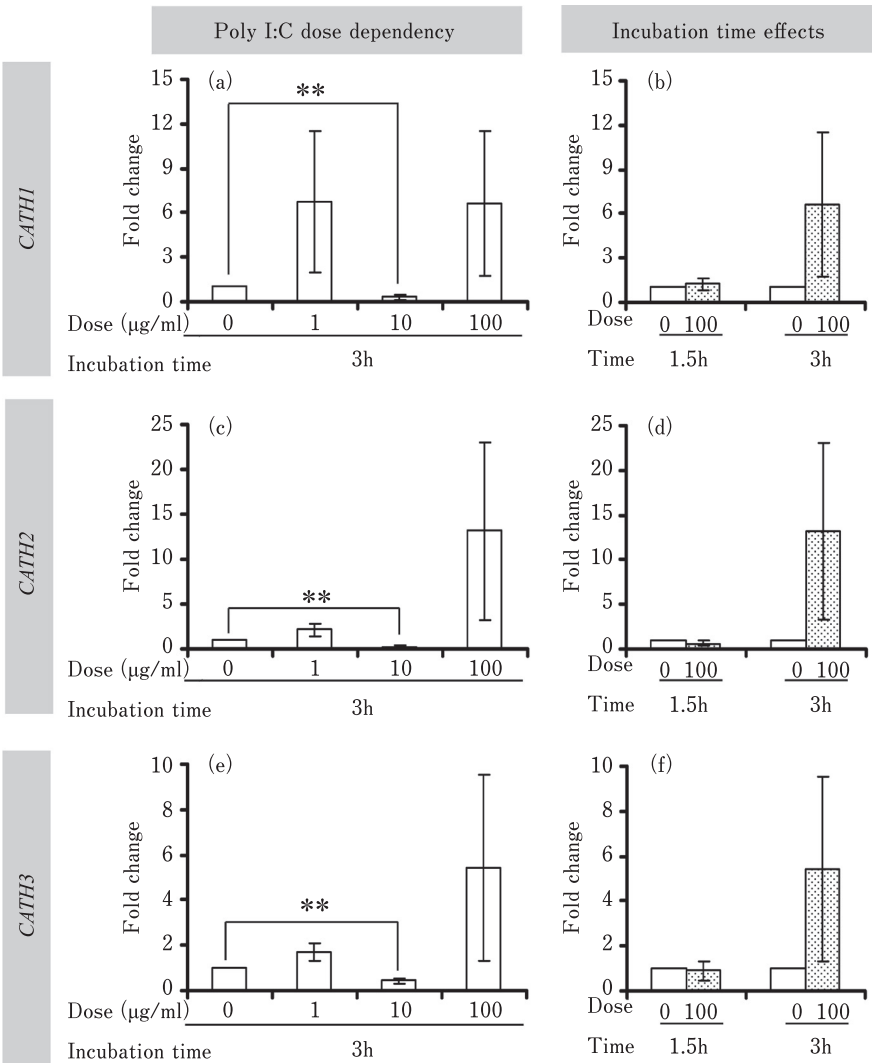


Fig. 2. **Changes in the expression of *CATH1* (a and b), *CATH2* (c and d) and *CATH3* (e and f) in the cultured mucosal tissues of the vagina in response to poly I:C stimulation.** (a, c and e) Tissues were stimulated with 0−100 μg/m*l* poly I:C for 3 h. (b, d and f) Tissues were stimulated with 0 or 100 μg/m*l* poly I:C for 1.5 h or 3 h. Values are mean ±SEM ($n$=5). * and ** Values are significantly different at $P<0.05$ and 0.01.

ligands to the standard sample.

***Statistical Analysis***

The fold change of *CATH1, CATH2* and *CATH3* was expressed as the mean±SEM ($n$=5). Paired T-test was performed to examine the significance of difference in the fold changes between the groups treated with poly I:C-, R848-, or CpG-ODN and the control groups incubated without those substances. Values were considered significantly different when $P<0.05$.

## Results

The expression of *CATH1* and *CATH3* was identified in the mucosal tissues of all segments of the oviduct from the infundibulum to vagina. The PCR products of *CATH2* were identified in the infundibulum, isthmus, uterus and vagina, but not in the magnum. No PCR products of *CATHB1* were identified at any segments of the oviduct (Fig. 1).

The expression of *CATH1, CATH2* and *CATH3* in the cultured vaginal tissues was significantly down-regulated by stimulation for 3 h with 10 μg/m*l* Poly I:C, but not with 1 or 100 μg/m*l* Poly I:C, compared with the control tissues exposed to no Poly I:C (Fig. 2a, c, e). There were no significant differences in the expression of *CATH1, CATH2* and *CATH3* between the control tissues incubated without Poly I:C and tissues incubated with 100 μg/m*l* Poly I:C for 1.5 h or 3 h (Fig. 2b, d, f).

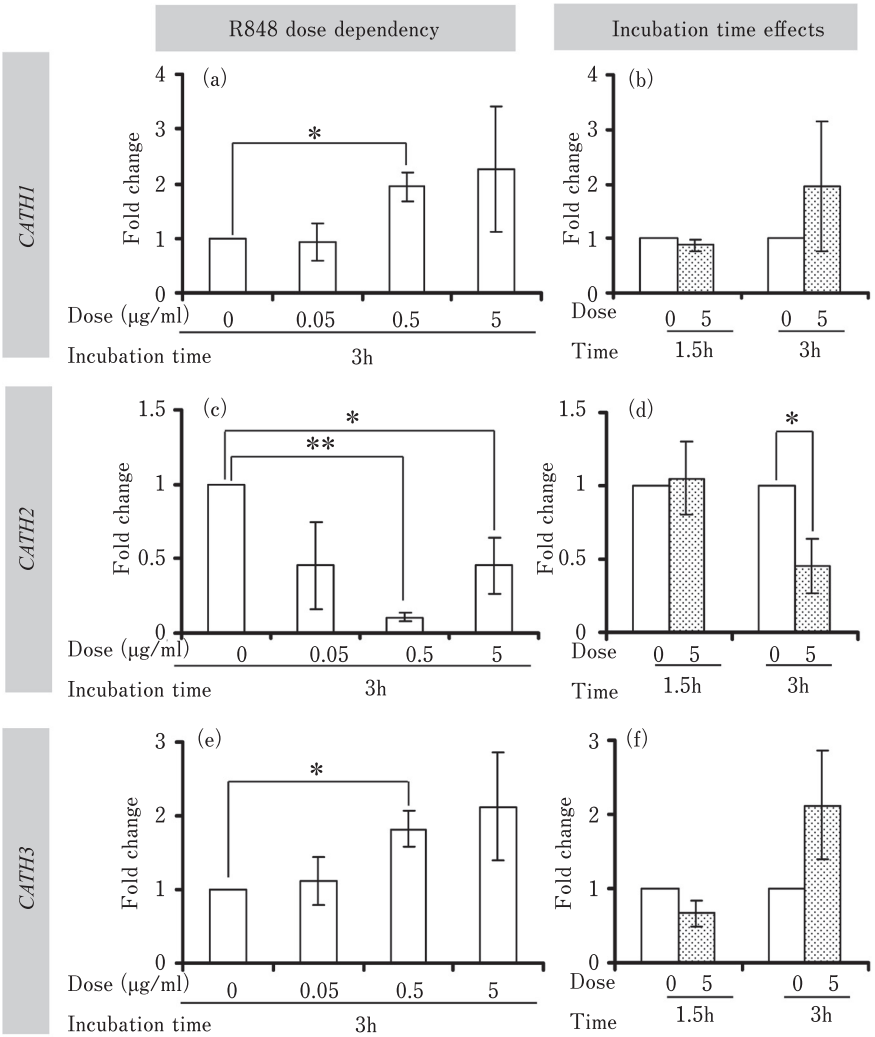


Fig. 3. **Changes in the expression of *CATH1* (a and b), *CATH2* (c and d) and *CATH3* (e and f) in the cultured mucosal tissues of the vagina in response to R848 stimulation.** (a, c and e) Tissues were stimulated with 0−5 $\mu$g/m*l* R848 for 3 h. (b, d and f) They were stimulated with 0 or 5 $\mu$g/m*l* R848 for 1.5 h or 3 h. Values are mean±SEM ($n$ =5). * and ** Values are significantly different at $P<0.05$ and 0.01.

The expression of *CATH1* and *CATH3* in the cultured vaginal tissues was up-regulated by the incubation with 0.5 $\mu$g/m*l* R848 for 3 h, but not by incubation with 0.05 or 5 $\mu$g/m*l* R848 (Fig. 3a and e). No effect on their expression was found in the tissues incubated with 5 $\mu$g/m*l* R848 for 1.5 or 3 h (Fig. 3d). On the other hand, the expression of *CATH2* was significantly down-regulated by incubation with 0.5 and 5 $\mu$g/m*l* R848 for 3 h (Fig. 3c); whereas the down-regulation by 5 $\mu$g/m*l* R848 was not found in the tissues incubated for 1.5 h (Fig. 3d).

Treatment of the vaginal cultured tissues by CpG-ODN did not affect the expression of *CATH1, -2* and *-3* in both time and dose courses (Fig.4a-f).

## Discussion

We report herewith the expression profile of the *CATH*s in the oviductal tissues and the effects of virus molecular patterns, namely the ligands of TLR3 (Poly I:C), 7 (R848) and 21 (CpG-ODN), on *CATH*s expression in the cultured vaginal mucosal tissues. The expression of TLR3, 7 and 21 in the vagina has been reported by the previous studies (Michailidis *et al*., 2011; Ozoe *et al*., 2009). The major findings were: 1) the expression of *CATH1* and *CATH3* was identified in all oviductal segments, and *CATH2* was expressed in all the segments except for the magnum; 2) Poly I:C down-regulated the expression of these three *CATH*s, whereas R848 up-regulated the expression of *CATH1* and

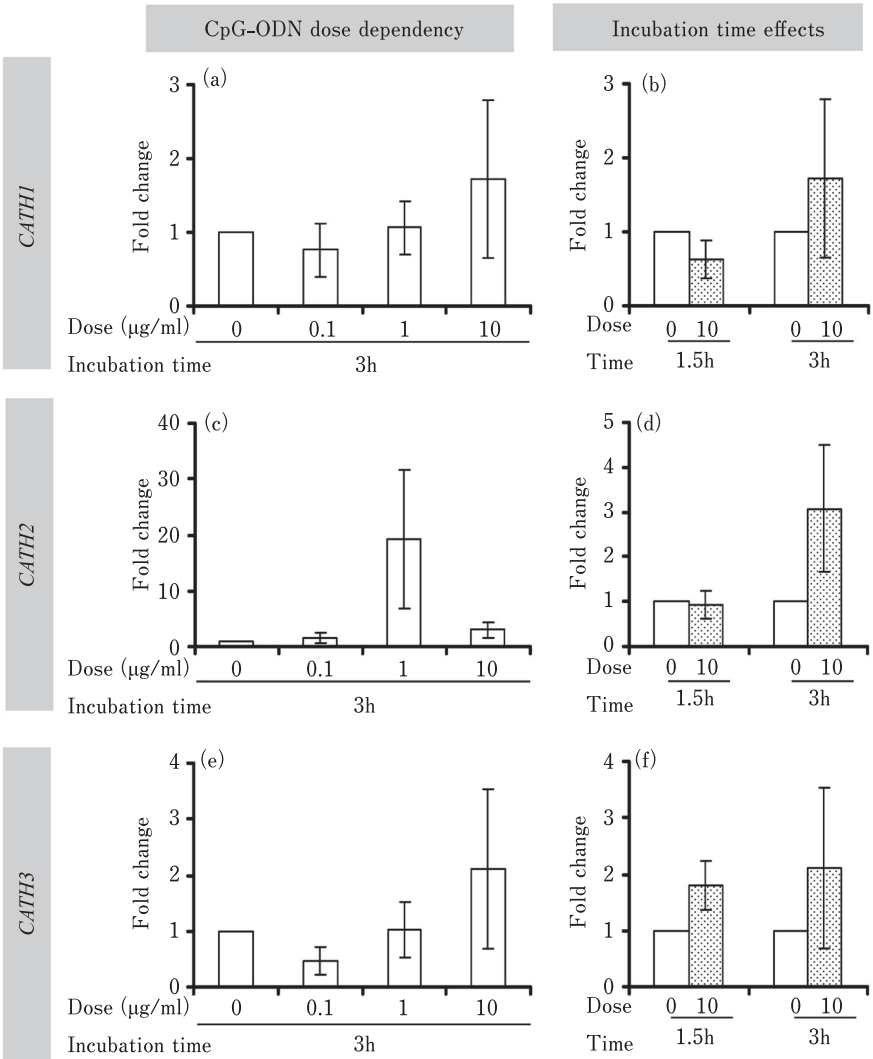


Fig. 4. **Changes in the expression of *CATH1* (a and b), *CATH2* (c and d) and *CATH3* (e and f) in the cultured mucosal tissues of the vagina in response to CpG-ODN stimulation.** (a, c and e) Tissues were stimulated with 0−10 $\mu$g/m*l* CpG-ODN for 3 h. (b, d and f) They were stimulated with 0 and 10 $\mu$g/m*l* CpG-ODN for 1.5 h or 3 h. Values are mean±SEM ($n$=5).

*CATH3* but down-regulated the expression of *CATH2*, and CpG-ODN did not affect the *CATH*s expression in the vaginal tissue. The expression of *CATH*s has been identified in various tissues of the chicken including digestive, respiratory and immune systems (Van Dijk *et al*., 2005; Xiao *et al*., 2006; Goitsuka *et al*., 2007). The current study reports that *CATH*s were also expressed in the oviduct.

The expression of *CATH1* and *CATH3* was identified in the mucosal tissues of all segments of the oviduct, and *CATH2* was expressed in the oviductal mucosal tissues except for the magnum. The expression of *CATHB1* could not be identified in any segment of the oviduct. The expression profile of *CATH*s may be an indicator for their phylogenetic relationship (Xiao *et al*., 2006; Goitsuka *et al*., 2007). Previous reports described that the expression profile was resembled to *CATH1*, *-2* and *-3*, and the expression profiles of those *CATH*s differed from that of *CATHB1*. The expression level of *CATH1, -2* and *-3* was high in the lung and digestive tract, whereas the high expression of *CATHB1* was identified in the bursa of Fabricius (Goitsuka *et al*., 2007; Achanta *et al*., 2012). Thus, it is likely that the differences in the expression profiles between *CATHB1* and the other *CATH*s in the vagina resemble to the patterns of *CATH*s expression in other organs. We have reported that the density of immunoreactive AvBD-3 and -12 in the uterus was reduced when the eggs stayed there, suggesting that these AvBDs were secreted into the eggs (Mageed *et al*., 2009). However, the effects of egg location on the *CATH*s ex-

pression is probably negligible in this study since the vaginal mucosa was collected 6 h after oviposition when the egg located at the caudal end of the isthmus or in the uterus, not in the vagina.

It was reported that stimulation of the human neonatal foreskin keratinocytes by Poly I:C up-regulated the expression of *LL37*, a member of cathelicidin family (Howell *et al*., 2006; Hau *et al*., 2013). The current results indicated that Poly I:C at a specific dose (10 $\mu$g/m*l*) down-regulated the expression of *CATH1*, *-2* and *-3* in the vaginal tissue. Thus, the response of *CATH*s expression to Poly I:C may differ among the different cells types. Although the mechanism by which *CATH*s expression was down-regulated by Poly I:C in the chicken vaginal cells is not known, we assume that infection by dsRNA virus in the vagina does not stimulate cathelicidin synthesis.

Imiquimod, an agonist of TLR7, up-regulated the expression of *CATH*s in the CD11c+ cells of mice (Sainathan *et al*., 2012), whereas, to our knowledge, there is no available report describing the effect of R848, another TLR7 agonist, on the expression of *CATH*s. In the current study, the expression of *CATH1* and *CATH3* was up-regulated, but *CATH2* was down-regulated by R848 in the vaginal mucosa. The increase of *CATH1* and *CATH3* by R848 suggests that infection by the ssRNA virus may induce the synthesis of these cathelicidins in the vagina. The CpG-ODN is the molecular pattern of bacterial genome and DNA virus. Since CpG-ODN did not affect the expression of *CATH*s in the current study, the effects of DNA virus infection on the cathelicidins induction may be limited.

Based on the current results, it is likely that the infection by ssRNA viruses up-regulates the expression of *CATH*s, especially *CATH1* and *CATH3*, and the dsRNA have suppressive effects on the induction of *CATH*s expression. We have reported that the expression of proinflammatory cytokines, including *IL1$\beta$* and *IL6*, was up-regulated by R848 in the vagina; however, the expression of *AvBD*s, another members of antimicrobial peptides, was not affected (Abdel-Mageed *et al*., 2014). It was also reported that challenge by *avian infectious bronchitis* virus, one of the major ssRNA viruses causing eggshell malformation in hens, up-regulated the proinflammatory cytokine expression and cytotoxic cell influx in the oviduct (Nii *et al*., 2014; Nii *et al*., 2015). The cathelicidins synthesized in the vagina in response to the ssRNA visues may play role to attack those viruses together with proinflammatory cytokines. Although antiviral properties of cathelicidins had received little attention, recent studies suggest they may attack DNA and RNA viruses by causing damage of the viral envelope (Barlow *et al*., 2014).

In conclusion, this study suggests that the mucosal tissues of the oviduct expressed *CATH*s to provide a protection against pathogenic microbes, and infection by ssRNA viruses may up-regulate the expression of *CATH1* and *CATH3*. The role of cathelicidins in the antiviral properties against dsRNA and DNA viruses remains unknown because the *CATH*s expression was suppressed or unaffected by the molecular patters of these viruses.

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