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# Antiviral potential and stability analysis of chicken interferon-α produced by Newcastle disease virus in chicken embryo fibroblast cells

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**Abstract:** Chicken interferon-α (chIFN-α) is an important antiviral cytokine and represents one of the first lines of the chicken's innate immune system. The current study is the first-ever report of chicken IFN (chIFN) production in Pakistan. In this study, we have used live and UV-irradiated Newcastle disease virus (NDV) to induce the expression of chIFN-α in chicken embryo fibroblast (CEF) cells. ChIFN-α was partially purified in a two-step protocol; ultracentrifugation followed by treatment with anti-chIFN-β antibodies. The purified chIFN-α was analysed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the *in vitro* antiviral potential of chIFN-α was determined against the H9N2 avian influenza virus (AIV) via a cytopathic inhibition assay. The relative mRNA level of the IFN-stimulated genes (ISGs) in the IFN-stimulated CEF cells was measured at various time intervals by a quantitative polymerase chain reaction (qPCR). The stability of natural chIFN-α to the temperature, pH, and ultraviolet (UV) light was also determined. The *in vivo* therapeutic potential of chIFN-α was determined in 7-day-old broiler chickens challenged with AIV. We found that a higher chIFN-α expression level was induced by the UV-irradiated NDV in the CEF cells as compared to the live NDV. The UV-irradiated NDV induced the maximum IFN production in the CEF cells at 24 h post-infection. Two bands of 21 kDa on SDS-PAGE confirmed the presence of the chIFN-α protein. The cytopathic inhibition assay indicated the strong antiviral activity of chIFN-α against AIV. Our results of the stability analysis showed that chIFN-α was stable at a wide range of temperatures and pH levels. However, a little exposure to UV-light resulted in a significant loss of antiviral activity. We also observed that the antiviral activity of chIFN-α is related to the expression levels of the antiviral ISGs. The results of the *in vivo* study showed that the chIFN-α therapy via the oral route resulted in a significant improvement in the tracheal pathology of chickens challenged with AIV. In conclusion, we suggest that chIFN-α could be an important therapeutic tool to control avian influenza infection in poultry.

**Keywords:** antiviral; chicken embryo fibroblast; chicken type I IFNs; cytopathic inhibition assay; IFN-stimulated genes; innate immunity; Newcastle disease virus

Type I interferon (IFN) was first discovered by Isaacs and Lindenmann during the cultivation of the avian influenza virus (AIV) in embryonated chicken eggs (Isaacs and Lindenmann 1957). Type I IFNs are an integral part of the chicken's innate immune mechanisms and provide the first line of innate immune protection against a variety of viruses (Anjum et al. 2020b). To date, three sub-

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types of type I IFNs have been discovered in chickens named as chicken IFN-α (chIFN-α), chicken IFN-β (chIFN-β) and chicken IFN-κ (chIFN-κ) (Santhakumar et al. 2017a; Anjum et al. 2020a). Chicken type I IFNs are primarily produced by fibroblast cells in response to viruses (Santhakumar et al. 2017b). In general, viral nucleic acids (RNA/DNA) are identified by the chicken pattern recognition receptors (i.e., chicken MDA5, chicken TLRs or chicken DNA sensors) and result in the downstream intracellular signalling and activation of transcription factors such as IFN regulatory factors 7 (IRF7), NF-κB, and AP-1. The activation of IRF7, NF-κB, and AP-1 results in their intranuclear localisation followed by the induction of type I IFNs from host cells (Anjum et al. 2020a). Despite the structural diversity, all three subtypes of chicken type I IFNs (chIFN-α, chIFN-β, and chIFN-κ) bind to the same complex of heterodimer receptors comprised of IFNAR1 and IFNAR2 chains (Santhakumar et al. 2019; Anjum et al. 2020a). Type I IFNs binding to their receptors activate the JAK-STAT pathway followed by the activation and intranuclear localisation of the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex.

This transcription factor on the binding to IFN-stimulated response elements (ISREs) induces the transcription of several IFN-stimulated genes (ISGs) such as *PKR, 2'-5' OAS, STAT1,* and *Mx1* (Santhakumar et al. 2017b). These ISGs encode proteins that inhibit the virus replication in several ways, i.e., blocking the transcription and translation of viral proteins and the degradation of viral nucleic acids (Goossens et al. 2013; Schneider et al. 2014; Santhakumar et al. 2017b).

Among the type I IFNs, chIFN-α is a more potent antiviral agent and has a high *Mx* promoter-inducing activity as compared to chIFN-β (Mo et al. 2001). Many RNA viruses have been reported as potent inducers of type I IFNs. Ellis and his colleagues reported on the ability of avian reoviruses for the induction of chIFN in chicken embryo fibroblast (CEF) cells (Ellis et al. 1983). Fournier et al. (2012) reported the Newcastle disease virus (NDV) as an efficient inducer of chicken IFNs. Previously, some studies have reported the use of NDV for the induction of chicken type I IFNs in several cell culture systems including CEF cells (Ho and Breinig 1965; Youngner et al. 1966; Lomniczi 1973; Heller et al. 1997). The differential IFN inducing ability of various NDV strains (lentogenic, mesogenic, and velogenic) has also been reported in some earlier reports (Youngner et al. 1966; Lomniczi 1973; Heller et al. 1997). In the present study, we have produced natural chIFN-α (nchIFN-α) by the LaSota strain of NDV and then the ability of nchIFN-α to induce the transcription of four ISGs, i.e., *myxovirus* (*Mx*), *protein kinase R* (*PKR*), *2'-5' oligoadenylate synthetase* (*2'-5' OAS*), and *signal transducer and activator of transcription 1* (*STAT1*), was assessed in a time-dependent manner. Furthermore, a stability analysis and the *in vitro* and *in vivo* antiviral potential of nchIFN-α against AIV was also assessed.

## MATERIAL AND METHODS

### Chickens

Sixty (7-day-old) broiler chicks were procured from a local hatchery in Faisalabad, Pakistan and housed under standard management conditions as described by the Institutional Bioethics Committee (IBC), University of Agriculture, Faisalabad, Pakistan. The birds were provided with feed and water *ad libitum* with a standard broiler starter and grower diet plan throughout the entire experiment.

### Cell culture

Chicken embryo fibroblasts (CEF) cells were derived from specific pathogen-free (SPF) chicken embryos (9-day-old) and cultured in a complete Eagle's minimal essential medium (MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS), 1X gentamicin sulfate, tryptose phosphate broth (5%), and L-glutamine (0.02%) according to the standard procedure as described by Hernandez and Brown (2010).

### Viruses

The NDV (LaSota strain) and low pathogenic AIV (H9N2) were propagated in the CEF cells. Briefly, CEF cells were grown in 25 $cm^2$ cell culture flasks. After the monolayer was formed, the CEF cells in each flask were infected with 1 ml of NDV and AIV individually followed by uniform spreading. The flasks were incubated for 1 h at 37 °C in a humidified $CO_2$ incubator to allow the adsorption

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of the viruses with irregular shaking after every 15 minutes. A maintenance media (4 ml per 25 $cm^2$ flask) was added in flasks and, again, incubation was provided for 5 days (3 days in the case of AIV) at 37 °C in a humidified $CO_2$ incubator until complete cytopathic effects (CPEs) appeared in the virus-infected flasks (Kang et al. 2016). When the full CPE appeared, the virus-infected flasks were subjected to three cycles of freezing and thawing for the virus harvesting. The virus inoculum from the flasks was transferred into falcon tubes and centrifuged at 3 000 × *g* for 15 min at 4 °C. This supernatant containing the virus inoculum was supplemented with gentamicin (30 µg/ml) and stored at –80 °C until further use. The $TCID_{50}$ value of both viruses (NDV and AIV) was calculated using Karber's method (Karber 1931).

## UV-irradiation of NDV

For the UV-irradiation of NDV, 0.2 ml of the inoculum containing NDV was taken in a Petri dish (60 mm) placed on a horizontal shaker and exposed to an electric UV lamp (254 nm) at 50 cm for 30 minutes. After every 3 min, the inoculum was transferred to another petri dish to ensure a uniform exposure of UV light (Pohuang et al. 2011).

## ChIFN-α production, purification and quantification

CEF cells were seeded in the cell culture flasks and incubated at 38 °C in a humidified (80%) incubator under 5% $CO_2$ for 48 hours. After the confluent monolayer was formed, the cell culture flasks were inoculated with live and UV-irradiated NDV, individually. The inoculation dose of NDV used was 200 µl ($10^{5.5}$ $TCID_{50}$/100 µl) per flask.

After adsorption of one hour at 37 °C, the virus inoculum was removed, and a fresh media was added after washing the cell sheet. At various time intervals (6, 12, 24, 36 and 48 h) post-stimulation by NDV, the cell culture supernatant was harvested and centrifuged at 2 000 × *g* for 30 min at 4 °C to remove the cellular debris. The supernatant was acidified (pH: 2) and, again, centrifuged at 30 000 × *g* for 1 h at 4 °C and the pellets containing the residual virus and the precipitated proteins were discarded (Lai and Joklik 1973). A haemagglutination test was performed to determine the presence of any residual NDV in the cell culture supernatant containing the IFN. To remove any chIFN-β fractions from the cell culture supernatant, fractions containing the concentrated IFN protein were treated with anti-chIFN-β antibodies (Biorad, Hercules, CA, USA) followed by centrifugation and an analysis of the supernatant on SDS-PAGE. The partially purified chIFN-α obtained from the above steps was assayed for titration through a cytopathic inhibition assay as described by Ramakrishnan (2016).

## *In vitro* antiviral activity of chIFN-α against AIV

The partially purified chIFN-α was tested against H9N2 AIV through a cytopathic inhibition assay (Jiang et al. 2011). Briefly, CEF cells ($2 \times 10^4$ per flask) were grown in cell culture plates. After the formation of a confluent monolayer, the cells were washed with Hank's solution followed by pre-treatment with chIFN-α (100 IU/0.1 ml) for 15 h before infection. The positive control was left untreated (no IFN-pre-treatment). After 15 h, the cell culture plates were again washed with Hank's solution and the cells in the IFN-treated and positive control were infected with AIV (100 $TCID_{50}$/100 µl). The non-adsorbed virus was removed after one hour of incubation (37 °C and 5% $CO_2$). The CEF cells were washed and supplemented with a fresh MEM media and, again, incubated at 37 °C in a humidified $CO_2$ (5%) incubator till the appearance of the CPEs. The reduction in the cytopathic effects was observed under an inverted microscope (Nikon, Tokyo, Japan).

## Expression analysis of ISGs in response to the chIFN-α treatment

The ability of chIFN-α to stimulate the ISGs was investigated by treating the CEF cells with chIFN-α. After various time intervals (0, 6, and 12 h) post-stimulation by chIFN-α, the total RNA was harvested using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) and processed for a quantitative real-time PCR analysis of the selected ISGs (*PKR*, *2'-5' OAS*, *Mx1*, *STAT1*) previously demonstrated to be stimulated by chIFN-α. To measure

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Table 1. List of primers used for the amplification of the *2′-5′ OAS*, *Mx1*, *STAT1*, and *PKR* genes

| Genes | Primer sequences (5' to 3') | Size of amplicon | References |
|---|---|---|---|
| *OAS* | F: CAC GGC CTC T TC TA C GAC A<br>R: T GG GCC ATA CGG TG T AGA CT | 103 | Li et al. (2007) |
| *Mx1* | F: AGACCTTGCTTTTGGATGTGCT<br>R: TTGCTCAGGCGTTTATTTGCT | 105 | Meng et al. (2011) |
| *STAT1* | F: TCAGGCAGTACTTGGCACAGTGG<br>R: GATCATCAAGCTGTGACAGCAGG | 111 | Thanthrige-Don et al. (2010) |
| *PKR* | F: CCT CTG CTG GCC TTA CTG TCA<br>R: AAG AGA GGC AGA AGG AAT AAT TTG CC | 151 | Daviet et al. (2009) |
| β-actin | F: GAGAAATTGTGCGTGACATCA<br>R: CCTGAACCTCTCATTGCCA | 152 | Meng et al. (2011) |

the relative expression of the selected ISGs, the untreated CEF cells harvested at 0 h were used as a calibrator. For the internal control, β-actin was used. The specific sets of primers used for all the genes, i.e., *PKR*, *2'-5' OAS*, *Mx1*, *STAT1*, and β-actin are described in Table 1.

The reaction was carried out using the following conditions; 50 °C for 5 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 seconds. The melting curve was determined at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 seconds. The Ct values of the mRNA were determined and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

## Stability analysis of chIFN-α produced from CEF cells

To determine the stability analysis, the partially purified chIFN-α with a known biological activity was exposed to various conditions, i.e., temperature, pH, and UV-light. To determine the effect of the temperature, chIFN-α was incubated for 1 h at a wide range of temperatures, i.e., –20, 4, 25, 37, 65, and 75 °C.

The stability of chIFN-α was also assessed by incubating chIFN-α under various pH conditions (2, 5, 7 and 10) at room temperature for 1 hour. Similarly, the effect of UV-light on the activity of chIFN-α was tested by exposing chIFN-α to UV-light (254 nm) for 1, 2, and 5 minutes.

The reduction in the antiviral potential of chIFN-α was measured in terms of the percentage loss in the IFN activity via the IFN bioassay as described previously by Ramakrishnan (2016).

## Determination of therapeutic potential of chicken IFN-α against low pathogenic AIV

A total of 60 (7-day-old) broiler chicks were divided into four groups as A, B, C and D with each group comprised of 15 birds. The birds in group A, B, and C were challenged with the H9N2 AIV ($1 \times 10^6$ $EID_{50}$/bird) via an intranasal route. No infection was given to the group D (negative control) birds. Eight hours post virus challenge, the birds in groups A and B were orally fed with 500 IU and 1 000 IU of chIFN-α, respectively, once a day for three consecutive days. To determine the protective effect of chIFN-α, a histopathological examination of the trachea was performed and the results were compared with the day-matched positive control (challenged with AIV only) and the negative control (without any treatment). For this purpose, the tracheal tissues were collected from the birds of the control groups (C and D) and the experimental groups (A and B). All the tracheal tissues were first fixed in a neutral buffer formalin (10%) followed by processing via a paraffin embedding technique as described by Suvarna et al. (2018). Tissue blocks were cut into 5 µm thickness and mounted on frosted glass slides followed by staining of tissue sections with haematoxylin and eosin (H&E) stains. To determine any cellular degenerative changes in all the IFN-treated and untreated groups, the stained tissue sections were examined under the microscope at × 100 magnification.

The assessment of the histopathological lesions in response to the AIV infection was performed descriptively and the severity level (mild, moderate or severe) of the degenerative changes was based on the presence of degeneration and haemorrhages.

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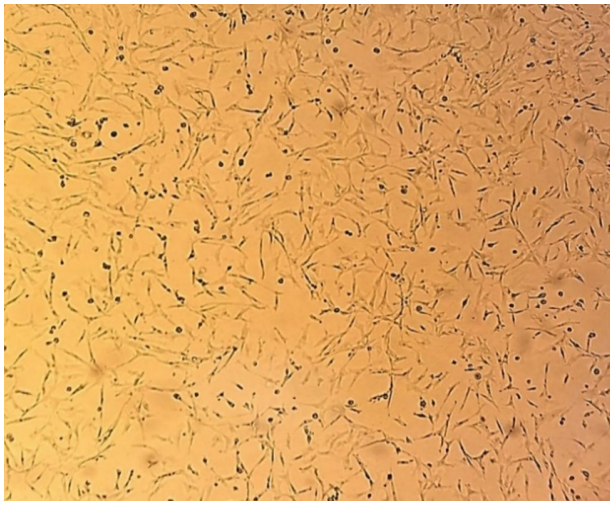
## RESULTS

### ChIFN-α induction by live and UV-irradiated NDV in CEF cells

In the current study, we have determined the IFN-inducing ability of the live and UV-irradiated NDV (LaSota strain) in the CEF cells. Figure 1A highlights the normal morphology of the CEF cells derived from the 9-day-old SPF embryonated chicken eggs. After 24 h of incubation, the CEF cells were examined under an inverted microscope for their morphology and appeared spindle-shaped with long fibres extending out from the ends. Figure 1B describes the concentration of chIFN-α (IU/100 µl) in the cell culture supernatant determined via the IFN bioassay at 6, 12, 24, 36, and 48 h post-stimulation of the CEF cells with the live and UV-irradiated NDV. Although both the live and UV-irradiated NDV stimulated the chIFN-α production in the CEF cells, the UV-irradiated NDV induced a significantly higher chIFN-α expression as compared to the live NDV at 6, 12, 24, 36 and 48 h post-NDV infection. Both viruses (live and irradiated NDV) yielded the highest chIFN titre from the CEF cells at 24 h post-NDV inoculation. After that, the titre began to decrease with the lowest titre at 48 h post-infection (Figure 1B).

(A)



(B)

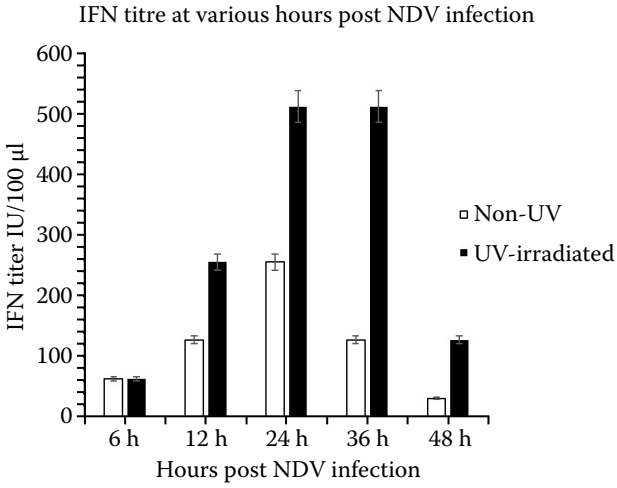



Figure 1. (A) Normal cell morphology of the chicken embryo fibroblast (CEF) cells at 24 h observed under an inverted microscope (× 100). (B) Crude chIFN-α yield in the cell culture supernatant harvested at 6, 12, 24, 36, and 48 h post-stimulation of the CEF cells by the live and UV-irradiated NDV

After partial purification through ultracentrifugation, the protein fractions containing the IFN were treated with anti-chIFN-β antibodies to remove the chIFN-β followed by analysis on SDS-PAGE. Figure 2 highlights the two protein bands of approximately 21 kDa, indicating the presence of chIFN-α. These protein bands were eluted and further determined for their antiviral activ-

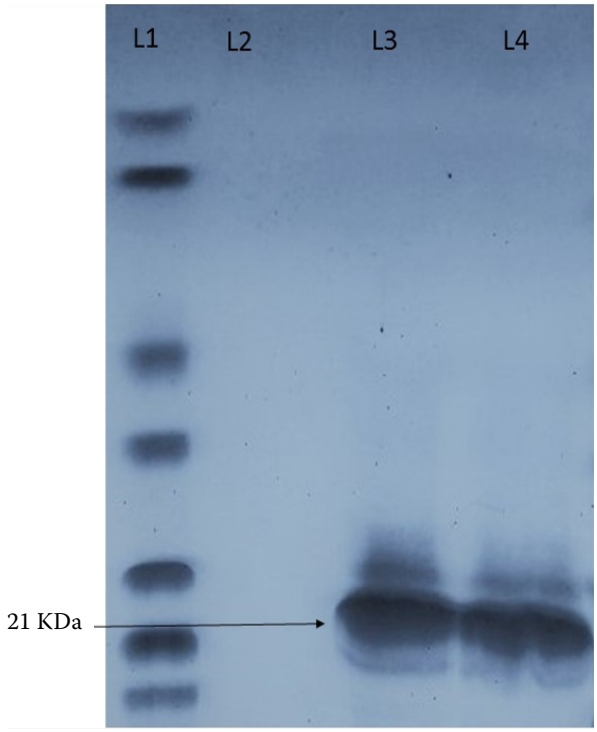



Figure 2. SDS-PAGE analysis of the protein fractions containing IFN

L1 is the protein ladder, while L3 and L4 represent the 21 kDa protein bands of chIFN-α obtained after the purification of the CEF supernatant while L2 indicates the blank control

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ity via a cytopathic inhibition assay. It is visible in Figure 3 that no CPEs were observed in the CEFs pre-treated with chIFN-α. However, H9N2 AIV produced strong CPEs (i.e., the fusion of cells resulting in large aggregates of cells) in the CEF cells that were left untreated (positive control). These results demonstrate that the chIFN-α pre-treatment of CEF cells protected the cells against virus-induced CPEs.

## ChIFN-induced ISGs expression in CEF cells

The relative expression of various ISGs in the IFN-treated and untreated cells was quantified by qPCR. Our results showed that the chIFN-α treatment induced the expression of the *PKR*, *2'-5' OAS*, *STAT1*, and *Mx1* genes in a time-dependent fashion (Figure 4). After treatment of the CEF cells with chIFN-α, the mRNA levels of all four selected genes gradually increased reaching a peak at 12 h post-treatment. The relative mRNA level of *2'-5' OAS* at 6 and 12 h post-IFN-treatment was 9.33-fold and 14.58-fold higher as compared to the calibrator (non-treated CEF cells) (Figure 4D). In response to the chIFN-α treatment, the mRNA level of *PKR* was slightly upregulated (2-fold) at 6 h post-treatment, while a modest (~3.52-fold) increase in the expression was observed from 6 h to 12 h post-treatment (Figure 4B). A higher and efficient *Mx1* expression of 45.67-fold and 89.10-fold was induced by the chIFN-α treatment at 6 and 12 h, respectively (Figure 4C). The relative mRNA of *STAT1* increased from 3.52-fold to 7.92-fold from 6 h to 12 h post-IFN-treatment, respectively (Figure 4E). Among all the four genes, the expression of *Mx1* was more prominent with *PKR* showing the weakest induction followed by the chIFN-stimulation of the CEF cells.

## Effect of temperature, pH, and UV-light on the stability of chIFN-α

Figure 5 highlights the percentage loss in the IFN-activity after exposure to various conditions of temperature, pH, and UV-light. No loss in the IFN-activity was observed at –20 °C and 4 °C while only a slight (30%) loss in the IFN-activity was observed at 25 °C. The IFN activity was found stable from 25 °C to 65 °C. A significant reduction in the IFN-activity was witnessed at 75 °C (Figure 5A). When exposed to extreme acidic (pH 2) and basic (pH 10) environments, a slight decrease in the IFN-activity was measured. While the IFN activity was

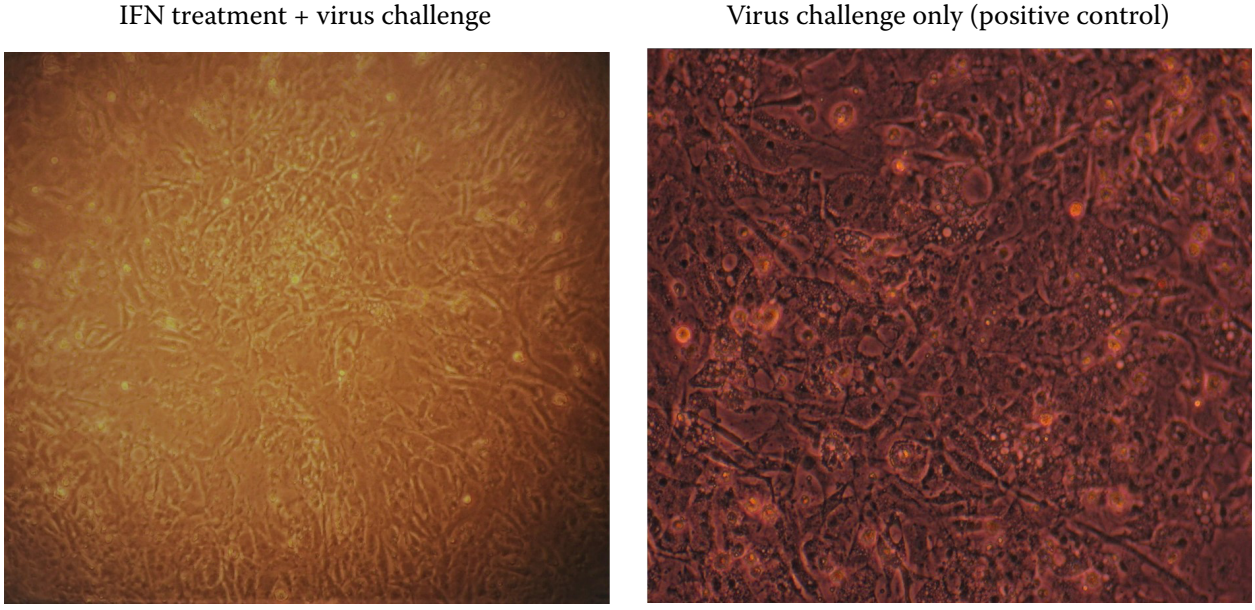


Figure 3. No cytopathic effects (CPEs) were observed in the CEF cells pre-treated with chIFN-α in comparison to the untreated CEF, i.e., positive control

Protection from CPEs was observed in the cells pre-treated with chIFN-α compared with the non-treated cells that were infected with AIV only

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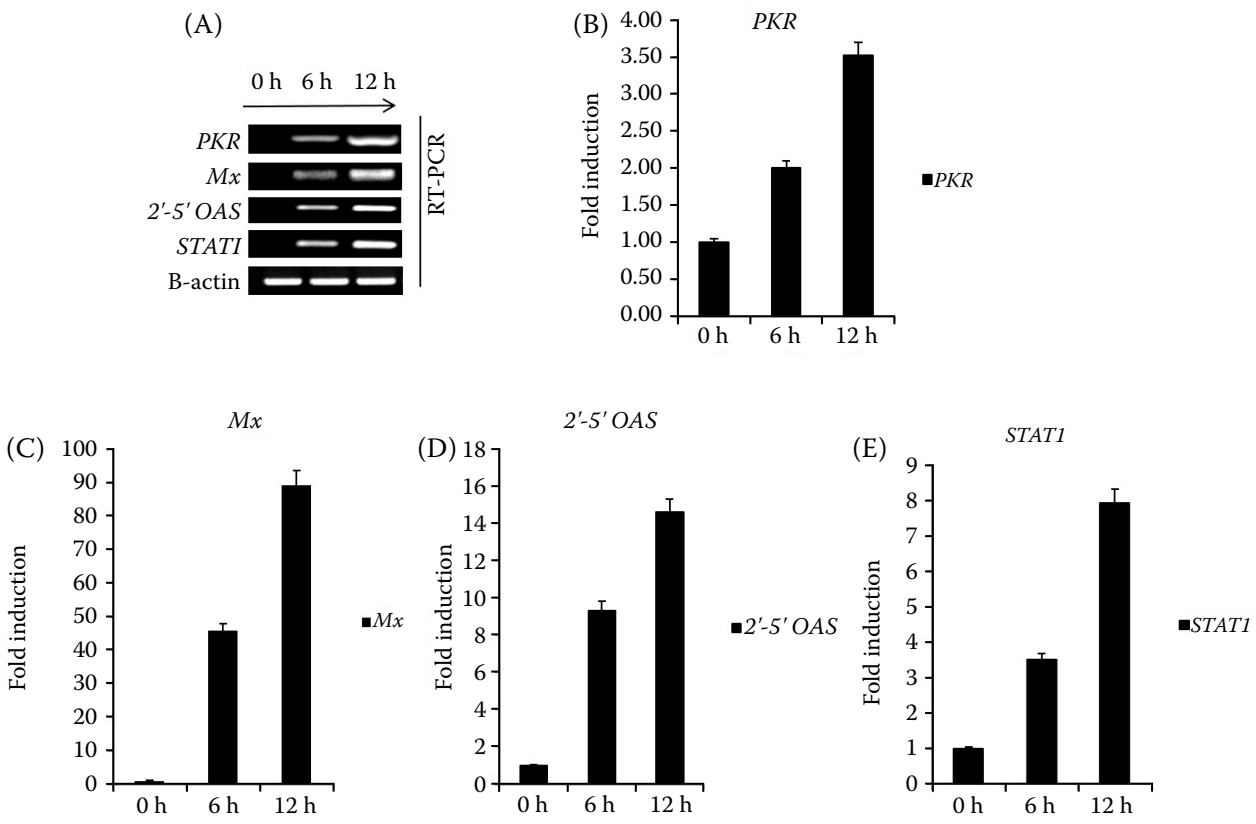


Figure 4. The relative mRNA levels of the IFN-stimulated genes assayed by qPCR at various time intervals post-stimulation by chIFN-α

After the CEF cells were incubated with chIFN-α, the CEF cells were harvested at the indicated time intervals for the RNA extraction and cDNA preparation. The transcriptional levels of *PKR* (B), *Mx* (C), 2'-5' *OAS* (D), and *STAT1* (E) were assayed by qPCR

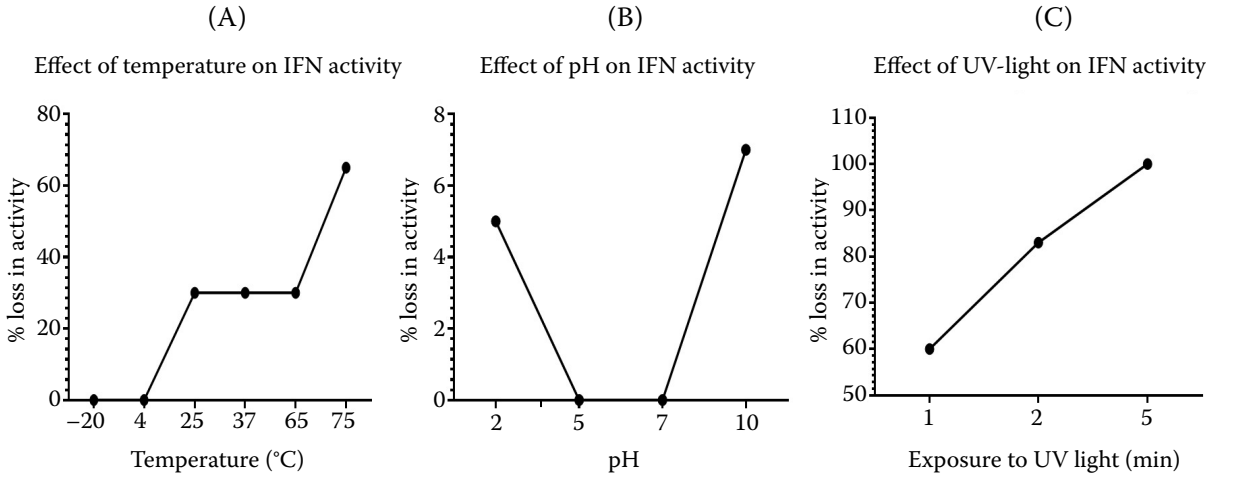


Figure 5. Effect of the temperature, pH and UV-light on the biological activity of chIFN-α produced by NDV in the CEF cells

observed to be stable from pH 3 to 9 (Figure 5B). A rapid loss in the biological activity of chIFN-α was observed with an increase in the exposure time to UV-light. Exposure of chIFN-α to UV-light for 5 min resulted in the complete (100%) destruction of the IFN activity (Figure 5C).

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## Results of histopathological examination

The histopathological examination of the tracheal specimens collected from all four groups (A, B, C and D) of the *in vivo* experiment is described in Figure 6. The chIFN-α ameliorated tracheal histopathology in the chickens challenged with AIV. Both the group A and group B birds administered with 500 IU and 1 000 IU of chIFN-α, respectively, showed a marked amelioration in the tracheal histology as compared to the positive control birds (group C). Histopathologically, the group B birds showed no lesions in the trachea. Only mild histopathological changes were observed in the group A chickens treated with 500 IU of chIFN. Severe degenerative changes, i.e., sloughing of the tracheal epithelium, haemorrhages, oedema, and a lymphocyte infiltration were observed in the tissue sections from the group C birds. A normal histological structure of the trachea was observed in the negative control (group D).

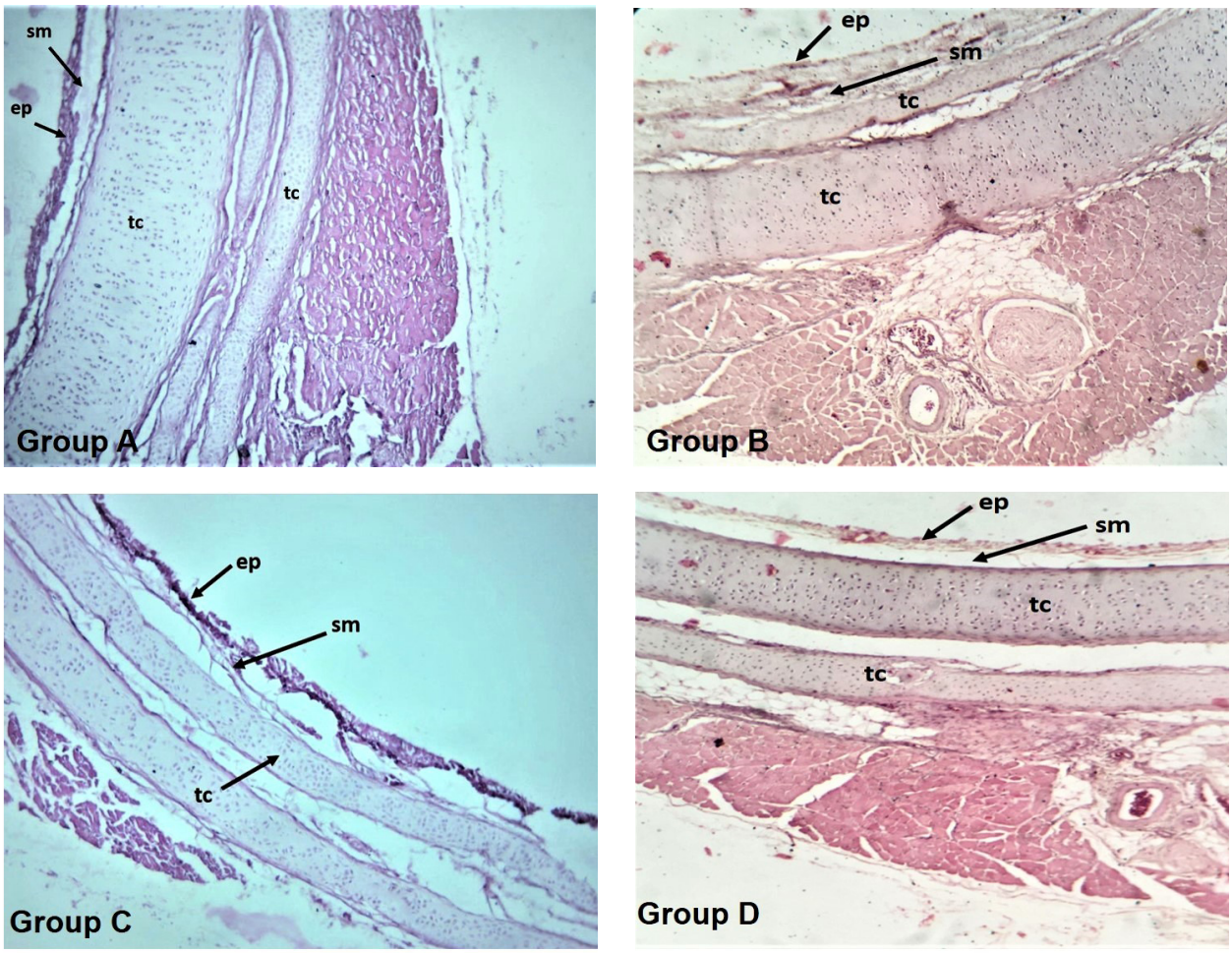


Figure 6. H&E stained histological micrograph (× 100) of the trachea collected from various chicken groups of the *in vivo* study

Ep = epithelium; sm = submucosa; tc = tracheal cartilage

Group A = AIV challenged + chIFN-α therapy (500 IU for 3 consecutive days); Group B = AIV challenged + chIFN-α (1 000 IU for 3 consecutive days); Group C = AIV challenged only (positive control); Group D = PBS treatment only (negative control)

A mild disruption of the epithelium and submucosa was seen in the trachea of the group A birds while the trachea of the group B birds exhibited a normal tracheal histology. Severe degenerative changes, i.e., mucosal sloughing of the epithelium and submucosal oedema along with the infiltration of inflammatory cells were observed in the virus challenged group C birds. A normal trachea with mucosal epithelium, cilia and plates of cartilage was observed in the group D birds. The submucosa contains normal glands and connective tissue

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## DISCUSSION

In the current study, we have utilised the LaSota strain of NDV to produce chIFN-α and determined its antiviral potential against AIV. ChIFN-α was produced by the stimulation of the CEF cells with two different stimuli, i.e., a live and UV-irradiated NDV. Both the live and UV-irradiated NDV stimulated the chIFN-α production in the CEF cells. The maximum chIFN-α was induced by the UV-irradiated NDV as compared to the live NDV. Previous studies have also reported that UV-irradiated NDV induced a higher IFN titre in CEF cells (Ho and Breinig 1965). Studies on the IFN production and IFN sensitivity of various NDV strains showed that UV-irradiation, for a short period, enhanced the IFN-inducing ability of the lentogenic NDV strains in CEF cells (Lomniczi 1973). Previously, a comparison of UV-light and heat inactivation on the IFN inducing ability of NDV demonstrated that the UV-irradiated NDV was a good inducer of chIFN as compared to the heat-inactivated NDV which completely failed to induce the IFN in the CEF and mouse L cells. Interestingly, the heat-inactivated NDV induced a significant IFN production in the mouse model (Youngner et al. 1966). This difference in the IFN inducing the ability of live, UV-irradiated and heat-inactivated NDV could be attributed to either the virulence of the virus or the host cells that are stimulated.

A two-step protocol was employed to purify chIFN-α from the cell culture supernatant. First, the cell culture supernatant was partially purified through ultracentrifugation. In the second step of purification, the eluent was treated with anti-chIFN-β antibodies to remove the chIFN-β in the final fraction. The appearance of active protein bands (21 kDa) on SDS-PAGE confirmed the presence of chIFN-α. The cytopathic inhibition assay indicated the strong antiviral activity of chIFN-α in the CEF cells infected with AIV. A previous study by Jiang et al. (2011) also reported that the pre-treatment of CEF cells with recombinant chIFN-α abrogated the CPEs in comparison to virus-infected cell cultures (non-IFN treated).

In the current study, the expression of various ISGs was also quantified via qPCR. Type I IFNs mediate antiviral activities through the upregulation of several hundred ISGs in the host cells (Santhakumar et al. 2017b). The upregulated level of ISGs acts as a molecular marker and highlights the antiviral state of the cells. In our study, a significant upregulation of the mRNA levels of *2'-5' OAS*, *Mx1*, *PKR*, and *STAT1* showed the antiviral status produced by chIFN-α in the CEF cells. A previous study also demonstrated an increase in the mRNA level of *2'-5' OAS*, *PKR*, *Mx1*, and *STAT1* followed by the stimulation of DF-1 cells with chicken type I IFNs (Qu et al. 2013). The selected ISGs in this study encode important proteins that participate in the host cell defence against viruses. A previous report by Reemers et al. (2009) also suggests that both *PKR* and *2'-5' OAS* are upregulated and participate in the host defence against H9N2 infection in chickens. The transcription of *PKR* in response to the H5N1 infection has also been reported by Daviet et al. (2009). In another study, PKR resulted in the inhibition of HBV (Hepatitis B virus) replication in a human hepatoma culture (Han et al. 2011).

All these studies reflect the important role of *2'-5' OAS* and *PKR* in limiting the virus replication. Also, an increase in the *STAT1* transcription was observed in the current study. The upregulated level of *STAT1* was also reported by Qu et al. (2013) followed by the chIFN-α stimulation of DF-1 cells. STAT1 plays a central role in the type I IFN mediated signalling mechanism to establish an antiviral environment in the cell via the transduction of cytoplasmic signals to the nucleus (Schindler et al. 2007). The critical role of STAT1 in transducing the IFN responses was also demonstrated by Schindler and Darnell (1995), Stark et al. (1998). Mx is an IFN-inducible protein and a significantly higher expression of Mx was measured in the current experiment. However, previous studies have reported that chicken Mx does not inhibit virus replication (Benfield et al. 2008; Schusser et al. 2011). Previous reports regarding the transcriptional analysis of ISGs in CEFs demonstrate that chicken type I IFNs provoke antiviral actions via the regulation of at least MDA5, OAS, and Mx-mediated antiviral pathways (Santhakumar et al. 2017b). Our results of the chIFN-α stability, when exposed to various conditions were quite similar to those previously reported by Lampson et al. (1963) and Santhakumar et al. (2017b). The current study demonstrated that the decrease in the IFN activity was not gradual with an increasing temperature. ChIFN-α showed 100% activity when kept at low temperatures, i.e., –20 °C and 4 °C. Although the IFN activity was reduced by 30% at 25 °C, this activity was observed to be stable onwards till

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65 °C. After that, a sudden reduction in activity was observed with an increase in temperature.

Similar findings regarding the thermal stability of IFNs have also been reported by Lampson et al. (1963). Our results showed that at a highly acidic (pH 2) and basic pH (10), a slight loss in the chIFN-α activity was observed suggesting that chIFN-α is stable to a wide range of pH environments, which is in accordance with previous studies (Heller et al. 1997; Santhakumar et al. 2017b). ChIFN-α was found to be highly sensitive to UV-light as little exposure to UV-light resulted in a significant loss in the IFN activity. This rapid loss in IFN activity when exposed to UV-light was also reported by Lampson et al. (1963). The results of the histopathology showed that chIFN-α protected the chickens from the AIV infection *in vivo*.

In our study, the oral treatment of 500 IU of chIFN-α for three consecutive days significantly ameliorated the AIV-induced pathology in the trachea.

However, a complete restoration of the tracheal histology was observed in birds treated with 1 000 IU of chIFN-α for three consecutive days. Previous findings of Xia et al. (2004) also indicate protection from H9N2 AIV when 1 000 IU of recombinant chicken interferon-α (rChIFN-α) were administered to chickens. Marcus et al. (1999) also described an amelioration in the pathology of the trachea in response to a chIFN-α treatment via an oral route.

We conclude that the UV-irradiated NDV is a potent inducer of the heat and pH resistant chIFN-α in the CEF cells and that the chIFN-α substantially induced the expression of ISGs in the CEF cells. We propose that chIFN-α could be an effective option for controlling the avian influenza and may have clinical implications for treating other viral diseases in the poultry industry.

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## Conflict of interest

The authors declare no conflict of interest.

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