FULL PAPER

Virology

Schmallenberg virus induces apoptosis in Vero cell line via extrinsic and intrinsic pathways in a time and dose dependent manner

Emel AKSOY[1) and Ahmet Kursat AZKUR[1)]*

[1)Department of Virology, Faculty of Veterinary Medicine, Kirikkale University, 71450 Yahsihan, Kirikkale, Turkey

ABSTRACT. Schmallenberg virus (SBV), discovered in 2011 in Germany, is associated with clinical manifestations of fever, diarrhea, reduced milk yield, abortions and congenital malformations in ruminants. Despite many studies performed for SBV, there is no detailed research on *in vitro* apoptotic effect of SBV. This study is aimed to determine apoptosis pathways and role of pro-apoptotic and anti-apoptotic molecules in Vero cells infected with SBV. The study results showed that SBV induced apoptosis via both extrinsic and intrinsic pathways by activating both caspase-8 and caspase-9, respectively. Expression analyses of pro-apoptotic (*Bax*, *Bak* and *Puma*) and anti-apoptotic (*Bcl-2* and *Bcl-XL*) genes revealed that SBV-induced apoptosis causes upregulation of pro-apoptotic genes, dominantly via *Puma* gene, whereas *Bcl-2* and *Bcl-XL* genes were downregulated. In conclusion, this is the first detailed report about SBV induced apoptosis in the Vero cells via both extrinsic and intrinsic cascades and apoptosis induction is seem to be regulated by *Puma*.

KEY WORDS: apoptosis, caspase, DNA fragmentation, flow cytometry, Schmallenberg virus

Schmallenberg virus (SBV) was discovered in 2011 in Germany in cattle with fever, diarrhea and reduced milk yield symptoms. SBV is a negative-sense single stranded RNA virus which is classified into the Simbu serogroup in genus *Orthobunyavirus* in *Peribunyaviridae* family in order *Bunyavirales*. The SBV genome structure resembles other Orthobunyaviruses and consists of 3 segments of S (small), M (medium) and L (large). SBV S segment (830 nt) encodes nucleocapsid (N) and a nonstructural (NSs) proteins, M segment (4415 nt) encodes envelope glycoproteins Gn, Gc and nonstructural (NSm) protein. RNA-dependent RNA polymerase of SBV is encoded in L segment (6865 nt) [11, 12]. SBV is transmitted by *Culicoides* biting midges and semen [23, 31]. SBV infection reported in domestic and wild ruminants [4, 11]. The clinical signs include fever, reduce in milk yield, diarrhea, abortion and congenital malformations such arthrogryposis, hydranencephaly, torticollis, scoliosis, kyphosis, brachignatia inferior, hydrocephalus, porencephaly and these occur in cattle, sheep and goat infected by SBV [10, 11]. SBV was spread through Europe including Turkey [4, 30].

The several types of cell death, which include apoptosis, autophagy, necrosis, cornification and pyroptosis, are defined by some morphological, biochemical and molecular criteria. Apoptosis is active, metabolic and genetically encoded type of programmed cell death without causing any inflammations which has specific biochemical and morphological properties. Autophagy is characterized by formation of autophagosomes which includes cytoplasmic organelles and/or cytosol. Necrosis is a type of cell death which plasma membrane of cell rupture and intracellular contents are released [13, 29]. Detection of cell death is based on characteristic biochemical, molecular and morphological features, such as, activation of pro-apoptotic Bcl-2 family proteins, activation of caspases, phosphatidylserine exposure, DNA fragmentation could be used for detection of apoptosis. Caspases are cysteine proteases which have many roles on such as apoptotic cell death, cell differentiation, cell signaling and inflammation. Caspases are classified in two main groups: initiator caspases such as caspase-8 and -9 and effector caspases such caspase-3, -6 and -7. Caspase-8 is mainly responsible for apoptosis triggered by the extrinsic pathway which is related with death receptors located on cell surface, whereas caspase-9 has a role on intrinsic pathway initiated apoptosis that is connected with mitochondria and related proteins [2, 9, 15].

Bcl-2 family proteins are divided into two basic groups: one is associated with apoptosis inhibition named anti-apoptotic subfamily and other is pro-apoptotic subfamily which is responsible for triggering apoptosis. Anti-apoptotic subfamily consists of such Bcl-2, Bcl-XL, Bcl-W, Mcl-1 proteins while pro-apoptotic subfamily contains Bax, Bak, Bid, Bim, Bad, Puma, Noxa etc. proteins [29].

*Correspondence to: Azkur, A. K.: azkurkursat@hotmail.com, kursatazkur@kku.edu.tr

Apoptosis could occur in both physiological conditions such embryonic development, immune cell selection and in pathological status like mutations, DNA damage and certain infections [9]. Some viruses can induce or inhibit apoptosis in cells by their proteins. Inhibition of apoptosis could be needed for persistent infection and viral replication whereas induction of apoptosis could be because of lytic infection and viral spread without any inflammation. African swine fever virus, fowl poxvirus, bovine herpesvirus-5 are some viruses that inhibit apoptosis following infection of cells [5, 14, 19]. Certain apoptosis inducing viruses including Crimean-Congo hemorrhagic fever virus, bluetongue virus, Akabane virus, Aino and Chuzan viruses [16, 18, 24]. Although apoptotic induction of CPT-Tert and HEK-293T cell lines by SBV was detected with caspase-3/-7 activation [7] there was no information about apoptotic effect of SBV on Vero cell line. Vero cell line is widely used for SBV isolation, plaque titration and plaque reduction neutralization tests and antigen preparation [17, 31]. There is one study about apoptosis and SBV in pubmed according to our knowledge [7]. Because of inadequacy of detailed information on SBV-induced apoptosis, in present study, apoptosis in Vero cell line was investigated with DNA fragmentation, caspase activation, Annexin-V/PI staining and expression analyses of pro- and anti-apoptotic genes, further time and dose dependent analyses were performed.

MATERIALS AND METHODS

Cell and virus

Vero cell line is maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin at 37°C incubator with 5% CO_2. Schmallenberg virus is kindly provided by Dr. Wim H. M. van der Poel (Wageningen University in the Netherlands). Vero cells were infected with 0.1 and 0.01 MOI of SBV and excess virus not adsorbed was removed after 1 hr of incubation. Cell viability examined with trypan blue (15250061, Thermo Fisher) exclusion method.

Plaque titration

SBV titration is performed on Vero cells as described previously [17] with some modifications. Briefly, Vero cell line was inoculated in 12-well cell culture plate and after 18 hr cells were infected with SBV dilutions. After 3 hr of incubation of virus-infected cells in 37°C incubator with 5% CO_2 virus dilutions, inocula were removed and 1:1 mixture of 2× DMEM and 2% carboxymethyl cellulose (CMC) were dispersed to all wells. After incubation for 6 days cells were fixed with 10% neutral buffered formalin solution and stained with crystal violet. Plaques were counted and stock virus titration was calculated as PFU/m*l*.

DNA fragmentation assay

Cells infected with 0.1 and 0.01 MOI of SBV were collected on 0, 2, 6, 12, 18, 24, 36, 48 hr Staurosporine (35385.02, Serva, Heidelberg, Germany) was used as positive control for apoptosis induction with final concentration of 1 μM/m*l*. Apoptotic DNA ladder kit (K170, Biovision, California, U.S.A.) was used for detection of DNA fragmentation as following the instructions of manufacturer's. Briefly, approximately 2×10^6 cells were collected and cell pellets were suspended with extraction buffer followed by centrifugation at 1,600 × g. RNase and proteinase were added to supernatants of each samples and incubated at 50°C for 30 min. After incubation samples were incubated at −20°C for 10 min after ammonium acetate and isopropanol addition. Samples were centrifuged at 16,000 × g for 10 min for DNA precipitation after incubation. DNA pellet were washed with 70% ethanol and centrifugation was repeated at 16,000 × g for 10 min. Ethanol was removed and tubes were incubated at room temperature for 15 min for evaporation of ethanol residues. DNA samples were suspended in suspension buffer and loaded on 2% agarose gel stained with ethidium bromide and visualized under UV light.

Flow cytometry analysis

Cells infected with 0.1 and 0.01 MOI of SBV were harvested on 0, 2, 6, 12, 18, 24, 36, 48 hr and were stained with Annexin V-FITC and PI with using Annexin V-FITC Apoptosis Kit (K101, Biovision, CA, U.S.A.) for detection of apoptosis in flow cytometry. Briefly, cell pellets were suspended with 1× binding buffer. Cell groups were determined as unstained, FITC, PI and FITC+PI stained. After cells were stained with related dye, samples were incubated in room temperature for 5 min. Flow cytometry analyses were maintained with BD FACSAria II (Franklin Lakes, NJ, U.S.A.) after calibration with beads. Data analysis of samples was carried out with BD FACSDIVA software.

Caspase assays

Caspase-3, caspase-8 and caspase-9 activities of cells infected with 0.1 and 0.01 MOI of SBV were determined in Vero cells harvested on 0, 2, 6, 12, 18, 24, 36, 48 hr pi. Caspase-3 (K106, Biovision), caspase-8 (K113, Biovision) and caspase-9 (K119, Biovision) colorimetric assay kits were used for determination of caspase activations. After harvesting, cell pellets were suspended in 50 μl of cell lysis buffer followed by incubation on ice for 10 min. Samples were centrifuged at 10,000 × g for 1 min and cytosolic extracts were transferred to fresh tubes. Protein concentration of samples was determined and 250 μg of protein from each sample were dilute in total volume of 50 μl of cell lysis buffer in a 96-well microplate. Into each well 50 μl of 2× reaction buffer with 10 mM of dithiothreitol (DTT) was added. DEVD-*p*NA, IETD-*p*NA and LEHD-*p*NA substrates were added with final concentration of 200 μM to each sample for determination of caspase-3, caspase-8 and caspase-9 activation, respectively. Plates were incubated at 37°C for 2 hr and read at 405 nm using a microplate reader.

RNA isolation and reverse transcription

RNA samples were isolated from cells with using High Pure RNA isolation kit (11 828 665 001, Roche, Mannheim, Germany). Briefly, cells pelleted and re-suspended in 200 μl PBS thereafter 400 μl of lysis/binding buffer added to each sample and vortexed. Samples were transferred to filter tubes and centrifuged at 8,000 × g for 15 sec. Ninety μl DNase incubation buffer and 10 μl DNase I were mixed per sample and transferred to filter tubes followed by incubation at room temperature for 15 min. Five hundred μl of wash buffer I was added to tubes and centrifuged at 8,000 × g for 15 sec. After centrifugation, 500 μl of wash buffer II was added to tubes and centrifuged again at 8,000 × g for 15 sec. Two hundred μl of wash buffer II was added to tubes and centrifuged at 13,000 × g for 2 min. Filter tubes were transferred into microcentrifuge tubes and 100 μl of elution buffer were added to upper reservoir of filter tubes. After centrifugation at 8,000 × g for 1 min, eluted RNA samples were stored at −80°C until used for reverse transcription. Reverse transcription of cDNAs were performed with Transcriptor First Strand cDNA synthesis kit (04 897 030 001, Roche, Mannheim, Germany) using anchored-oligo $(dT)_{18}$ and random hexamer primers. Briefly, total RNA, anchored-oligo $(dT)_{18}$ and random hexamer primers were mixed in total volume of 13 μl and heated to 65°C for 10 min in thermal cycler. Thereafter tubes were immediately cooled on ice. Mix of 4 μl transcriptor reverse transcriptase reaction buffer, 0.5 μl protector RNase inhibitor, 2 μl dNTP mix, 0.5 μl transcriptor reverse transcriptase were prepared for each samples and dispersed to tubes in final volume of 20 μl. Reverse transcription reaction was performed in following condition, 25°C for 10 min followed by 55°C 30 min.

PCR and real-time PCR

Vero cells infected with SBV were confirmed by PCR using two different primers sets for amplification of S segment of SBV after reverse transcription (Table S1). Real-time PCR was used to analyze expression levels of anti-apoptotic genes *Bcl-2* and *Bcl-XL* and pro-apoptotic genes *Bak*, *Bax* and *Puma*. GAPDH and Beta actin were selected as reference genes. Primer sets for these apoptosis-related genes were designed (Table S2) and real-time PCR optimization were performed for all sets. cDNAs were subjected to real-time PCR with SYBR Green I master mix (04707516001, Roche, Mannheim, Germany) in total volume of 20 μl for each reactions and 95°C 10 sec, 60°C 10 sec, 72°C 10 sec profile was used. Each sample was studied in duplicate. Analyses were performed with LightCycler 96 instrument (Roche, Mannheim, Germany). Normalized gene expression levels of apoptosis-related genes were calculated with $2^{-(Cq \text{ gene of interest } -Cq \text{ reference gene}) \text{ infected sample } -Cq \text{ gene of interest } -Cq \text{ reference gene}) \text{ noninfected sample}}$ formulation [22, 26] with distinct normalization both with GAPDH and Beta actin.

Statistical analyses

One-way ANOVA and independent *t*-test were used for statistical analyses in IBM SPSS Statistics 21 software (Chicago, IL, U.S.A.). Results were considered as statistically significant at $P<0.01$. Graphics were created with GraphPad Prism software (San Diego, CA, U.S.A.).

RESULTS

Infection of Vero cells with SBV

Vero cells were infected with SBV on Vero cells and were confirmed with RT-PCR and plaque titration that was also carried out to determine titer of virus stocks (Figs. S1, S2, Table S1). Vero cells infected with 0.1 and 0.01 MOI of SBV were visualized under inverted microscope (DM IL LED, Leica, Wetzlar, Germany) and cells were controlled daily for cytopathic effect (cpe). Cells infected with 0.1 MOI of SBV showed cpe first in 18 hr post-infection (pi). In infection of 0.01 MOI of SBV first cpe were seen in 24 hr pi and cpe of cells were much less in comparison to those cells infected with 0.1 MOI of SBV.

Schmallenberg virus causes DNA fragmentation in Vero cell line

To detect whether DNA fragmentation is induced in SBV infection, cells were collected on 0, 2, 6, 12, 18, 24, 36, 48 hr post-infection and DNA samples were isolated. DNA fragmentation assay of 0.1 and 0.01 MOI of SBV infected cells were visualized on 2% agarose gel. DNA fragmentation of SBV infected Vero cells was dependent on infection time and dose of virus, as DNA fragmentation of 0.1 MOI of SBV infected cells initiated on 18 hr pi while 0.01 MOI of SBV infected cells showed first DNA fragmentation on 24 hr pi. Positive control of apoptosis (Staurosporine) was positive in DNA fragmentation and mock infected control cells showed no DNA fragmentation (Fig. 1).

Phosphatidylserine exposure on Schmallenberg virus infected Vero cell line

To address if SBV infected cells culminate in phosphatidylserine translocation, cells stained with Annexin V-FITC/PI were analyzed in flow cytometry. The highest ratio of phosphatidylserine exposure of Vero cells was detected in 48 hr pi in 0.1 MOI of SBV infection with total rate of 37.84% (Fig. 2A), whereas in 0.01 MOI infected 48 hr pi cells showed 22.7% apoptotic cells in total (Fig. 2B).

Schmallenberg virus activates caspase-3, caspase-8 and caspase-9

To determine whether the infected cells show significantly more caspase activation than the mock infected in the different time and dose, effects of SBV infection on caspase-3, -8 and-9 activation in Vero cells were measured by using colorimetric kits. According to caspase activity assays, caspase-3, -8 and -9 activations in SBV infected Vero cell were detected. All caspase activation data sets were shown as the mean ± standard error of the mean (SEM) versus the mock infected group for both 0.1 and

0.01 MOIs (Fig. 3).

Significant differences are determined for the infection time points versus the mock infected cells and between dose groups. According to results, caspase-3 activation was significantly different at 36 hr pi and 48 hr pi in both of 0.1 MOI and 0.01 MOI infected cells compared to the mock infected group ($P<0.01$) (Fig. 3A). For caspase-3, there is statistically significance between 0.1 MOI and 0.01 MOI groups of cells at 2, 6 and 12 hr pi ($P<0.01$) (Fig. 3A). Results indicate that SBV induce apoptosis in Vero cells with activation of caspase-3 in both viral doses.

In activation of caspase-8 there are significant differences determined between mock infected group and 2, 6, 36 and 48 hr pi cells in 0.1 MOI ($P<0.01$). Caspase-8 was activated significantly at 0.01 MOI of 12, 18, 24, 36 and 48 hr pi cells versus the mock infection cells ($P<0.01$). Caspase-8 is slightly activated in SBV infected cells but there is statistically significance between all doses except for those of 36 hr pi ($P<0.01$). Unexpectedly, proportions of activated caspase-8 at 2, 6, 12, 18 and 24 hr pi of 0.01 MOI are higher than 0.1 MOI groups (Fig. 3B). Caspase-8 activation in SBV infected cells indicates that extrinsic pathway is triggered by SBV.

Activation of caspase-9 indicated moderate but significant increases at 0.01 MOI and interestingly, stayed almost unchanged during the time. At 0.01 MOI in 2, 12, 18, 24, 48 hr pi cells there was statistical significance compared to mock infection ($P<0.01$) (Fig. 3C). Statistically significance is determined at 0.1 MOI for 2, 6, 18, 36 and 48 hr pi groups in comparison to mock infected group ($P<0.01$). Infected cells with 0.1 MOI of SBV has higher proportion of activated caspase-9, supporting SBV induce intrinsic cascade (Fig. 3C).

Gene expression analyses of SBV infected Vero cells

After the induction of apoptosis in SBV infected Vero cells was confirmed by DNA fragmentation, flow cytometry analyses and caspase activation assays, expression analyses of pro-apoptotic (*Bax*, *Bak*, *Puma*) and anti-apoptotic (*Bcl-2*, *Bcl-XL*) genes of Vero cell were performed. GAPDH and Beta actin reference genes were used to normalize gene expression levels of apoptosis related genes (Table S3). Expressions of *Bak*, *Bax*, *Bcl-2*, *Bcl-XL* and *Puma* genes of Vero cells were analyzed following SBV infection. It was seen that increasing levels of gene expression has higher ratio in those of which are normalized to GAPDH reference gene than those were normalized to Beta actin reference gene (Figs. 4 and 5).

In normalization to GAPDH reference gene, *Bak*, *Bax*, and *Puma* gene expressions were upregulated, whereas *Bcl-2* and *Bcl-XL* genes were downregulated gradually by time (Fig. 4). Statistically significance ($P<0.01$) is determined for 0.1 MOI infected cells in comparison to mock infected group for *Bak* (at 2, 6, 12, 18, 24, and 36 hr pi), *Bax* (at 24 and 48 hr pi), *Puma* (at 48 hr pi), *Bcl-2* (at all time points) and *Bcl-XL* (at all time points) genes. In cells infected with 0.01 MOI of SBV, there is statistically significance ($P<0.01$) in different MOI doses and in between time points and mock infected group. Statistically significance ($P<0.01$) is determined in GAPDH normalization for 0.01 MOI infected cells in comparison to mock infected group in *Bak* (at 2, 12, 18, 24, 36 and 48 hr pi), *Bax* (at 6 and 48 hr pi), *Puma* (at 36 hr pi), *Bcl-2* (at 2, 6, 24, 36, and 48 hr pi) and *Bcl-XL* (at 2, 12, and 48 hr pi) genes.

In normalization to Beta actin reference gene, only *Puma* gene expression was upregulated (Fig. 5). There is statistically significance ($P<0.01$) determined for both 0.1 MOI and 0.01 MOI infected cells at all time points in comparison to mock infected group for *Bak*, *Bcl-2* and *Bcl-XL* genes, and for *Bax* gene at 6, 12, 18, 24, 36, and 48 hr pi. For *Puma* gene, the cells infected with 0.1 MOI (at 18 and 48 hr pi) and 0.01 MOI (at 2, 6, 18, 24, 36, and 48 hr pi) have shown statistically significance ($P<0.01$) and when compared to mock infected group. The Beta actin normalization results demonstrated that SBV causes upregulation of *Puma* gene in Vero cells, however does not increase the levels of pro-apoptotic genes *Bak* and *Bax* and SBV infection culminated in downregulation of anti-apoptotic genes *Bcl-2* and *Bcl-XL*.

According to both GAPDH and Beta actin reference genes, Bcl-2/Bax rate was 0.19 and 0.16 in 48 hr pi in 0.1 and 0.01 MOI infections, respectively. Similarly, Bcl-XL/Bax rates were identical when calculated according to GAPDH and Beta actin reference genes. Rate of Bcl-XL/Bax at 48 hr pi was 0.88 and 0.45 for 0.1 and 0.01 MOI infections, respectively.
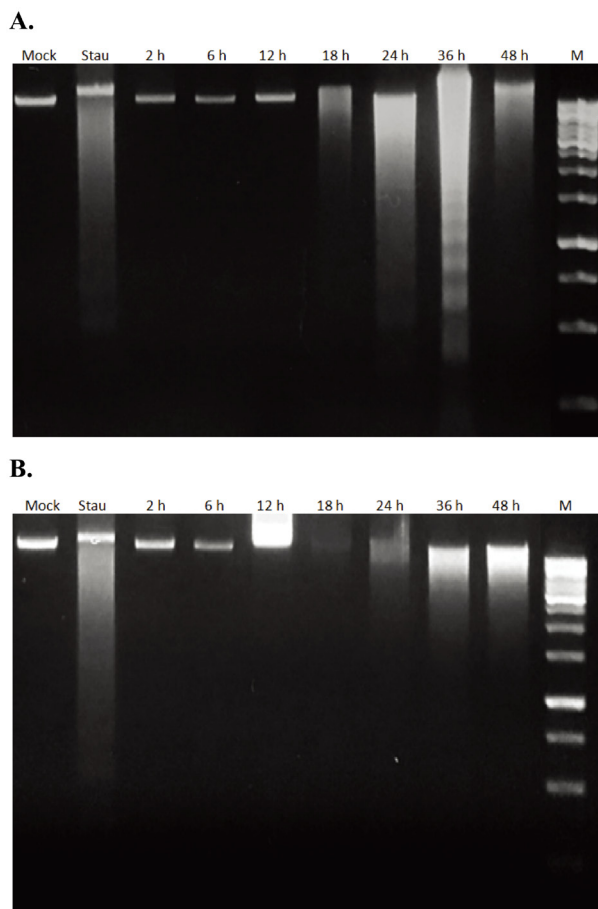
Fig. 1. DNA fragmentation assay of 0.1 MOI (A) and 0.01 MOI (B) of SBV infected Vero cells collected on 0, 2, 6, 12, 18, 24, 36, 48 hr post-infection. DNA samples were applied to 2% agarose gel and visualized under UV light. Mock: mock infected negative control cells, Stau: positive control cells inoculated with staurosporine, M: 1 kb DNA ladder (SM0311, Thermo Fisher).

A.

ANNEXIN V-FITC

B.

ANNEXIN V-FITC
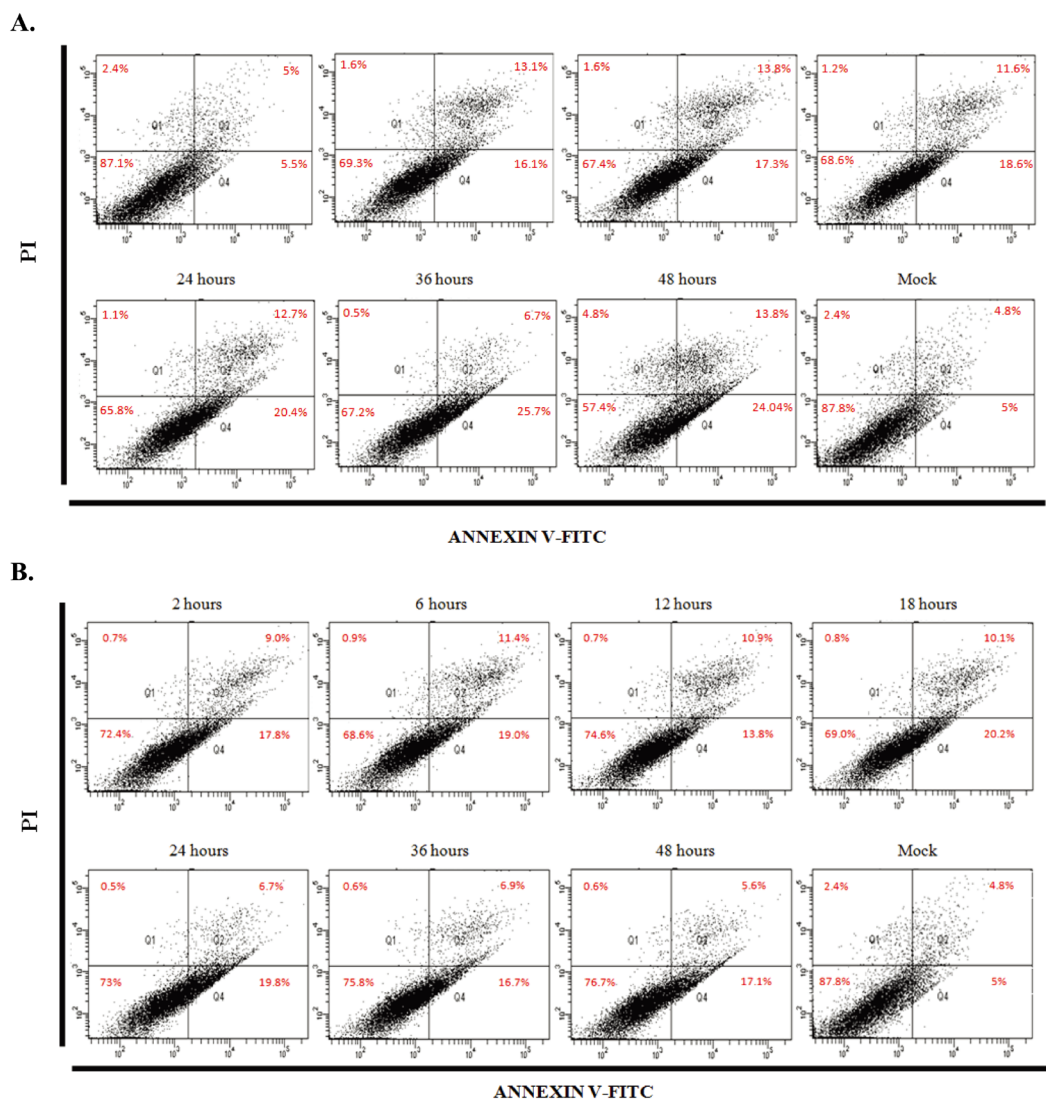
Fig. 2. Flow cytometry analyses of Vero cells infected with 0.1 MOI (A) and 0.01 MOI (B) of SBV infected cells which are collected in 2, 6, 12, 18, 24, 36, and 48 hr post-infection. Mock infected cells are uninfected Vero cells. Cells were determined as live (Annexin V−/PI−), early apoptotic (Annexin V+/PI−), late apoptotic (Annexin V+/PI+) and necrotic (Annexin V−/PI+) depending on staining.

DISCUSSION

Cell death in viral infections could be directed by some viral proteins both for induction and inhibition of apoptosis. Inhibition of apoptosis by certain viruses can be related with persistent infection and achievement of viral replication. Some viruses which induce apoptosis in cells could be for lytic infection and viral spread without any inflammation and immune response. To date, some members of *Peribunyaviridae* family were found to induce apoptosis in several cell lines. Akabane, Aino, Crimean-Congo hemorrhagic fever, Oropouche viruses of *Peribunyaviridae* family reported as triggering apoptosis in different cell lines such HeLa, Huh7 and Vero [16, 24]. SBV, another member of *Peribunyaviridae*, was shown to induce apoptosis in CPT-Tert and HEK-293T cell lines [7] but there was no data about induction or inhibition of apoptosis by SBV in different cell lines except these. This lack of information on Vero cell line, one of the most preferred and susceptible cell line for SBV was eliminated by data of this study.

Induction of apoptosis could be detected by many features of cells *in vitro*. DNA fragmentation, caspase activation, cleavage of apoptosis related proteins such as caspases, PARP etc., translocation of cytochrome c from mitochondria to cytoplasm, increasing of expression of pro-apoptotic genes, phosphatidylserine exposure on outer leaflet of cells are the most reliable and commonly used characteristics of apoptotic cells and could be detected *in vitro* by using different methods. In present study, DNA fragmentation, phosphatidylserine exposure, caspase activation and apoptosis related gene expression levels were analyzed with agarose gel electrophoresis, Annexin V/PI staining in flow cytometry, colorimetric caspase activation assays and real-time PCR methods, respectively.
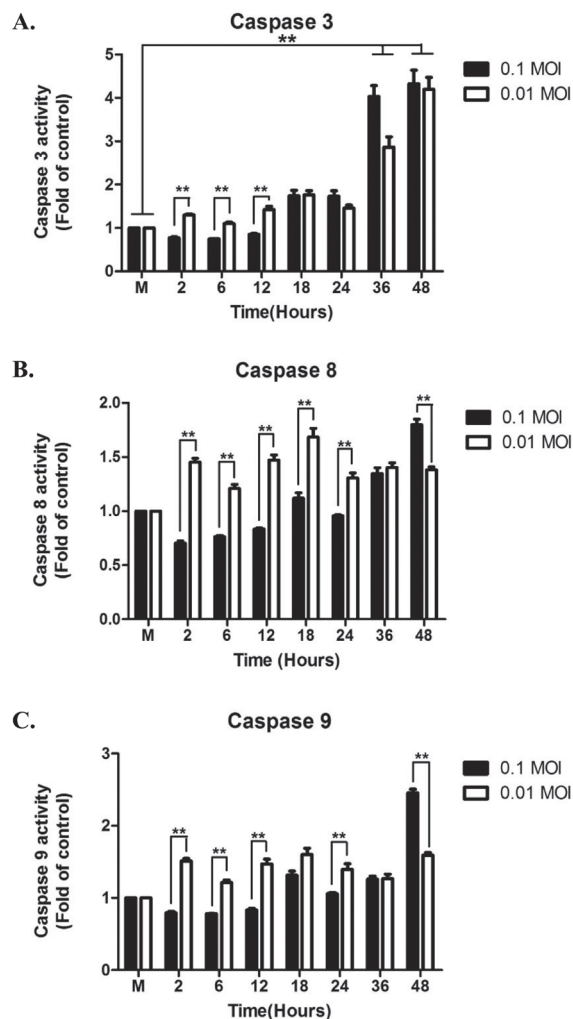
DNA fragmentation is one of the basic characteristics of apoptosis/necrosis and can be detected in agarose gel electrophoresis.

Results of this study on DNA fragmentation of SBV infected Vero cells was detected as being dependent on infection time and dose of virus. First DNA fragmentation of SBV infected cells were seen on 18 hr pi at 0.1 MOI and on 24 hr pi at 0.01 MOI of SBV (Fig. 1). Virus dose and infection time can affect the first DNA fragmentation appearance on virus-infected cells. A detectable DNA laddering at the 2 MOI of H9N2 virus infected A549 cells was detected at 16 hr pi [28] whereas all 0.1, 1 and 10 MOI of chikungunya virus infected cells showed DNA fragmentation at 48 hr pi [1].
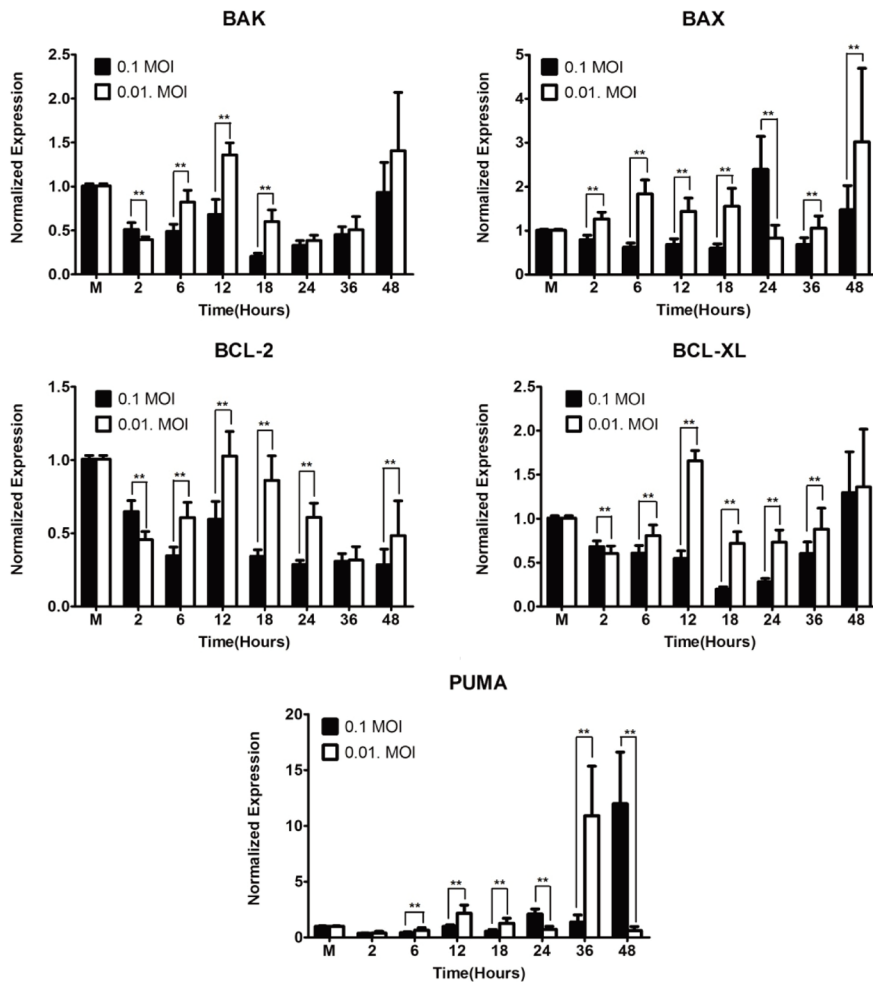
Cells under physiological conditions have phosphatidylserine on inner leaflet of plasma membrane. During apoptosis induction in cells phosphatidylserine become exposed on outer leaflet of plasma membrane and display "eat me" signal to phagocytes [27]. Annexin V, an anticoagulant protein which binds to phosphatidylserine, is used for detection of apoptosis incidence in cells. Propidium iodide (PI) which is used widely with combination of Annexin V is a plasma membrane permeability marker. When used combined together cells could be seperated into 4 groups: live (Annexin V−/PI−), early apoptotic (Annexin V+/PI−), late apoptotic (Annexin V+/PI+) and necrotic (Annexin V−/PI+) cells [33]. Flow cytometry results showed that highest apoptotic cell rate were in 48 hr pi samples in infection of 0.1 MOI (Fig. 2A). Early apoptosis rates were 24.04% and 17.1% while late apoptosis rates were 13.8% and 5.6% in 0.1 and 0.01 MOI of SBV infected cells, respectively (Fig. 2). This is the first results of apoptosis detection of SBV infected Vero cells by using Annexin V/PI staining in flow cytometry. Apoptotic cell percentage could be affected by virus species/strain, virus dose, cell line and time of infection. Early and late apoptotic cells were detected as 61–75 and 13–19%, respectively, in HeLa cells transfected with canine parvovirus-2 NS1 encoding vector [25]. It is surprising that phosphatidylserine externalization starts in the early time in the flow cytometry results but it is not seen caspase-3 activation until 36 hr pi.

Caspases, cystein proteases that modulates apoptosis, cleave their specific substrates and activate downstream molecules and apoptotic cell death occurs via mainly two pathways: extrinsic and intrinsic pathways. In present study, caspase-3 which is one of the effector caspases, caspase-8 and caspase-9 which have roles on initiation of extrinsic and intrinsic pathways respectively had been studied on Vero cells infected with SBV. To date whether SBV leads to caspase activation of infected cells had investigated in only CPT-Tert and HEK-293T cells with result of slight activation of caspase-3/7 [7]. Paucity of information in Vero cell line for caspase activation has eliminated in present study with caspase-3 activation and further investigation of pathway related caspases. Caspase-3 was activated following SBV infection in both MOIs, but statistically significance is determined for 36 and 48 hr pi cells compared to mock infected group (*P*<0.01), confirming that SBV induces apoptosis in Vero cells (Fig. 3).

To determine whether SBV modulates extrinsic and intrinsic signaling cascade, caspase-8 and caspase-9 activation was analyzed, respectively. Caspase-8 was slightly but significantly (*P*<0.01) activated in SBV infected cells. However, unexpectedly, at 2, 6, 12 and 18 hr pi of 0.01 MOI proportions of activated caspase-8 are higher than 0.1 MOI groups (Fig. 3). Caspase-8 activation in cells at 0.1 MOI initiated from 18 hr pi and reached maximum level at 48 hr pi. Activated caspase-8 levels were slightly high at first hours of

Fig. 3. Effects of SBV infection on activation of caspase-3, -8 and -9 in Vero cells. Activation of caspases are detected by using colorimetric kits. Samples were collected at 2, 6, 12, 18, 24, 36, 48 hr post-infection. Results are calculated as the mean ± SEM versus mock infected group. Asterisk indicates statistically significance (*P*<0.01). M stands for mock infected cells. Caspase-3 activation was significantly different at 36 hr pi and 48 hr pi of 0.1 MOI and 0.01 MOI infected cells compared to the mock infected group (*P*<0.01) (A). For caspase-8 activation, there are significant differences determined between mock infected group and 2, 6, 36 and 48 hr pi cells in 0.1 MOI (*P*<0.01). Caspase-8 was activated significantly at 0.01 MOI of 12, 18, 24, 36 and 48 hr pi cells versus the mock infection cells (*P*<0.01) (B). Statistically significance is determined for caspase-9 at 0.1 MOI for 2, 6, 18, 36 and 48 hr pi groups in comparison to mock infected group (*P*<0.01). There was statistical significance for caspase-9 in 0.01 MOI in 2, 12, 18, 24, 48 hr pi cells compared to mock infection (*P*<0.01) (C).

infection and interestingly peaked at 18 hr pi at 0.01 MOI (Fig. 3). Caspase-9 activation of SBV infected cells was investigated to find out if intrinsic pathway is triggered. Likely caspase-8, activation of caspase-9 at 0.1 MOI has reached to highest level at 48 hr pi. Cell group infected with 0.1 MOI of SBV has higher proportion of activated caspase-9, while activation of caspase-9 indicated moderate but significant increases at 0.01 MOI and interestingly, stayed almost unchanged during the time (Fig. 3). These data
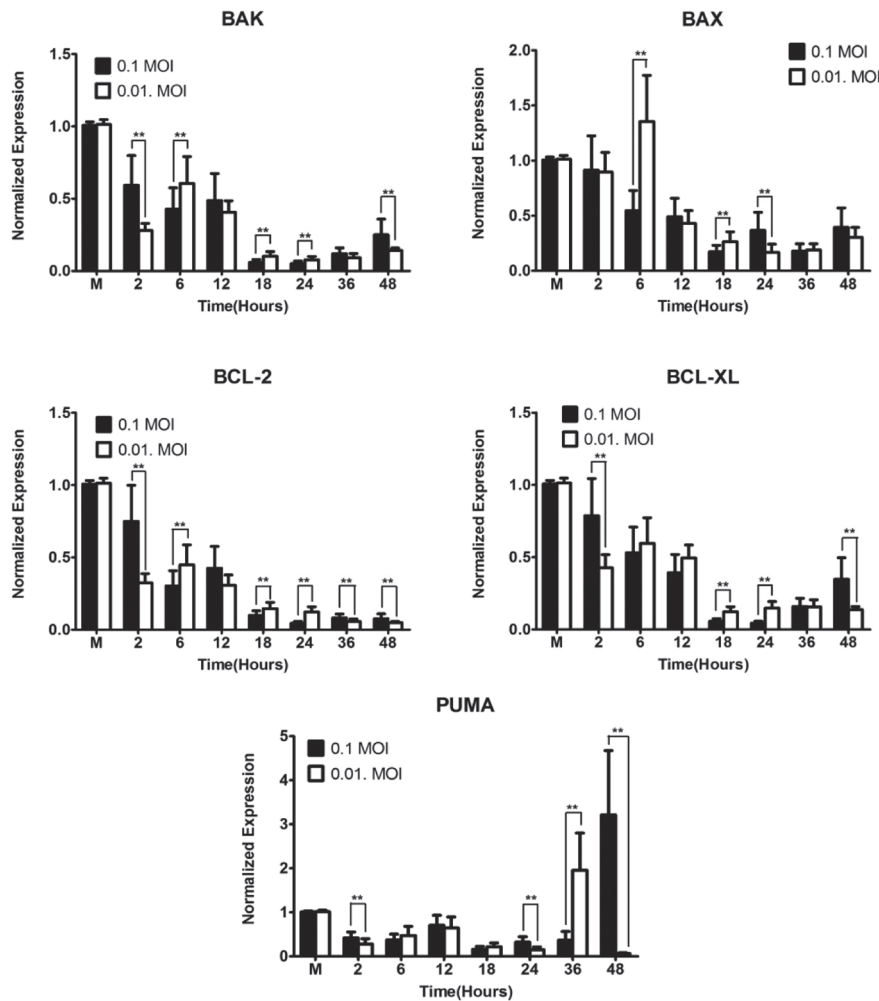
Fig. 4. Real-time PCR results of SBV infected Vero cells. Samples were collected in 0, 2, 6, 12, 18, 24, 36, 48 hr post-infection. Relative expression analyses of *Bax*, *Bak*, *Bcl-2*, *Bcl-XL* and *Puma* apoptosis-related genes of Vero cells infected with 0.1 MOI and 0.01 MOI of SBV are normalized to *GAPDH* reference gene. Asterisks indicate statistically significance (*P*<0.01) between 0.1 MOI and 0.01 MOI groups of SBV infected cells. M stands for mock infected cells.

indicate that SBV induce intrinsic cascade. Crimean-Congo hemorrhagic fever virus (CCHV) which is another member of family *Peribunyaviridae* induces apoptosis via both extrinsic and intrinsic pathways [6].

Many research reported that pro-apoptotic genes like *Bak*, *Bax* and *Puma* are upregulated when apoptosis induced in cells. *Bak* gene was upregulated in Hepatitis C virus infected tissues and HepG2 cell lines associated with apoptosis induction [34]. Chicken fibroblast cells infected with infectious bronchitis virus (IBV) showed increased *Bak* gene levels as 1.39 and 2.09 fold after 8 and 16 hr pi, respectively. *Bak* levels were 6.41 fold increased in IBV infected chicken embryos after 48 hr pi [35]. In this study, at 48 hr pi *Bak* gene levels normalized to GAPDH were found increased approximately as 0.93 fold in 0.1 MOI and 1.40 fold in 0.01 MOI (Fig. 4), whereas levels normalized to Beta actin were found approximately 0.25 fold in 0.1 MOI and 0.14 fold in 0.01 MOI (Fig. 5). In normalization to GAPDH reference gene, *Bak* gene expression was upregulated, whereas the gene was downregulated according to Beta actin normalization. These data indicates that SBV infection has minor effect on *Bak* gene. Although certain viruses can affect or interact with Bak [32, 35], there is no study that investigate whether SBV interact with Bak protein in cellular level. *Bax* gene expression level was found to be increased in canine distemper virus infected dog's cerebellum as approximately 9.8 fold [8]. According to GAPDH normalization in this study *Bax* gene were increased approximately 1.47 fold in 0.1 MOI and 3.02 fold in 0.01 MOI of SBV in 48 hr pi in Vero cells (Fig. 4). *Bax* gene was upregulated as 0.39 fold in 0.1 MOI and 0.30 fold in 0.01 MOI in 48 hr pi of SBV infection when expression level was normalized to Beta actin (Fig. 5).

Bcl-2 gene which is an anti-apoptotic gene blocks apoptosis induction in healthy cells. When apoptosis is triggered in cells *Bcl-2* gene expression starts to decrease. Bcl-2/Bax rate in West Nile virus infected Neuro2a cells were detected as 3.50 in 0 hr and 0.40 in 6 hr pi [20]. In this study Bcl-2/Bax rate was 0.82 in 2 hr pi and 0.19 in 48 hr pi in 0.1 MOI of SBV, whereas 0.36 in 2 hr pi and decreasing to 0.16 in 48 hr pi in 0.01 MOI, according to both normalization with GAPDH and Beta actin, indicating that SBV induce apoptosis by affecting apoptosis related genes. Another anti-apoptotic gene *Bcl-XL* inhibits mitochondrial permeabilization in cells and prevents apoptosis. Bcl-XL/Bax rate in 0.1 MOI of SBV infected Vero cells was 0.86 in 2 hr pi and 0.88 in 48 hr pi. For 0.01 MOI, Bcl-XL/Bax rate was 0.48 in 2 hr pi and decreasing to 0.45 in 48 hr pi. Bcl-XL/Bax rate was same with both GAPDH and Beta actin normalizations and results indicated that SBV does not affect Bcl-XL/Bax rate significantly.

Puma is pro-apoptotic gene which has critical role on apoptosis induced by wide range of stimuli such as toxins, endoplasmic reticulum stress, ischemia and infections. CCHV causes upregulation of *Puma* gene in Huh7 cell line at both 1 and 0.1 MOI [24].

Fig. 5. Real-time PCR results of SBV infected Vero cells. Samples were collected in 0, 2, 6, 12, 18, 24, 36, 48 hr post-infection. Relative expression analyses of *Bax*, *Bak*, *Bcl-2*, *Bcl-XL* and *Puma* apoptosis-related genes of Vero cells infected with 0.1 MOI and 0.01 MOI of SBV are normalized to *Beta actin* reference gene. Asterisks indicate statistically significance ($P<0.01$) between 0.1 MOI and 0.01 MOI groups of SBV infected cells. M stands for mock infected cells.

Puma is found to be induced in HIV envelope transfected HeLa cells [21]. In present study *Puma* is upregulated in Vero cells at the highest level in comparison to other pro-apoptotic genes investigated (Figs. 4, and 5). In GAPDH normalization statistically significance ($P<0.01$) is determined for 0.1 MOI and 0.01 MOI infected cells in comparison to mock infected group at 48 hr pi and 36 hr pi, respectively, for *Puma* (Fig. 4). The results of Beta actin normalization indicated that the cells at 18 and 48 hr pi of 0.1 MOI and the cells at 2, 6, 18, 24, 36, and 48 hr pi of 0.01 MOI have shown statistically significance ($P<0.01$) when compared to mock infected group, causing upregulation of *Puma* gene in Vero cells (Fig. 5). These results are thought to be related with apoptosis of Vero cells by SBV could be induced dominantly via *Puma* gene, as given that Rift Valley fever virus (RVFV), another member of *Peribunyaviridae*, is shown to use p53 pathways and cause upregulation of *Puma* [3]. Activation and/or utilization of p53 pathways upon SBV infection still remain unclear.

This is the first detailed study of apoptosis induction in Vero cells caused by SBV infection. Results of this study showed that SBV induce apoptosis via both intrinsic and extrinsic pathways and moreover intrinsic pathway induction could be modulated over *Puma* gene. To understand the whole molecular mechanisms used by SBV in both Vero and other cell lines, more detailed studies should be performed in future.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

REFERENCES

1. Abraham, R., Mudaliar, P., Padmanabhan, A. and Sreekumar, E. 2013. Induction of cytopathogenicity in human glioblastoma cells by chikungunya virus. *PLoS One* **8**: e75854. [Medline] [CrossRef]

2. Ashkenazi, A. and Dixit, V. M. 1998. Death receptors: signaling and modulation. *Science* **281**: 1305–1308. [Medline] [CrossRef]
3. Austin, D., Baer, A., Lundberg, L., Shafagati, N., Schoonmaker, A., Narayanan, A., Popova, T., Panthier, J. J., Kashanchi, F., Bailey, C. and Kehn-Hall, K. 2012. p53 Activation following Rift Valley fever virus infection contributes to cell death and viral production. *PLoS One* **7**: e36327. [Medline] [CrossRef]
4. Azkur, A. K., Albayrak, H., Risvanli, A., Pestil, Z., Ozan, E., Yılmaz, O., Tonbak, S., Cavunt, A., Kadı, H., Macun, H. C., Acar, D., Özenç, E., Alparslan, S. and Bulut, H. 2013. Antibodies to Schmallenberg virus in domestic livestock in Turkey. *Trop. Anim. Health Prod.* **45**: 1825–1828. [Medline] [CrossRef]
5. Banadyga, L., Veugelers, K., Campbell, S. and Barry, M. 2009. The fowlpox virus BCL-2 homologue, FPV039, interacts with activated Bax and a discrete subset of BH3-only proteins to inhibit apoptosis. *J. Virol.* **83**: 7085–7098. [Medline] [CrossRef]
6. Barnwal, B., Karlberg, H., Mirazimi, A. and Tan, Y. J. 2016. The Non-structural Protein of Crimean-Congo Hemorrhagic Fever Virus Disrupts the Mitochondrial Membrane Potential and Induces Apoptosis. *J. Biol. Chem.* **291**: 582–592. [Medline] [CrossRef]
7. Barry, G., Varela, M., Ratinier, M., Blomström, A. L., Caporale, M., Seehusen, F., Hahn, K., Schnettler, E., Baumgärtner, W., Kohl, A. and Palmarini, M. 2014. NSs protein of Schmallenberg virus counteracts the antiviral response of the cell by inhibiting its transcriptional machinery. *J. Gen. Virol.* **95**: 1640–1646. [Medline] [CrossRef]
8. Del Puerto, H. L., Martins, A. S., Moro, L., Milsted, A., Alves, F., Braz, G. F. and Vasconcelos, A. C. 2010. Caspase-3/-8/-9, Bax and Bcl-2 expression in the cerebellum, lymph nodes and leukocytes of dogs naturally infected with canine distemper virus. *Genet. Mol. Res.* **9**: 151–161. [Medline] [CrossRef]
9. Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35**: 495–516. [Medline] [CrossRef]
10. Herder, V., Wohlsein, P., Peters, M., Hansmann, F. and Baumgärtner, W. 2012. Salient lesions in domestic ruminants infected with the emerging so-called Schmallenberg virus in Germany. *Vet. Pathol.* **49**: 588–591. [Medline] [CrossRef]
11. Hoffmann, B., Scheuch, M., Höper, D., Jungblut, R., Holsteg, M., Schirrmeier, H., Eschbaumer, M., Goller, K. V., Wernike, K., Fischer, M., Breithaupt, A., Mettenleiter, T. C. and Beer, M. 2012. Novel orthobunyavirus in cattle, Europe, 2011. *Emerg. Infect. Dis.* **18**: 469–472. [Medline] [CrossRef]
12. ICTV 2017. Virus Taxonomy: 2017 Release. Virus Taxon. 2017 Release.
13. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green, D. R., Hengartner, M., Knight, R. A., Kumar, S., Lipton, S. A., Malorni, W., Nuñez, G., Peter, M. E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino G., Nomenclature Committee on Cell Death 2009 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ.* **16**: 3–11. [Medline] [CrossRef]
14. Ladelfa, M. F., Kotsias, F., Del Médico Zajac, M. P., Van den Broeke, C., Favoreel, H., Romera, S. A. and Calamante, G. 2011. Effect of the US3 protein of bovine herpesvirus 5 on the actin cytoskeleton and apoptosis. *Vet. Microbiol.* **153**: 361–366. [Medline] [CrossRef]
15. Li, J. and Yuan, J. 2008. Caspases in apoptosis and beyond. *Oncogene* **27**: 6194–6206. [Medline] [CrossRef]
16. Lim, S. I., Kweon, C. H., Yang, D. K., Tark, D. S. and Kweon, J. H. 2005. Apoptosis in Vero cells infected with Akabane, Aino and Chuzan virus. *J. Vet. Sci.* **6**: 251–254. [Medline]
17. Mansfield, K. L., La Rocca, S. A., Khatri, M., Johnson, N., Steinbach, F. and Fooks, A. R. 2013. Detection of Schmallenberg virus serum neutralising antibodies. *J. Virol. Methods* **188**: 139–144. [Medline] [CrossRef]
18. Nagaleekar, V. K., Tiwari, A. K., Kataria, R. S., Bais, M. V., Ravindra, P. V. and Kumar, S. 2007. Bluetongue virus induces apoptosis in cultured mammalian cells by both caspase-dependent extrinsic and intrinsic apoptotic pathways. *Arch. Virol.* **152**: 1751–1756. [Medline] [CrossRef]
19. Nogal, M. L., González de Buitrago, G., Rodríguez, C., Cubelos, B., Carrascosa, A. L., Salas, M. L. and Revilla, Y. 2001. African swine fever virus IAP homologue inhibits caspase activation and promotes cell survival in mammalian cells. *J. Virol.* **75**: 2535–2543. [Medline] [CrossRef]
20. Parquet, M. C., Kumatori, A., Hasebe, F., Morita, K. and Igarashi, A. 2001. West Nile virus-induced bax-dependent apoptosis. *FEBS Lett.* **500**: 17–24. [Medline] [CrossRef]
21. Perfettini, J. L., Roumier, T., Castedo, M., Larochette, N., Boya, P., Raynal, B., Lazar, V., Ciccosanti, F., Nardacci, R., Penninger, J., Piacentini, M. and Kroemer, G. 2004. NF-kappaB and p53 are the dominant apoptosis-inducing transcription factors elicited by the HIV-1 envelope. *J. Exp. Med.* **199**: 629–640. [Medline] [CrossRef]
22. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**: e45. [Medline] [CrossRef]
23. Rasmussen, L. D., Kristensen, B., Kirkeby, C., Rasmussen, T. B., Belsham, G. J., Bødker, R. and Bøtner, A. 2012. Culicoids as vectors of Schmallenberg virus. *Emerg. Infect. Dis.* **18**: 1204–1206. [Medline] [CrossRef]
24. Rodrigues, R., Paranhos-Baccalà, G., Vernet, G. and Peyrefitte, C. N. 2012. Crimean-Congo hemorrhagic fever virus-infected hepatocytes induce ER-stress and apoptosis crosstalk. *PLoS One* **7**: e29712. [Medline] [CrossRef]
25. Saxena, L., Kumar, G. R., Saxena, S., Chaturvedi, U., Sahoo, A. P., Singh, L. V., Santra, L., Palia, S. K., Desai, G. S. and Tiwari, A. K. 2013. Apoptosis induced by NS1 gene of Canine Parvovirus-2 is caspase dependent and p53 independent. *Virus Res.* **173**: 426–430. [Medline] [CrossRef]
26. Schmittgen, T. D. and Livak, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**: 1101–1108. [Medline] [CrossRef]
27. Segawa, K. and Nagata, S. 2015. An apoptotic 'Eat Me' signal: Phosphatidylserine exposure. *Trends Cell Biol.* **25**: 639–650. [Medline] [CrossRef]
28. Shahsavandi, S., Ebrahimi, M. M., Sadeghi, K., Mosavi, S. Z. and Mohammadi, A. 2013. Dose- and time-dependent apoptosis induced by avian H9N2 influenza virus in human cells. *BioMed Res. Int.* **2013**: 524165. [Medline] [CrossRef]
29. Shamas-Din, A., Kale, J., Leber, B. and Andrews, D. W. 2013. Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harb. Perspect. Biol.* **5**: a008714. [Medline] [CrossRef]
30. Tonbak, S., Azkur, A. K., Pestil, Z., Biyikli, E., Abayli, H., Baydar, E., van der Poel, W. H. M. and Bulut, H. 2016. Circulation of Schmallenberg virus in Turkey, 2013. *Turk. J. Vet. Anim. Sci.* **40**: 175–180. [CrossRef]
31. Van Der Poel, W. H. M., Parlevliet, J. M., Verstraten, E. R. A. M., Kooi, E. A., Hakze-Van Der Honing, R. and Stockhofe, N. 2014. Schmallenberg virus detection in bovine semen after experimental infection of bulls. *Epidemiol. Infect.* **142**: 1495–1500. [Medline] [CrossRef]
32. Underbrink, M. P., Dupuis, C., Wang, J. and Tyring, S. K. 2016. E6 proteins from low-risk human papillomavirus types 6 and 11 are able to protect keratinocytes from apoptosis via Bak degradation. *J. Gen. Virol.* **97**: 715–724. [Medline] [CrossRef]
33. Wlodkowic, D., Skommer, J. and Darzynkiewicz, Z. 2009. Flow cytometry-based apoptosis detection. *Methods Mol. Biol.* **559**: 19–32. [Medline] [CrossRef]
34. Zekri, A. R. N., Bahnassy, A. A., Hafez, M. M., Hassan, Z. K., Kamel, M., Loutfy, S. A., Sherif, G. M., El-Zayadi, A. R. and Daoud, S. S. 2011. Characterization of chronic HCV infection-induced apoptosis. *Comp. Hepatol.* **10**: 4. [Medline] [CrossRef]
35. Zhong, Y., Liao, Y., Fang, S., Tam, J. P. and Liu, D. X. 2012. Up-regulation of Mcl-1 and Bak by coronavirus infection of human, avian and animal cells modulates apoptosis and viral replication. *PLoS One* **7**: e30191. [Medline] [CrossRef]