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RESEARCH ARTICLE

Intravaginally CpG-ODN and *Salmonella* enteritidis on *TLR21*, cytokines, and *AvBD10* gene expressions in the reproductive tract of native chicken

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar Enteritidis (SE) is a global concern for the poultry industry due to its association with foodborne illnesses. The transmission occurs through the transovarial route which initiates from colonization in oviducts and ascending to ovaries. Though there are studies on cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) and the increase of innate immune response, there is limited research on the intravaginal treatment using CpG-ODN. Previous studies have shown that stimulating CpG-ODN can induce the production of antimicrobial peptide avian beta-defensins (*AvBDs*) in vaginal cell cultures, there is limited information on the use of intravaginal treatment to induce the innate immune system, particularly in the Kampung Unggul Balitbangtan (KUB-1) chickens (*Gallus gallus domesticus*). This study investigates the impact of intravaginal CpG-ODN stimulation on the innate immune response in KUB-1 chicken ovaries and oviducts when challenged to SE. A total of 39 KUB-1 chickens were divided into four groups namely T1 (treated with CpG-ODN, n=12), T2 (SE group, n=12), T3 (CpG-ODN and SE, n=12), and Control (without CpG-ODN and SE, n=3). Chickens were observed from day 1 to 4 post-intravaginal (PI) inoculation. The results suggest that intravaginal CpG-ODN treatment modulates *AvBD10* production through toll-like receptor (*TLR)21*, with interleukin *(IL)1B* and *IL10* playing reciprocal roles, providing insights into the potential of this treatment to prevent transovarial Salmonellosis in poultry. The novelty of this study adds valuable insights to the current body of knowledge.

Keywords: *AvBD10*; *IL1B*; *IL10*; *Salmonellosis*; *TLR21*.

INTRODUCTION

Salmonellosis outbreaks in humans worldwide are associated with the consumption of contaminated eggs, as well as infected or carrier chickens (Sodagari *et al*., 2020). *Salmonella enterica* is the most important foodborne pathogen, causing ± 93 million cases of gastroenteritis and 155,000 deaths worldwide yearly, with the most common serotype being *Salmonella* Enteritidis (SE) (Castro-Vargas *et al*., 2020). One of the international strategies to prevent foodborne illness is through the control of *Salmonella* in poultry, with the major source being contaminated chicken eggs (Popa & Popa, 2021)

Transovarial transmission of SE occurs through colonization in oviducts, which then ascends to ovaries (Osowski *et al*., 2019). Consequently, reproductive tissue becomes the initial site of innate immune activation (Wigley, 2014). Chickens reproductive tissue contains various toll-like receptors (TLRs) and avian beta-defensins (AvBDs) antimicrobial peptides (Wigley *et al*., 2021). TLR signaling is the first line of defense against SE (Gou *et al*., 2012). In humans and mice, four TLRs (TLR4, TLR2, TLR5, and TLR9) are responsible for SE antigen recognition, while in chickens, TLR1–TLR5, TLR7, TLR15, and TLR21 have been identified (Rehman *et al*., 2021).

Among these, TLR4 and TLR5 recognize LPS and bacterial flagellin, respectively (Wu *et al*., 2016), while TLR21 recognizes bacterial CpG-ODN (Lai *et al*., 2019) a functional homolog of mammalian TLR9 (Li *et al*., 2017). The interaction between TLRs and their ligands leads to the synthesis of pro-inflammatory cytokines and host defense molecules such as AvBD (Sonoda *et al*., 2013; Taha-Abdelaziz *et al*., 2017). Chickens have 14 *AvBDs* (Lyu *et al*., 2020) with 11 being expressed in the reproductive system (Abdelsalam *et al*., 2011). In ovaries, *AvBDs* are gradually upregulated and their synthesis may increase in response to SE infection (Michailidis *et al*., 2011). *AvBDs* also exhibit upregulation after being treated with lipopolysaccharide (LPS) (Rengaraj *et al*., 2018). Ovaries express *AvBD10* (Yoshimura, 2015), and stimulation with LPS increased their expression in oviducts, while CpG-ODN increased interleukin *(IL)1B* (Sonoda *et al*., 2013).

The immune response against SE is influenced by cytokines (Kaiser *et al*., 2022), and requires a balance between pro- and anti-inflammatory responses, with IL10 playing a crucial role (Fu & Harrison, 2021). In response to LPS, *IL10* is expressed by macrophages and myeloid dendritic cells (Mittal *et al*., 2015) after exposure to pathogens to regulate TLR-dependent immune response

that recognizes CpG-ODN (Fitzgerald & Kagan, 2020). TLR activation leads to the production of cytokines and antimicrobial peptides (Juul-Madsen *et al*., 2013). In ovarian follicles, LPS stimulation induces *IL1B* expression (Abdelsalam *et al*., 2011), while *IL10* inhibits pro-inflammatory cytokines formation (Fu & Harrison, 2021). In oviducts, LPS promotes the upregulation of pro-inflammatory cytokines (Parvizi *et al*., 2014) and cytokines induce antimicrobial agents like *AvBDs* (Yoshimura & Barua, 2017). The anti-inflammatory IL10 limits the immune response of the host to pathogens, thereby preventing damage and maintaining normal tissue homeostasis (Neumann *et al*., 2019). *IL10* products are antagonistic to TLR signaling (Mittal & Roche, 2015), which promotes the upregulation of *IL10,* a suppressor of immunity *in vivo* (Du *et al*., 2016). Innate immune cells are primary sources, with epithelial cells also exhibiting expression in response to infections (Fu & Harrison, 2021).

An immune response leading to regulation differences between pro- and anti-inflammatory cytokines may be produced by CpG-ODN stimulation, as observed in the caecal tonsils (Taha-Abdelaziz *et al*., 2017). A lack of balance between these two classes of cytokines can disable the appropriate function of the immune system (Trifunoviт *et al*., 2015). Inoculation of SE led to an increase in *AvBD* expression (Michailidis *et al*., 2011) upregulated by the *IL1B* in the vagina (Sonoda *et al*., 2013) and ovarian theca (Abdelsalam *et al*., 2011). *AvBD* in oviductal tissue was upregulated after *Salmonella* infection and stimulation with CpG-ODN (Sonoda *et al*., 2013).

This study aimed to investigate the impact of intravaginal CpG-ODN stimulation on the innate immune response in KUB-1 chickens ovaries and oviducts when exposed to SE. It was hypothesized that *TLR21* activation would promote cytokines *IL1B* and *IL10* to modulate *AvBD10* regulation. The results are expected to contribute to the understanding of innate immune responses initiated by CpG-ODN as PAMP and its interaction with TLR21 receptor in KUB-1 chickens and the potential future implication of this study's outcome is contributing to advancement or development in vaccine design.

MATERIALS AND METHODS

Experimental design and animal groups

Animal experiments were carried out according to the ethical guidelines and regulation stipulated by the Bandung Institute of Technology Ethics Commission with the issuance of Decree No. 01/KEPHP-ITB/3-2019. All procedures complied with internationally accepted principles for the use and care of test animals. The samples were 39 60-week-old female KUB-1 chickens that were *Salmonella*-free. After a week of acclimation, chickens were divided into four groups: T1 (GpG-ODN), T2 (SE group), T3 (CpG-ODN and SE), and Control (received only PBS). Intravaginal CpG-ODN treatment was prepared from 5 µg/500 µl of ODN 2007 Class B CpG oligonucleotide with the sequence 5'-TCGTCGTTGTCGTTTTGTCGTT-3' (InvivoGen), while SE $5x10^7$ CFU was prepared from Culti-Loops™ ATCC-13076. The administration of CpG-ODN and SE infections intravaginally involved utilizing a needle-free 1 cc syringe. The syringe was carefully inserted through the cloaca and then injected upon reaching the chicken's vagina. Tissue samples from ovaries and oviducts were collected aseptically at four time points (Day 1 to Day 4) post-intravaginal (PI) inoculation, under BSL2+ conditions.

RNA extraction and cDNA synthesis

Ovarian and oviducts samples were collected and processed for RNA extraction using the Quick-RNA Miniprep Plus Kit (Zymo) and synthesized into cDNA by reverse transcription with the SensiFAST cDNA Synthesis Kit (Bioline) following the manufacturer's protocol. PCR amplification was conducted at 25°C: 10 minutes (m), 42°C:15 m, and 85°C:5 m, then the resulting cDNA products were stored at -20°C.

Real-time qPCR and analysis of relative gene expression levels

Real-time qPCR was performed to analyze expression of target gene including *TLR21, IL1B, AvBD10,* and *IL10*, while *ACTB* and *GAPDH* served as housekeeping gene. Primers were designed using the Primer3 Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and Oligocalculator (http://biotools.nubic.northwestern.edu/OligoCalc.html). The synthesis (Table 1) was conducted by IDT, Singapore. The cDNA was mixed with a primer and SYBR Green in SensiFAST SYBR Lo-Rox Kit (Bioline) following the manufacturer's protocols. Real-time qPCR used the setting outlined in Table 2. Cycle Threshold (CT) values were determined in six technical replicates and RGE was assessed using the $2^{-\Delta\Delta Ct}$ formula (Livak & Schmittgen, 2001)

Statistical analysis

The data from replicated experiment trials were analyzed using IBM SPSS version 25.0 for One-way ANOVA and Duncan's test was used to determine the statistical significance between samples at $P \leq 0.05$. Data graphics were generated using GraphPad Prism 9.4.0.

RESULTS

Partial RGE of *TLR21, IL1B, AvBD10,* and *IL10* in KUB-1 chickens ovaries and oviducts

Intravaginal administration of CpG-ODN increased *TLR21* RGE in ovaries and oviducts (Figure 1a). In particular, T1 exhibited a significantly higher expression level in both ovaries ($P \leq 0.01$) and oviducts ($P \leq 0.001$), while T2 did not. As for T3, a significant increase was observed in both locations ($P \leq 0.05$). The treatment also substantially elevated expression of *IL1B* RGE as shown in Figure 1b. T1 exhibited a significant increase in oviducts ($P \leq 0.01$), but not in ovaries. In T2 and T3 a significant elevation was observed only in ovaries ($P \leq 0.05$). Expression of *AvBD10* RGE was also affected by intravaginal CpG-ODN treatment (Figure 1c). T1 showed a significant rise only in oviducts ($P \leq 0.05$) and T2 exhibited an increase in both locations but the result was not significant. In the T3, a significant elevation was observed in both locations ($P \leq 0.05$). The treatment also significantly raised *IL10* RGE expression within ovaries and oviducts in T1 and T2 but the increase was not significant in T2. In T3, significant elevation was found only in oviducts ($P \leq 0.05$) as shown in Figure 1d.

Table 1. Primers of target and housekeeping genes

Identity	Primer	Size	Accession number NCBI
GAPDH	F: AGCCATTCCTCCACCTTTGA R: CAACAAAGGGTCCTGCTTCC	190 bp	NM_204305.1
ACTB	F: ATGAAGCCCAGAGCAAAAGA R: GGGGTGTTGAAGGTCTCAAA	244 bp	NM_205518.1
TLR21	F: GCAGGTGTTGTGGCTCAAT R: GCAGTCTGTGGAGGTCAATG	148 bp	NM_001030558.2
IL1B	F: TCCTCCAGCCAGAAAGTGAG R: GTCCAGGCGGTAGAAGATGA	228 bp	NM_204524.1
AvBD10	F: TGTTAAACTGCTGTGCCAAGATTC R: TGTTGCTGGTACAAGGGCAAT	98 bp	NM_001001609.1
IL10	F: CAGCACCAGTCATCAGCAGAGC R: GCAGGTGAAGAAGCGGTGACAG	94 bp	NM_001004414.2

Table 2. Setting conditions of qPCR real-time

Cycle	Temperature (°C)	Time	Activity
1	95	2 min	*Polymerase action*
40	95	5 sec	*Denaturation*
	58	30 sec	*Annealing/Extention*
	60 for *IL10*		

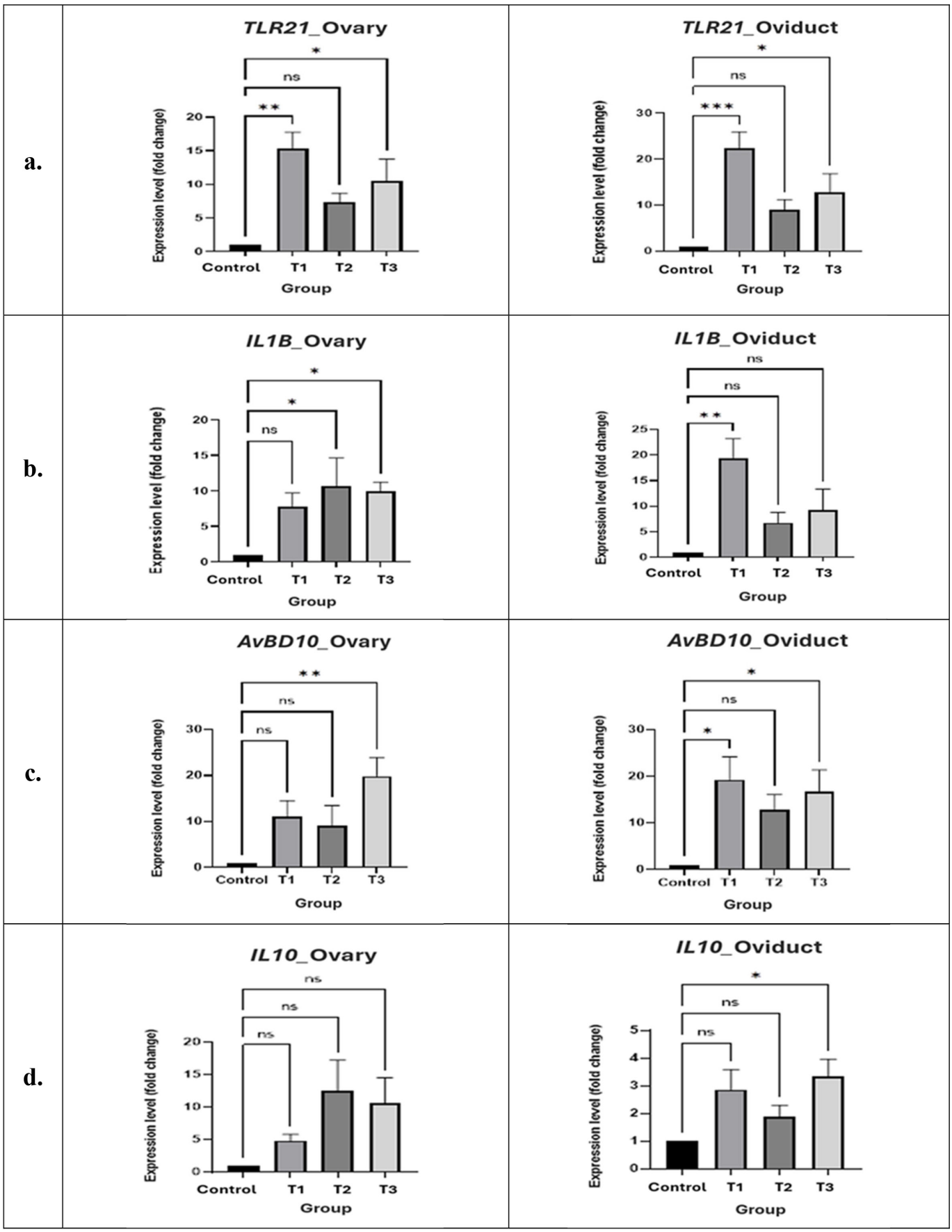

Figure 1. The RGE of *TLR21*(1a), *IL1B* (1b), *AvBD10* (1c), and *IL10* (1d) in the ovaries and oviducts of KUB-1 chickens in T1, T2, and T3 compared to the Control group through real-time qPCR normalized by *ACTB* and *GAPDH,* analyzed with One-way ANOVA and each bar represents the mean ± SEM. Sign * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

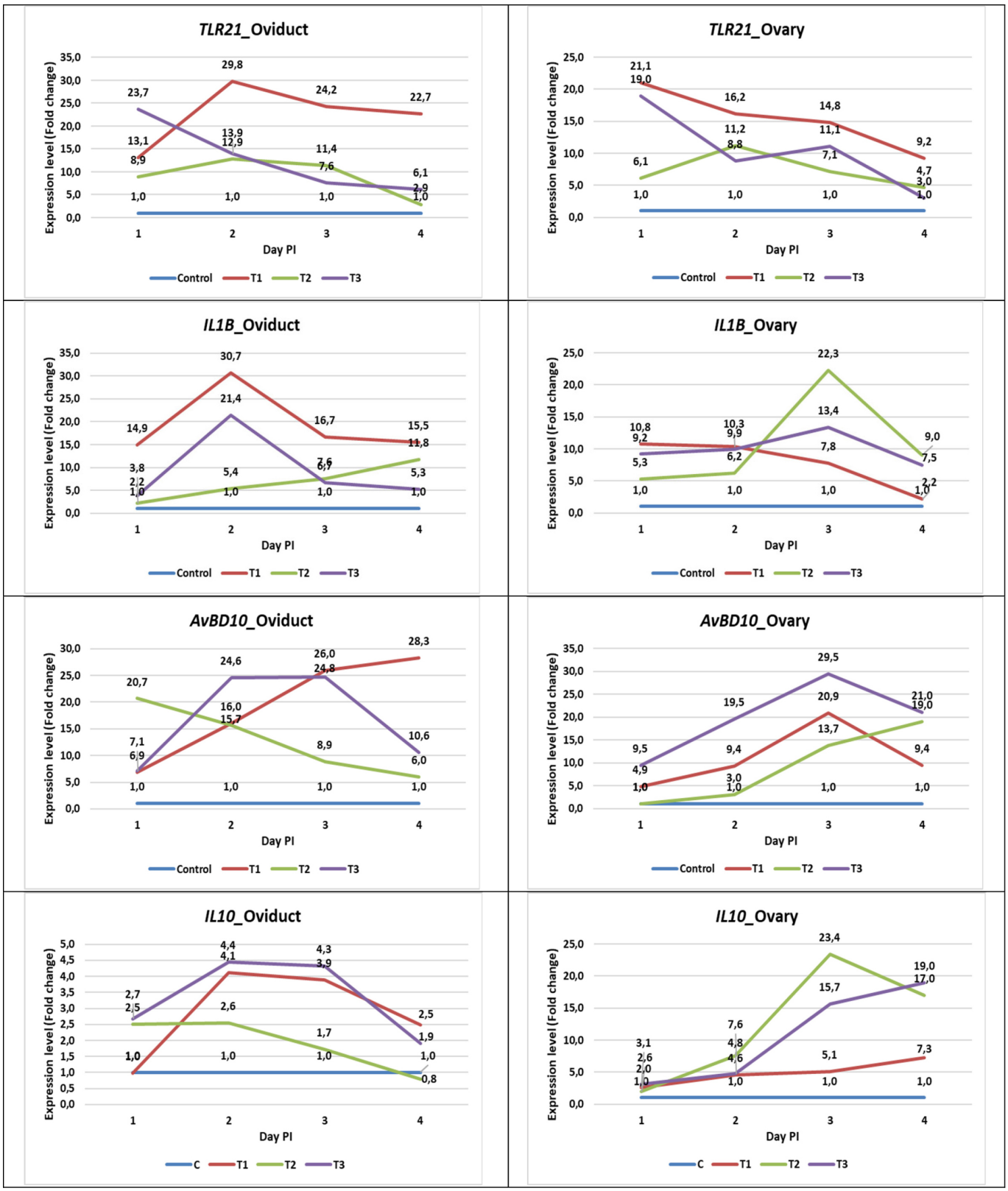

Figure 2. Fluctuation levels of *TLR21*, *IL1B*, *AvBD10*, and *IL10* from day 1 to day 4 PI in the ovaries and oviducts of KUB-1 chickens in T1, T2, and T3 compared to Control group through real-time qPCR normalized by ACTB and GAPDH. Each plot represents the mean ± SEM).

Temporal RGE of *TLR21, IL1B, AvBD10,* and *IL10* in KUB-1 chickens ovaries and oviducts

TLR21 RGE showed the highest level on day 1 PI, with fluctuations observed from day 1 to 4 PI (Figure 2a). The maximal level in T1 was 21.1-fold in ovaries on day 1 PI and 29.8-fold in oviducts on day 2 PI, while in T3, there was a 19.0-fold and 23.7-fold increase respectively. Similarly, T2 showed its highest level on day 2 PI, although the increase was not as pronounced compared to T3.

Expression of *IL1B* RGE exhibited a decreased level after day 2 PI. The results (Figure 2b) showed that on day 2 PI, the highest increase in T1 was 30.7-fold in oviducts and 10.3-fold in ovaries. However, the peak increase was observed on day 3 PI reaching 22.3-fold in ovaries. The T1, T2, and T3 treatments exhibited increased expression on day 2 PI, which subsequently decreased on the following day, except in oviducts of T2.

AvBD10 RGE showed an increase on day 3 PI followed by a subsequent decrease on day 4 PI. As shown in Figure 2c, the maximal *AvBD10* level observed in the T1 across ovaries and oviducts was raised by 21-fold and 26-fold respectively, until day 4 PI, reaching 28.3-fold in oviduct. Furthermore, T2 showed differing trends, in oviducts, expression tended to increase from day 1 to day 4 PI, while the opposite occurred in ovaries. The highest increase occurred on day 1 PI (18.9-fold), and in oviducts on day 4 PI (20.1-fold). T3 also reached its maximum level on day 3 PI, increasing by 29.5-fold and 24.8-fold in ovaries and oviducts, respectively.

Figure 2d showed that *IL10* in ovaries increased, reaching a maximal level of 7.3-fold (T1), 23.4-fold (T2), and 19-fold (T3) on days 2 PI, 3 PI, and 4 PI respectively. However, in oviducts, the increase was less significant, with the highest level of 4.1-fold (T1), 2.6-fold (T2), and 4.4-fold (T3) on days 2 PI. On day 4 PI, the *IL10* levels decreased at notable 2.5-fold at T1. Subsequently, at T2, the levels further diminished, dropping below the control levels, and at T3 observed a decrease to 1.9-fold.

DISCUSSION

The partial analysis of RGE sowed an upregulated *TLR21* in the group treated with intravaginal CpG-ODN (T1 and T3). This implied that reproductive tissue could recognize CpG-ODN as its ligand as reported in a previous study that an interaction with LRR in the extracellular domain of TLR21 (Wu *et al*., 2016). This result suggested the ability of *TLR21* expressed in the ovaries and oviducts to act as a receptor as identified in a similar phenomenon but *TLR21* level was not addressed in their investigation (Chuang *et al*., 2020). In another study, CpG-ODN was administrated to expression vectors and co-transfected cells of the reporter gene led to the measurement of the induced luciferase reporter activity, indicating stimulation of *TLR21* (Yeh *et al*., 2017). The upregulated expression at T2 was consistent with an increase in *TLR21* RGE following PO of SE in 104-week-old Red Rhode Island laying hens, an effect not observed in 28-week-old hens (Michailidis *et al*., 2011).

The elevated *IL1B* RGE at T1, T2, and T3 indicated that the ovaries and oviducts express *IL1B.* This result was consistent with previous studies which found similar expression after CpG-ODN administration in vaginal cell cultures (Sonoda *et al*., 2013; Kamimura *et al*., 2017). The immunomodulatory cascade also triggered by CpG-ODN activated the secretion of pro-inflammatory cytokines (Ichikawa *et al*., 2023). The increased *AvBD10* RGE corresponded with earlier investigation showing upregulated expression in oviductal tissue due to CpG-ODN stimulation (Sonoda *et al*., 2013), and flagellin of bacteria (Abdel-Mageed *et al*., 2014). As for T2, the elevated expression but not significant contrast with a previous study where 0.1-10 µg/ml CpG-ODN tended to downregulated *AvBD10* in uterine and vaginal cell cultures of White Leghorn after LPS stimulation (Abdel-Mageed *et al*., 2014). This study used 5 µg/0.5 ml CpG-ODN intravaginally in KUB-1 chickens, while SE infection induced *AvBD10* expression in the vagina, as well as *AvBD5* and *AvBD11* in oviducts (Anastasiadou & Michailidis, 2016). The elevated *IL10* RGE was consistent with the prior finding wherein broiler chickens treated by PO of CpG-ODN and *C. jejuni* exhibited significantly increased expression in the caeca tonsils ($P \leq 0.05$) (Taha-Abdelaziz *et al*., 2017).

The temporal analysis of upregulated *TLR21* levels on day 1 PI in the T1 and T3 indicate a strong initial response to intravaginal CpG-ODN. The increased expression at T1 which was higher in oviducts than the ovaries suggested a location-based response. This was consistent with a previous study wherein the highest *TLR* level was observed at the bottom part of the reproductive tract (Wigley *et al*., 2021). In T2, the ovaries exhibited the highest *TLR21* expression of 12.9-fold on day 2 PI, lower than T1 and T3. Nevertheless, the increased level in T2 suggested the possibility of TLR21 recognizing the PAMP of SE bacteria (Parvizi *et al*., 2014). The declining trend over time was consistent with a previous report which levels peaked on day 1 post-infection but decreased subsequently, remaining above the Control (Gou *et al*., 2012). Based on the results, *TLR21* fluctuations showed the highest increase on day 1 PI, gradually decreasing until day 4 PI. This decrease could be associated with a half-life of CpG-ODN (Hanagata, 2017; Bavananthasivam *et al*., 2019). Chickens reproductive tissues had significantly increased *TLR21* levels ($P \leq 0.001$) at three hours post-stimulation (Chrząstek & Wieliczko, 2014).

The increased *IL1B* level was in line with previous finding, where induced expression was indetified in vaginal cell culture following CpG-ODN stimulation (Sonoda *et al*., 2013). Similarly, in a separate investigation, chickens infected with *A. paragallinarum* had increased levels on days 1, 3, and 5 post-stimulation (Gou *et al*., 2012). Furthermore, CpG-ODN stimulation induced *IL6, IL8*, and *IFNγ* pro-inflammatory cytokines after four hours (Yeh *et al*., 2017). In this study, *IL1B* levels increased on day 2 PI in T1, T2, and T3, but decreased on day 3 PI or day 4 PI, except for T2 oviducts. The decline after reaching a peak may be attributed to anti-inflammatory cytokines, stating that pro-inflammatory cytokines were responsible for initiating inflammation, but at a controlled rate (Moon *et al*., 2021). Therefore, the sustained elevation of *IL1B* levels in T2 oviducts on day 4 PI suggests that the pro-inflammatory response is not yet countered by anti-inflammatory cytokines.

AvBD10 levels in oviducts of T1 increased 26-fold on day 3 PI and 28.3-fold on day 4 PI indicating that expression was induced by intravaginal CpG-ODN treatment. Fluctuation levels in T3 which tended to increase on day 3 PI and then decrease on day 4 PI facilitated a reduction in *AvBD10* after the effective elimination of bacteria. In a prior investigation, the innate immunity formation rapidly decreased after infection was halted (van der Meer *et al*., 2015), and the increased *AvBD* eliminated microbes during the infection process (Sonoda *et al*., 2013). Therefore, the level in oviducts of T3 which initially increased by 20.7-fold and then decreased to 10.6-fold could be in response to SE. Based on previous reports, bacterial infection modulated *AvBD* expression, whereas *Salmonella* infection increased the levels in oviducts (Yoshimura, 2015).

Regarding *IL10* levels, the fluctuation observed in this study suggests its role as a feedback regulator, contributing to the modulated immune response. This aligns with earlier research that assessed the functional analysis using ELISA for vaccine efficacy (Wu *et al*., 2016). Additionally, a separate study demonstrated the ability of *IL10* isolated from chicken jejunum, to inhibit the pro-inflammatory genes *IL1B, IL6, IL17,* and *IL8* after LPS induction (Hu *et al*., 2021).

Figure 3 illustrates the fluctuations in *TLR21, IL1B, AvBD10,* and *IL10* levels within the reproductive tract of KUB-1 chickens. The highest significant increase in *TLR21* levels occurred on day 1 PI at 23.7-fold ($P \leq 0.001$). The *IL1B* levels had the highest elevation

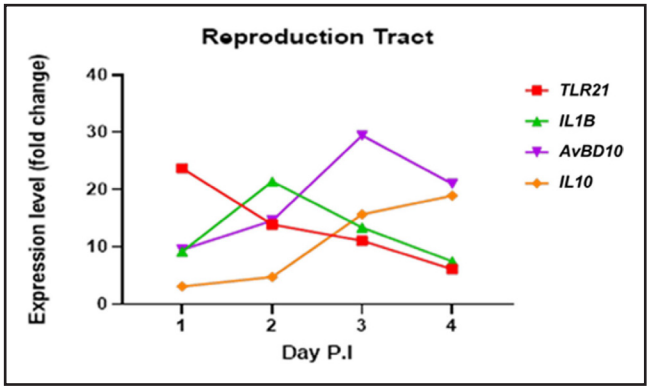

Figure 3. Trendline of *TLR21, IL1B, AvBD10,* and *IL10* expression levels in the reproductive tract of KUB-1 chickens. Red = *TLR21*, green = *IL1B* pro-inflammatory cytokine, purple = *AvBD10*, and orange = *IL10* anti-inflammatory cytokine.

on day 2 PI by 21.4-fold ($P \leq 0.05$). *AvBD10* exhibited the most significant increase on day 3 PI by 29.5-fold ($P \leq 0.01$), while *IL10* levels showed the highest rise on day 4 PI by 19-fold significantly ($P \leq 0.05$). The temporal trend *TLR21* fluctuations, acting as a CpG-ODN receptor, indicated a consistent decline over time, paralleling the decrease in levels within Harderian glands decreased at 1-18 hours post-stimulation (Chrząstek & Wieliczko, 2014). The decreased expression after day 1 PI aligns with findings indicating that *TLR21* levels in chicken leukocytes increased on day 1 post-SE infection and subsequently decreased until day 12 PI (Gou *et al*., 2012).

From day 2 to 4 PI, a reciprocal pattern was observed between pro-inflammatory cytokine *IL1B* and anti-inflammatory cytokine *IL10*. This phenomenon resonates with findings from a study where the per oral (PO) administration of CpG-ODN to Ross broiler chickens induced an immune response that generated a reciprocal pattern of regulation between *IL1B* and *IL13* in the caeca tonsils at 6 hours, 1 day, 2 days, and 3 days post-administration (Taha-Abdelaziz *et al*., 2017). Earlier investigations have demonstrated that *IL10* acted as an antagonist to pro-inflammatory cytokine signaling, with *TLR* activation increasing *IL10* secretion as an anti-inflammatory response (Mittal & Roche, 2015; Du *et al*., 2016).

AvBD10 levels fluctuation pattern paralleled those of the *IL1B*, but the peak expression was observed on day 3 PI and 2 PI, respectively. Correspondingly, *IL1B* declined on day 3 PI and decreased *AvBD10* levels on day 4 PI. A previous study indicated the impact of *IL1B* on *AvBD12* levels in chicken ovaries (Abdelsalam *et al*., 2011) as well as *AvBD1* and *AvBD3* levels in oviducts (Sonoda *et al*., 2013). In another study, *IL1B* expression was influenced by *TLR21*, with CpG-ODN acting as its ligand and stimulating *IL1B* and *AvBD7* levels in ileal and caecal cell cultures of broiler chicken (Terada *et al*., 2020). This study suggested a reciprocal role between the *IL1B* pro-inflammatory and *IL10* anti-inflammatory cytokines in the *AvBD10* modulation of KUB-1 chicken ovaries and oviducts.

CONCLUSION

Intravaginal CpG-ODN treatment significantly ($P \leq 0.05$) upregulated *TLR21, IL1B, AvBD10*, and *IL10* in ovaries and oviduct of KUB-1 chickens challenged by SE. The interplay between *IL1B* pro-inflammatory and *IL10* anti-inflammatory cytokines reciprocally modulated *AvBD10*, contributing to the immune response against SE. Therefore, intravaginal CpG-ODN could be developed in vaccine development as a preventive method for transovarial Salmonellosis.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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