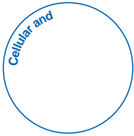

Canine Distemper Virus Causes Apoptosis in HEK-293 Cells by both Extrinsic and Intrinsic Pathways

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Abstract

Background: Apoptosis is a form of natural or stress induced cell death that plays a pivotal role in many cellular

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signs and symptoms such as fever, cough, coryza and conjunctivitis. It has been observed that CDV infection induces apoptosis of di erent

detailed study of majority of the apoptotic proteins need to be done. To our knowledge, this is the rst report of apoptosis by canine distemper virus in HEK-293 cell line. e aim of this study was to elucidate the

Results

Culture of HEK-293 cells and infection with CDV

HEK-293 cells available in the laboratory was grown in 25cc asks using DMEM media supplemented with 5% FBS. At 60-70% con uence, the cells were infected with 100ul of CDV-SH, observed for 4-5 days and were harvested at 7 days' post infection. Morphological changes in cells such as aggregation and degeneration could be observed without any characteristic CPE (Figure 1). Diagnostic RT-PCR could detect CDV growth in all passages which as observed by an amplicon size of 267 bp in agarose gel electrophoresis (Figure 2).

MTT Assay

MTT assay was carried out in HEK-293 cells at 6, 12, 24, 48 hrs post CDV-SH infection. MTT assay showed 100%, 84.78%, 79.21% & 76.95% of cell viability in HEK-293 cells at 6, 12, 24 and 48 hrs post CDV infection respectively (Figure 3). Zhau reported decline of ovarian cancer cells survival following infection with live-attenuated measles vaccine virus in both time and dose dependent manner. e Measles virus vaccine resulted in 2.7%, 4.3% and 17.6% cell death at 12, 24 and 48 hrs post infection respectively in SKOV-3 cells by MTT assay.

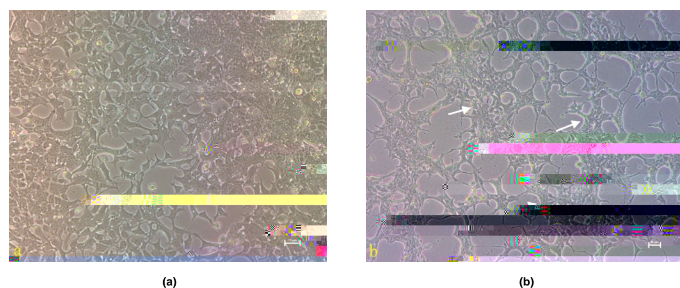
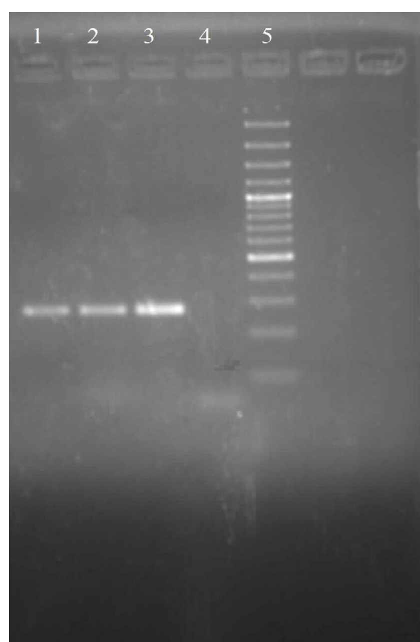


Figure 1:



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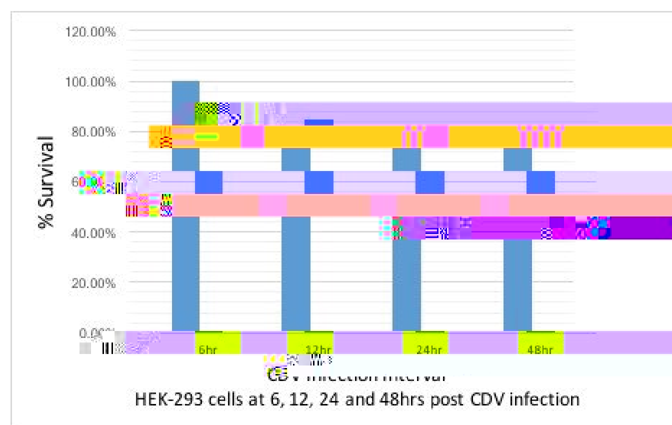

Figure 3:

DNA Laddering Assay

DNA isolated from CDV infected HEK-293 cells at 6, 12, 24 and 48 hrs post infection was electrophoresed in agarose gel at low voltage and a laddering pattern of DNA was observed. DNA laddering in HEK-293 cells showed a faint laddering pattern at 24 & 48 hrs post CDV infection indicated small amounts of DNA fragmentation [5].

Gene expression studies following CDV infection in HEK-293 cells using qPCR array

A panel of 48 genes associated with di erent apoptotic pathways listed in Table 1, were selected to study their expression pro le following CDV infection in HCT-15 cells. Brie y, we assayed the expression of genes from the extrinsic pathway (5), genes related to the intrinsic pathway (10), genes from the MAVS-MKK7-JNK2 pathway (4), genes from RIG-1/MAVS pathway (4), execution pathway genes (3), genes from ER stress induced apoptosis pathway genes (4) and genes from PTEN induced pathway (2). e list of selected genes was sent to Qiagen for custom designing of the qPCR arrays. For apoptotic gene expression studies, HEK- 293 cells infected with CDV-SH were harvested at di erent time intervals (6, 12, 24 & 48 hrs). RNA was extracted and cDNA was prepared. A diagnostic RT-PCR was rst carried out to detect CDV growth in the harvested samples which showed a 267 bp amplicon in agarose gel electrophoresis con rming the virus growth. en the cDNAs synthesized from di erent samples were used to perform the qPCR array to study the apoptotic gene expression pro le. e qPCR was performed in duplicates for each target gene and the mean C_t values obtained were recorded for analysis of the test samples (infection intervals) against the control sample (non-infected cells). e hourly infection intervals (6, 12, 24 & 48 hrs) were named into groups (group 1, 2, 3 & 4), respectively. e qPCR data was checked for quality control to check the PCR array reproducibility which shows that if the average PPC (PCR positive control) C_t is 20 ± 2 and no two arrays have average PPC Ct that are > 2 away from one another then the sample and group pass. Data normalization was done by manually selecting the housekeeping gene, and selecting the use of geometric or arithmetic mean by considering only genes with small changes in expression across di erent sample groups (di erences in CT values less than 1). Fold change was calculated for relative quanti cation by comparing non- infected controls with infected samples of di erent time intervals. Fold-change values greater than one indicates a positive or an up-regulation and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative

or down-regulation, and the fold- regulation is the negative inverse of the fold-change [6].

e p values were calculated based on a student's t-test of the replicate 2 ^ (-Delta CT) values for each gene in the control group and treatment groups. e fold change (cut o set to 2) was calculated for the upregulated and downregulated genes against the control (non-infected sample). e gene expression data was used to generate scatter plots of each group as compared to the control group (non-infected group). e scatter plot compares the normalized expression of every gene on the array between the two selected groups by plotting them against one another to quickly visualize large gene expression changes. e central line indicates unchanged gene expression. e dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper le and lower right sections meet the selected fold regulation threshold [7].

e expression data was used to generate a heat map with cluster analysis of CDV infected HEK-293 cells. e clustergram in shows the qPCR array gene expression pro le of CDV infected HEK-293 cells which shows group clustering analysis of di erentially expressed genes identi ed in control versus CDV infected HEK-293 cells at speci c time intervals (6, 12, 24 & 48 hrs). Table 2 shows the list of overexpressed genes in di erent groups and Table 3 shows the list of under expressed genes in the di erent groups.

Expression pro le

e expression analysis of CDV infected HEK-293 cells at 6, 12, 24 and 48 hr post infection as compared with the non-infected controls, showed di erent genes that were overexpressed or under-expressed. In group 1 (6 hr PI), 2 genes (TRAIL & TNF) were overexpressed and no genes were under-expressed. In group 2 (12 hr PI, 2 genes (TNF & TRAIL) were overexpressed and 3 genes (Calreticulin, Caspase-8 & Smac) were under-expressed. In group 3 (24 hr PI), 9 genes (IFN- , TRAIL, TNF , IRF-3, Foxo3a, Bak1, Puma, p53 & cytochrome C) were overexpressed and no genes were under-expressed. In group 4 (48 hr PI) vs. control group, 19 genes (TRAIL, CHOP, IFN- , RIG-1, TNF , Noxa, Caspase-9, Caspase-3, Puma, Fas, Calreticulin, PERK, IRE1, JNK1, IRF-3, IFN- , Calnexin, FasLG & Bad) were overexpressed and no genes were under-expressed [8, 9].

Analysis of CDV infected HEK-293 cells using IPA

To investigate the possible biological interactions of the di erently expressed genes upon CDV infection of HEK-293 cells, all the datasets as described in with expression fold change values obtained from the qPCR array analysis studies were imported into the IPA tool. e list of di erentially expressed genes analyzed by IPA revealed 12 signi cant networks and among them the top three networks identi ed by IPA were Death receptor signaling; Apoptotic signaling and Induction of apoptosis by HIV-1 comprising of 29 focused molecules and signi cance score of 27. Further, the IPA analysis also shows groups of di erently expressed genes into biological mechanisms that are related to Cell Death and Survival 2.45E-04, Cellular Function and Maintenance 2.45 E-04, Cell Morphology 1.17 E-04 and Cellular Compromise 1.75 E-04. IPA also revealed in CDV infected HCT-15 cells the important upstream regulators are TP 53, Cisplatin & POU5F1. Since there were 4 groups (infection intervals) the data set was analyzed for each individual group compared to the control group in IPA. In group 1 (6 hr PI) fold change, the most important canonical pathway that was found was of the apoptotic signaling (p-value -6.27E-27) with a z-score of 3 and a ratio of 0.232 consisting of sixteen upregulated molecules. A graphical representation of the pathway with the molecules involved in apoptotic signaling was generated by IPA where (TNF/FasL, TNFR/Fas,

Caspase 8, Bid, tBid, p53, JNK1, Bak, Bcl-2, Bad, Diablo, Cytochrome C, Caspase 9, Caspase 3, CAD, PARP) no. of genes were involved in sequential activation of each downstream molecule leading to caspase activation. In group 2 (12 hr PI) fold change, the apoptotic signaling was found to be signi cant (p-value -3.74E-15) with a z- score of 1.667 and a ratio of 0.13 consisting of nine upregulated molecules. IPA generated a graphical representation of the pathway with the molecules involved in apoptotic signaling in the group 2 (12 hr pi) cells where (TNF/FasL, TNFR/Fas, p53, Bak, Bcl-2, Caspase 9, Caspase 3, PARP) no. of genes were found to be upregulated and directly participated in the process of apoptosis. In group 3 (24hr PI) fold change, the apoptotic signaling was found to be signi cant (p-value- 1.69E-25) with a z-score of 3.5 and a ratio of 0.232 consisting of sixteen upregulated molecules. IPA showed a graphical representation of the molecules involved in apoptotic signaling pathway where (TNF/FasL, TNFR/Fas, Caspase 8, MKK4/7, JNK1, Bak, Bcl-2, Bad, Bax, Cytochrome C, Caspase 9, Caspase 3, CAD) no. of genes were found upregulated and interacted in a sequential manner leading to activation of downstream caspases. In group 4 (48hr PI) fold change, the signi cant canonical pathway was the apoptotic signaling (p-value -7.18E-30) with a z-score of 3.3 and a ratio of 0.261 consisting of eighteen upregulated molecules. A graphical representation of the pathway with the molecules involved in apoptotic signaling was generated by IPA where (TNF/FasL, TNFR/Fas, Caspase 8, Bid, tBid, p53, JNK1, Bak, Bcl-2, Bax, Diablo, Cytochrome C, Caspase 9, Caspase 3, PARP) no. of genes were found to be upregulated and in uenced the subsequent molecules for execution of apoptosis. Further, the upstream regulator analysis showed that TP53, POU5F1, Cisplatin, Foxo3a are the important upstream regulator in all the groups with the possible targets and prediction activation states as shown by evidence for e ects. e analysis of possible genes also revealed many downstream e ects which correlate with diseases or functions that are induced upon CDV infection of Hek-293 cells. e IPA showed the expression pattern of apoptotic genes similar to that of predicted activation states of up to nine 'diseases and function annotation' like "Apoptosis; Cell death; Apoptosis of tumor cell lines; Cell death of tumor cell lines; Necrosis; Cell death of carcinoma cell lines and Degradation of DNA" with a higher p-value and a z-score in the CDV infected HEK-293 cells. Further, in group 1 the downstream e ects analysis showed that 23 out of 27 genes have the measurement direction consistent with the increase in "Apoptosis". In group 2, the downstream e ects analysis showed that 13 out of 16 genes have the measurement direction consistent with the increase in "Apoptosis". In group 3, the downstream e ects analysis showed that 26 out of 30 genes have the measurement direction consistent with the increase in "Apoptosis". In group 4, the downstream e ects analysis showed that 27 out of 29 genes have the measurement direction consistent with the increase in "Apoptosis". As described by IPA, the most highly rated network as shown is the "Apoptosis of Embryonic cell lines" with di erent genes that are predicted to increase and regulate the process of apoptosis [10-12].

Discussion

Prediction of apoptotic pathway activated upon CDV infection of HEK-293 cells

According to the list of overexpressed genes, in group 1 & 2 the expression of TNF & TRAIL was seen to be upregulated. TNF is a pro-in ammatory cytokine that is involved in plays a role in immune modulation, in ammation, viral replication. TNF binds to cellular receptors such as TNFR1 and TNFR2 leading to conformational changes and binding to downstream death domains and activating death signaling. TNF-Related Apoptosis Inducing Ligand (TRAIL) is a trans membrane protein that belongs to the TNF family and is involved in the induction of apoptosis. TRAIL binds to receptors DR4 and DR5 which leads to its trimerization and clustering of the receptors

chromatin condensation and DNA fragmentation [13].

As observed by the gene expression analysis by qPCR array and Ingenuity Pathway database, it was found that in the group 1 (6 hrs PI), TNF overexpression was observed which acts as a ligand for binding to the death receptor TNFR1. Downstream of the death receptor activation, activation of caspase-8 was observed which is an initiator caspase involved in extrinsic pathway. Upon ligand binding to death receptors, an adapter molecule FADD is recruited which binds to inactive caspase-8 thus forming a complex called Death Inducing Signaling Complex (DISC). e activated caspase further cleaves downstream caspases or associates in the cleavage of proapoptotic protein Bid, thus forming a cross-link with the intrinsic pathway of apoptosis. It was observed that in CDV infected HEK-293 cells, caspase- 8 led to the cleavage of Bid into tBid which further moves to the mitochondria leading to activation of Bcl-2 proapoptotic proteins. Bid, a BH3 only pro-apoptotic protein was found to be upregulated with the activation of tBid. It is known that following the death receptor pathway Bid is cleaved by caspase-8, this cleavage results in the formation of a truncated protein tBid. It is further suggested that tBid is capable of activating Mitochondrial Outer Membrane Permeabilization (MOMP) as a result of the imbalance in the pro and anti-apoptotic proteins that enables it to occur. Next, Bak activation was proposed in the pathway, which is Bcl-2 pro-apoptotic protein which localizes to the mitochondria and functions to induce apoptosis.

e Bcl-2 proteins are a crucial checkpoint in the apoptosis at the mitochondria. During apoptosis, the BH3s such as tBid activate Bak and Bax, thus inducing cytochrome C release and nally leading to caspase activation. Further in the pathway analysis, the expression of Bax and Diablo was upregulated downstream of tBid and Bak. Upon apoptotic stimuli, it is believed that Bax and Bak get activated upon upstream signals and get oligomerized at the Mitochondrial Outer Membrane (MOM) and causes its permeabilization, leading to initiation of downstream apoptotic cascades. us, Bax and Bak work at appropriate levels to facilitate the release of cytochrome C and Smac/ Diablo into the inter membrane space. Direct IAP Binding Protein with Low Isoelectric Point (Diablo) release from the mitochondria has been shown to repress the inhibitor of apoptosis proteins and further activates caspase-9. As the pathway progressed the activation of Bad, Bcl-2 associated death promoter, which induces apoptosis by inhibiting anti-apoptotic proteins Bcl-2 & Bcl-xl. e expression of Bax, Diablo and cytochrome C was seen to be upregulated which describes the permeabilization of mitochondria with the release of Diablo. Cytochrome C is a heme protein which is associated with the inner membrane of mitochondria, which is involved in the initiation of apoptosis by its release from the mitochondria upon mitochondrial membrane permeabilization or sensing due to increase in intracellular calcium e ux. With the over- expression of proapoptotic proteins Bax, Bad & Bak, and the release of Diablo and cytochrome C from the mitochondria, there was upregulation of Apoptotic Protease Activating Factor (APAF1) which serves as a key molecule in the intrinsic apoptosis, which is known to oligomerize upon Cytochrome C release from the mitochondria resulting in the formation of a complex called the apoptosome. In this study, Downstream to APAF1 activation, activation of caspase-9 and caspase-3 was observed in CDV infected HEK-293 cells. It is known that activated APAF1 binds to the procaspase 9 to form the apoptosome complex through monomer interactions, which leads to the dimerization and formation of catalytically active caspase 9. Active caspase 9 leads to the cleavage and activation of the executioner caspases 3, 6 & 7 resulting in chromatin condensation, DNA fragmentation, cell shrinkage and nally apoptosis. IPA revealed the upregulation of major apoptotic proteins (Bak1, Bid, FasLG, MAP2K7, MAPK8, p53, Bcl-2, Cytochrome C, TNF, Bax, Diablo, Bad, APAF1, Caspase-8, Caspase-3, Caspase-9, PARP1) from both extrinsic and intrinsic pathway of apoptosis along with important upstream regulator and downstream regulators. As in this study, upregulation of caspase 8, 9 and 3 could detected which play major roles in the classical xtrinsic and intrinsic apoptotic pathways. erefore, it is hypothesized that CDV induced apoptosis in HEK-293 cells occurs by both intrinsic and extrinsic pathways of apoptosis [14, 15].

Ethics Declarations

Con ict of Interest

e authors declare that they have no known competing nancial interests or personal relationships that could have appeared to in uence the work reported in this paper.

Ethical Approval

is article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

All the authors agree to submit for publication.

Acknowledgement

None

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