Enhancement of protective efficacy of innate immunostimulant based formulations against yolk sac infection in young chicks

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ABSTRACT This study was conducted to characterize and compare the protective effects of various innate immune stimulants against yolk sac infection (**YSI**) caused by an avian pathogenic *Escherichia coli* in young chicks. The immune stimulants were administered alone or in various combinations of unmethylated CpG oligodeoxynucleotides (**CpG**), polyinosinic:polycytidylic acid (**Poly I:C**), and avian antimicrobial peptides (**AMPs**). Routes included in ovo or in ovo followed by a subcutaneous (**S/C**) injection. CpG alone and in combination with Poly I:C, truncated avian cathelicidin (**CATH**)-1(6-26), avian beta defensin (**AvBD**)1, and CATH-1(6-26) + AvBD1, were administered in ovo to 18-day-old embryonated eggs for gene expression and challenge studies. Next, CpG alone and the potentially effective formulation of CpG + Poly I:C, were administrated via the in ovo route using 40 embryonated eggs. At 1 day post-hatch, half of each group also received their respective treatments via the S/C route. Four hours later, all chicks were challenged using *E. coli* strain EC317 and mortalities were recorded for 14 d. The first challenge study revealed that amongst the single use and combinations of CpG with different innate immune stimulants, a higher protection and a lower clinical score were offered by the combination of CpG + Poly I:C. The second challenge study showed that this combination (CpG + Poly I:C) provides an even higher level of protection when a second dose is administered via the S/C route at 1 day post-hatch. The current research highlights the efficacy of a combination of CpG + Poly I:C administered either in ovo or in ovo along with a S/C injection and its potential use as an alternative to antibiotics against yolk sac infection in young chicks.

Key words: CpG, Poly I:C, combination, in ovo, innate immune stimulant

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INTRODUCTION

Unmethylated CpG oligodeoxynucleotides (**CpG**), polyinosinic-polycytidylic acid (**Poly I:C**), and avian antimicrobial peptides (**AMPs**) are the most common innate immune stimulants investigated for control of bacterial and viral diseases in chickens (Zou et al., 2017; Bavananthasivam et al., 2018; Nguyen et al., 2021; Sarfraz et al., 2022). Innate immunity is the first line of host defense and includes rapid responses for instant control of invading microbes or to instruct adaptive immune responses (Janeway and Medzhitov, 2002; Kogut, 2009). Host cellular toll-like receptors (**TLRs**) play a pivotal role in pathogen recognition in the innate immune system, triggering signal transduction and gene expression networks (Kogut, 2009). Several TLRs agonists, including CpG, Poly I:C, lipopolysaccharide (**LPS**), Pam3CSK4, and *Bacillus subtilis* spores, are reported to be effective against a number of bacterial and viral infections in birds (Dar et al., 2009; Mackinnon et al., 2009; Liang et al., 2013; Alkie et al., 2017; Yuan et al., 2017; Abdul-Cader et al., 2018; Allan et al., 2018; Bavananthasivam et al., 2018; Gunawardana et al., 2019).

CpG, consisting of CpG motifs that are highly prevalent in bacterial DNA and recognized by TLR9 (Hemmi et al., 2000) and TLR21 (Brownlie et al., 2009) in mammals and chickens, respectively, has shown protection against some common avian pathogens (Abdul-Cader et al., 2018). For instance, administration of CpG via subcutaneous (**S/C**), intramuscular (Gomis et al., 2003), or in ovo routes (Allan et al., 2018; Gunawardana et al., 2019; Nguyen et al., 2021) or through intrapulmonary delivery (Goonewardene et al., 2017) has shown protection against *Escherichia coli* infection in chickens. In addition, following intraperitoneal or in ovo

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administration of CpG, it has exhibited protection against *Salmonella* Enteritidis (He et al., 2005; Mackinnon et al., 2009) and *Salmonella* Typhimurium infections (Taghavi et al., 2008). Previous studies reported that in ovo delivery of CpG resulted in reduced mortality and morbidity and induced protection from viral infections including infectious laryngotracheitis virus (**ILTV**) (Abdul-Cader et al., 2018) and reticuloendotheliosis virus (**REV**) infections (Yuan et al., 2017). Similarly, application of nanoparticle encapsulated CpG enhanced immune activity and increased the half-life and cellular uptake of CpG (Bavananthasivam et al., 2018). Notably, oral administration of poly D, L-lactic-co-glycolic acid (**PLGA**)-encapsulated CpG reduced colonization of *Campylobacter jejuni* in cecal contents of chickens (Taha-Abdelaziz et al., 2018). Likewise, in ovo delivery of CpG formulated with carbon nanotubes or liposomes showed protection from *E. coli* infection in broiler chickens (Gunawardana et al., 2015). Taken together, CpG has potential as an alternative to antibiotics and as a vaccine adjuvant for induction of protective immunity against infectious diseases in poultry.

Poly I:C is a double-stranded RNA and a TLR3 ligand that exhibits antiviral and antibacterial activities. In a previous experiment, administration of Poly I:C enhanced *E. coli* phagocytosis in murine microglial cells through elevated cytokine induction (Ribes et al., 2010). In addition, enhanced expression of *IL-6, IL-8, IL-1α*, and monocyte chemoattractant protein-1 in murine lungs was reported following intranasal spray of Poly I:C solution leading to protection against *Francisella tularensis* infection (Pyles et al., 2010). Moreover, administration of Poly I:C in chicken-derived cells has led to rapid induction of pro-inflammatory cytokines and nitrite production in chicken spleenocytes and monocytes (Villanueva et al., 2011; He et al., 2012; St Paul et al., 2013). Notably, in ovo administration of Poly I:C protected against an *E. coli* challenge (Allan et al., 2018). In addition, Poly I:C has been used as a vaccine adjuvant for improving humoral and cellular immune responses. Administration of Poly I:C as an adjuvant in a H5N1 influenza vaccine significantly reduced virus shedding in mice (Ichinohe et al., 2009). Similarly, the combination of Poly I:C and H9N2 vaccine significantly induced higher anti-influenza antibody titers, reduced virus shedding, and elevated mRNA levels of *IFN-α*, *IFN-γ*, *IL*-6, and MHC-II in ducks (Zhang et al., 2017). In addition, Poly I:C triggered production of pro-inflammatory cytokines and interferon-β, leading to restriction of Marek's disease virus infection in chicken embryo fibroblasts (**CEFs**) (Zou et al., 2017). In another study, Poly I:C combined with gp90 recombinant proteins significantly reduced the viremia and immunosuppressive effects caused by reticuloendotheliosis virus (**REV**) challenge in chickens (Yuan et al., 2017). Hence, Poly I:C is not only a potential anti-infection agent, but also a good adjuvant to enhance immune responses for its incorporated vaccines.

Antimicrobial peptides are important signaling molecules of the innate immune system and demonstrate high potency as immunostimulants and antibacterial agents in birds. Besides being directly bactericidal, AMPs can modulate host immunity through 1) activation or recruitment of immunocytes; 2) neutralization of bacterial products, including lipopolysaccharide (**LPS**) or lipoteichoic acid (**LTA**) to suppress inflammation; or 3) enhancement of nucleic acid recognition to promote autoinflammation (van Dijk et al., 2011; Zhang and Gallo, 2016; Nguyen et al., 2021). Regarding antibacterial activity, AMPs have shown in vitro killing effects against food-borne pathogens including *E. coli, C. jejuni, Clostridium perfringens, S.* Typhimurium, and other important pathogenic bacteria and fungi such as *Staphylococcus aureus, Streptococcus pyogenes*, and *Candida albicans* (Xiao et al., 2006; van Dijk et al., 2007, 2009; Zhao et al., 2014; van Dijk et al., 2016; Nguyen et al., 2021). In ovo administration of Cathelicidin-2 (**D-CATH-2**) showed protection against *E. coli* infection in chickens by reducing mortality and respiratory bacterial load (Cuperus et al., 2016). Furthermore, recombinant avian CATH and β-defensin (**AvBD**) have been produced using an *E. coli* system and proven their antimicrobial activities against multiple microorganisms (Zhao et al., 2014; Yu et al., 2015; Tanhaiean et al., 2018). In our recent study, various peptides, including CATH-1(6-26), CATH-2(1-15), AvBD1, AvBD2, AvBD6, AvBD9 were tested in vitro. CATH-1 (6−26) and AvBD1 were determined to have the most potential as candidates against avian pathogens including *E. coli* strain EC317, *Salmonella* sp., *C. jejuni, C. perfringens* (Nguyen et al., 2021). Notably, in ovo administration of AvBD1 offered a comparable anti-*E. coli* effect to CpG in young chicks (Nguyen et al., 2021). Used alone, these immunostimulants have potential for prevention of bacterial infections in birds; however, the effects of their combinations are still not fully understood.

Yolk sac infection (**YSI**) or omphalitis has emerged as the most common bacterial infection that leads to high rates of early chick mortality (**ECM**) and economic losses in poultry production (Saskatchewan, 2021). Avian pathogenic *E. coli* (**APEC**) is a frequently isolated pathogen from poultry farms, followed by various bacteria such as *Enterococcus, Staphylococcus, Salmonella, Pseudomonas, Proteus, Streptococcus*, and *Klebsiella* (Olsen et al., 2012; Amare et al., 2013; Swelum et al., 2021). YSI induces mortality or low weight gains and leaky navels in survivor birds (Fasenko and O'Dea, 2008), and increases susceptibility to other infections (Allan et al., 2018). Currently, YSI is managed by heavy culling and administration of antibiotics via in ovo injection or post-hatch (Allan et al., 2018). However, consumer demand for antibiotic-free products and reduction of antibiotic-resistant bacteria has prompted the poultry industry to search for alternative products. Although multiple studies have been performed for development of vaccines as well as antimicrobial alternatives, there are no commercially available alternatives for prevention and control of mortalities due to YSI and other bacterial infections in young chicks (Swelum et al.,

2021). The goal of the present investigation was to determine the immune response and protective effects of CpG alone and in combination with Poly I:C and AMPs when administered in ovo or in ovo followed by a S/C injection at one day of age for control of YSI.

MATERIALS AND METHODS

Reagents

CpG ODNs 2007 was purchased from Biospring (Frankfurt, Germany) with >85% purity and endotoxin level below 1,000 EU/g. Poly I:C (P1530) was purchased from Sigma_Aldrich (MO). AMPs [AvBD1 and CATH-1(6-26)] were synthesized in linear forms by Genscript (NJ) using the Fmoc solid-phase peptide synthesis (**SPPS**) method with >95% purity assessed by mass spectrometry (MS). Sequences of CpG ODNs 2007, AvBD1, and CATH-1(6-26) used in this study are listed in Table 1. CpG, Poly I:C, and AMPs were suspended in sterile, endotoxin-free water (Millipore Sigma, MA) as stock solutions. Working solutions for in ovo experiments were prepared by mixing stock solutions with phosphate-buffered saline (**PBS**; Millipore Sigma, MA).

In Vitro Cytotoxicity Assay

Cytotoxicity of CpG, AvBD1, CATH-1(6-26), and their combinations were evaluated using chicken macrophage cells (HD11 cells) following the protocols of van Dijk et al. (2009) and Nguyen et al. (2021). Briefly, HD11 cells (confluency 80−90%) harvested from a T75 flask were dispensed into Nunc 96-well fat-bottom plates (ThermoFisher scientific, OH) at a concentration of 1×10^5 cells/well/100 μL in RPMI-1640-glutaMAX medium (Gibco, ThermoFisher scientific) supplemented with 10% fetal bovine serum (**FBS**; Sigma-Aldrich), 1% v/v HEPES (ThermoFisher scientific), 1% v/v Na Pyruvate (ThermoFisher scientific, OH), and 0.1% gentamycin (v/v) (Sigma-Aldrich). Cells were incubated for 24 h at 37°C and 5% CO_2 in a 3110 Thermo Forma II humidified CO_2 incubator (Thermo). The following day, the old medium was replaced with 90 μL of new DMEM/F12 medium (Gibco, ThermoFisher scientific) without phenol red, FBS, and Gentamicin, followed by addition of 10 μL of CpG, AvBD1, CATH-1(6-26), or their combinations including 1) AvBD1 + CATH-1(6-26), 2) CpG + CATH-1(6-26), 3) CpG + AvBD1, 4) CpG + Poly I:C, 5) CpG + CATH-1(6-26) + AvBD1 in respective wells to obtain 30, 15, 7.5, 3.25, and 1.65 μg/mL as final concentrations of each immune stimulant. The treated cells were incubated at 37°C in a 5% CO_2 incubator. After 24 h incubation, old media were replaced with 100 μL of new DMEM/F12 medium (without phenol red, FBS, and Gentamicin) and 10 μL WST-1 assay reagent (Abcam, MA) was added to each well and incubated for 60 min at 37°C in a 5% CO_2 incubator. The absorbance was measured at 440 nm using a SPECTRAmax 340 PC Microplate Reader (Molecular Devices, CA). The average OD of 3 replicates was calculated for the test and control samples. Cytotoxicity effect (%) was calculated following the formula:

$$\%Cytotoxicity = \frac{(100\,x\,(Control - Sample))}{Control}$$

Animal Experiments

Experiment 1 For the investigation of gene expression following in ovo administration of innate immune stimulants, 185 eighteen-day-old embryonated eggs (from Lohmann LSL-lite layers) were obtained from the breeder operation of the Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. Yolk sacs and livers were collected from 5 embryos and cultured on MacConkey plates to ensure they were free from *E. coli* infection. The remaining 180 eggs were randomly divided into 6 equal groups as follows: Group A (CpG, 30 μg /embryo); Group B (CpG + AvBD1, 15 μg each/embryo); Group C [CpG + CATH-1(6-26), 15 μg each/embryo]; Group D [CpG + AvBD1 + CATH-1(6-26), 10 μg each/embryo]; Group E (CpG + Poly I:C, 15 μg each/embryo); Group F (PBS Control). Each embryo received 100 μL of treatment via the in ovo route into the amniotic sac. Following in ovo injection, spleen samples were collected from 6 embryos/group at 6, 24, 48, 72, and 96 h postadministration.

Experiment 2 This experiment aimed at studying protective efficacy of the combinations of innate immune stimulants described in Experiment 1. A total of 240 eighteen-day-old embryonated eggs were randomly divided into 6 groups (A-F, n = 40 eggs/group) and received the same formulations as described in Experiment 1 via the in ovo route. The hatching rate was recorded, and yolk sacs and livers from 3 birds/group were collected for *E. coli* isolation using MacConkey plates. Twenty-four hours post-hatch all groups were subjected to *E. coli 317* challenge.

Experiment 3 In order to enhance the protection achieved by administration of the combination CpG + Poly I:C, we designed an experiment using a total of 145 eighteen-day-old embryonated eggs. Prior to innate immune stimulant administration, 5 eggs were

Table 1. Sequence and purity of CpG and AMPs used in this study.

Immunostimulant	Sequence	Purity
CpG ODNs 2007	5’-TCGTCGTTGTCGTTTTGTCGTT-3’	>85%
CATH-1(6-26)	WPLVIRTVIAGYNLYRAIKKK-NH2	>95%
AvBD1	GRKSDCFRKSGFCAFLKCPSLTLISGKCSRFYLCCKRIW	>95%

Abbreviations: AMPs, avian antimicrobial peptides; CpG, unmethylated CpG oligodeoxynucleotides.

Table 2. Target genes and primer sequences used for quantitative RT-PCR in this study.

Target gene	Primer name	Primer sequence (5'-3')	Reference
β-actin	β-actin-F	CAACACAGTGCTGTCTGGTGGTA	(St Paul et al., 2011)
	β-actin-R	ATCGTACTCCTGCTTGCTGATCC	
IL-8	Ch-IL8-F	CAGCTGCTCTGTCGCAAG	(Dar et al., 2009)
	Ch-IL8-R	GTGGTGCATCAGAATTGAGCT	
IL-1β	Ch-IL1β-R	GTTGGAGCGGGCAGTCAG	(Dar et al., 2009)
	Ch-IL1β-F	GGCATCAAGGGCTACAAGC	
IFN-γ	Ch-IFN-γ-F	CCAAGAAGATGACTTGCCAGA	(Dar et al., 2014)
	Ch-IFN-γ-R	ACCTTCTTCACGCCATCAGG	

Abbreviations: *IL*, chicken interleukin; *IFN-γ*, chicken interferon-gamma.

checked for *E. coli* contamination as described above. The remaining 140 embryos were randomly divided into 3 groups (A, B, and C) with n = 40 embryos/group and group D with 20 embryos. Each in ovo treatment was 100 μL of the respective stimulant or sham solution/embryo. Group A was treated with CpG, 30 μg/embryo, whereas group B received CpG + Poly I:C, 15 μg each/embryo. Group C was given PBS in ovo as a control, while group D received no in ovo injection. One day post-hatch, all birds in groups A, B, and C were further divided into 2 subgroups with 20 birds per group. Birds in one of the subgroups from treatments A, B, and C received a second dose (same dosages as in ovo injection) via the S/C route. Birds in Group D were injected with the combination (CpG + Poly I:C, 15 μg each/embryo) via the S/C route only. Four hours after S/C injection, all birds were challenged with APEC *E. coli* strain EC317 as described below.

E. coli *Challenge*

Prior to challenge, birds were tagged individually and placed into an isolation room in the animal care unit of VIDO, University of Saskatchewan. Liver and yolk sac samples from 3 birds per group were collected for isolation of *E. coli* as described previously. Each bird was challenged with 35 CFU/100 μL of *E. coli* strain EC317 into the yolk sac via the intranavel route. For first 6 days postchallenge (**dpc**), the birds were checked and clinically scored 4 times daily while later clinical observations and scoring was done twice daily up to 14 dpc as reported previously (Allan et al., 2018; Nguyen et al., 2021). Dead and euthanatized birds were necropsied, and livers and yolk sacs were swabbed for *E. coli* isolation using MacConkey plates. The cumulative clinical scores (**CCS**) were determined as a sum of the clinical scores over the 14 dpc observational period. All animal experiments were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol#20160079) following the Canadian Council on Animal Care guidelines for animal use.

Gene Expression Study

RNA Isolation and cDNA Synthesis Spleen samples collected from embryos were directly placed into 1 mL Trizol tubes, homogenized and kept at −80°C until further processing for RNA isolation, which was performed as per manufacturer's instruction. The NanoDrop-ND1000 Spectrophotometer (Thermo Scientific, Canada) was used to determine RNA quality and quantity. Genomic DNA contamination was removed from the isolated RNA samples using DNase I (Thermo Scientific, Canada). cDNA synthesis was carried out using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.) as described previously (Nguyen et al., 2021).

Quantitative Reverse Transcription Polymerase Chain Reaction Quantitative reverse transcription polymerase chain reaction (**qRT-PCR**) was performed using SsoAdvanced Universal SYBR Green Supermix (ThermoFisher scientific) and the primers listed in Table 2. The reaction was performed in a CFX96 System thermocycler (Bio-Rad Laboratories, Inc.) and conditions were as follows: 95°C for 3 min; then 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Target gene expression was normalized to the expression of β-actin using the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001).

Statistical Analysis

Data was analyzed and graphs were generated using the Prism 8 software (GraphPad Software Inc., San Diego, CA). The survival proportions were compared between treated and control groups at all time-points using the Gehan-Breslow-Wilcoxon test. Cumulative clinical scoring (**CCS**) data was analyzed by Kruskal-Wallis nonparametric analysis of variance in Prism 8. Significant differences of gene expression in spleen samples at each time point among groups were analyzed using one-way ANOVA (and nonparametric or mixed) with Tukey's post-hoc test. P value < 0.05 represents significance difference.

RESULTS

Cytotoxicity of Immunostimulants and Their Combinations to HD11 Cells

The cellular toxicity of stand-alone and combinations of CpG, AvBD1, CATH-1(6-26), and Poly I:C with a dosage ranging from 1.56 to 30 μg/mL was tested in HD11 cells. Twenty-four hours post exposure, 2 to 36% of treated cells showed effects when treated with peptides, however, enhanced cytotoxicity to HD11 cells (32 to 81%) was observed in cells treated with CpG and its

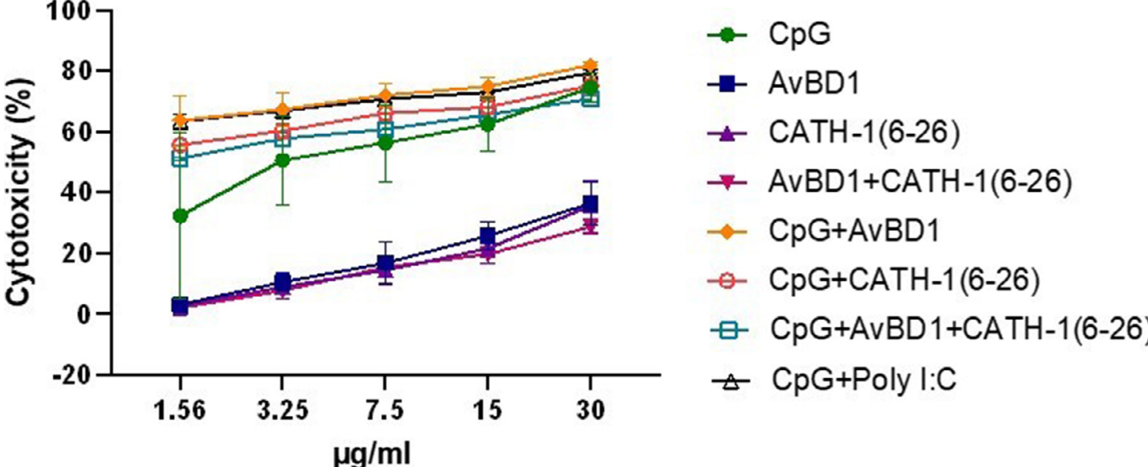

Figure 1. In vitro toxicity of studied immunostimulants and their combinations to HD11 cells. Cytotoxicity to HD11 cells was determined by WST-1 assays. Cells were exposed to stimulants with dosages ranging from 1.56 to 30 μg/mL for 24 h. The graph represents data from three independent experiments on different days with each experiment carried out using three replicates for each group.

combinations. Stand-alone use of CpG showed a dose dependent increase in cell toxicity. For instance, CpG at a dose of 1.56 μg/mL caused ~32% cell death, whereas, this percentage was increased to 74% at the highest dose of 30 μg/mL. The combinations of CpG with peptides or with Poly I:C also induced toxicity to HD11 cells with an average mortality of 50 to 81% with a dose ranging from 1.56 to 30 μg/mL (Figure 1).

Experiment 1: Cytokine Expression Following In Ovo Injection

To understand effects of innate immune stimulants on cytokine gene expression, spleen samples were collected at 6, 24, 48, 72, and 96 h post in ovo administration. Transcriptional changes in 3 pro-inflammatory cytokine genes, namely *IL-1β, IL-8*, and *IFN-γ* were investigated (Figure 2). In most of the treatment groups, upregulation of the *IL-1β* gene was noticed at various time points. Significantly higher up-regulation of *IL-1β* was observed in CpG treated birds at 72 h postinjection (**PI**) compared to the PBS group (1.06-fold vs. 3.6-fold respectively), whereas, CpG + AvBD1 + CATH-1(6-26) treated groups showed expression similar to the PBS group. At 96 h post administration of the immunostimulants, *IL-1β* gene expression was significantly higher in birds treated with CpG + AvBD1 (2.4-fold) compared to those treated with CpG + CATH-1(6-26) (1.24-fold), CpG + Poly I:C (1.27-fold), and PBS (1.03-fold). Upregulation of the *IL8* gene occurred at the early time points post in ovo injection (PI) in birds treated with CpG (6-fold and 3.3-fold at 6 h and 24 h, respectively) and CpG + Poly I:C (2.1-fold at 6h PI). The *IFN-γ* gene showed higher gene expressions in CpG and CpG + Poly I:C treated groups at 6 h (6.9-fold and 2.96-fold, respectively), 48 h (9.1-fold and 3.1-fold, respectively), and 72 h PI (4.5-fold and 2.8-fold, respectively). However, statistically significant expression of *IFN-γ* compared to the control group was only noticed at 48 h PI.

Experiment 2: Effects of In Ovo Administration of CpG Alone and in Combination With Different Immunostimulants

Hatching Rate The lowest hatching rate was noticed in the PBS group with 82.05%, while higher rates were found in all treatment groups including 89.47% (CpG), 84.62% (CpG + CATH-1(6-26) and CpG + AvBD1), 89.47% (CpG + CATH-1(6-26) + AvBD1), and 94.87% (CpG + Poly I:C) (Figure 3A).

Survival Rate Following administration of CpG alone or in combination with other immunostimulants, the CpG + Poly I:C combination provided a significant difference in protection represented by 72.41% survival rate after 14 dpc. This was followed by CpG alone and CpG + CATH-1(6-26) injected groups which showed 65.38% and 60% survival, respectively. Birds receiving CpG + AvBD1 (52% survival) and CpG + AvBD1 + CATH-1(6-26) (50% survival) showed similar mortality proportions to the control group (50% survival; Figure 3B).

Clinical Score Means of CCS for Group F (PBS Control) was 18.71, whereas, these values in the CpG alone, CpG + AvBD1, CpG + CATH-1(6-26), CpG + AvBD1 + CATH-1(6-26), and CpG + Poly I:C groups were 11.96, 15.6, 13.32, 14.54, and 10.07, respectively. Similar to survival rate, CpG + Poly I:C had the lowest CCS in comparison with the remaining groups (Figure 3C).

Experiment 3: Enhancement of Protection Following In Ovo and a Subcutaneous Administration of CpG + Poly I:C Combination

The combination of CpG (15μg) + Poly I:C (15 μg) was chosen to be administered via different routes including in ovo only, in ovo plus S/C, and S/C only.

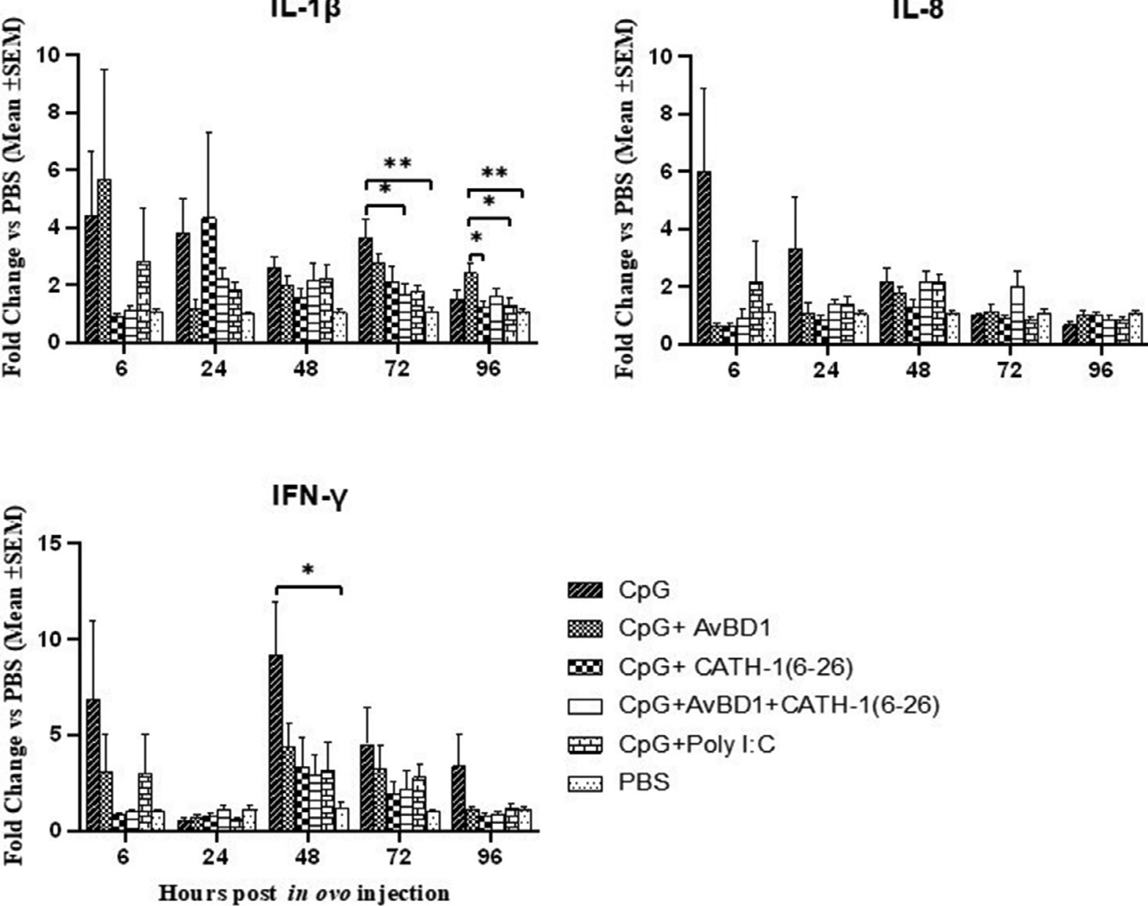

Figure 2. Cytokine expression in spleen tissues at 6, 24, 48, 72, and 96 h post in ovo administration of immunostimulants. Six embryos from each group per time point were randomly collected for RNA isolation and qRT-PCR. Data represents mean ± SEM. Asterisks (*), (**) mean significant differences with $P < 0.05$ and $P < 0.01$, respectively, using one-way ANOVA analysis for comparing data at each time point.

In addition, administration of CpG alone (30 μg) in ovo and in ovo + S/C in two different groups was included as standard controls. Following 14 dpc with *E. coli* via the intra-navel route, birds in the group treated with CpG + Poly I:C via the in ovo plus S/C route showed the highest protection (100% survival rate), followed by CpG alone (93.33%) and CpG + Poly I:C (89.47%) injected in ovo only. Similar survival rates were found in birds receiving CpG via in ovo + S/C (67%), CpG + Poly I:C S/C only (65%) and those receiving PBS (53 and 64% for in ovo and in ovo + S/C, respectively; Figure 4).

DISCUSSION

Antibiotics have played a pivotal role in prevention and treatment of infectious diseases worldwide, however, their overuse and inappropriate application have led to progressively increasing antibiotic resistance. Emergence of various antibiotic-resistant microorganisms has resulted in multiple setbacks for disease treatment, leading to health and economic burdens on health care systems (Li and Webster, 2018). Recently, innate immune stimulants have been evaluated and proven efficacious as alternatives to antibiotics in several human and animal trials. Immunostimulants including CpG, Poly I:C, and AMPs have shown many benefits including antimicrobial activity, wound recovery efficacy, and ability to reduce sepsis (Ichinohe et al., 2009; Ribes et al., 2010; Allan et al., 2018; Nguyen et al., 2021). In chickens, stand-alone use of these stimulants has produced positive effects against various bacterial pathogens such as avian pathogen *E. coli* (APEC), *Campylobacter* sp., and *Salmonella* sp. and shown good adjuvant potential against viral diseases (Ichinohe et al., 2009; Ribes et al., 2010; van Dijk et al., 2016; Zhang et al., 2017; Allan et al., 2018; Nguyen et al., 2021). In this study, we aimed to characterize the protective efficacies of combinations of these immunostimulants using different administrated approaches for control of YSI in young chicks.

Although the antimicrobial and immunostimulatory effects of CpG, Poly I:C, and AMPs have been investigated in multiple in vitro as well as in vivo experiments, the potential toxicity of these immunostimulators has not been fully understood. In a previous study, we found that among 6 tested antimicrobial peptides, CATH-1(6-26) showed the highest in vitro antibacterial activities and the highest toxicity to chicken macrophage cells (HD11) at concentrations of 7.5 to 30 μg/mL (Nguyen et al., 2021). In the current study, AMPs showed lower

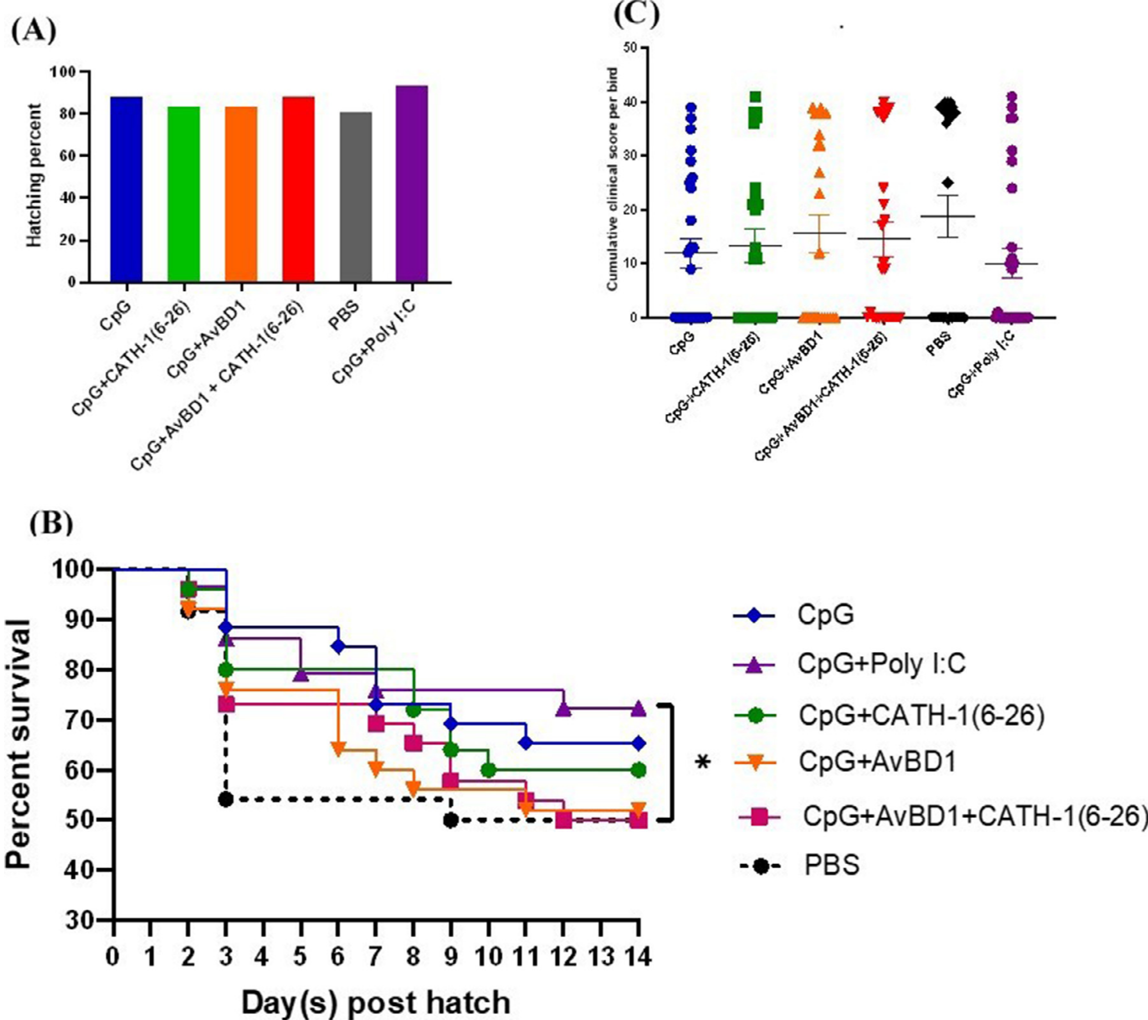

Figure 3. Hatching, cumulative clinical scores and survival rates of the experiment 2. (A) Hatching rates of birds after receiving in ovo treatments of CpG and its combinations. Hatching percent was identified by division of number of healthy hatched birds to total embryonated eggs in each group. (B) Survival proportions following the challenge with 35 CFU *E. coli*/bird via the intra-navel route at one-day post hatch (n = 30). Survival rates were compared at all time-points between treated and control groups using the Gehan-Breslow-Wilcoxon test. (*) means a significant difference with $P < 0.05$. (C) Cumulative clinical scores during 14-d post *E. coli* challenge. Each bird was assigned a daily clinical score as follows: 0 = normal; 0.5 = slow to move; 1 = ruffled feathers, sitting, reluctant to stand, and mouth breathing; 2 = unable to stand or walk, unable to reach feed or water, wings extended, and difficult breathing; and 3 = found dead. Birds with clinical score of 2 were euthanized for bacterial isolation and recorded lesion score. Clinical scoring was performed four times a day for the first 6 d postchallenge, and then twice a day until the experiment termination.

toxic effects to HD11 cells compared to CpG and its combinations. HD11 cells treated with AvBD1, CATH-1(6-26), or AvBD1 + CATH-1(6-26) showed up to 36% cell mortality, however, cells receiving CpG and CpG combined with Poly I:C, AvBD1, CATH-1(6-26), or AvBD1 + CATH-1(6-26) had 32% to 81% cell death at 1.56 to 30 μg/mL. In contrast, in ovo administration of these immune stimulants indicated no or very low toxic effects to the chicken embryos as indicated by the improved hatching rates. For instance, the lowest hatching rate (82.05%) was found in the control chicks receiving PBS, whereas these rates were higher in the treated groups (84.62−94.87%). These results are in agreement with our previous report which showed non-significant differences in weights and hatchability of chicks treated with AMPs at a dose of 30 μg/embryo (Nguyen et al., 2021). Therefore, despite in vitro toxicity shown in an HD11 cell line, CpG, Poly I:C, AMPs, and their combinations were shown to be safe for in ovo administration in chicken embryos within the tested dose ranges in the current study.

Previous reports suggest that CpG could promote strong immune responses against pathogens due to the enhanced expression of cytokines and chemokines. CpG administrated via the in ovo route promoted inflammatory responses through enhanced expressions of pro-inflammatory cytokines such as *IL-1β, IL-6, IL-18*, and lipopolysaccharide induced tumor necrosis factor (***LITAF***) (mainly produced by macrophages when stimulated by bacterial products) in lungs and spleen and it was suggested that it could stimulate both Th1 and Th2 types of cytokines (Gunawardana et al., 2019). In addition, the induction of Th1 type responses following intramuscular administration of CpG ODNs in chicken was also reported (Patel et al., 2008). In our study, we evaluated the effects of in ovo administration

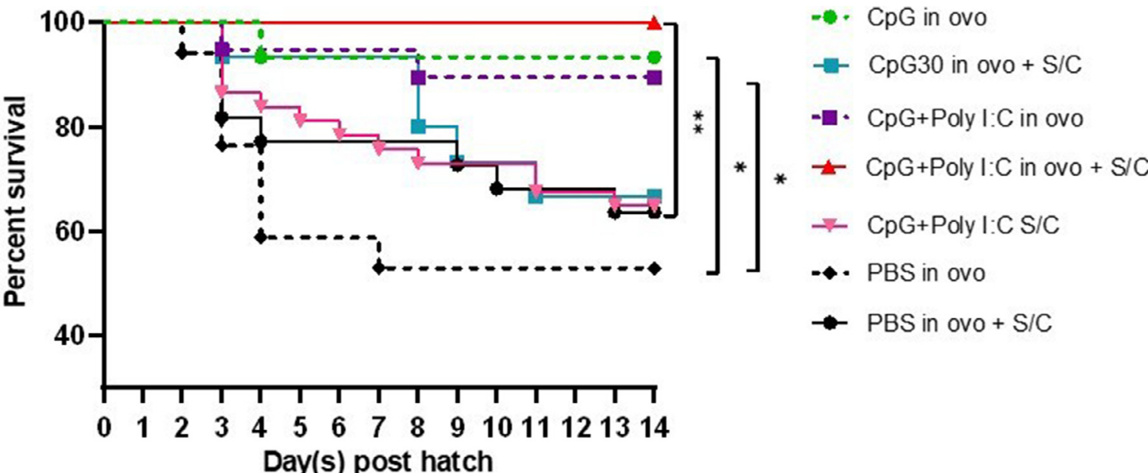

Figure 4. Survival proportions of birds receiving CpG or CpG + Poly I:C via different routes. Survival proportions between treated and control groups were compared at all time-points using the Gehan-Breslow-Wilcoxon test in the Prism 8 sofware (GraphPad Sofware Inc., San Diego, CA). (*), (**) mean differences with $P < 0.05$ and $P < 0.01$, respectively. CpG vs. PBS in ovo ($P = 0.0115$); CpG + Poly I:C vs. PBS in ovo ($P = 0.0126$); CpG + Poly I:C vs. PBS in ovo + S/C ($P = 0.0041$).

of CpG and its combinations on the expression of three important cytokines (*IL-1β, IL-8, IFN-γ*) in spleen cells. In agreement with previous reports, our data showed that CpG alone could stimulate higher expression levels of all the examined genes than in combination with AMPs and Poly I:C. In addition, we found that the expression levels of these cytokines in the CpG group decreased from 6 h to 96 h postadministration (PA). Due to the variations in expression among the individual embryos, CpG injected birds showed only significant differences in expression of *IL-1β* at 72 h PA and *IFN-γ* at 48 h PA. Co-administrations of CpG with other immune stimulants induced upregulation of these cytokines in a different manner. For instance, CpG + AvBD1 caused an increase in *IL-1β* gene expression at 6 h PA, however, a statistically significant difference was only noticed at 96 h PA. Two combinations including CpG + CATH-1 (6-26) and CpG + AvBD1 + CATH-1(6-26) showed increasing expressions of *IL-1β* and *IFN-γ*, although no statistical difference was observed. The CpG + Poly I:C combination also elicited increased expression of these genes; however, relatively lower expression levels were observed compared to embryos that received CpG alone. This difference in gene expression might be a result of the distinct amounts of each delivered TLR agonists or the interaction between them. During a bacterial infection, multiple nonspecific immune responses are involved such as the recruitment of neutrophils, dendritic cells, and macrophages and the activation of complement and cytokine production to eliminate pathogens (Tosi, 2005). Pro-inflammatory cytokines and chemokines such as *IL-1β* and *IL-8* rapidly induce pro-inflammatory mediators that play pivotal roles in the first line of host defenses (Tosi, 2005). *IFN-γ*, a type II interferon, has both antiviral activity and immunoregulatory functions (Lee and Ashkar, 2018). Therefore, we assume that induction of these cytokines and chemokines following in ovo injection might have led to reduced *E. coli* infection in the experimentally challenged birds.

It is of note that the differences in administration routes and ages of animals may contribute to differences in antimicrobial responses of toll-like receptor ligands (**TLR-Ls**). In a previous study, antiviral effects of Poly I:C against avian influenza virus H4N6 LPAIV strain was observed following in ovo and in vitro administration, whereas this antiviral effect was absent when Poly I:C was administered in post-hatched chicks (Ahmed-Hassan et al., 2018). Administration of CpG in chickens via a single route such as in ovo, S/C, intramuscular, or intrapulmonary was previously reported (Gomis et al., 2003;Goonewardene et al., 2017; Allan et al., 2018; Nguyen et al., 2021). However, the combination of these administration methods has not yet been studied. In the present study, we investigated the protective efficacies of these immune stimulants and their combinations administered in ovo and in ovo followed by a S/C injection at hatch. Our results from the second in ovo treatments demonstrated that administration of CpG + Poly I:C (15 μg each/embryo) provided the highest protective efficacy among various combinations of CpG with AMPs and Poly I:C. The CpG + Poly I:C treatment resulted in 72.41% survival rate compared to 50% survival rate in the control group (PBS) at 14 dpc with *E. coli* via the intra-navel route. In addition, the third experiment showed that CpG + Poly I:C injected in ovo and then again S/C at hatch induced a significantly higher protection (100% survival rate) than when given in ovo alone (89.47% survival proportion). Interestingly, we also found that CpG in ovo + S/C and CpG + Poly I:C only via the S/C route proved to be poorly protective in the treated chicks (67% and 65% survival rates, respectively). Thus, CpG treatment was more effective via in ovo injection than in ovo + S/C, whereas the combination of CpG + Poly I:C resulted in the highest protection when given both in ovo + S/C, and was only moderate efficacy when delivered by in ovo only.

Poly I:C has been well characterized as an antiviral agent by activation of both innate and adaptive immune

responses in animals. However, Poly I:C has also shown protection against multiple bacterial infections. For instance, Ribes et al., 2020 demonstrated that administration of Poly I:C induced protection of immunocompromised mice against *E. coli* meningitis (Ribes et al., 2020). Poly I:C increased expression of RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted, also known as CCL5), IFN-γ, recruitment of natural killer (NK) cells, and also increased microglial numbers, resulting in a more effective clearance of the pathogen (Ribes et al., 2020). Similarly, feeding turbot (*Scophthalmus maximus*) a low-dose of 0.00125% Poly I:C for 4 wk induced a stronger inflammatory response, long-term protection against *Edwardsiella piscicida*, and alleviated white feces syndrome after 3 to 7 wk resting period (He et al., 2021). In chickens, recent reports showed that chicks receiving Poly I:C via in ovo injection had higher survival rates than those in the control group after an *E. coli* infection (Allan et al., 2018; Sarfraz et al., 2022). A previous study in chickens showed that after Poly I:C treatment, *AvBD2, 11, 12, 13, CATH2, CATHB1,* and *LEAP2* (liver-expressed antimicrobial peptide 2) significantly increased in bone marrow-derived cells (**BMCs**), and *AvBD2, 3, 6, 9, 11, 12, 13, 14, CATH1, CATH3, CATHB1, GNLY* (chicken NK-lysin), and *LEAP2* increased in chicken embryonic fibroblasts (**CEFs**) (Jang et al., 2020). Moreover, Poly I:C stimulates the activity of various immune cells, including macrophages (Reimer et al., 2008), dendritic cells (**DCs**; Pulko et al., 2009), natural killer (**NK**) cells (Akazawa et al., 2007), and $\gamma\delta$T lymphocytes (Shojaei et al., 2009). Therefore, a combination of Poly I:C and CpG could enhance the protection against *E. coli* challenge in day-old chicks. Moreover, we believe this is the first report showing that repeated CpG + Poly I:C injection after in ovo administration was more effective than a single dose. These results will facilitate further studies to evaluate protective efficacy of the combination of CpG + Poly I:C for control of other bacterial and viral pathogens in the poultry industry. In addition, this combination may be useful as a vaccine adjuvant for chickens as well as other animals.

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DISCLOSURES

The authors declare no conflict of interest.

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