Newcastle disease virus-induced caspase-independent apoptosis pathway in BHK-21 cells

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Research

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Newcastle disease virus-induced caspase-independent apoptosis pathway in BHK-21 cells

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27 **Abstract**

28 **Background:** Newcastle disease virus (NDV) is an important virus for
29 humans. It is highly lethal in fowl and is newly identified as an oncolytic
30 virus for cancer treatment. *In vivo,* NDV induces spleen, thymus, bursa,
31 and mesenteric gland cell apoptosis, thereby causing immunosuppression.
32 *In vitro*, NDV can induce apoptosis by caspase-dependent pathways
33 including the mitochondria-mediated pathway and death
34 receptor-mediated pathway.

35 **Methods:** In this study, the major materials were baby hamster kidney
36 (BHK-21) cells, NDV virus (*Miyadera* strain; $10^{3.8}$ $TCID_{50}$/μl), and
37 pan-caspase inhibiter Z-Val-Ala-DL-Asp-fluoromethylketone
38 (z-VAD-fmk). All of the experiments used a viral infection MOI of 1.
39 Apoptosis was confirmed using DNA fragmentation and TUNEL assay.
40 Finally, the apoptosis-independent pathway was confirmed by western
41 blot analysis and immunofluorescence.

42 **Results:** In this study, we differentiate between the caspase group and
43 caspase inhibition group (added 100 μM pan-caspase inhibitor
44 [z-VAD-fmk]) in BHK-21 cells treated with NDV at 0, 12, and 24 h. In

the DNA fragmentation and TUNEL assays, the apoptosis appears at 12 h and apoptosis increases over time, regardless of caspase or not. In protein level determination, the antiapoptotic protein Bcl-2 decreased over time, which is the opposite of how the proapoptotic proteins Bax, cytochrome C, Mst3, and AIF behaved. Further, using western blot and immunofluorescent staining, we checked the AIF and Endo G translocation from the mitochondria (cytoplasmic) to the nucleus. In addition to apoptosis, we found that NDV treatment of BHK-21 cells decreased actin, regardless of caspase. The actin always decreased in the NDV-treated BHK-21 cells at 12 and 24 h.

Conclusions: NDV-mediated BHK-21 cell apoptosis mechanisms involve complex pathway; when in the normal state, the caspase-dependent pathway is main apoptosis pathway; when the caspase is suppressed, the BHK-21 can switch on the caspase-independent pathway by AIF, Endo G, or Mst3 to allow apoptosis to continue.

Keywords: *Newcastle disease virus, Apoptosis, Caspase-independent pathway*

Background

Newcastle disease virus (NDV) is a single-stranded, negative-sense, non-segmented RNA genome of the genus *Avulavirus* in the family *Paramyxoviridae* [1]. It is virus with great significance; it has caused more outbreaks than any other virus and has a high lethality in fowl worldwide. However, the virus' oncolytic efficacy is emerging as a novel cancer treatment and may give NDV a new research direction [2-4]. NDV was first used to treat cancer in in the early 1950's; NDV treatment induced tumor apoptosis or partial necrosis and sloughing in uterine carcinoma [5]. In 2013, Shobana et al. altered the viral fusion protein for exclusive cleavage by a prostate-specific antigen, making it the target of a tumor-specific signal for induction of apoptosis [6].

NDV *in vivo* can induce spleen, thymus, bursa, and mesenteric glands cell apoptosis for immunosuppression [7-9]. Most studies that have focused on apoptosis have determined whether or not it occurred, without further study of the *in vivo* mechanism. In this study, NDV has been discovered as a good oncolytic virus. NDV safely and effectively infects and kills

cancer cells. NDV is considered to be a good potential oncolytic agent in a clinical setting and is an experimental oncolytic agent [10]. Researchers have change NDV in vitro to target cancer cell specific markers and were confirmed in HT1080, KHOS, KB8-5011, HCV29T, IMR32, and G104 cell lines to cause cell apoptosis and lack of apoptosis in normal cells [5, 11, 12].

Apoptosis is a programmed multistep cell death that is intrinsic in all cells of the body; it is a necessary biological defense mechanism. Apoptosis has two pathways: a cysteine-aspartyl specific protease activation pathway (caspase-dependent) and an inactivated-caspase pathway (caspase-independent) [13]. Most apoptosis occurs because of multiple pathway interactions those that utilize a receptor, enzyme, regulatory protein, and signal transduction molecules. In the apoptosis research, the different cell induce cell death program are difference [14]. The viruses that induce apoptosis have two types. Direct pathways include the caspase-dependent pathway, mitochondria-mediated pathway, death receptor-mediated pathway, endoplasmic reticulum stress pathway, and

mitogen-activated protein kinases pathways. Indirect pathways utilize secretion of proinflammatory cytokines and chemokines and induction of the innate and acquired immune responses. The natural killer cells, lymphocytes, and macrophages are then stimulated to remove cells from the diseased area [15].

In 2006, Elankumaran et al. showed that NDV can use a caspase-dependent pathway for 16 different types of cell apoptosis, the major pathways being the mitochondria-mediated and death receptor-mediated pathway. Interestingly, when caspase is inhibited and cells are treated with NDV, apoptosis can still occur. It appears that NDV can induce apoptosis through a caspase-independent pathway [16]. After this, the other apoptosis pathways investigated were p53, Bax, and p3 MAPK during 2009 to 2011 [17-20]. However, no other signals from NDV induce apoptosis a caspase-independent pathway in the cell. Therefore, we wanted to examine the mechanism by which NDV induces cell apoptosis through a caspase-independent pathway and identify the proteins involved in it.

Methods

Cells and virus

The BHK-21 (baby hamster kidney) cell line was grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and streptomycin at 37°C in a 5% CO_2 humidified incubator. NDV was isolated from a wild strain; after sequencing, it was determined to be *Miyadera* (gbjM 18456.1) and was then amplified. The final sample was quantified at a concentration of $10^{3.8}TCID_{50}/\mu l$, and the virus was stored at −80°C for further use. The samples were divided into two groups for this research; the normal NDV infection group (caspase group) and the caspase inhibition group. The caspase inhibition group was treated with 100μM pan-caspase inhibiter z-VAD-fmk (Z-Val-Ala-DL-Asp-fluoromethylketone) in the culture medium 1 h before virus treatment.

DNA fragmentation

The BHK-21 cells at a concentration of 2×10^6 were infected with an NDV MOI of 1. The samples were separated as caspase and caspase

135 inhibition groups. After the 0, 12, and 24 h of incubation, the detached
136 cells in the medium were collected. The pellet was washed with PBS
137 twice, and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 20 mM
138 EDTA, and 1% Nonidet P-40). The supernatant was collected and
139 incubated with RNaseA at a final concentration 5μg/μl for 2 h at 56°C.
140 Proteinase K was added to a final concentration of 2.5 μg/μl, and the
141 mixture was incubated at 37°C for 2 h. Furthermore, 3 M acetic acid (10
142 μl), 20 μl saturated sucrose, and cold 100% ethanol at a 2.5x volume was
143 added, and the sample was stored at −80°C overnight. The sample pellet
144 was collected and washed with cold 80% ethanol. The samples were dried
145 at room temperature for 10 min, dissolved in deionization and distilled
146 water, and diluted to a concentration of 5 μg per well. Finally,
147 electrophoresis was performed using 2% agarose gel.
148
149 *TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end*
150 *labeling assay)*
151 The BHK-21 cells were diluted at a concentration of 3×10^5 and infected
152 with NDV MOI of 1. The sample was separated into the caspase and

153 caspase inhibitor groups. After the 0, 12, and 24 h of incubation at 37°C
154 in an atmosphere of 5% CO_2, the TUNEL assay was carried out using a
155 FragELTM DNAFragmentation Detection Kit (CALBIOCHEM,
156 QIA33-1EA). The cover slip was sealed with 40% glycerol, and
157 observation and counting were done under a microscope.
158
159 *Western blot analysis*
160 The western blot sample was applied to the two groups of caspase and
161 caspase inhibition at 0, 12, and 24 h. In addition, the samples were
162 differentiated between groups using total protein, cytoplasm protein, and
163 nuclear protein groups at 0 and 24 h. The BHK-21 cells at a concentration
164 of 2×10^6 were infected with an NDV MOI of 1. The cytoplasm protein
165 was extracted using buffer I (10 mM HEPES, 1.5 mM MgCl2, 10 mM
166 KCl, 0.5 mM OTT, 0.05% NP-40, prepared at pH 7.9) and the other
167 groups were extracted using RIPA lysis buffer. All samples were
168 quantified using a Bio-Rad protein assay reagent with a protein of a
169 uniform weight of 50 μg. All samples were separated by 12% SDS-PAGE
170 and transferred onto a PVDF membrane (Millipore, USA). The

171 membranes were blocked and incubated with antibody with NET buffer
172 (Gelatin 2.5 g, NaCl8.75 g, DETA·2Na 1.8 g, Tris 6.05 g, Tween 20 0.5
173 ml in 1 L water, pH8.0). The primary antibody was incubated overnight
174 at 4°C with α-tubulin (SC8035, Santa Cruz Bioechnology, CA), actin
175 (MAB1501, Millipore, USA), AIF (SC1946, Santa Cruz Bioechnology,
176 CA), Bax (SC493, Santa Cruz Bioechnology, CA), Bcl-2 (SC7382, Santa
177 Cruz Bioechnology, CA), cleaved caspase-3 (SC22171, Santa Cruz
178 Bioechnology, CA), cytochrome C (SC7159, Santa Cruz Bioechnology,
179 CA), Endo G (SC26923, Santa Cruz Bioechnology, CA), GADPH
180 (ab9483, Abcam®, UK), lamin B (SC6216, Santa Cruz Bioechnology,
181 CA), Mst3 (SC21400, Santa Cruz Bioechnology, CA), and NDV
182 (ab138719, Abcam®, UK). After washing, horseradish peroxidase
183 (HRP)-conjugated secondary antibodies were added and the abundance of
184 proteins was quantified via chemiluminescence assay using an ECL kit
185 (WBULS0500, Millipore, USA). The image was acquired using an
186 illumination imaging system (UVP, Upland, CA, USA) and the amount
187 of proteins was estimated via densitometric analysis using the
188 Bio-imaging camera system. The quantity of each protein was calculated

189 according to the GAPDH detected in each sample. The relative
190 abundance of each protein was determined by dividing the arbitrary unit
191 over that of their 0 h protein.
192
193 *Immunofluorescence*
194 The BHK-21 cells at a concentration of 3×10^5 and infected with an
195 NDV MOI of 1. The cells were challenged with NDV at 0 and 24 h, the
196 caspase and caspase inhibitor groups were incubated separately. The
197 sample was added to Mitotracker (Invitrogen, M-7512) (50 μg/mL) and
198 incubated at 37°C for 30 min, after which the medium was removed and
199 the cover slip was washed with cold PBST. After that, the cells were
200 fixed with 3.7% ice-cold paraformaldehyde for 10 min. Following, the
201 slip washed three times with cold PBST. The sample was added to the
202 primary antibodies (goat anti-AIF and goat anti-Endo G Santa Cruz, USA;
203 chicken anti-ND Abcam®, UK) in PBST for at least 4 h at room
204 temperature. After washing, the cells were incubated with
205 FITC-conjugated donkey anti-goat IgG (Santa Cruz, USA) or goat
206 anti-chicken IgY (Santa Cruz, USA) in PBST at room temperature for 1 h

207 in the dark. Then, the cells were added to DAPI 1 μg/ μL in PBST at
208 room temperature for 25 min in the dark. Finally, the cells were placed
209 under the cover slip sealed with 40% glycerol, and visualization was
210 carried out using a fluorescence microscope.
211
212 **Results**
213 *Induction of apoptosis by NDV-mediated caspase and*
214 *caspase-independent pathway in BHK-21 cells*
215 The NDV-treated BHK-21 cells had been divided into two groups, one
216 group was directly challenge by NDV, and the other group was treated
217 with pan-caspase inhibiter z-VAD-fmk (final concentration 100 μM) to
218 inhibit caspase-dependent pathway apoptosis before challenged with
219 NDV. Cell apoptosis was induced by a caspase-dependent pathway with
220 activation of caspase-3 by entering the nuclei; the caspase protein will cut
221 the DNA according to nucleosome nucleotides length, causing DNA
222 fragmentation. In Figure 1a, the caspase group indicated that the caspase
223 family protein cut the DNA according to nucleosome nucleotides length
224 that became the DNA markers in the 2% agarose gel. The caspase

225 inhibition group did not take the shape of DNA ladders after NDV
226 infection, as the caspase group did (Figure 1b). The rate of cell apoptosis
227 and cells fusion increased as time passed. The ratios of apoptosis of the
228 caspase group from 0, 12, and 24 h were 6.50 ± 1.69%, 29.59 ± 3.52%,
229 and 62.48 ± 3.69%, respectively. On the other hand, for the caspase
230 inhibitor group, the apoptosis ratios from 0, 12, and 24 h were 5.94 ±
231 0.75%, 22.92 ± 1.66%, 32.57 ± 1.31%, respectively. The data are shown
232 in Figure 3a–g. In addition, we observed that NDV infection increased
233 cell fusion depending on the infection time. The ratios of cell fusion of
234 caspase group at 0, 12, and 24 h were 0 ± 0%, 7.15 ± 1.32%, and 35.81 ±
235 3.53%, respectively, and in caspase inhibitor group, the ratios of cell
236 fusion at 0, 12, and 24 h were 0 ± 0%, 4.85 ± 2.77%, and 17.55 ± 1.84%,
237 respectively (Fig 2h).
238
239 *NDV mediates BHK-21 cell apoptosis caspase-dependent and*
240 *-independent pathway.*
241 NDV induced apoptosis via caspase-dependent and caspase-independent
242 pathways in BHK-21 cells. In the two groups, we used 0 h protein as a

starting point to confirm protein expression at 12 and 24 h. The results reveal that the proapoptotic proteins Bax, cytochrome C, Mst3, and AIF were increased over time and the antiapoptotic protein Bcl-2 decreased over time, regardless of the caspase-dependent or -independent pathway. Among them, Endo G was special; it was increased at 24 h in the caspase-dependent pathway and increased over time in the caspase-independent pathway; however, only the cleaved caspase-3 was different. It was inhibited by z-VAD-fmk in the caspase-independent pathway. In addition to apoptosis, we found that NDV-treated BHK-21 cells had less actin, regardless of having caspase or not. The actin always decreased in the NDV-treated BHK-21 cells at 12 and 24 h. This was because we used GAPDH to do protein internal control, not used actin. The western blot data were shown in Figure. 3.

In apoptosis caspase-independent pathway, Mst3, AIF, and Endo G are transferred from the mitochondria to the nucleus

In Figure 4a and 4b, the sample was separated it two groups; one was caspase apoptosis group that had 0 h total protein (0 h T), 0 h cytoplasmic

protein (0 h C), 0 h nucleus protein (0 h N), 24 h total protein (24 h T), 24 h cytoplasmic protein (24 h C), and 24 h nucleus protein (24 h N). The other group were caspase inhibited group that had same protein samples. The total protein internal control was actin, the cytoplasmic protein sample was α-tubulin, and the nucleus protein sample was lamin B. In Figure 4a, we confirm the transfer of Mst3, AIF, and Endo G from cytoplasm to the nucleus; Figure 4b shows that the caspase inhibition group was similar to the caspase group in terms of current protein expression. After that, we showed the image data in immunofluorescent staining in Figure 5a and 5b. In Figure 5a and 5b, the green color is target protein (FITC), red color is mitochondria (Mitoracker), and blue marks the nucleus (DAPI). The FITC three different target proteins were AIF, Endo G, and NDV. In Figure 5a and 5b, the 0 h, all samples show the protein of AIF and Endo G in cytoplasmic, and the color overlaps appear yellow. AIF and Endo G are transferred from the mitochondria to the nucleus 24 h after NDV infection. NDV replicated in cell cytoplasm and induced cell aggregation and fusion.

Discussion

NDV infect BHK-21 for 24 h caused growth inhibition in MMT assay. At 36 h, the MTT illustrated negative growth but this cannot explain how NDV can induce BHK-21 cell apoptosis, let alone through an apoptotic pathway. The findings must be confirmed by more specific experiments, like DNA fragmentation and TUNEL assay. When caspase-dependent pathway would be inhibited, NDV can induce BHK-21 apoptosis by caspase-independent pathway (Figure 3). In the tests, we used NDV added to BHK-21 cell before treatment with caspase inhibitor z-VAD-fmk to control caspase-dependent or independent pathway. NDV induced BHK-21 apoptosis via caspase-dependent and caspase-independent pathways (Figure 3). This result is the same as that obtained with THP-1, leukemia cell lines, HL cells, and HEG2 cell lines treated with NDV [15, 16].

Studies on the pathways of apoptosis induction in specific cells by NDV can cause different cells to undergo apoptosis in different pathways. Most NDV apoptosis papers showed that both the extrinsic and intrinsic pathways are activated in NDV-induced apoptosis. In 2011 the paper

297 confirmed that PC12 rat pheochromocytoma cell mitogen-activated
298 protein kinase pathway was not involved in NDV-induced apoptosis, but
299 in A549 human lung cancer, can induce apoptosis using the p38 and
300 MAPK pathway, in addition, the endoplasmic reticulum apoptosis
301 pathway no effect [17, 20]. When NDV induces cell apoptosis, Bax
302 transfers from cytoplasm to mitochondria and cytochrome C transfers
303 from mitochondria to cytoplasm. But Bcl-2 of protein expression is not
304 any increase and decrease in NDV-induced apoptosis. If the caspase
305 would be suppressed, the cell would be induced caspase-independent
306 apoptosis pathway [16, 18, 21]. In summary, NDV-induced apoptosis has
307 two mechanisms; one is NDV has different apoptosis pathways induced
308 by different cells, and there is no absolute path followed. The other is
309 NDV-induced apoptosis is a complex multiple pathway response. Our
310 study showed that cytochrome C, AIF, Mst3, and Endo G were important
311 because their proteins express increasingly form 0 to 24 h. Bax in
312 caspase-dependent or independent pathway is increase protein expression,
313 but the Bcl-2 is decrease.
314

315 The Bax protein configuration changes and aggregation can promote
316 apoptosis; Bcl-2 can prevent that when the Bcl-2 protein expressed more
317 than Bax and there is no apoptosis [22-25]. His study of NDV-induced
318 HeLa cell apoptosis showed that Bax configuration changed before NDV
319 infected the cell and that the Bax protein changed location and caused
320 cytochrome C from the mitochondria to be released into the cytoplasm;
321 the activity of Bcl-2 remained unchanged [18]. We observed the state of
322 the Bax and the Bcl-2 was different. The Bax continued to increase with
323 or without added inhibitors and Bcl-2 was exactly the opposite in our
324 study. Expression of the other proteins Mst3, AIF, Endo G, and
325 cytochrome C protein increased over time. Bax is an upstream regulatory
326 protein in apoptosis that mainly affects the release of apoptotic proteins in
327 the mitochondria and ultimately promotes the release of Endo G, AIF,
328 and cytochrome C from the mitochondria, in which immunofluorescence
329 staining results show that AIF and Endo G are two apoptotic proteins that
330 are transferred to the nucleus because of NDV infection of BHK-21 cells.
331
332 During NDV infection, the cell surface often accumulates highly fused F

protein implants into infected cells to form a multinucleate state to facilitate virus transmission [26]. This phenomenon can be easily identified using a TUNEL assay and immunofluorescence, and the state of cell fusion has also been shown to effectively enhance NDV-induced cancer cell apoptosis in vitro or in vivo. How did the cell structure change affect our experiment? In the case of BHK-21 infected with NDV, the most special change was actin. Originally in 1977, NDV infection of BHK-21 was mentioned in a paper that actin will change with the infection. The paper uses immunofluorescence light staining to mark actin, and the results showed that actin has an increasing trend after adding virus [27]. However, the results of our experiment show that the actual performance of actin is declining, because the 1977 paper did not use western blotting and other more accurate protein detection methods to confirm; NDV can promote the infection of cells *in vitro* to cause cell fusion. When multiple cells are aggregated into one, the amount of actin should higher than that of a single cell. Actin is one of the cytoskeletal proteins in cells. Actin is involved in the various processes of virus infection, assembly, and release. Among the viruses of the

351 *Paramyxoviridae* family, actin is involved in the viral fusion protein (F
352 protein). In the case of the matrix protein (M protein), when a large
353 amount of F protein appears on the surface of the host cell, it will initiate
354 the cell fusion between cells, and the M protein will transport the F
355 protein and HN protein to the assembly process. The inner side of the cell
356 membrane is fixed, and the fixation process also requires the participation
357 of actin [27-30]. Actin will also be affected by caspase. When the cell
358 breaks down, caspase-3, -6, and -9 can directly cleave poly (ADP-ribose)
359 polymerase-1 (PARP), lamins, CAD, and actin, which may explain the
360 lower actin content over time, coupled with cell fusion and various effects
361 of virus assembly. This results in decreased actin protein content in
362 BHK-21 cells after 24 h following challenge with NDV; the content is
363 reduced to 0.60 times of 0 h.
364
365 **Conclusions**
366 In this study, we show the NDV-mediated BHK-21 cell apoptosis
367 mechanisms involve complex pathway; when in the normal state, the
368 caspase-dependent pathway is main apoptosis pathway; when the caspase
369 is suppressed, the BHK-21 can switch on the caspase-independent

pathway by AIF, Endo G, or Mst3 to allow apoptosis to continue. And actin will also be affected by caspase, which may explain the lower actin content over time.

List of abbreviations

NDV Newcastle disease virus

BHK Baby hamster kidney

z-VAD-fmk Z-Val-Ala-DL- Asp-fluoromethylketone

caspase cysteine aspartyl specific proteases

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All relevant data are within the paper and its Supporting Information files.

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Authors' contributions: Conceptualization: Wang CW, Wu HY; Data curation: Wang CW, Wu HY; Formal analysis: Wang CW, Wu HY , Lin

CY; Funding acquisition: Wu HY; Investigation: Wang CW, Wu HY; Methodology: Wang CW, Shih WL, Wu HY; Project administration: Wang CW, Wu HY; Resources: Wu HY; Software: Wang CW; Supervision: Wang CW, Lin CY, Wu HY; Validation: Wang CW, Thongchan D; Chung SC, Lin TC, Wu HY; Visualization: Wang CW, Wu HY; Writing-original draft: Wang CW, Wu HY; Writing-review & editing: Wang CW, Wu HY

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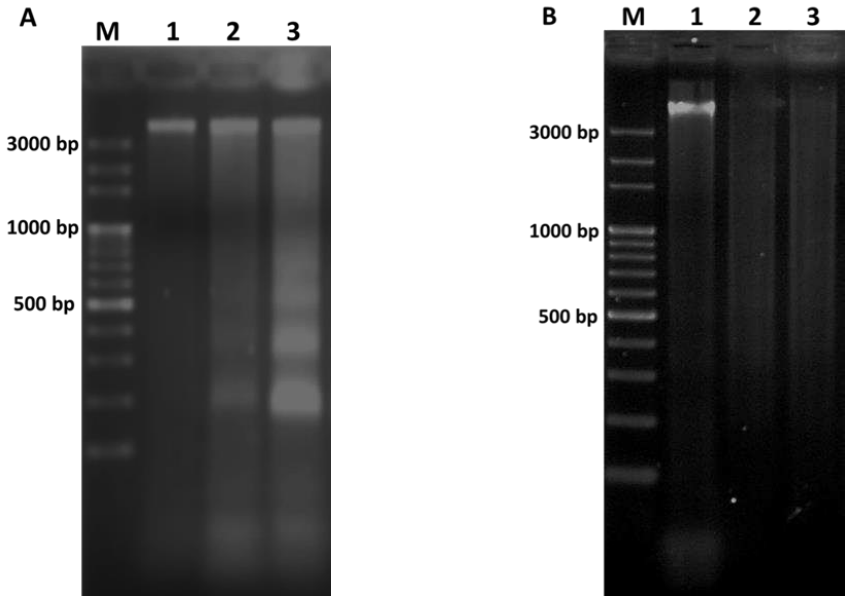

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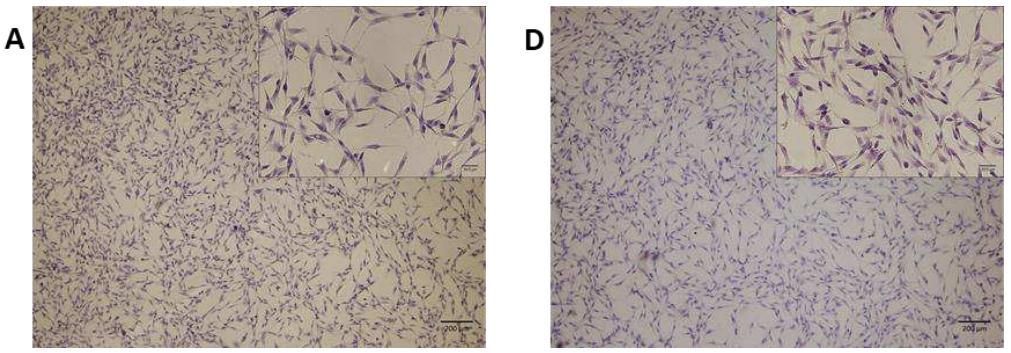

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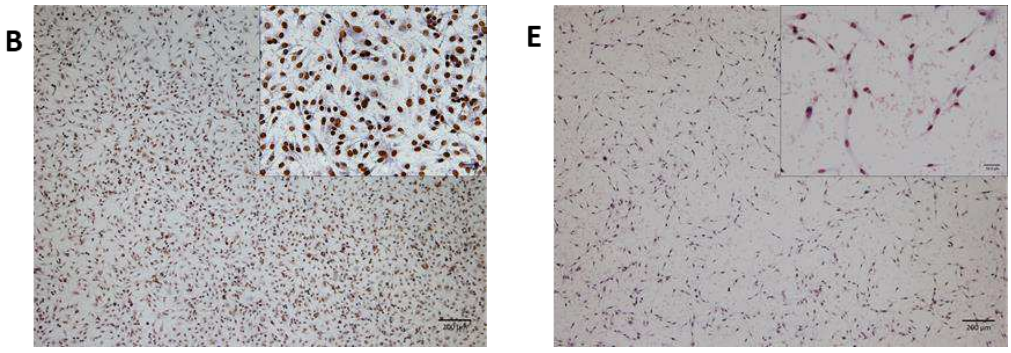

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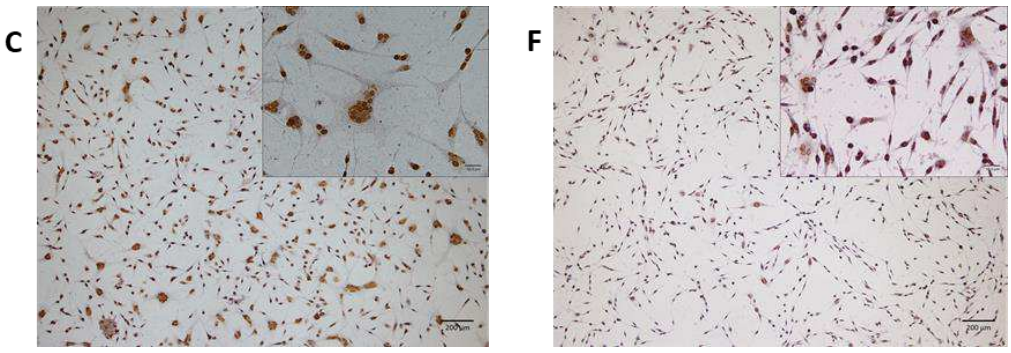

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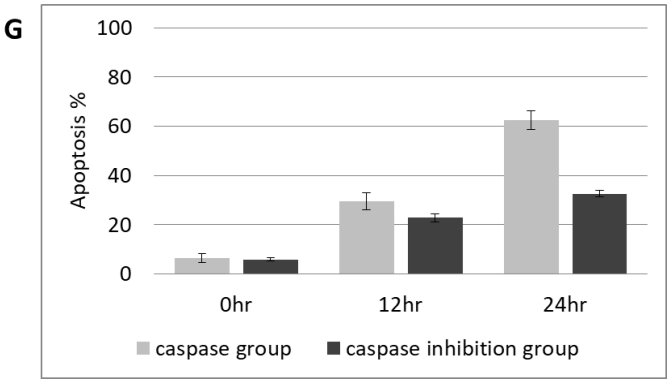

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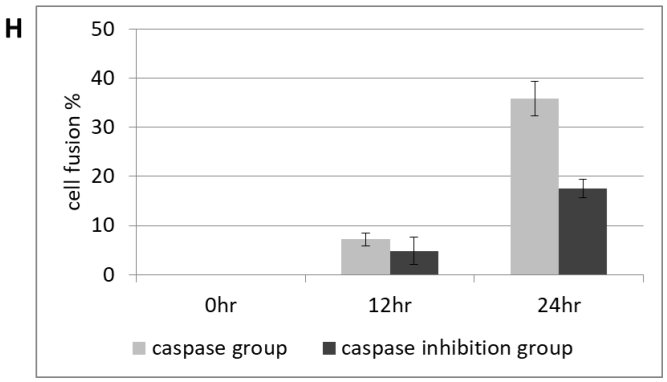
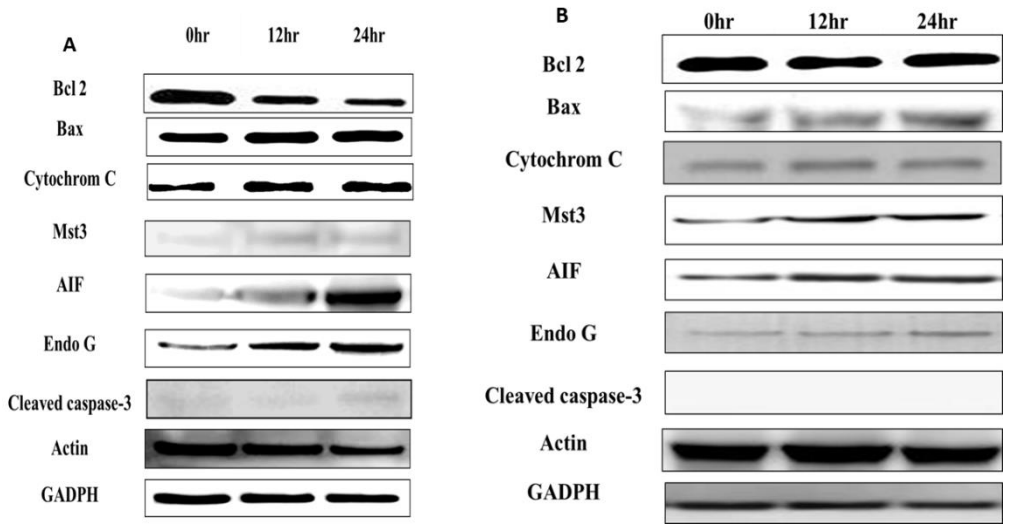
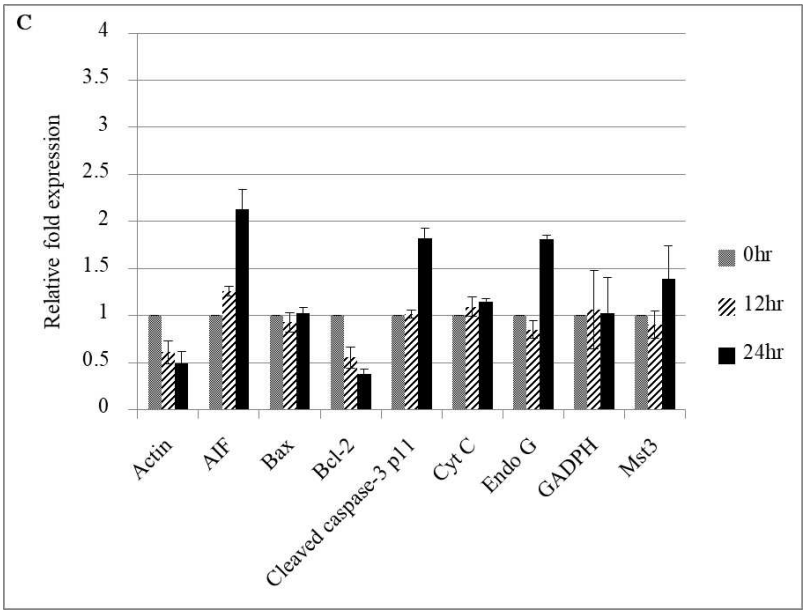


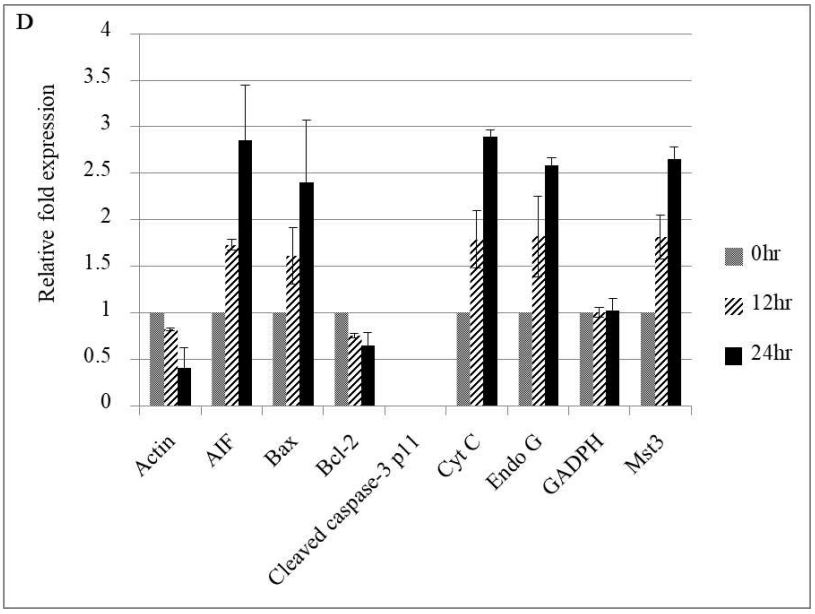
555

556 Figure 2a – f. Microscopic views of two groups of BHK-21 cells after
557 NDV challenged for 0, 12, and 24 h; samples were treated with
558 TdT-FragEL™ DNA Fragmentation Detection Kit, and cells with nuclear
559 showing brownish color are undergoing apoptosis, while the cells with
560 blue nuclear are living cells. 2a–2c are images of the caspase group and
561 2D–2F are images of the caspase inhibition group. Figure 2a – 2f is
562 magnified, scale bar = 200.0 μm (big) and scale bar = 50.0 μm (small).
563 2g and 2h. The trend of apoptosis rate in two groups of BHK-21 cells
564 under different time periods of NDV challenge, the increasing tendency
565 of apoptosis and cell fusion with or without addition of caspase inhibitor
566 z-VAD-fmk is confirmed by TUNEL assay. Figure 2g x-axis represents
567 time after NDV challenge while y-axis is percentage of cells undergoing
568 apoptosis. Results are shown in the form of mean value ± SD for the three
569 experiments. Figure 2h is the x-axis representing time after NDV

challenge while y-axis stands for percentage of cell undergoing fusion. The results are shown in the form of mean value ± SD for the three experiments.







577

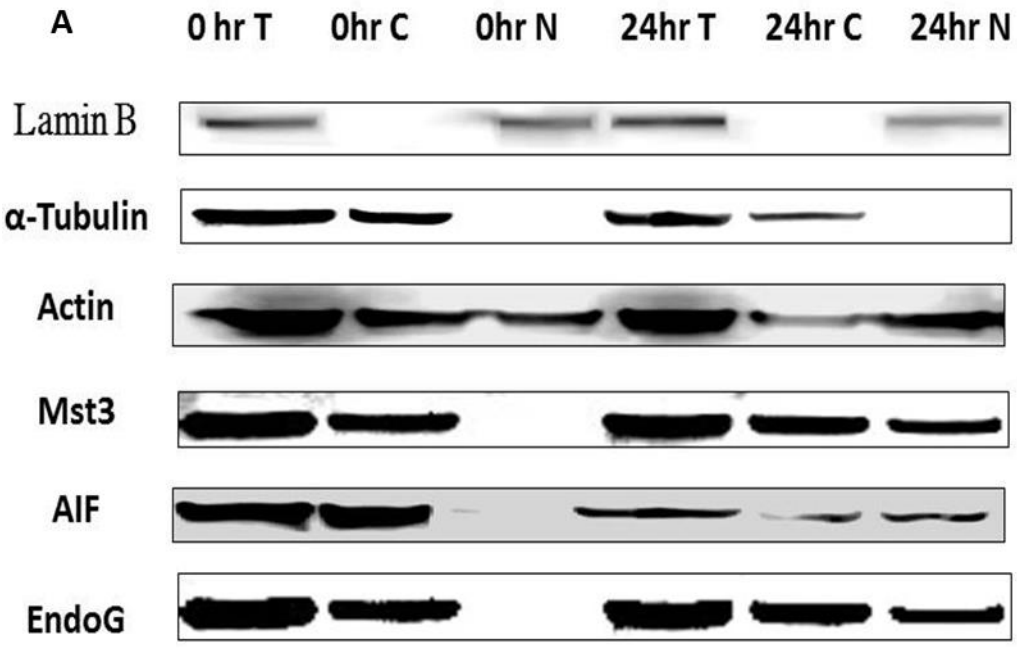
578 Figure 3 Influence of the expression of apoptosis-associated proteins at
579 different times in BHK-21 cells treated with NDV and the caspase
580 inhibitor z-VAD-fmk at the final concentration of 100 μM. The proteins
581 detected were the proapoptotic proteins Bax, cytochrome C, Mst3, AIF,
582 Endo G, and cleaved caspase-3, the antiapoptotic protein Bcl-2, internal
583 control GADPH, and actin.

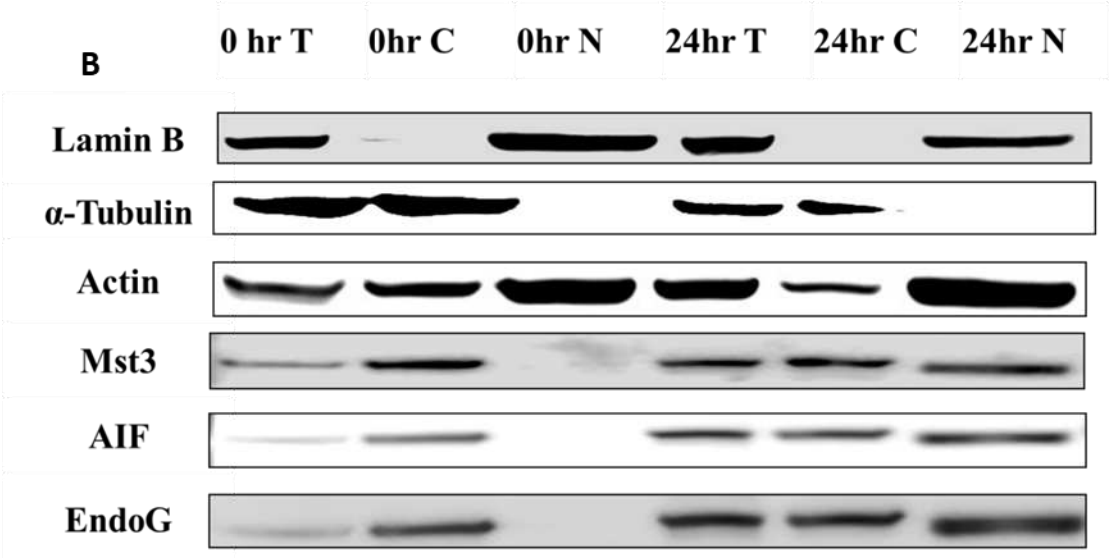
584 (3a) After BHK-21 treatment with NDV for 0, 12, and 24 h, the
585 caspase-dependent apoptosis-associated proteins were expression.

586 (3b) After BHK-21 treatment with NDV for 0, 12, and 24 h, the
587 caspase-independent apoptosis-associated proteins were expression.

588 (3c) The graph represents protein expression in the caspase-dependent
589 group. The x-axis represents apoptosis-associated proteins and the y-axis

represents the relative fold expression, challenge time as 0, 12, and 24 h. (3d) The graph represents protein expression in the caspase inhibition group. The x-axis represents apoptosis-associated proteins and the y-axis represents the relative fold expression, challenge time as 0, 12, and 24 h.





598 Figure 4 The cytoplasm and nucleus are shown separately to depict the
599 changes in the positions of proteins. Lamin B is the internal control for
600 the nuclear fraction, α-tubulin is the internal control for the cytoplasmic
601 fraction, and actin is the internal control for the total cell. T: total protein;
602 C: cytoplasmic protein; N: nucleus protein.
603 (4a) NDV challenged BHK-21 cells at 0 and 24 h (caspase group). Mst3,
604 AIF, and Endo G were transferred from the cytoplasm to the nucleus in
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606 (4b) NDV challenged BHK-21 cells at 0 and 24 h (caspase inhibition
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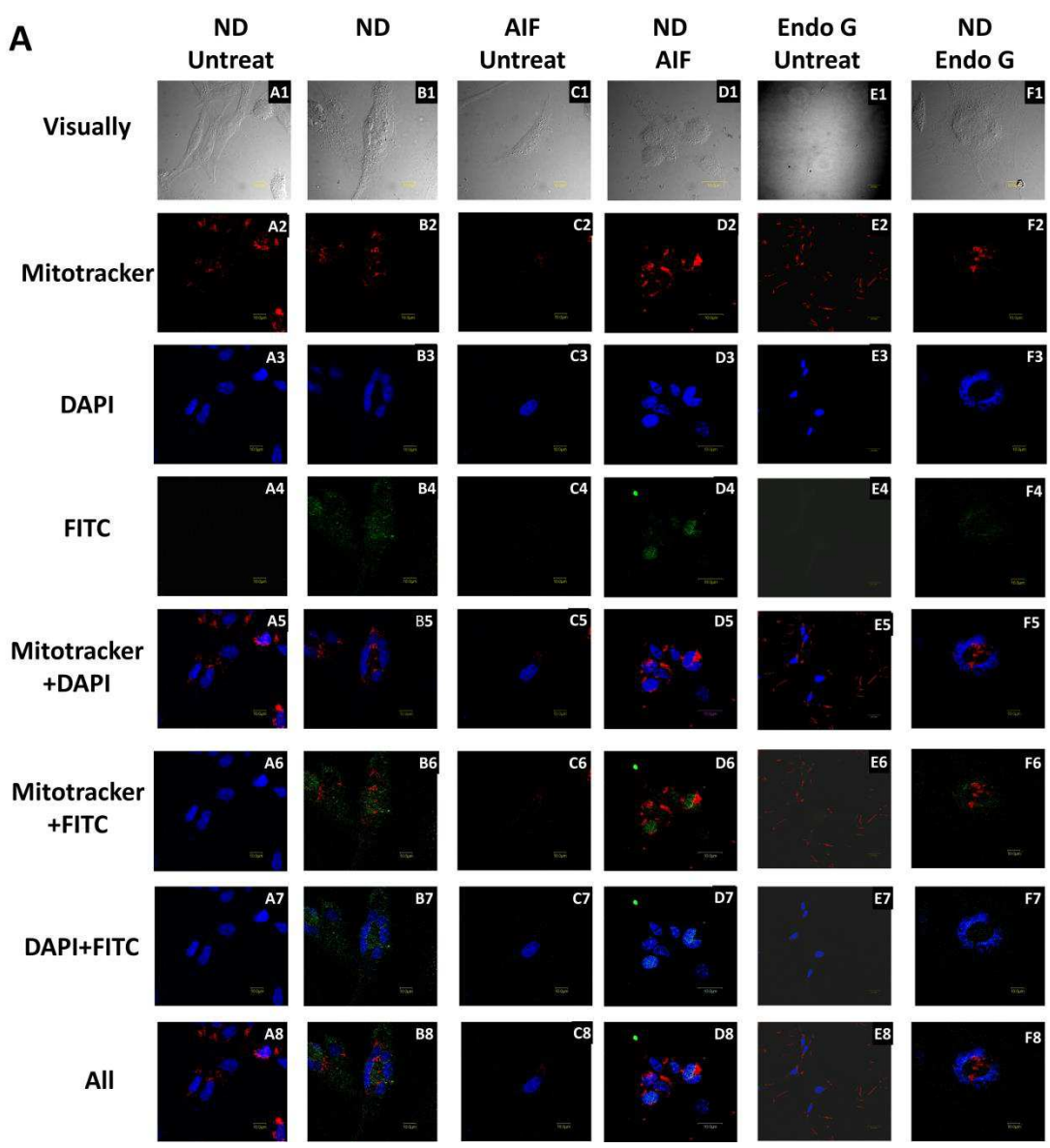
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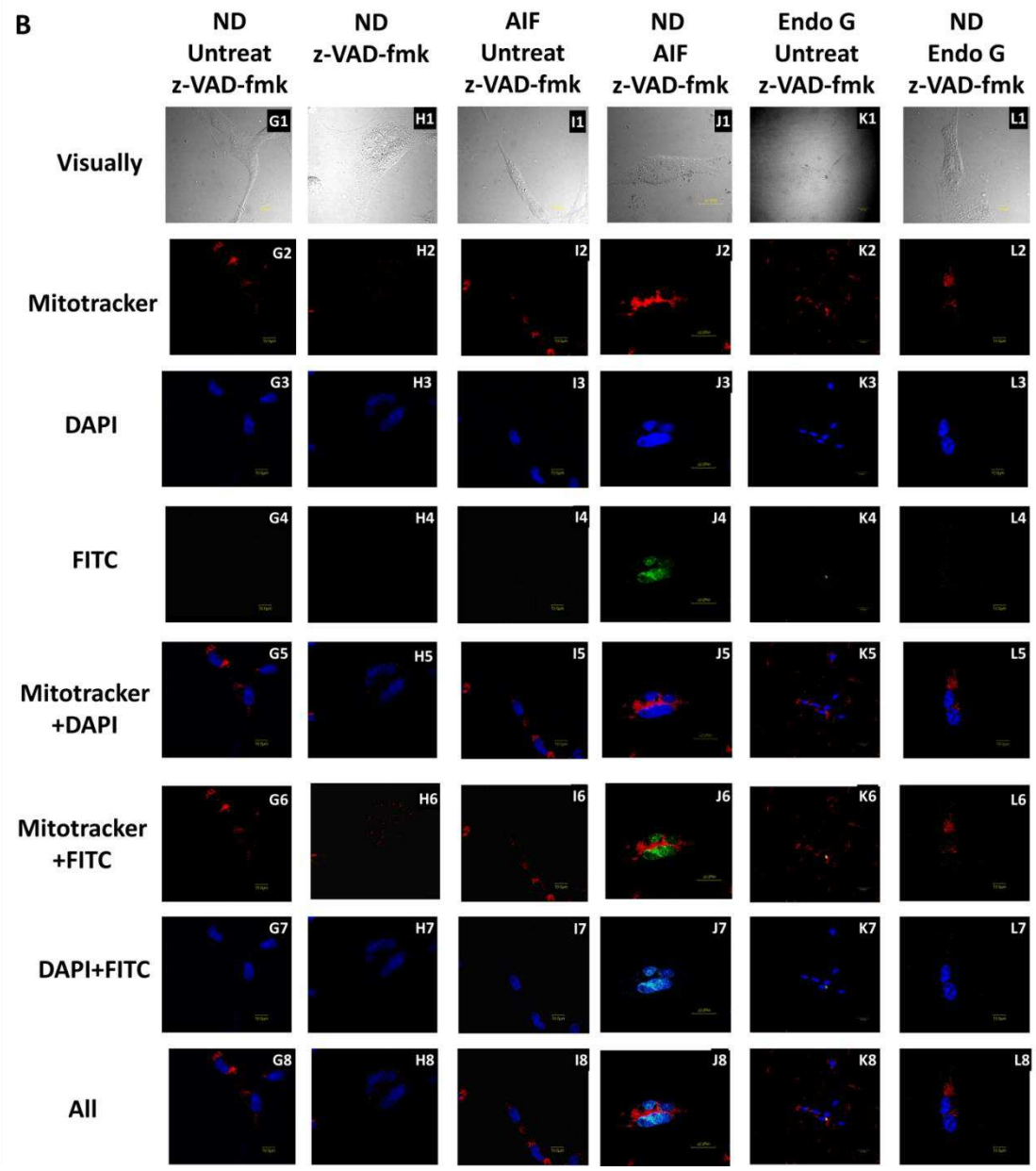

Figure 5 The Endo G and AIF were transferred from the cytoplasm to the nucleus. The green color represents NDV, Endo G, and AIF (FITC). The red color represents the mitochondria (Mitoracker). The blue color represents the nucleus (DAPI). (5aA) The BHK-21 was treated NDV 0 and 24 h in apoptosis caspase-dependent study. (5bB) The BHK-21 was treated NDV 0 and 24 h in apoptosis

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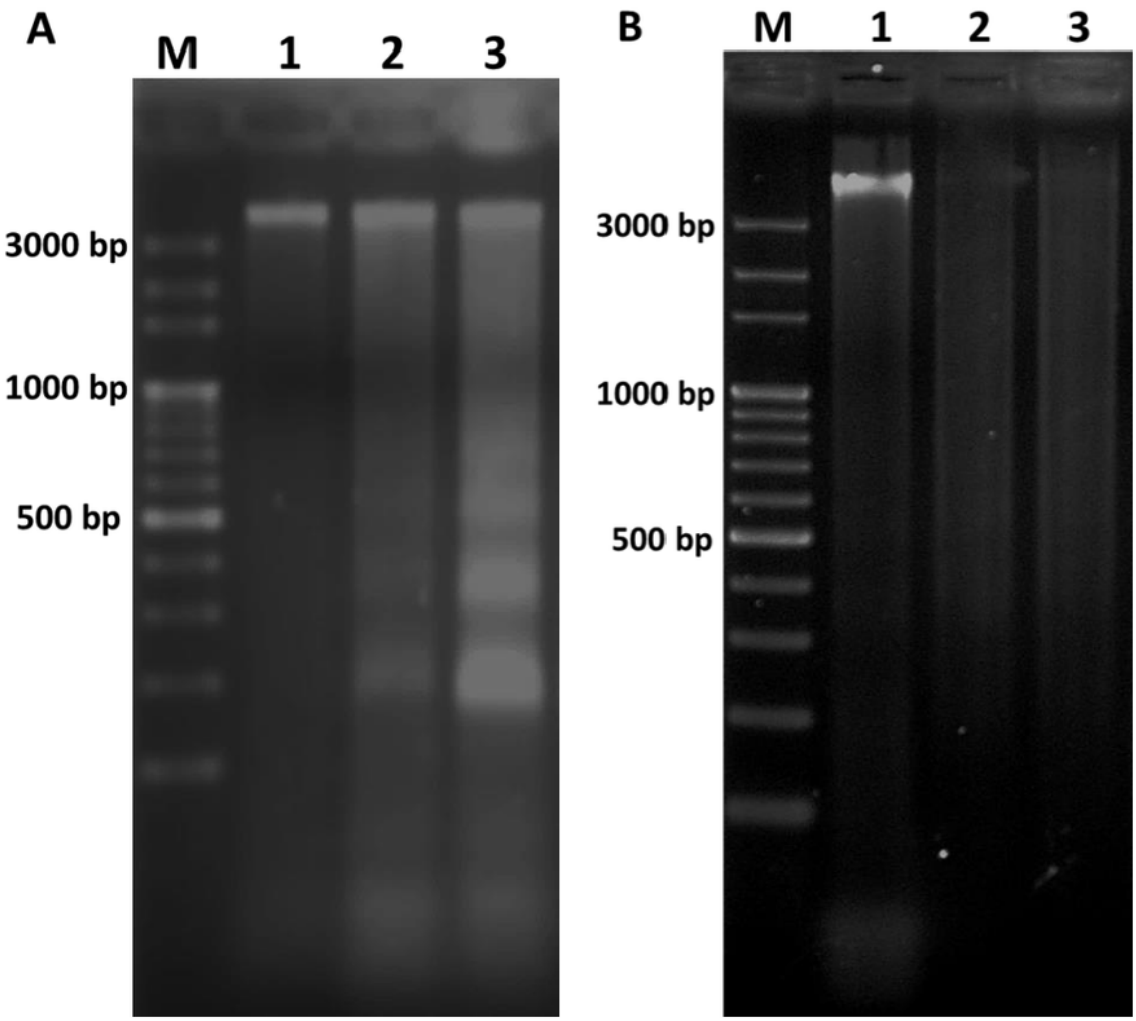
Figures



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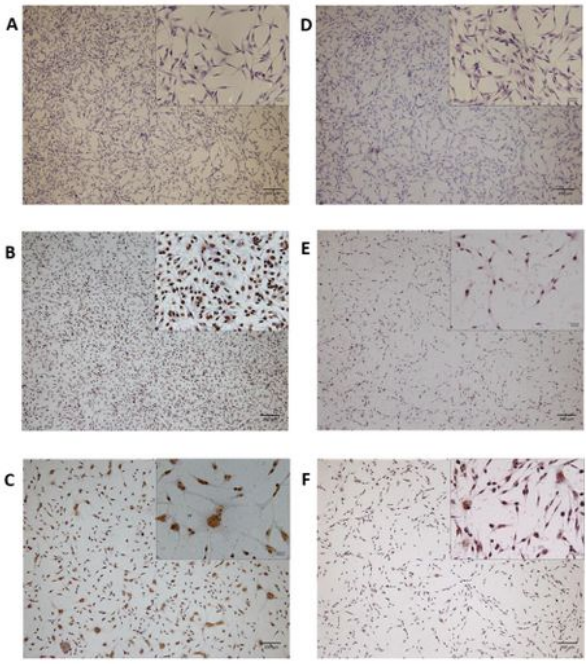

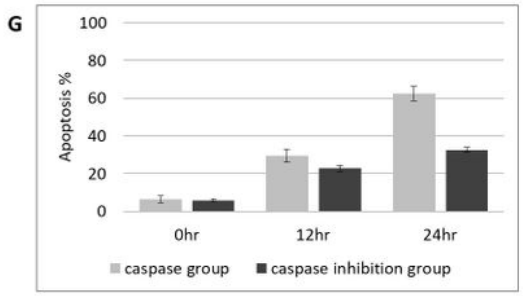

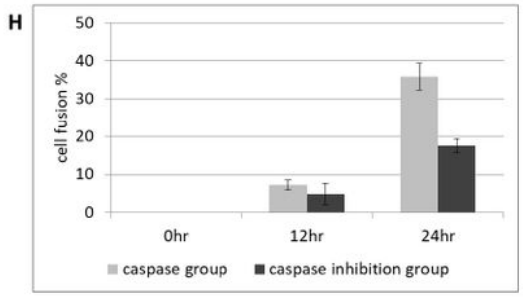

Figure 2

2a–f. Microscopic views of two groups of BHK-21 cells after NDV challenged for 0, 12, and 24 h; samples were treated with TdT-FragEL™ DNA Fragmentation Detection Kit, and cells with nuclear showing brownish color are undergoing apoptosis, while the cells with blue nuclear are living cells. 2a–2c are images of the caspase group and 2D–2F are images of the caspase inhibition group. Figure 2a–2f is magnified, scale bar = 200.0 μm (big) and scale bar = 50.0 μm (small). 2g and 2h. The trend of apoptosis

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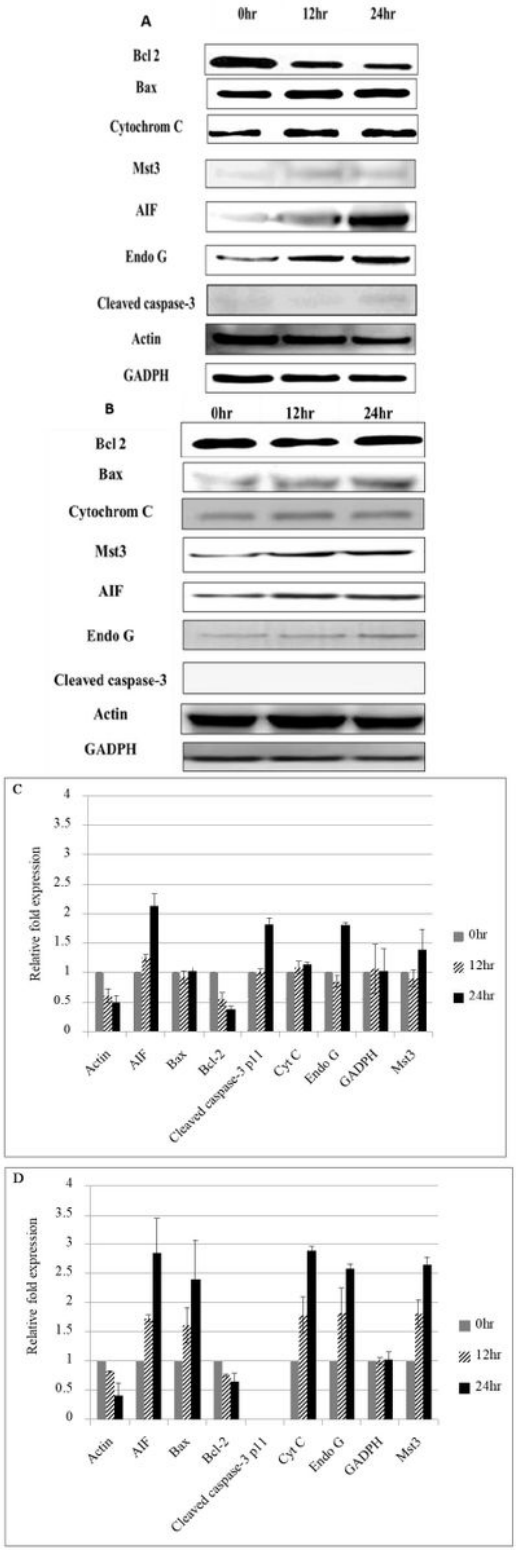

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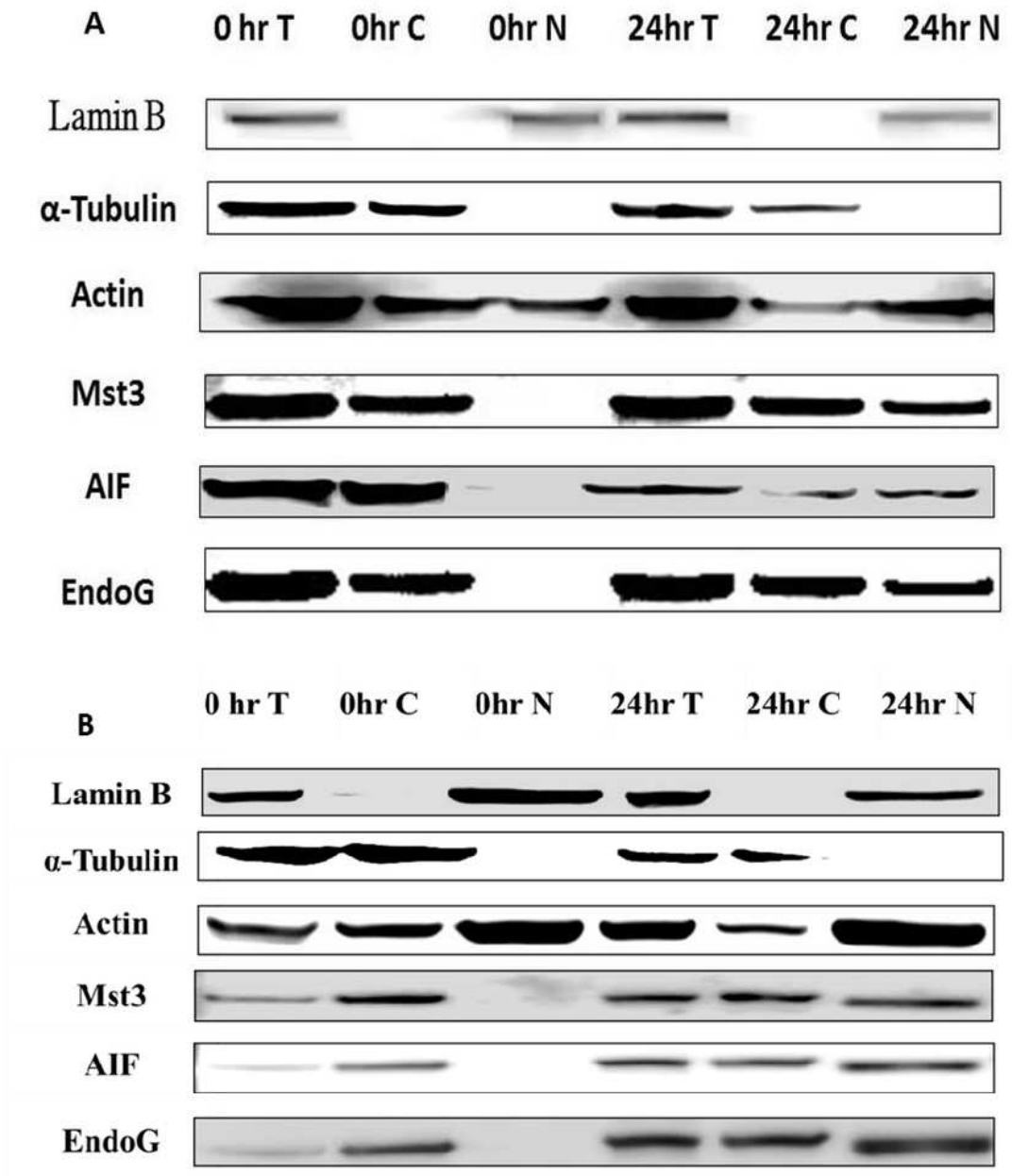

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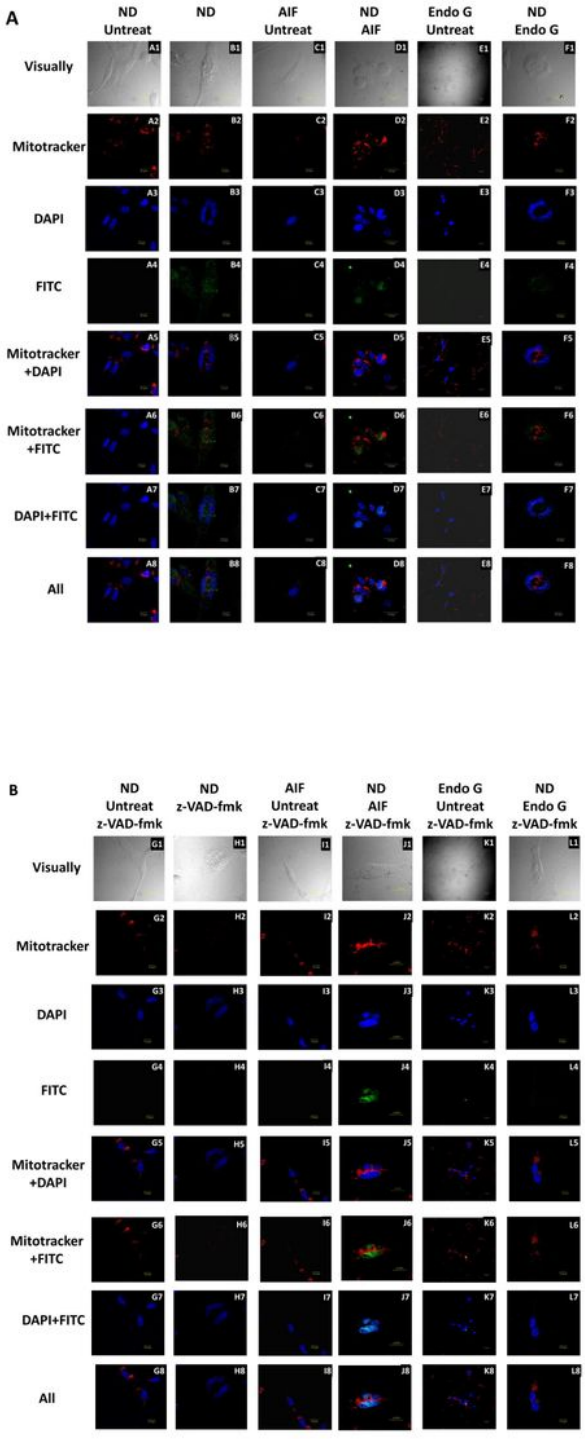

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