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1 Equine Arteritis Virus gP5 Protein Induces Apoptosis in Cultured Insect Cells

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12

13 **Abstract**

14 Equine Arteritis Virus (EAV) has been shown to induce apoptosis *in vitro* but the
15 induction of this mechanism has not been previously associated with any viral gene
16 product. In this work, we found a cytotoxicity effect of the EAV gP5 protein on
17 baculovirus-insect cells and a low yield of protein recovery. Besides, different
18 morphological features by electron transmission microscopy, DNA fragmentation in
19 agarose gel, TUNEL analysis and caspase 3 activity were found. All these findings
20 indicate that the EAV gP5 protein induces apoptosis in insect cells.

21 **Keywords:** Equine Arteritis Virus, gP5 protein, apoptosis, insect cells.

22

23 **Short communication**

24 Equine Viral Arteritis (EVA) is a respiratory and reproductive disease of
25 horses caused by Equine Arteritis Virus (EAV). EAV was first isolated in Ohio,
26 USA, in 1953 (Doll et al., 1957a, 1957b) and has been classified as a member of
27 the order *Nidovirales*, family *Arteriviridae*, and grouped together with lactate
28 dehydrogenase-elevating virus (LDV), porcine reproductive and respiratory
29 syndrome virus (PRRSV), and simian hemorrhagic fever virus (SHFV) (Cavanagh,
30 1997; Snijder, 2001).

31 EAV is a small enveloped virus with a 12.7-kb positive-sense single-
32 stranded RNA genome which includes nine functional open reading frames (ORFs)
33 (Snijder and Meulemberg, 1998). ORFs 1a and 1b encode two replicase
34 polyproteins (Snijder, 2001) and the remaining seven ORFs (2a, 2b, and 3 to 7)
35 encode the structural proteins of EAV. These structural proteins include four
36 membrane glycoproteins (GP2 (25 kDa), GP3 (36 to 42 kDa), GP4 (28 kDa), and
37 GP5 (30-44 kDa), encoded by ORFs 2b, 3, 4, and 5, respectively), two
38 unglycosylated membrane proteins (E (8 kDa) and M (17 kDa), encoded by ORFs
39 2a and 6), and the phosphorylated nucleocapsid protein N (14 kDa), encoded by
40 ORF 7 (Snijder et al., 1999; Wieringa et al., 2002).

41 In our laboratory, we have been studying the expression of EAV gP5
42 proteins as immunogen using baculovirus-insect cell system. The EAV gP5 gene
43 was amplified using cDNA from the EAV strain LP02/C (GenBank reported partial
44 ORF: DQ435441.1) (Echeverría et al., 2007). A pairs of primers with a restriction
45 site sequence were then designed to allow the subsequent cloning into the

46 pFastBacHT-B vector. The sense and antisense primers designed were:
47 pFastgP5*Bam*HI:5'-GGGGATCCGGCTCAACGATGTTATCT-3' (nucleotides 11137
48 to 11154) and pFastgP5*Hind*III:5'-GGAAGCTTATGAATCTATGGCTCCCA-3'
49 (nucleotides 11902 to 11919). The underlined nucleotides denote the restriction
50 enzyme sites added to the primers.

51 EAV gP5 PCR product was digested with the corresponding restriction
52 enzyme and cloned into the pFastBacHT-B vector previously digested with the
53 same restriction enzyme to generate the pFastBac-gP5 recombinant vector. This
54 construction was confirmed by sequencing, using pFastBac Forward/Reverse
55 Sequencing Primers.

56 Competent *Escherichia coli* DH10Bac cells, containing bacmid (baculovirus
57 shuttle vector plasmid) and helper plasmid, were used to transform with pFastBac-
58 gP5 and generate the recombinant bacmid (Bac-gP5) according to the Bac-to-Bac
59 Baculovirus Expression System Manual (Invitrogen). The recombinant Bac-gP5
60 was transfected into a *Spodoptera frugiperda* cell line (*Sf9*) in serum-free medium
61 using Cellfectin reagent (Gibco BRL).

62 Three days after transfection, the culture supernatant containing
63 recombinant viruses (Bac-gP5) was harvested and subjected to standard plaque
64 purification methods (Brown and Faulkner, 1977; King et al., 2007). Expression of
65 recombinant gP5 protein was determined in High Five cells by SDS-PAGE and
66 Western Blot with Anti-Histidine Antibody (GE Healthcare) (Fig. 1).

67 In our attempt to recover gP5 protein from Bac-gP5 recombinant
68 baculovirus, we had problems of very low yields of protein expression as shown in

69 SDS-PAGE and an unusual cytopathic effect observed in cultured insect cells as
70 compared with recombinant baculoviruses carrying the EAV M (Bac-M). Infected
71 High Five cells with Bac-gP5 did not show the rounded refringent cells
72 characteristic of successful infection. We found no previous reports of problems
73 in the expression of EAV gP5 protein in a baculovirus system (Hedges et al., 1998)
74 or other expression systems used to express this protein (MacLachlan et al., 1998;
75 Weiland et al., 2000). Nevertheless, we found that previous reports have shown
76 that gP5 protein from Porcine Reproductive and Respiratory Syndrome Virus
77 (PRRSV) is associated with a strong cytotoxicity in cultured cells. This
78 phenomenon has been associated with the induction of apoptosis (Suárez et al.,
79 1996) by PRRSV gP5 protein. Also, other authors have reported that the first 119
80 amino acids, especially amino acids 90-119, constitute a fundamental region
81 capable of inducing this mechanism (Fernández et al., 2002).

82 Apoptosis is one of the mechanisms by which nucleated eukaryotic cells die
83 (Elmore, 2007) and is the innate mechanism by which organisms eliminate
84 unwanted cells. Apoptosis is characterized by chromatin condensation, plasma-
85 membrane blebbing, cell shrinkage, and DNA fragmentation into membrane-
86 enclosed vesicles or apoptotic bodies (Häcker, 2000) and is triggered by a variety
87 of stimuli such as UV radiation, chemicals and infectious agents.

88 Several viruses have been shown to be associated with induction of
89 apoptosis (Del Puerto et al., 2011; Sur et al., 2000; Tran et al., 2013; Xu et al.,
90 2012). Archambault and St-Laurent (1999) found that EAV induces this mechanism
91 *in vitro* but they could not identify the virus genes responsible for its induction.

92 As mentioned above, apoptotic cells exhibit characteristic morphological
93 features. Consequently, Bac-gP5-infected High Five cell monolayers were
94 prepared for examination by transmission electron microscope. Bac-gP5-infected
95 cells showed condensation of chromatin, nuclear fragmentation into apoptotic
96 bodies and plasma membrane blebbing, observed also in sorbitol-induced
97 apoptosis cells. Non-infected and Bac-M-infected cells did not show any of these
98 features (Fig. 2). These morphological features were used as a first evidence of
99 apoptosis in Bac-gP5 infected culture.

100 The incidence of nucleosome fragmentation by activation of intracellular
101 endonucleases is associated with morphological changes in cells undergoing
102 apoptosis (Rogalinska, 2002). Consequently, DNA was extracted from Bac-gP5
103 and Bac-M-infected cultures and from non-infected-cultured High Five cells using a
104 DNA Purification Kit (Promega). The results were analyzed by agarose gel
105 electrophoresis, using sorbitol as a positive control of DNA fragmentation. The gel
106 showed DNA fragmentation in Bac-gP5 and in sorbitol positive control. In contrast,
107 no evidence of DNA fragmentation was observed in non-infected and in High Five
108 cells infected with wild-type baculovirus (Bac) and with Bac-M (Fig. 3). This
109 observed evidence of cellular apoptosis is due to the EAV gP5 expression in insect
110 cells but has no correlation with the expression of EAV M protein or with
111 baculovirus backbone.

112 Further, we analyzed the fragmentation of DNA in situ by using DeadEnd™
113 Colorimetric TUNEL System (Promega) to complement the above results. Briefly,
114 in this system, biotinylated nucleotides are incorporated at the 3'-OH DNA ends by

115 using the terminal deoxynucleotidyl transferase. Horseradish peroxidase-labeled
116 streptavidin is then bound to these biotinylated nucleotides, which are detected
117 using peroxidase and diaminobenzidine (DAB) and with methyl green as a
118 counterstain after completion of the immunohistochemistry. Using this procedure,
119 apoptotic nuclei are stained dark brown. As it showed in figure 4, Bac-gP5-infected
120 cells and sorbitol-induced apoptosis cells showed evidence of precipitated brown
121 staining after 48hs post-infection compatible with colorimetric detection of DNA
122 fragmentation. These observations were also evidence in DNase-treated cells
123 used as positive control. No evidence of apoptotic nuclei were found in any of the
124 other assays made.

125 These results provide evidence that morphological changes previously
126 observed in transmission electron microscopy are due to the activation of
127 endonucleases. These endonucleases are cysteine proteases called caspases that
128 have a central function in the amplification of cell death signal and consequently to
129 the induction of apoptosis mechanism (Kumar, 2007). Caspase-3 is required for
130 some typical hallmarks of apoptosis as chromatin condensation and DNA
131 fragmentation in all cell types examined (Porter and Jänicke, 1999).

132 An immunodetection of caspase-3 in cultured High Five cells was made
133 using Purified Rabbit Anti-Active Caspase-3 (BD-Biosciences-Pharmingen). The
134 figure 5 showed a band at a predicted molecular weight of ~ 20 kDa corresponding
135 to the activate caspase-3 in Bac-gP5 infected cells and in sorbitol positive control.

136 We speculated that EAV gP5 protein could play a role in the induction of
137 apoptosis, as its counterpart, gP5 protein from PSRRV (Suárez et al., 1996). The

138 expression of EAV gP5 protein in insect cells evidences a poor accumulation and
139 strong cytotoxicity in infected cells due to apoptotic death, as observed with
140 expression of PRSSV gP5 (Fernández et al., 2002).

141 Collectively, the results obtained in this study indicate that EAV gP5 protein
142 induces apoptosis in insect cells. This is the first report that associates the
143 induction of apoptosis by an EAV viral product. The first evidence of EAV apoptosis
144 induction was observed in *in vitro* cultured Vero cells (Archambault and St-Laurent,
145 2000). It has been shown that this induction is initiated by caspase-8 activation and
146 subsequent mitochondria-dependent caspase-9 activation (St Louis and
147 Archambault, 2007) and that this mechanism of induction is mediated through the
148 intrinsic signaling pathway (Cholleti et al., 2013). Nevertheless, none of these
149 studies identified any viral gene product that could be a key factor in the induction
150 of apoptosis by EAV.

151 It has been documented that insect cells induce apoptosis as an antiviral
152 defense because of their lack of an adaptive immune response (Clarke and Clem,
153 2003). Baculoviruses are known to inhibit apoptosis in host cells (Clem et al.,
154 1991), a fact that has been correlated with at least two different types of
155 antiapoptotic genes, *p35* and *iap*, which prevent this mechanism in insect cells
156 (Clem, 2001). Normally, insect cells infected by baculoviruses do not exhibit
157 evident cytopathic effect until three days post-infection (Clem et al., 1991) because
158 these baculovirus-encoded proteins block apoptosis in early stages of virus
159 infection (Lacount et al., 1997), thus prolonging virus replication. We have found
160 DNA fragmentation in cells infected with baculovirus harboring EAV gP5 after 24hs

161 of infection (data not shown) correlating this results with the expression of this
162 protein.

163 Ours results evidence that EAV gP5 protein is sufficient to trigger an
164 apoptotic response in insect cells, despite the presence of these antiapoptotic
165 proteins. Cultured-infected insect cells with wild-type baculovirus and baculovirus
166 expressing EAV M protein did not show evidence of apoptosis with any of the
167 methods used in this study indicating that the observed effect were due to the
168 expression of gP5 protein in High Five cells. However, the mechanism of action in
169 this activation is not clear. We hypothesize that the expression of EAV gP5 protein
170 could inhibit the action of any of the antiapoptotic baculovirus proteins or induce
171 apoptosis by a distinct apoptotic signal cascade that is not inhibited by these
172 baculovirus proteins in insect cells. This induction of apoptosis may contribute to
173 the low expression level of EAV gP5 recovery. Similar results have been found in
174 the expression of Hepatitis C Virus E protein in insect cells (Ciccaglione et al.,
175 2003).

176 As mammalian and insect cells share similarities in their apoptotic pathways,
177 encouraged by this results our future experiments will attempt to identify and
178 characterize this mechanism of EAV gP5 apoptosis induction in mammalian cells.

179

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185

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274 **Figure1:** [A] 12% SDS-PAGE. Total protein from High Five cells lysates transfected
275 with recombinant baculoviruses. (L) Low Molecular Weight Marker ; (1, 3) Non-
276 infected High Five cells; (2, 4) Bac-gP5 and Bac-M infected High Five cells,
277 respectively. [B] Western Blot revealed with 1:3000 Anti-Histidine Antibody . (1)
278 Bac-M-infected High Five cells; (2) Page Ruler Marker ; (3) Bac-gP5- infected High
279 Five cells.

280

281 **Figure 2:** Transmission electron micrographs taken from different infected and
282 non-infected High Five cells at 72 h post-infection. Representative fields are
283 shown. (A, B, C) Bac-gP5-infected High Five cells and (D) Sorbitol-induced
284 apoptosis High Five cells show morphological hallmarks of apoptosis included
285 condensation of chromatin, the formation of apoptotic bodies and plasma
286 membrane blebbing. (E, F) Non-infected cells and (G) Bac-M infected cells do not
287 show distinguishable signs of apoptosis.

288

289 **Figure 3:** Agarose gel electrophoretic pattern of DNA extracted from different
290 cultured cells taken at 48h post-infection: (L) 100bp DNA ladder ; (1) Sorbitol-
291 induced apoptosis High Five cells; (2) Bac-gP5-High Five infected cells; (3) Bac-M-
292 High Five infected cells; (4) Non-infected-High Five cells; (5) Wild-type baculovirus-
293 High Five infected cells

294

295 **Figure 4:** Several apoptotic cells by using Colorimetric TUNEL System showing
296 the distinct condensation of the nuclear chromatin. (A, B, C) Bac-gP5-infected High
297 Five cells; (D) Sorbitol-induced apoptosis High Five cells; (E) High Five cells
298 incubate with DNase. No evidence of apoptosis in non-induced cells (F) and Bac-
299 M-infected High Five cells (G). Photographs were taken with 40x magnification.

300

301

302 **Figure 5:** Western Blot analysis using Purified Rabbit Anti-Active Caspase-3 . (L)
303 Page Ruler Marker ; (1) Bac-gP5-High Five infected cells; (2) Non-infected High
304 Five cells; (3) Bac-M-High Five infected cells; (4) Wild-type baculovirus-infected
305 cells; (5) Sorbitol-induced apoptosis cells. The highlight bands of ~20 kDa
306 corresponding to the active caspase-3. The bands observable at ~25 kDa
307 represent the light chains of Ig used for the immunoprecipitation.

308

308 **Highlights**

309 A recombinant baculovirus expressing EAV gP5 was constructed.

310 The expression of EAV gp5 protein showed cytotoxicity effect in insect cells.

311 PRRSV gP5 protein was associated with a strong cytotoxicity in culture cells.

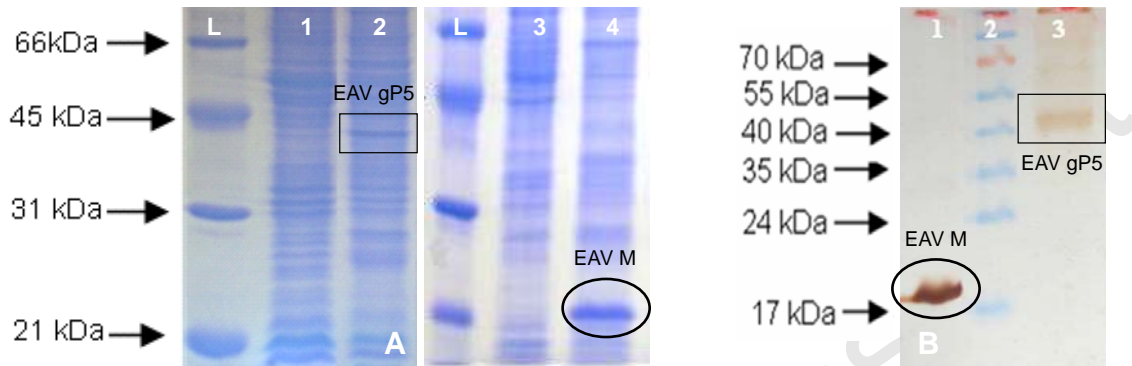
312 Several observed features in insect cultures were correlated with the induction of
313 apoptosis.

314 We identify a viral gene product that could be a key-factor in apoptosis induction.

315

315 FIGURE 1

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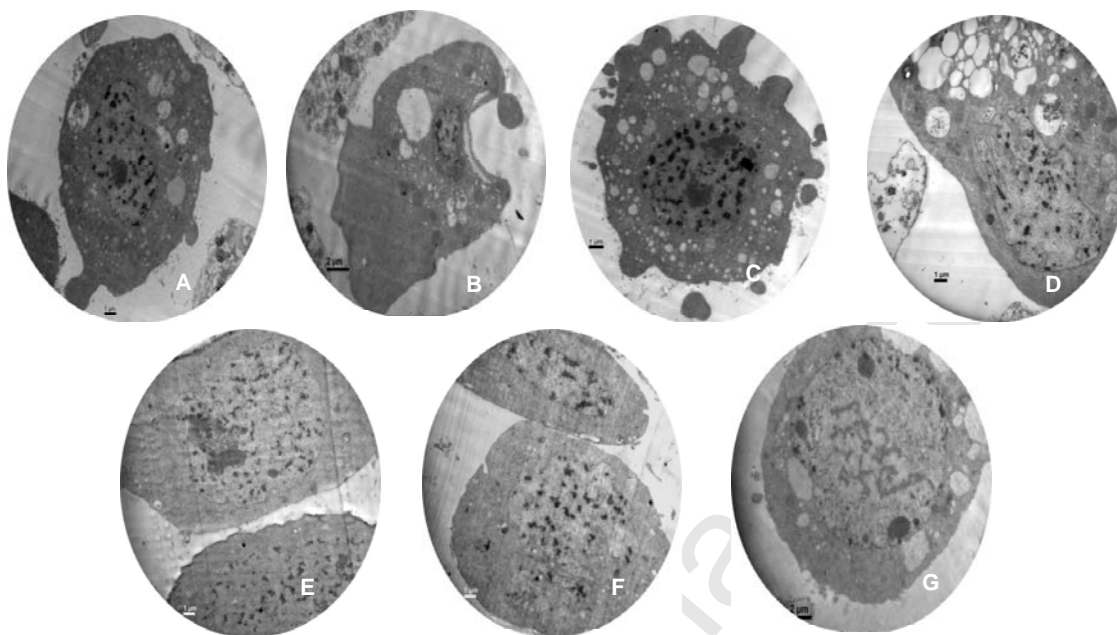
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319 FIGURE 2

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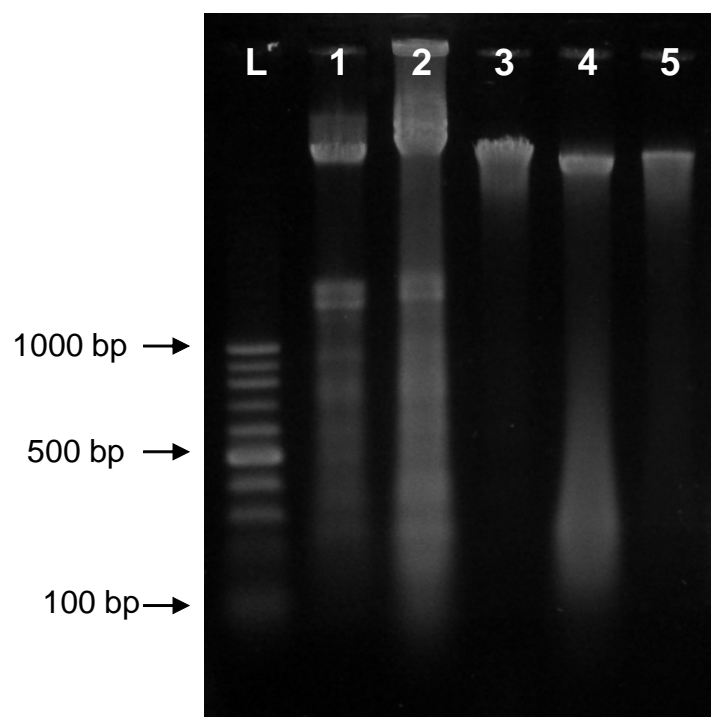


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322 FIGURE 3

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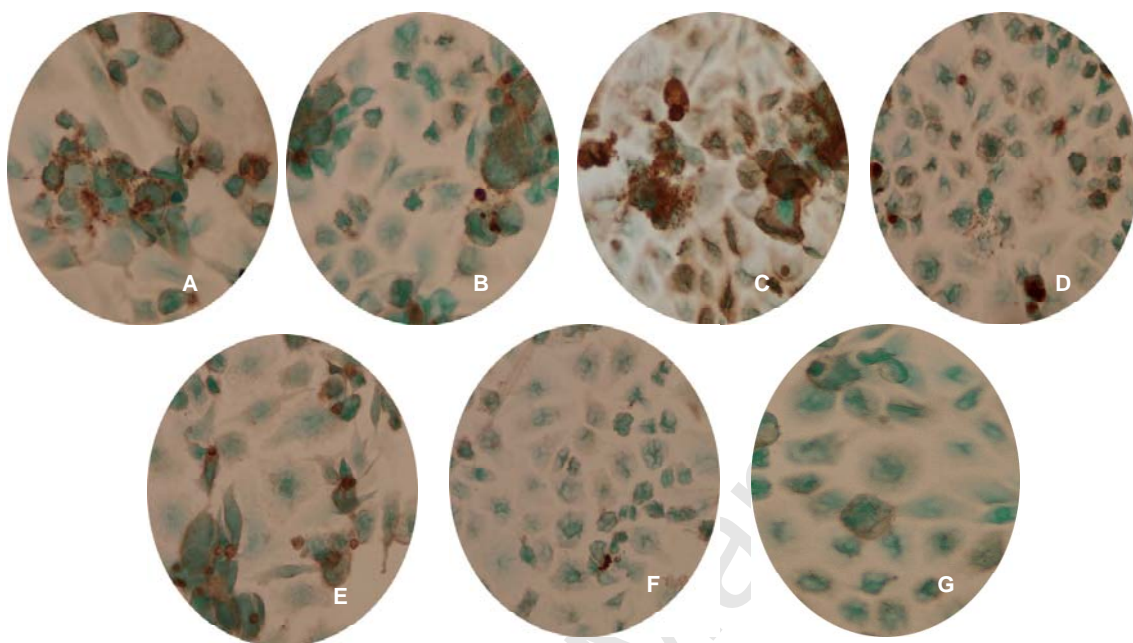
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328 FIGURE 4

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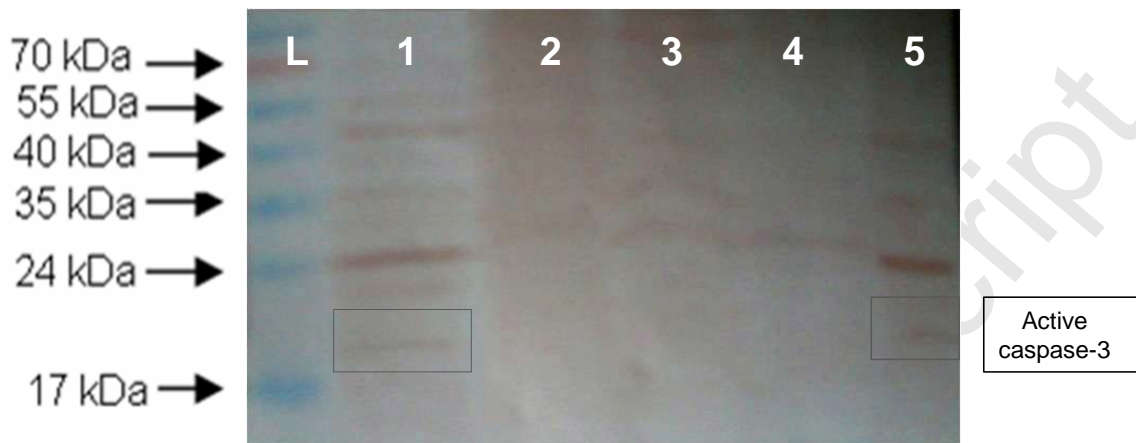
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337 FIGURE 5

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