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1 **Bid cleavage, cytochrome *c* release and caspase activation in canine coronavirus-induced**  
2 **apoptosis.**

3

4

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30

31 **Abstract**

32 A previous study demonstrated that infection of a canine fibrosarcoma cell line (A-72 cells)  
33 by canine coronavirus (CCoV) resulted in apoptosis (Ruggieri et al., 2007). In this study, we  
34 investigated the cell death processes during infection and the underlying mechanisms. We found  
35 that CCoV-II triggers apoptosis in A-72 cells by activating initiator (caspase-8 and -9) and  
36 executioner (caspase-3 and -6) caspases. The proteolytic cleavage of poly(ADP-ribose) polymerases  
37 (PARP) confirmed the activation of executioner caspases. Furthermore, CCoV-II infection resulted  
38 in truncated bid (tbid) translocation from the cytosolic to the mitochondrial fraction, the cytochrome  
39 *c* release from mitochondria, and alterations in the pro- and anti-apoptotic proteins of bcl-2 family.  
40 Our data indicated that, in this experimental model, both intrinsic and extrinsic pathways are  
41 involved. In addition, we demonstrated that the inhibition of apoptosis by caspase inhibitors did not  
42 affect CCoV replication, suggesting that apoptosis does not play a role in facilitating viral release.

43

44 *Keywords:* Canine coronavirus type II; apoptosis; caspases; PARP; bcl-2 family members.

45

## 46 **Introduction**

47

48 Apoptosis is a particular type of cell death that is characterized by distinctive changes in  
49 cellular morphology, including cell shrinkage, nuclear condensation, chromatin margination and  
50 subsequent degradation that are associated with inter-nucleosomal DNA fragmentation. Apoptosis  
51 may be initiated by a wide variety of cellular insults, including death receptor stimulation and  
52 cytotoxic compounds. Induction or inhibition of apoptosis is an important feature of many types of  
53 viral infection, both in vitro and in vivo (McLean et al., 2008; Hay and Kannourakis, 2002; Barber,  
54 2001; Derfuss and Meinel, 2002; Benedict et al., 2002; Garnett et al., 2006). Despite this fact, the  
55 mechanisms of virus-induced apoptosis remain largely unknown.

56 Canine coronavirus (CCoV), a member of antigenic group 1 of the family *Coronaviridae*, is  
57 a single positive-stranded RNA virus responsible for enteric disease in young puppies (Decaro and  
58 Buonavoglia, 2008). CCoV, first described in diarrhoeic dogs (Binn et al., 1974), is responsible for  
59 diarrhoea, vomiting, dehydration, loss of appetite and occasional death in puppies. Two serotypes of  
60 CCoVs were described (Pratelli et al., 2003), CCoV-I and -II, sharing about 90% sequence identity  
61 in most of their genome. In the present study, we evaluated the processes implicated in cell death  
62 induced by CCoV, in particular of CCoV type II, which is the only CCoV that grows in cell cultures  
63 (Pratelli et al., 2004).

64 An important regulatory event in the apoptotic process is represented by the caspases  
65 activation which regulates two major relatively distinct pathways, the extrinsic and intrinsic  
66 pathways. The extrinsic pathway is mediated by activation of caspase-8 that is initiated mainly by  
67 binding of death receptors to their ligands. The intrinsic pathway involves the release of cytochrome  
68 c from mitochondria; subsequently, cytochrome c binds to the adaptor molecule Apaf-1 (apoptotic  
69 protease activating factor-1) causing autocleavage of caspase-9 (Adrain et al., 1999). This pathway  
70 known also as mitochondrial pathway involves pro and anti-apoptotic members of the proteins bcl-2  
71 family which can be a key to trigger mitochondrial apoptosis (Hildeman et al., 2002). Both  
72 pathways, extrinsic and intrinsic, converge at the activation of executioner caspases (caspase-3, -6  
73 and -7), which are responsible for the characteristic morphological changes of apoptosis  
74 (Budihardjo et al., 1999; Elmore, 2007). Generally, both death receptor and mitochondrial apoptosis  
75 signalling pathways were shown to be implicated in apoptosis induced by viruses. Induction of  
76 caspase-dependent apoptosis has been observed during infection by other coronaviruses, including  
77 transmissible gastroenteritis coronavirus (Eleouet et al., 1998), avian coronavirus infectious  
78 bronchitis virus (Liu et al., 2001), human coronavirus strain 229E (Collins, 2002), and equine  
79 coronavirus (Suzuki et al., 2008).

80 During infection of CCoV, Ruggieri et al. (2007), demonstrated activation of caspase 3. The  
81 aim of this study was to provide a better characterization of the pattern of caspase activation  
82 following infection with CCoV-II. We showed that CCoV infection results in the activation of the  
83 initiator caspases, caspase-8 and -9, and of the effector caspases, caspase-3 and -6. The data  
84 demonstrated that both death receptor and mitochondrial pathways can play an essential role in  
85 CCoV-induced apoptosis. Furthermore we observed no variation of CCoV release inhibiting  
86 apoptosis by caspase inhibitors.

87

88

## 89 **2. Materials and methods**

90

### 91 *2.1. Cell culture and virus preparation*

92 A canine fibrosarcoma cell line (A-72 cells) was grown and maintained in complete medium  
93 consisting of Dulbecco Minimal Essential Medium (D-MEM) supplemented with 2 mM L-  
94 glutamine, 1% non essential amino acid, 5% heat-inactivated foetal calf serum (FCS), 100 IU of  
95 penicillin, and 100 µg of streptomycin per ml, at 37°C in a 5% CO<sub>2</sub> atmosphere incubator. This cell  
96 line was maintained free of mycoplasma and of bovine viral diarrhoea virus. Cells were trypsinized  
97 once a week.

98 CCoV type II strain S/378 (kindly provided by Prof. C. Buonavoglia, Faculty of Veterinary  
99 Medicine, University of Bari, Italy) was used for the study. For viral infection A-72 cells at 80 to  
100 90% confluence in complete medium as described above, were incubated with virus. One hour  
101 post-infection (p.i.) non internalized virus was removed by washing the cells three times with  
102 DMEM, and incubation continued in complete medium. Virus titers were determined by end point  
103 dilution tests using 96-well microtitre plates, and are given as 50% tissue culture infective doses  
104 (TCID<sub>50</sub>) according to the method of Reed and Muench (1938). Aliquots of CCoV-II were stored at  
105 -80°C until used.

106

### 107 *2.2. Cell viability and microscopy*

108 Cell viability after CCoV-II infection was monitored by evaluation of mitochondrial  
109 suffering through the MTT assay as previously described (Pagnini et al., 2004). Data are presented  
110 as a percentage of the control, and results are the mean ± SEM of three experiments performed in  
111 triplicate.

112 To identify apoptotic nuclei, cells uninfected or infected with CCoV-II, were stained with  
113 acridine orange (Sigma Chemical Co, St Louis, USA). Briefly, after 1 h of virus adsorption, the

114 inoculum was removed and fresh medium was added. After 24 and 48 h p.i., cells were washed with  
115 D-MEM and in each well were gently added 100  $\mu$ l of phosphate-buffered saline (PBS) with  
116 acridine orange (40  $\mu$ g/ml). The cells were incubated at room temperature for approximately 2 min  
117 in the dark. The preparations were washed with distilled water, covered with glass cover slips, and  
118 assessed on the same day using fluorescent microscope (Zeiss, Oberkochen, Germany) with a 460-  
119 nm filter. The duration of illumination was limited to 40 seconds per field. Each experiment was  
120 repeated at least three times to confirm the reproducibility of the results. Positive controls were  
121 obtained using camptothecin as previously described (Fiorito et al., 2008).

122

### 123 *2.3. Caspase detection by flow cytometry*

124 Caspase activation detection was performed by using the carboxyfluorescein FLICA Assay  
125 kits (B-Bridge International, Inc., CA, USA). Fluorochrome-labeled inhibitors of caspases (FLICA)  
126 included in kits were added during the final 1 h. Precisely, the fluorochrome-labeled inhibitors of  
127 caspase-3 (FAM-DEVD-FMK), caspase-8 (FAM-LETD-FMK) and caspase-9 (FAM-LEHD-FMK)  
128 were dissolved in DMSO following the manufacturer's instructions and then stored at -20°C in the  
129 dark until use. Solutions of each FLICA were added to cell cultures ( $1 \times 10^6$  cells/ml) at different  
130 times post infection; cells were then incubated for 1h at 37°C in a 5% CO<sub>2</sub> atmosphere incubator.  
131 After incubation, cells were washed twice with washing buffer, placed on ice and the cell  
132 fluorescence was measured within 15 min by a flow cytometer (Parteck Flow Cytometer). The  
133 amount of fluorescence detected was directly proportional to the amount of caspase activity. Results  
134 of all experiments are reported as mean  $\pm$  SEM of three experiments.

135

### 136 *2.4. Protein extraction and Western blot analysis*

137 Mock-infected and infected cells (MOI 10) were collected at different times p.i. and washed  
138 twice in PBS, then the cell pellets were homogenized directly into lysis buffer (50 mM HEPES, 150  
139 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl  
140 fluoride, 1  $\mu$ g/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate).  
141 The lysates were centrifugated at 14,000 x rpm for 10 min. Protein concentrations were determined  
142 by the Bradford assay (Bio-Rad, Hercules, CA). Equivalent amounts of proteins were loaded and  
143 electrophoresed on SDS-polyacrylamide gels. Subsequently, proteins were transferred to  
144 nitrocellulose membranes (Immobilon, Millipore Corp., Bedford, MA). After blocking with Tris-  
145 buffered saline-BSA (25 mM Tris (pH 7.4), 200 mM NaCl, and 5% BSA), the membrane was  
146 incubated with the following primary antibodies: anti-caspase-6 PAb (dilution 1:1,000) (Cell  
147 Signalling), anti-caspase-3 PAb (dilution 1:1,500) (Abcam), anti-caspase-8 PAb (dilution 1:2,000)

148 (Abcam), anti-caspase-9 PAb (dilution 1:2,000) (Stressgen), anti-bax PAb (1:1,000) (Aviva  
149 Systems Biology), anti-bcl-XL PAb (1:1,000) (Abcam), anti-bcl-2 PAb (1:1,000) (Abcam), anti-bid  
150 PAb (1:1000) (Abnova Corporation), anti-cytochrome *c* (1:1000) (Abcam), anti-bim PAb (1:1000)  
151 (Cell Signaling) and anti- $\beta$ -actin MAb (dilution 1:7,500) (Cell Signaling). Membranes were then  
152 incubated with the horseradish peroxidase-conjugated secondary antibody (dilution 1:1,000) (at  
153 room temperature), and the reaction was detected with an enhanced chemiluminescence system  
154 (Amersham Life Science). The relative amount of protein expression was quantified using Gel-Doc  
155 phosphorimager and Quantity One software (Bio-Rad) and normalized by the band intensity of  $\beta$ -  
156 actin.

157

### 158 2.5. Extraction of PARP

159 Mock infected and infected cells (MOI 10) were washed twice with ice-cold PBS and then  
160 once with ice-cold buffer A (100 mM Tris-HCl [pH 7.4], 10 mM MgSO<sub>4</sub>, 500 mM sucrose, 10 mM  
161 PMSF, 0.5  $\mu$ g of leupeptin per ml, 0.75  $\mu$ g of pepstatin per ml, and 5  $\mu$ g of antipain per ml). Cells  
162 were permeabilized by incubation on ice for 20 min with buffer B (100 mM Tris-HCl [pH 7.4], 10  
163 mM MgSO<sub>4</sub>, 500 mM sucrose, 10 mM PMSF, 1% NP-40; 0.25  $\mu$ g of leupeptin per ml, 0.35  $\mu$ g of  
164 pepstatin per ml, and 50  $\mu$ g of antipain per ml). PARP was extracted from permeabilized cells by  
165 incubation with cold buffer C (200 mM K<sub>2</sub>HPO<sub>4</sub>, 100 mM Tris-HCl [pH 7.4], 10 mM MgSO<sub>4</sub>, 500  
166 mM sucrose, 10 mM PMSF, 0.5  $\mu$ g of leupeptin per ml, 0.75  $\mu$ g of pepstatin per ml, and 5  $\mu$ g of  
167 antipain per ml) on ice for 20 min. The extract was centrifuged at 2,000  $\times$  g for 10 min at 4°C, and  
168 the supernatant was collected and mixed with 4 volumes of urea loading buffer (62.5 mM Tris-HCl  
169 [pH 6.8], 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, and 5%  $\beta$ -  
170 mercaptoethanol). The Western blot analysis of PARP was performed, as above reported, using  
171 anti-poly(ADP-ribose) polymerase (PARP) antibody with a dilution of 1:5,000 (BD Biosciences).  
172 The relative amount of protein expression was quantified using Gel-Doc phosphorimager and  
173 Quantity One software (Bio-Rad) and normalized by the band intensity of  $\beta$ -actin.

174

### 175 2.6. Cytosolic and mitochondrial protein extraction

176 Isolation of mitochondria and cytosolic fractions were carried out using a modified protocol  
177 from Kluck et al. [1997]. Mock-infected and infected cells were collected at different times p.i. and  
178 washed twice in PBS. The cell pellets were resuspended in lysis buffer (50 mM Tris [pH 7.5], 150  
179 mM NaCl<sub>2</sub>, 5 mM EGTA, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1% NP-40, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml  
180 aprotinin, 1  $\mu$ M PMSF, and 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>). Samples were then incubated on ice for 20 minutes  
181 and centrifuged at 14,000 rpm for 15 min. Equivalent amounts of proteins were loaded and

182 electrophoresed on SDS-polyacrylamide gels. The Western blot analysis of cytochrome *c* was  
183 performed, as above reported, using anti-cytochrome *c* PAb with a dilution of 1:1,000 (Abcam).  
184 The relative amount of protein expression was quantified using Gel-Doc phosphorimager and  
185 Quantity One software (Bio-Rad) and normalized by the band intensity of  $\beta$ -actin.

186

### 187 *2.7. Inhibition experiments of CCoV-II-induced apoptosis*

188 A-72 cells in 24-well plates, at confluency, were treated with 25  $\mu$ M of each of the caspase  
189 inhibitors Z-VAD-FMK (pan-caspase inhibitor), Z-IETD-FMK (caspase-8-specific inhibitor), Z-  
190 LEDH-FMK (caspase-9-specific inhibitor), Z-DEVD-FMK (caspase-3-specific inhibitor)  
191 (Calbiochem, EMD Biosciences), 2 h prior to infection with CCoV-II at MOI 10.

192 At 24 h p.i. cell viability was evaluated by MTT assay as above reported, and virus titers  
193 recovered from the culture medium were assayed by TCID<sub>50</sub> method according to Reed and  
194 Muench (1938).

195

### 196 *2.8. Statistical analysis*

197 The results are presented as mean  $\pm$  SEM of three experiments. One-way ANOVA with  
198 Turkey's post test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad  
199 Software, San Diego, CA, USA). *P* value < 0.05 was considered statistically significant.

200

## 201 **Results**

202

### 203 *3.1. Cell viability and morphological evidence of apoptosis induced in A-72 cells by CCoV-II* 204 *infection.*

205 Infection of A-72 cells with CCoV-II resulted in cell death in a time-dependent manner, as  
206 detected by MTT assay (Fig. 1). This loss of viability was also dependent on the MOI at which the  
207 cultures were infected. Moreover, a significant reduction of mitochondrial dehydrogenases activity  
208 was already detectable at 8 h p.i. using a MOI 10 ( $P < 0.05$ ). A significant decrease ( $P < 0.01$  and  
209  $P < 0.001$ ) in cell number was observed at all examined times post infection independently of the  
210 used MOI.

211 Using a MOI of 10, the typical cytopathic effects (CPE) characterized by cytoplasmic  
212 vacuolation, fusion, rounding up, and detachment of infected cells from the cultured plates were  
213 first detected in CCoV type II-infected A-72 cells at 24 h p.i. and the extent of CPE gradually  
214 increased by 48 h. (Fig. 2).

215 Cellular morphological changes were also investigated by acridine orange staining using  
216 fluorescence microscopy (Fig. 3). Cells at 80% confluence were mock-infected or infected with

217 CCoV-II at a MOI of 10. At various time p.i., cells were stained and observed. The results  
218 demonstrated that apoptotic morphological changes could be detected overall at 48 h p.i., when  
219 cells showed chromatin condensation. Thus, it could be seen clearly that the strong apoptotic signs  
220 were evident late in the infection. The data were in accordance with the results of flow cytometric  
221 assay (data not shown).

222

### 223 3.2. Activation of caspase cascade

224 To explore the cell death program, first we investigated caspase activation cascades during  
225 CCoV-II-induced apoptosis using a MOI of 10 and analyzing the cell lysates at different times p.i.  
226 (8, 12, 24 and 48 h). The activity of two initiator caspase-8 (death receptor-mediated) and caspase-9  
227 (mitochondrial-mediated) as well as the downstream effector caspase-3 were measured using  
228 fluorochrome-labeled inhibitors of caspases (FLICA) assay kits.

229 Fig. 4 shows at and after 12 h p.i. a significant increase ( $P < 0.01$  and  $P < 0.001$ ) of caspase-3,  
230 -8 and -9 activity in a time-dependent manner in CCoV-II-infected cells. These data suggested that  
231 both extrinsic and intrinsic pathways were involved in CCoV-II-induced apoptosis.

232 For further evidence, we investigated the initiator caspase-8, 9 activation as well as the  
233 executioner caspase-3 and -6 by Western blot analysis. A representative blot (Fig. 5a) and the  
234 relative densitometric analysis (Fig. 5b) show the involvement of the examined cleaved caspases.  
235 As described in Materials and Methods,  $\beta$ -actin was used as an internal loading control. The  
236 cleavage of caspase-8 and -9 was detected from 12 h p.i. in CCoV infected cells (Fig. 5a), and such  
237 result was in accordance with FLICA analysis. In particular, it was evident a higher expression level  
238 of cleaved caspase-8 compared to cleaved caspase-9 at 12 h p.i.. Then, whereas the cleaved  
239 caspase-8 showed a time-dependent downward trend, the cleaved caspase-9 didn't change over  
240 time.

241 The caspase-3 activation pattern was also well correlated with the FLICA analysis. In fact,  
242 we observed the caspase-3 proteolytic processing at and after 12 h p.i., and a significant peak at 24  
243 and 36 h p.i. (Fig. 5a and b). By Western blot analysis, we have also observed the cleaved caspase-6  
244 (Fig. 5a and b) and a higher activation of caspase-6 at and after 24 h p.i. than that of caspase-3.

245

### 246 3.3. PARP cleavage

247 We next assessed the cleavage of PARP in the induction of apoptosis in A-72 cells by  
248 CCoV-II. PARP is a substrate for activated caspase-3 and cleaved PARP is considered a hallmark  
249 of apoptosis (Elmore, 2007). PARP cleavage was monitored during CCoV-II infection by Western  
250 blot in order to provide additional evidence of caspase activation and apoptosis induced by CCoV-  
251 II. As shown in Fig. 6a, the full-length 116 kDa PARP was cleaved to active form of 85 kDa in

252 time-dependent manner. A representative blot (Fig. 6a) and its quantification (Fig. 6b) shows the  
253 involvement of proteolytic cleavage of PARP in virus-infected cells harvested at 12, 24, 36 and 48 h  
254 p.i., when the typical DNA laddering was also evident (data not shown). Furthermore, the Fig. 6c  
255 shows that the cleaved PARP/uncleaved PARP ratio was statistically significant ( $P < 0.01$ ) from 24 h  
256 p.i. until the end of infection compared to that of 12 h p.i.

257

### 258 3.4. Bid cleavage and cytochrome *c* release

259 In normal cells, the bid protein exists as an inactive form in the cytosolic fraction and  
260 becomes activated by caspase-8 in response to apoptotic stimuli. Then, the active form of bid  
261 translocates to the mitochondria, and triggers bax activation in correlation with the translocation of  
262 bax from the cytosol to the mitochondria. These events result in the cytochrome *c* release from the  
263 mitochondria to the cytosol (Korsmeyer et al., 2000; Roucou et al., 2002). Because CCoV-II  
264 infection, as shown above, induced caspase-8 activation in infected cells, we generated subcellular  
265 fractions to examine bid and cytochrome *c* cell localization. As shown in Fig. 7a and b, the full-  
266 length bid expression level evaluated by Western blot analysis in cytosolic fraction, decreased in  
267 time-dependent in virus-infected cells respect to mock-infected cells. The mitochondrial  
268 localization of 15 kDa fragment truncated bid (tbid) clearly appeared at 12 h p.i. Since this fragment  
269 contains the BH3 domain which is the functional part of bid for cytochrome *c* release, we examined  
270 whether the bid traslocation was accompanied by a mitochondrial release of cytochrome *c* into the  
271 cytosol. The relationships with bid-induced cytochrome *c* release is evident in Fig. 7c and d which  
272 shows the presence of cytochrome *c* in the cytosolic fraction from 12 h p.i. until the end of  
273 infection.

274

### 275 3.5. Effect of CCoV type II infection on the expression of Bcl-2 family of proteins

276 The Bcl-2 family of proteins, including both inhibitors and promoters of apoptosis, is  
277 involved in the controlling of mitochondrial permeability, as well as regulation of caspase  
278 activation. In this study, several Bcl-2 family proteins, including anti-apoptotic (bcl-2, bcl-xL) and  
279 pro-apoptotic members (bax, bim), were detected in response to CCoV-II infection in A-72 cells.  
280 The densitometric analysis of the blots demonstrated an increased expression of pro-apoptotic  
281 proteins, bim and bax, which presented a peak at 12 and 24 h p.i. respectively; but while the levels  
282 of bim protein slightly decreased after 36 h p.i., bax levels didn't change (Fig. 8a and b). Whereas  
283 the expressions of the anti-apoptotic members, bcl-2 and bcl-xL, were downregulated by CCoV-II  
284 infection (Fig. 8a). The densitometric analysis of the blots demonstrated a decreased expression of

285 bcl-2 and of bcl-xL after 8 h and 12 h p.i. respectively (Fig. 8b). The data suggest that bax, bim,  
286 bcl-2 and bcl-xL may play a role on arbitrating the CCoV type II-induced apoptosis.

287  
288 *3.2- Effects of caspase inhibitors on cell viability and viral replication*

289 To test if the apoptotic changes observed in CCoV type II-infected A-72 cells were caspase  
290 dependent, Z-VAD-FMK (pan-caspase inhibitor), Z-DEVD-FMK (caspase-3-specific inhibitor), Z-  
291 IETD-FMK (caspase-8-specific inhibitor), Z-LEHD-FMK (caspase-9 inhibitor) were added to the  
292 culture media before the infection. As shown in Fig. 9a, in presence of caspases inhibitors was  
293 observed a significant higher cell viability ( $P < 0.001$ ).

294  
295 The effects of apoptosis on the replication of CCoV-II were assayed by comparing virus  
296 titers in the presence of caspase inhibitors. As reported in Fig. 9b, the results showed that the virus  
297 release in presence of caspase inhibitors didn't change compared with control. It is conceivable that  
298 apoptosis is not required for the release of progeny virions. These observations therefore suggest  
299 that CCoV-II replication don't requires caspase activation to complete the virus life cycle.

## 301 Discussion

302  
303 It is believed that the modulation of apoptotic cell death, also known as programmed cell  
304 death, is relevant to diseases that are caused by various viruses. One of the main advantages of  
305 apoptotic cell death for virus infectivity is to facilitate the spread of virus progeny to the  
306 neighboring cells and to minimize the inflammatory reaction evoked by virus-infected cells on the  
307 host.

308 Coronaviruses are known to infect host cells by receptor-mediated endocytosis and some of  
309 them to cause cell-cell fusion during the late stages of infection, resulting in syncytium formation and  
310 CPE (Lai and Cavanagh, 1997). Apoptosis was observed in Vero E6 cells infected by SARS-CoV  
311 (Yan et al., 2004) and in a recent publication it has been reported that CCoV induces apoptosis in  
312 cultured cells (Ruggieri et al., 2007). It has been shown that in other coronavirus, e.g., avian  
313 infectious bronchitis virus (Liu et al., 2001), swine transmissible gastroenteritis virus (Eleouet et al.,  
314 2000) and murine coronavirus (Liu et al., 2006; Liu and Zhang, 2007), apoptosis is induced by a  
315 caspase-dependent mechanism.

316 The importance of caspase activation during apoptosis is well established (Hengartner,  
317 2000). Caspases play a central role in the effector phase during apoptotic cell death. To date, 14  
318 mammalian caspases have been described (Earnshaw et al., 1999).

319 In this study we show that CCoV type II infection leads to caspase-dependent apoptotic cell  
320 death in A-72 cells, *in vitro*. First, we examined by MTT assay and morphological evaluations the  
321 CCoV-II infection, demonstrating a dose- and time-dependent trend of the apoptotic process virus-  
322 induced. Then, in an attempt to understand the mechanism of induction of apoptosis in CCoV-II-  
323 infected cells we have investigated the caspase pathways. The results reported herein by FLICA  
324 method (Bedner et al., 2000) provide an unequivocal evidence that CCoV-II induces apoptotic cell  
325 death *in vitro*, involving caspase-dependent pathway of caspase-8 (death receptor-mediated) and  
326 caspase-9 (mitochondrial-mediated). This suggests that both the extrinsic and intrinsic pathways in  
327 the CCoV-II-induced apoptosis were involved. Our results match those Eleouet et al. (2000) who  
328 demonstrated that the transmissible gastroenteritis coronavirus (TGEV), also included in group 1  
329 coronaviruses as CCoV-II, triggers caspase activation events, involving both the extrinsic and  
330 intrinsic pathways, during infection. Further apoptosis-associated caspase activation has been  
331 documented also in other coronavirus groups such as SARS CoV (group 2) (Mizutani et al., 2004),  
332 MHV (group 2) (Chen and Makino, 2002), and IBV (group 3) (Liu et al, 2001).

333 However, in the present study, it was evident the activation of caspase-8 and -9 at and after  
334 12 h p.i. by FLICA and Western blot analysis. Furthermore, caspase 9 was only partially activated  
335 compared to the others investigated and not changing between times 24 -72 hours suggesting that  
336 viral proteins could interfere with such activation. A higher protein expression level of caspase-3  
337 and -6 compared to those of caspase-8 and -9, after 12 h p.i., was observed. These higher levels of  
338 activity achieved by caspase-3 and -6 could be related to the additive effect of both caspase-8 and -9  
339 pathways that activate these executioner caspases.

340 Caspase-8 has been shown to activate the intrinsic pathway following the cleavage of bid, a  
341 pro-apoptotic member of the Bcl-2 family of proteins (Li et al., 1998). The data herein presented are  
342 consistent with such mechanism, showing that the full-length bid expression level in cytosolic  
343 fraction decreased in time-dependent in virus-infected cells respect to mock-infected cells. We also  
344 described the relationships with bid-induced cytochrome *c* release in the cytosolic fraction from 12  
345 h p.i. until the end of infection.

346 Bid is part of the bcl-2 family of proteins, which includes both pro-apoptotic proteins (for  
347 example bid, bax, bak, bcl-XS, bad, bim), and anti-apoptotic proteins (for example bcl-2, bcl-XL  
348 bcl-w, mcl-1). Therefore, the bcl-2 family of proteins have been considered as pivotal players in  
349 apoptosis. Our study has shown that CCoV-II infection down-regulated bcl-2 but up-regulated bax  
350 expression. Furthermore, densitometric analysis of bim, which is one of the candidates for initial  
351 mitochondrial activation, indicated a marked increase of protein expression level up to 36 h p.i..  
352 Bim activates bax through interactions with mcl-1 and bcl-2 (Willis et al., 2007). Here we have

353 reported that bim significantly shifted the relative contributions of bax to cell death. Then, in  
354 response to apoptotic stimuli, bax has to translocate to mitochondria before it can oligomerize and  
355 form pores (Desagher and Martinou, 2000), and the relative decline in bcl-2 expression permits bax  
356 to gain dominance, the downstream effects of which most likely involve both direct and indirect  
357 activation of the cell death proteases that represent the final steps in the apoptotic pathway.

358 We have also shown additional evidence of downstream caspase activation evaluating the  
359 PARP cleavage during CCoV-II infection. PARP is a substrate for activated caspase-3 (Li and  
360 Darzynkiewicz, 2000), and here we have shown elevated expression of activated caspase-3 and  
361 cleaved PARP in the CCoV-II-infected cells in time-dependent manner. Thus, the CCoV-II-  
362 infection significantly increased PARP cleavage levels.

363 Furthermore, we observed the effects of apoptosis on both the replication of CCoV type II  
364 and virus titers in the presence of caspase inhibitors. The suppression of apoptosis by these  
365 inhibitors suggested that the activation of apoptosis doesn't represent an important step in virus  
366 release. These data are in agreement with previous studies, in fact, it was demonstrated that the  
367 inhibition of apoptosis, either by caspase inhibitors or by overexpression of the bcl-2 protein, did  
368 not affect SARS-CoV replication in Vero cells (Ren et al., 2005; Bordi et al., 2006), suggesting that  
369 apoptosis does not play a role in facilitating viral release.

370 Collectively, the results reported here demonstrate that: (i) apoptosis developing in a  
371 CCoV-II infection depended on the activation of caspase-3,-6 -8 and -9; (ii) apoptosis induced  
372 during *in vitro* infection of A-72 cells could occur both from the extrinsic pathway and the intrinsic  
373 pathway; (iii) CPE and cell death in A-72 cells, which are triggered by CCoV-II, are due to the  
374 induction of caspase activation, given the ability of caspase inhibitors to increase viability of  
375 CCoV-II-infected cell; and (iv) there is no interference between apoptosis and viral release.

376 These results suggest that this coordinated interplay among caspases is crucial in attaining  
377 full activation of apoptotic signal in A-72 infected cells; however, the first direct link between  
378 caspase activation and CCoV-II-induced apoptosis remains to be explored and studies are under  
379 way in order to identify targets of viral protein involved in the modulation of apoptosis during  
380 CCoV-II-infection.

381

382 **References**

383

384 Adrain, C., Slee, E.A., Harte, M.T., Martin, S.J., 1999. Regulation of apoptotic protease  
385 activating factor-1 oligomerization and apoptosis by the WD-40 repeat region. *J. Biol.*  
386 *Chem.* 274, 20855-20860.

387 Barber, G.N., 2001. Host defense, viruses and apoptosis. *Cell Death Differ.* 8, 113-126.

388 Bedner, E., Smolewski, P., Amstad, P., Darzynkiewicz, Z., 2000. Activation of caspases  
389 measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA):  
390 correlation with DNA fragmentation. *Exp. Cell Res.* 259, 308-313.

391 Benedict, C.A., Norris, P.S., Ware, C.F., 2002. To kill or be killed: viral evasion of apoptosis.  
392 *Nat. Immunol.* 3, 1013-1017.

393 Binn, L.N., Lazar, E.C., Keenan, K.P., Huxoll, D.L., Marchwicki, R.H., Strano, A.J., 1974.  
394 Recovery and characterization of a coronavirus from military dogs with diarrhoea. *Proc.*  
395 *Annu. Mtg US Anim. Health Assoc.* 78, 359-366.

396 Bordi, L., Castilletti, C., Falasca, L., Ciccocanti, F., Calcaterra, S., Rozera, G., Di Caro, A.,  
397 Zaniratti, S., Rinaldi, A., Ippolito, G., Piacentini, M., Capobianchi, M.R., 2006. Bcl-2  
398 inhibits the caspase-dependent apoptosis induced by SARS-CoV without affecting virus  
399 replication kinetics. *Arch. Virol.* 151, 369-377.

400 Budihardjo, I., Oliver, H., Lutter, M., Luo, X., Wang, X., 1999. Biochemical pathways of  
401 caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.* 15, 269-290.

402 Chen, C.J., Makino, S., 2002. Murine coronavirus-induced apoptosis in 17Cl-1 cells involves a  
403 mitochondria-mediated pathway and its downstream caspase-8 activation and bid cleavage.  
404 *Virology* 302, 321-332.

405 Collins, A.R., 2002. In vitro detection of apoptosis in monocytes/macrophages infected with  
406 human coronavirus. *Clin. Diagn. Lab. Immunol.* 9, 1392-1395.

407 Decaro, N., Buonavoglia, C., 2008. An update on canine coronaviruses: Viral evolution and  
408 pathobiology. *Vet. Microbiol.* 132, 221-234.

409 Derfuss, T., Mehl, E., 2002. Herpesviral proteins regulating apoptosis. *Curr. Top. Microbiol.*  
410 *Immunol.* 269, 257-272.

411 Desagher, S., Martinou, J.C., 2000. Mitochondria as the central control point of apoptosis.  
412 *Trends Cell Biol.* 10, 369-377.

413 Earnshaw, W.C., Martins, L.M., Kaufmann, S.H., 1999. Mammalian caspases : structure,  
414 activation, substrates, and functions during apoptosis. *Ann. Rev. Biochem.* 68, 383-424.

- 415 Eleouet, J.F., Chilmonczyk, S., Besnardeau, L., Laude, H., 1998. Transmissible gastroenteritis  
416 coronavirus induces programmed cell death in infected cells through a caspase-dependent  
417 pathway. *J. Virol.* 72, 4918-4924.
- 418 Eleouet, J.F., Slee, E.A., Saurini, F., Castagne, N., Poncet, D., Garrido, C., Solary, E., Martin,  
419 S.J., 2000. The viral nucleocapsid protein of transmissible gastroenteritis coronavirus  
420 (TGEV) is cleaved by caspase-6 and -7 during TGEV-induced apoptosis. *J. Virol.* 74, 3975-  
421 3983.
- 422 Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35, 495-516.
- 423 Fiorito, F., Pagnini, U., De Martino, L., Montagnaro, S., Ciarcia, R., Florio, S., Pacilio, M.,  
424 Fucito, A., Rossi, A., Iovane, G., Giordano, A., 2008. 2,3,7,8-Tetrachlorodibenzo-p-dioxin  
425 increases Bovine Herpesvirus type-1 (BHV-1) replication in Madin-Darby bovine kidney  
426 (MDBK) cells in vitro. *J. Cell. Biochem.* 103, 221-233.
- 427 Garnett, T.O., Filippova, M., Duerksen-Hughes, P.J., 2006. Accelerated degradation of FADD  
428 and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated  
429 apoptosis. *Cell Death Differ.* 13, 1915-1926.
- 430 Hay, S., Kannourakis, G., 2002. A time to kill: viral manipulation of the cell death program. *J.*  
431 *Gen. Virol.* 83, 1547-1564.
- 432 Hengartner, M.O., 2000. The biochemistry of apoptosis. *Nature* 407, 770-776.
- 433 Hildeman, D.A., Zhu, Y., Mitchell, T.C., Bouillet, P., Strasser, A., Kappler, J., Marrack, P.,  
434 2002. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim.  
435 *Immunity* 16, 759-767.
- 436 Kluck, R.M., Bossy-Wetzell, E., Green, D.R., Newmeyer, D.D., 1997. The release of  
437 cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*  
438 275, 1132-1136.
- 439 Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J., Schlesinger, P.H., 2000. Pro-  
440 apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in  
441 the release of cytochrome c. *Cell Death Differ.* 7, 1166-1173.
- 442 Lai, M.M.C., Cavanagh, D., 1997. The molecular biology of coronaviruses. *Adv. Virus Res.* 48,  
443 1-100.
- 444 Li, H., Zhu, H., Xu, C. J., Yuan. J., 1998. Cleavage of BID by caspase 8 mediates the  
445 mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94, 491-501.
- 446 Li, X., Darzynkiewicz, Z., 2000. Cleavage of Poly(ADP-ribose) polymerase measured in situ in  
447 individual cells: relationship to DNA fragmentation and cell cycle position during apoptosis.  
448 *Exp. Cell Res.* 255, 125-132.

- 449 Liu, C., Xu, H.Y., Liu, D.X., 2001. Induction of caspase-dependent apoptosis in cultured cells  
450 by the avian coronavirus infectious bronchitis virus. *J. Virol.* 75, 6402-6409.
- 451 Liu, Y., Pu, Y., Zhang, X., 2006. Role of the mitochondrial signaling pathway in murine  
452 coronavirus-induced oligodendrocyte apoptosis. *J. Virol.* 80, 395-403.
- 453 Liu, Y., Zhang, X., 2007. Murine coronavirus-induced oligodendrocyte apoptosis is mediated  
454 through the activation of the Fas signalling pathway. *Virology* 360, 364-375.
- 455 McLean, J.E., Ruck, A., Shirazian, A., Pooyaei-Mehr, F., Zakeri, Z.F., 2008. Viral manipulation  
456 of cell death. *Curr. Pharm. Des.* 14, 198-220.
- 457 Mizutani, T., Fukushi, S., Saijo, M., Kurane, I., Morikawa, S., 2004. Phosphorylation of p38  
458 MAPK and its downstream target in SARS coronavirus-infected cells. *Biochem. Biophys.*  
459 *Res. Commun.* 319, 1228-1234.
- 460 O'Brien, V., 1998. Viruses and apoptosis. *J. Gen. Virol.* 79, 1833-1845.
- 461 Pagnini, U., Montagnaro, S., Pacelli, F., De Martino, L., Florio, S., Rocco, D., Iovane, G.,  
462 Pacilio, M., Gabellino, C., Marsili, S., Giordano, A., 2004. The involvement of oxidative  
463 stress in bovine herpesvirus type 4-mediated apoptosis. *Front. Biosci.* 9, 2106-2114.
- 464 Pratelli, A., Decaro, N., Tinelli, A., Martella, V., Elia, G., Tempesta, M., Cirone, F.,  
465 Buonavoglia, C., 2004. Two genotypes of canine coronavirus simultaneously detected in the  
466 fecal samples of dogs with diarrhea. *J. Clin. Microbiol.* 42, 1797-1799.
- 467 Pratelli, A., Martella, V., Decaro, N., Tinelli, A., Camero, M., Cirone, F., Elia, G., Cavalli, A.,  
468 Corrente, M., Greco, G., Buonavoglia, D., Gentile, M., Tempesta, M., Buonavoglia, C.,  
469 2003. Genetic diversity of a canine coronavirus detected in pups with diarrhoea in Italy. *J.*  
470 *Virol. Methods* 110, 9-17.
- 471 Reed, L.J., Muench, H., 1938. A simple method of estimating 50 per cent end-points. *Am. J.*  
472 *Hyg.* 27, 493-497.
- 473 Ren, L., Yang, R., Guo, L., Qu, J., Wang J., Hung, T., 2005. Apoptosis induced by the SARS-  
474 associated coronavirus in Vero cells is replication-dependent and involves caspase. *DNA*  
475 *Cell Biol.* 24, 496-502.
- 476 Roucou, X., Montessuit, S., Antonsson, B., Martinou, J.C., 2002. Bax oligomerization in  
477 mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial  
478 protein. *Biochem. J.* 15, 915-921.
- 479 Ruggieri, A., Di Trani, L., Gatto, I., Franco, M., Vignolo, E., Bedini, B., Elia, G., Buonavoglia,  
480 C., 2007. Canine coronavirus induces apoptosis in cultured cells. *Vet. Microbiol.* 121, 64-72.
- 481 Ruggieri, A., Harada, T., Matsuura, Y., Miyamura, T., 1997. Sensitization to Fas-mediated  
482 apoptosis by hepatitis C virus core protein. *Virology* 229, 68-76.

- 483 Shen, Y., Shenk, T.E., 1995. Viruses and apoptosis. *Curr. Opin. Genet. Dev.* 5, 105-111.
- 484 Suzuki, K., Matsui, Y., Miura, Y., Sentsui, H., 2008. Equine coronavirus induces apoptosis in  
485 cultured cells. *Vet. Microbiol.* 129, 390-395.
- 486 Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H.,  
487 Lee, E.F., Douglas Fairlie, W., Bouillet, P., Strasser, A., Kluck, R.M., Adams, J.M.,  
488 Huang, D.C.S., 2007. Apoptosis initiated when BH3 ligands engage multiple Bcl-2  
489 homologs, not Bax or Bak. *Science* 315, 856-859.
- 490 Yan, H., Xiao, G., Zhang, J., Hu, Y., Yuan, F., Cole, D.K., Zheng, C., Gao, G.F., 2004. SARS  
491 coronavirus induces apoptosis in Vero E6 cells. *J. Med. Virol.* 73, 323-331.
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493 **Legends**

494

495 **Fig. 1** – Effect of various MOI of CCoV-II on A-72 cell viability. After the indicated times of post  
 496 infection, cell viability was evaluated with MTT assay. Values represent the mean ( $\pm$ SEM) of three  
 497 different experiments performed in triplicate. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to  
 498 mock-infected cultures.

499

500 **Fig. 2** – Photomicrographs at inverted microscope of CCoV-II-induced cytopathic effect (CPE)  
 501 using a MOI of 10, **A**, mock-infected A-72 cells; **B**, CCoV-II –infected cells at 24 h p.i.; **C**,  
 502 CCoV-II –infected cells at 48 h p.i.. Magnification: 100x..

503

504 **Fig. 3** – Photomicrographs under fluorescence microscopy showing morphological changes in  
 505 CCoV-II-infected cells (MOI 10) after orange acridine staining. **A**, mock-infected cells; **B**, CCoV-II  
 506 –infected cells at 24 h p.i.; **C**, CCoV-II –infected cells at 48 h p.i.. Magnification: 400x.

507

508 **Fig. 4** – Caspase-3, -8 and -9 activation by flow cytometric analysis. Caspase-3 FLICA (FAM-  
 509 DEVD-FMK), caspase-8 FLICA (FAM-LETD-FMK) and caspase-9 FLICA (FAM-LEHD-FMK)  
 510 was added at the last hour of treatment to evaluate their activation. The results of fluorescence  
 511 profiles of CCoV-II-infected cells (MOI 10) at 8, 12, 24 and 48 h p.i. are reported. The data are  
 512 presented as the means  $\pm$  SEM of the results of three separate experiments. Significant differences  
 513 between CCoV-II infected group and mock-infected groups are indicated by probability P.  
 514 \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

515

516 **Fig. 5** – Effects of CCoV-II infection on the caspase cascade activation. **a**) Western blot analysis of  
 517 cleaved caspase-8, -9, -6 and -3 in CCoV-II-infected cells (MOI 10). Cells were mock-infected  
 518 (lane cc) and CCoV-II infected for the indicated times (4, 8, 12, 24, 36 and 48 h p.i.). Cellular  
 519 extracts were analysed by Western blot using anti-caspase-8, -9, -6 and 3 antibodies.  $\beta$ -actin was  
 520 used as an internal loading control. The molecular weight (kDa) of protein size standards is shown  
 521 on the left hand side. Blot is representative of three separate experiments. **b**) Densitometric analysis  
 522 of blots relative to cleaved caspase-8, -9, -6 and -3. Results are expressed as the mean  $\pm$  SEM of  
 523 three separate experiments.

524

525 **Fig. 6** - Western blot analysis of PARP activation in CCoV-II-infected cells (MOI 10). **a**) Cells  
 526 were mock-infected (lane cc) and CCoV-II infected cells (4, 8, 12, 24, 36, and 48 h p.i.). Cellular  
 527 extracts were analyzed by Western blot using specific antibody.  $\beta$ -actin was used as an internal

528 loading control. The molecular weight (kDa) of protein size standards is shown on the left hand  
529 side. Blot is representative of three separate experiments. **b)** Densitometric analysis of blots relative  
530 to cleaved and uncleaved PARP. Results are expressed as the mean  $\pm$  SEM of three separate  
531 experiments. **c)** Cleaved PARP/uncleaved PARP ratio in infected-cells obtained by densitometric  
532 analysis. Significant difference between the value at 12 h p.i. and the others times of p.i. are  
533 indicated by probability *P*. \*\**P* < 0.01.

534

535 **Fig. 7** – Western blot analysis of bid and cytochrome *c* in CCoV-II-infected cells (MOI 10). **a)** Bid  
536 cleavage and translocation of bid to mitochondria. The cells were mock-infected (lane cc) and  
537 CCoV-II infected (4, 8, 12, 24, 36 and 48 h p.i.). Cell lysates were collected at the indicated times  
538 p.i., and equal amounts of protein from each sample were subjected to Western blot analysis.  $\beta$ -  
539 actin was used as an internal loading control. The molecular weight (kDa) of protein size standards  
540 is shown on the left hand side. Blot is representative of three separate experiments. **b)**  
541 Densitometric analysis of blots relative to bid. Results are expressed as the mean  $\pm$  SEM of three  
542 separate experiments. **c)** Cytochrome *c* release in cytosol fraction. Cell lysates were collected at the  
543 indicated times p.i., and equal amounts of protein from each sample were subjected to Western blot  
544 analysis, and probed for cytochrome *c*.  $\beta$ -actin was used as an internal loading control. The  
545 molecular weight (kDa) of protein size standards is shown on the left hand side. Blot is  
546 representative of three separate experiments. **d)** Densitometric analysis of blots relative to  
547 cytochrome *c*. Results are expressed as the mean  $\pm$  SEM of three separate experiments.

548

549 **Fig. 8** - Effects of CCoV-II infection (MOI 10) on bcl-2 family proteins. **a)** Analysis of bax, bim,  
550 bcl-2 and bcl-XL proteins expression. Whole-cell lysate was prepared from mock infected (lanes  
551 cc) and infected cells at the indicated times (4, 8, 12, 24, 36 and 48 h p.i.). Western blot analysis  
552 was performed with an antibody which specifically recognized bax, bim, bcl-2 and bcl-XL and  $\beta$ -  
553 actin.  $\beta$ -actin was used as an internal loading control. The molecular weight (kDa) of protein size  
554 standards is shown on the left hand side. Blot is representative of three separate experiments. **b)**  
555 Densitometric analysis of blots relative to bcl-2 family proteins. Results are expressed as the mean  
556  $\pm$  SEM of three separate experiments.

557

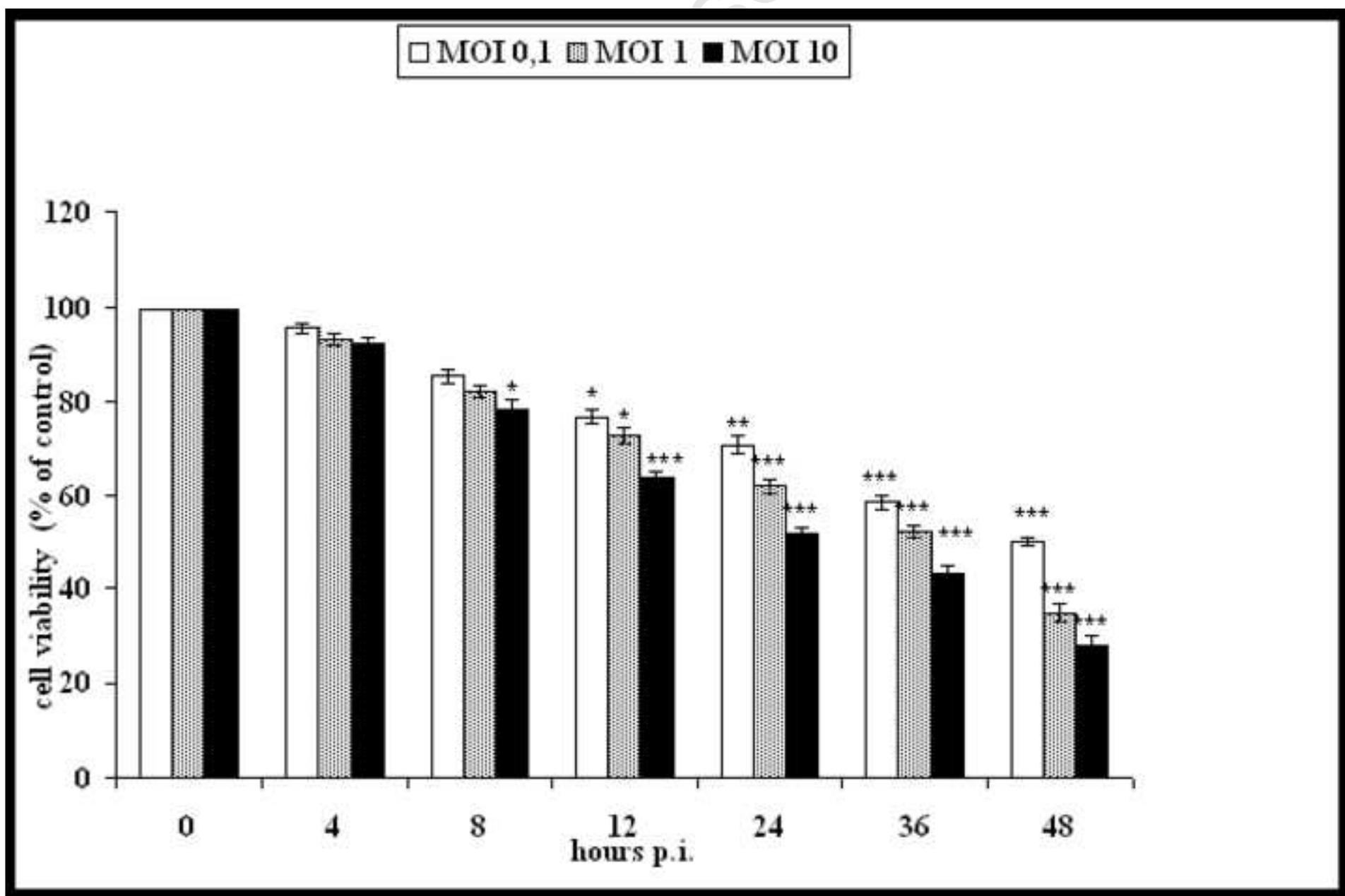
558 **Fig. 9** - Effect of caspase inhibitors on cell viability and virus release in CCoV-II-infected cells  
559 (MOI 10). **a)** A-72 cells were treated with Z-VAD-FMK (pan-caspase inhibitor), Z-DEVD-FMK  
560 (caspase-3-specific inhibitor), Z-IETD-FMK (caspase-8-specific inhibitor), Z-LEHD-FMK  
561 (caspase-9 inhibitor) 1 h prior to infection with CCoV-II. The cell viability were analyzed at 24 h

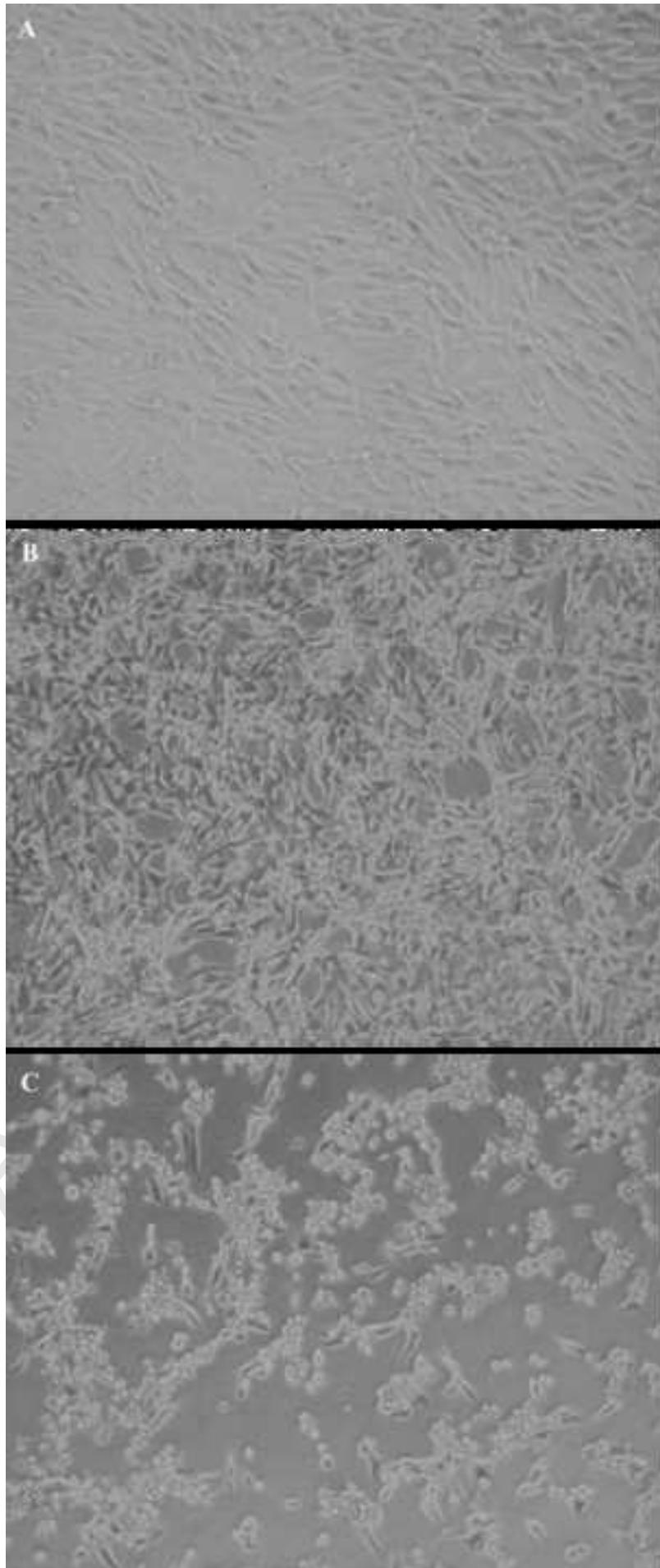
562 after infection by MTT assay. Data are presented as a percentage of the untreated cell control, and  
563 the results are the mean  $\pm$  SEM of three independent experiments. \*\*\*P < 0.001. **b)** Virus titration.  
564 The titer of virus released from cells treated with caspase inhibitors was estimated by TCID<sub>50</sub>. Data  
565 are presented as the mean  $\pm$  SEM of three separate experiments.

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Figure 1





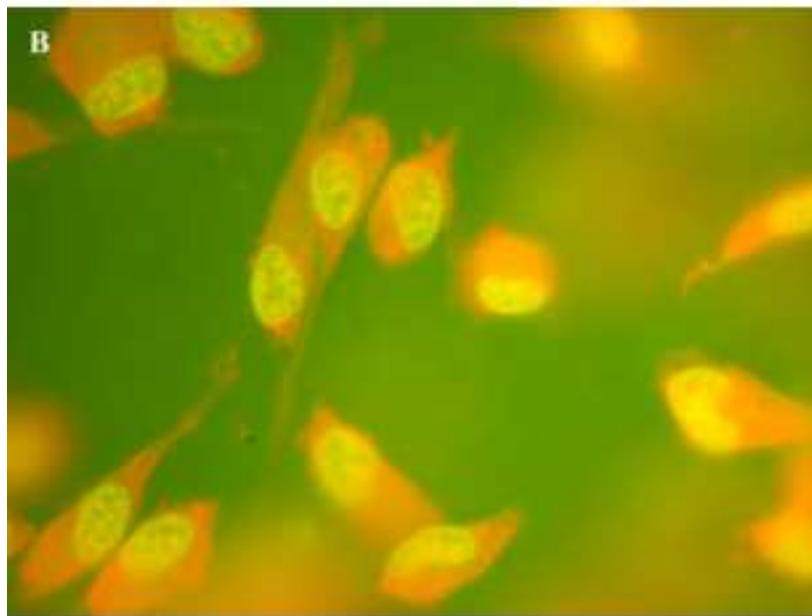
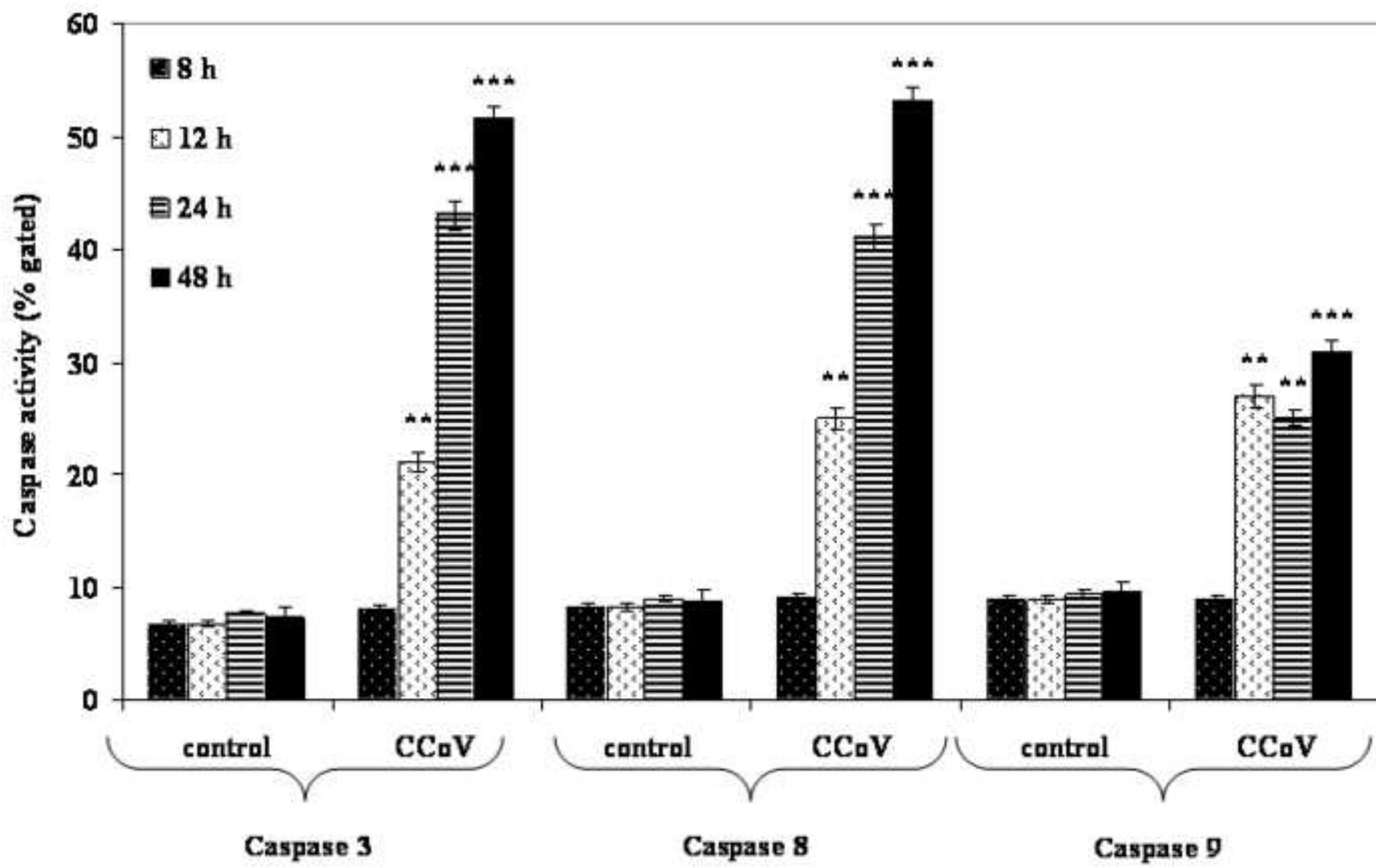
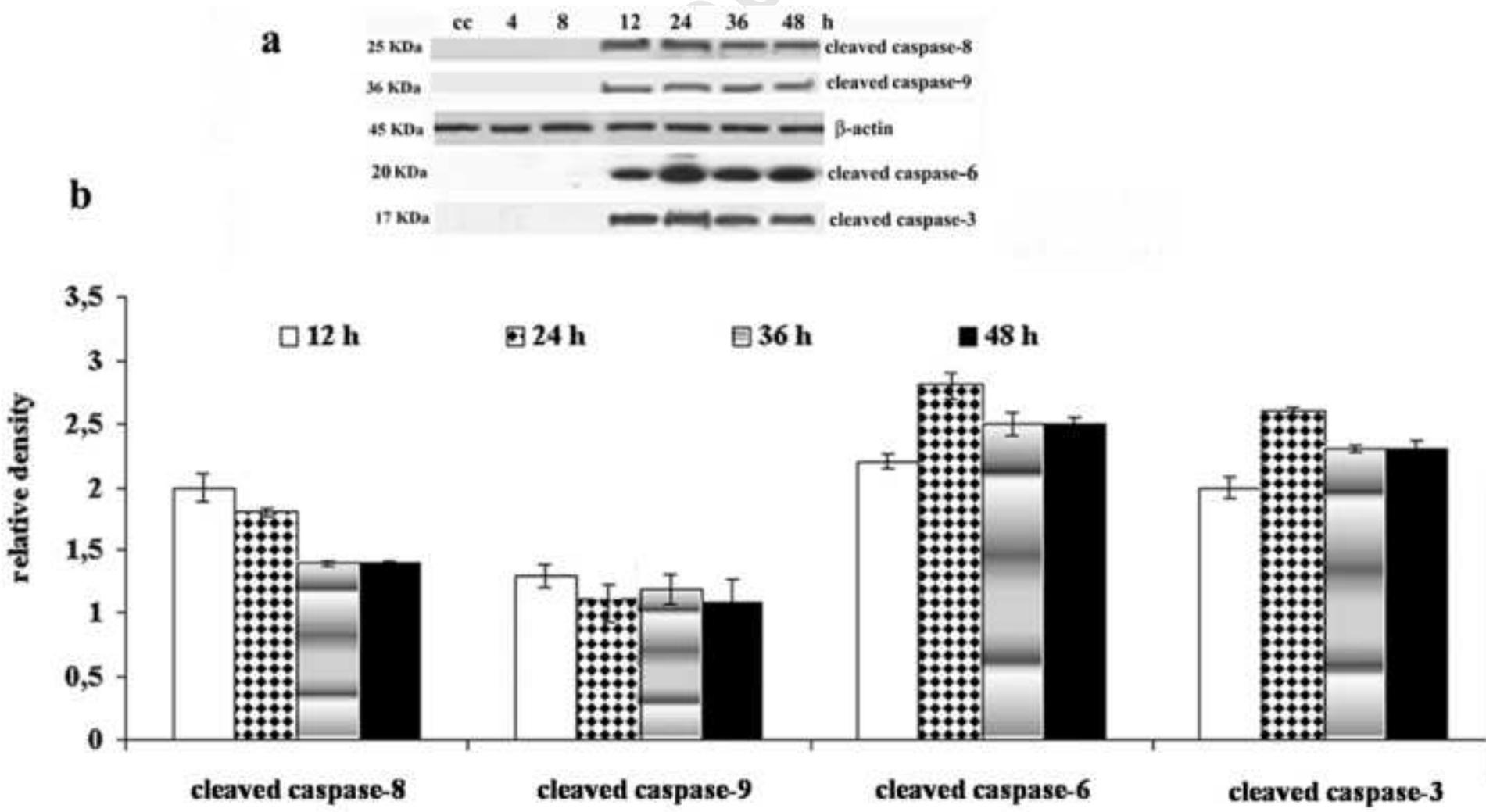
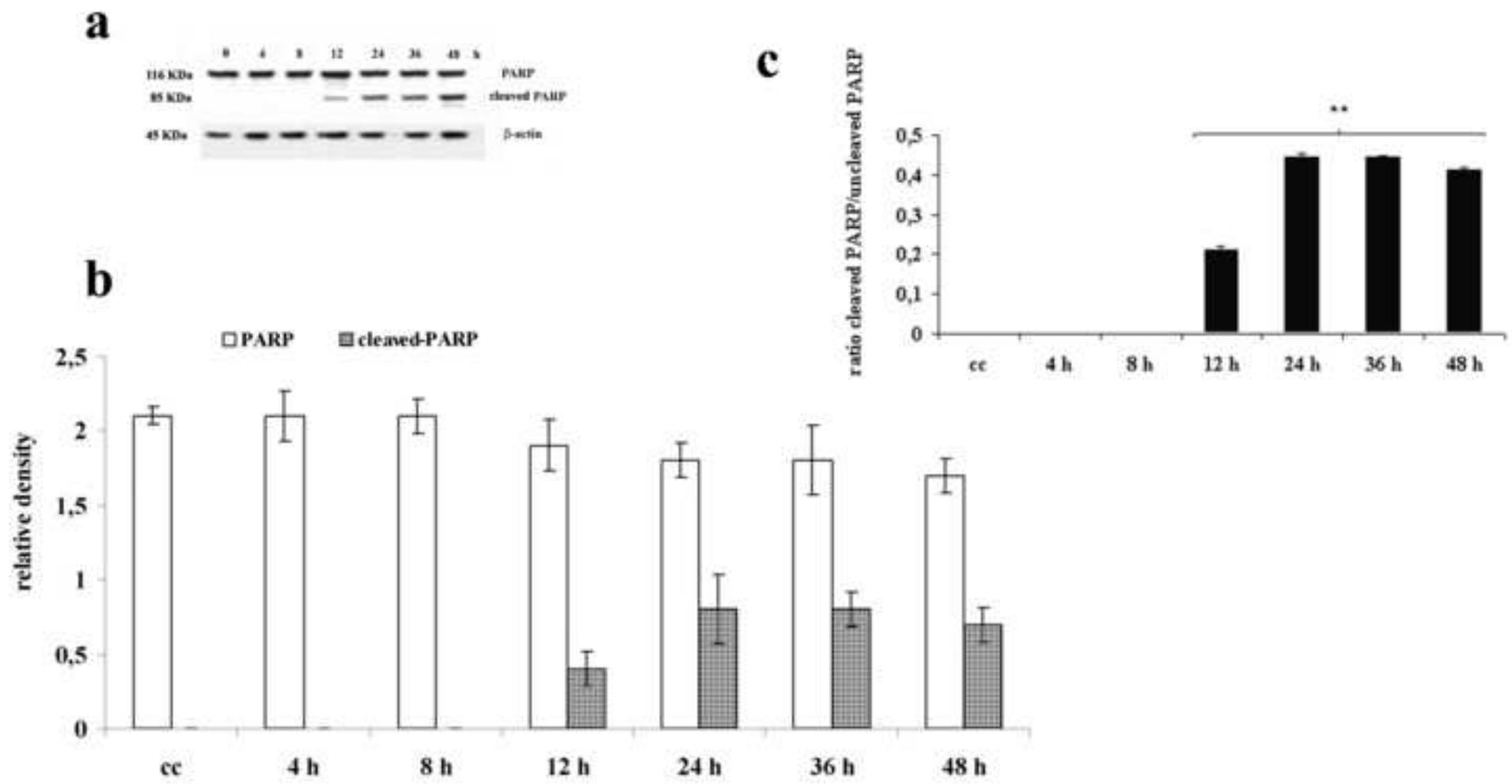


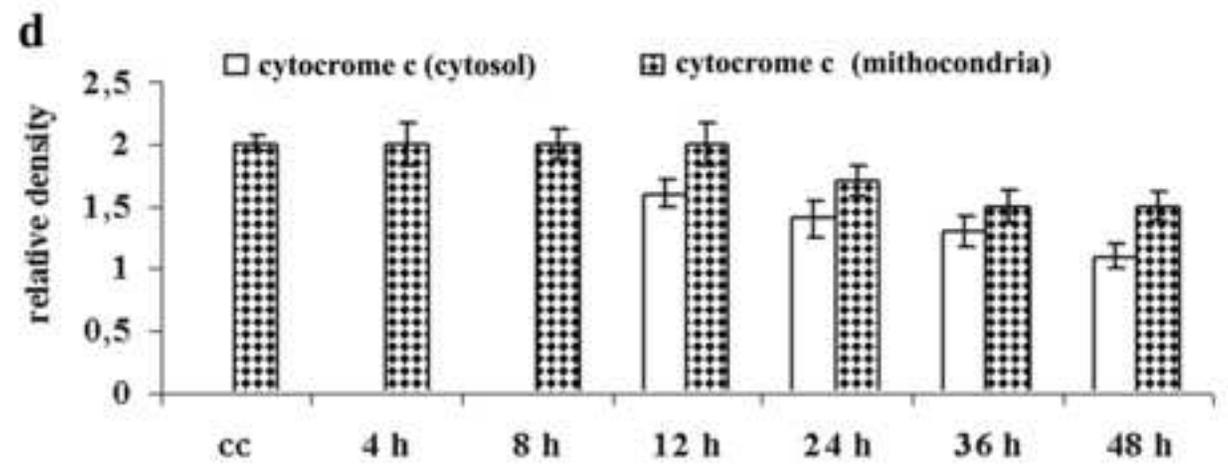
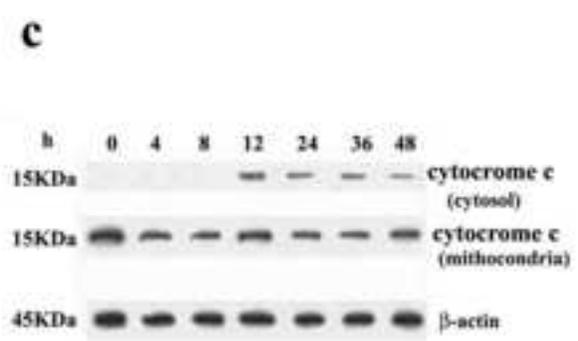
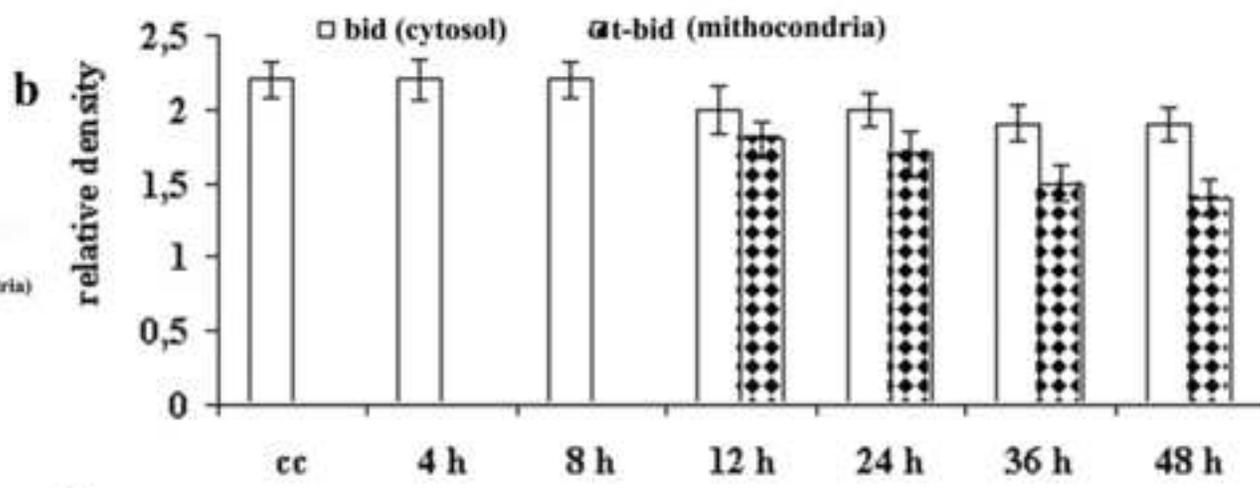
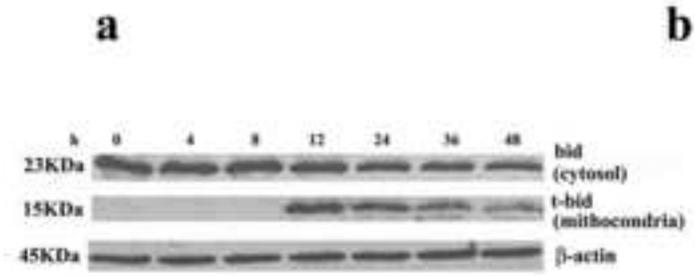
Figure 4





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