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EVALUATION OF APOPTOSIS AND AUTOPHAGY ACTIVITIES IN THE BRAIN OF DOGS NATURALLY INFECTED WITH CANINE DISTEMPER VIRUS BASED ON CHANGES IN APOPTOTIC AND AUTOPHAGIC MARKERS

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This study investigated the activation of apoptosis and autophagy in CDV infected brain tissues of dogs with acute neurological signs, by determining Cas-3, Cas-8, Cas-9, Bax, Bcl-2, LC3B, and Beclin-1 expression with real-time PCR. The expression levels of Beclin-1 and LC3B, autophagy markers, were significantly up-regulated in comparison with the control group ($p < 0.001$). The expression levels of apoptotic markers Cas-3, Cas-8, Cas-9 and Bax were slightly up-regulated, but Bcl-2 was slightly down-regulated in contrast to the control group ($p < 0.05$). Therefore, the autophagy markers were more activated than apoptotic markers in dogs with acute neurological signs. In conclusion, autophagy takes part in the pathogenesis of demyelination in canine distemper. Knowing this may be helpful to create new therapeutic strategies, such as new effective antiviral medicines.

Keywords: apoptosis, autophagy, canine distemper, real-time PCR

INTRODUCTION

Canine distemper is a worldwide disease caused by a member of the *Morbillivirus* genus, *Paramyxoviridae* family, closely related to measles and rinderpest virus [1]. The condition has been known to affect dogs and other carnivores and is one of the most infectious viral diseases with the highest fatality rate in dogs besides rabies [2]. Canine distemper virus (CDV) causes a multisystemic infection including the gastrointestinal, respiratory and nervous systems [3,4]. As a result of the viral spread to the central nervous system (CNS), multifocal demyelination occurs [3]. The demyelination-linked clinical findings of canine distemper encephalomyelitis vary [5] and include lethargy, seizures, behavioural disorders, ataxia, incontinence, muscle atrophy, hyperaesthesia and myoclonus [6]. The mechanism behind the formation of demyelination as the most

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impactful result of the disease is controversial as it is associated with viral replication in the white matter only in the early stage [7]. Additionally, Schobesberger et al. [8] have suggested that virus-induced demyelination was not a direct result of either apoptosis or necrosis in canine distemper. However, in recent studies, CDV has been shown to induce apoptosis [9] by activating cysteine aspartic acid-specific protease-8 (caspase-8) and caspase-3 gene expression [10] in the cerebellum and lymphoid tissue [11]. The exact mechanism was also observed in Vero cells [12,13]. In another study, Delpeut et al. [13] showed that autophagy was activated by morbilliviruses expeditiously and this activation was required for an effective spread to other cells. Similarly, Kabak et al. [15] underlined the increased autophagic activity in the cerebellum of CDV-infected dogs.

Under normal physiological conditions, apoptosis is responsible for regulated cell death. Apoptosis has an important function in the homeostasis of multicellular organisms through the cell and tissue regeneration, eradication of damaged and cancerous cells [16]. The way that apoptosis occurs is a complicated mechanism involving caspases, activating proapoptotic genes and inhibiting anti-apoptotic proteins. Apoptosis causes common morphological formations in the cell such as membrane overflow, condensation of chromatin, cell shrinkage and apoptotic bodies [17]. Apoptosis is mainly performed through two pathways, extrinsic and intrinsic. To initiate the extrinsic pathway, death-ligand binds with death receptors through activation of caspase-8. Activation of the intrinsic pathway, on the other hand, is based on a disparity between proapoptotic [Bcl-2-associated X-protein (Bax), Bcl-associated killer (Bak)] and antiapoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) from the Bcl-2 family in the mitochondria and cytosol, which leads to the release of cytochrome c from the mitochondria, which then activates caspase-9 [18]. Both initiator proteins caspase-8 and caspase-9, activate the executioner protein caspase-3. Caspase-3 besides the other caspase-effectors stick to critical cellular proteins which gives rise to apoptosis [18]. Cysteine aspartic acid-specific proteases are the most important effectors of apoptosis. Activation of caspase-3 is thought to play a pivotal role in apoptosis. It is responsible for the breakdown of several cellular components involved in DNA repair and regulation. This caspase is not directly activated by apoptotic stimulants, but is based on the proteolytic activity of upstream initiator caspase-8 and -10 [19].

Apoptosis can take part in the defence mechanism against viral infections. First and foremost, cells may detect the components of a virus, including the viral proteins, during the entry and replication of the virus. Then cells execute apoptosis to prevent being a production line of viral replication [20]. On the contrary, apoptosis can be a tool for viral infection as it can be activated or hindered by viral proteins [21]. Viral proteins can hinder apoptosis in order to augment viral replication and promote a persistent infection. In the later phase, when viral replication reaches its maximum, apoptosis is induced to facilitate the spread to the surrounding cells and eliminate the risk of host inflammatory responses.

Interestingly, some recent studies asserted that apoptosis and autophagy might be interconnected and concurrently occurring, with a possible transition from

an autophagic process at the beginning to apoptotic cell death at the end [22-24]. Both apoptotic and autophagic activities were reported to be induced by CDV in different studies [10-12,15,25]. Autophagy is a common mechanism responsible for maintaining homeostasis against cellular stress through reprocessing worn-out proteins and cytoplasmic organelles. Shortly, a membrane of autophagosome is formed around the damaged cellular components which then fuses with lysosomes to digest the components inside [26]. The ubiquitin E1-like enzyme autophagy-related gene 7 (Atg7) and the E2-like enzyme Atg10 take part in the conjugation of Atg5 and Atg12 in the autophagosome formation [27,28]. Subsequently, the cleavage of the microtubule-associated protein 1A / 1B-light chain 3 (LC3) in LC3A (LC3-I) and LC3B (LC3-II) takes place [29]. Those light chain forms (LC3A and LC3B) supervise the autophagic activity and are major components of the autophagosome. LC3A is localized intracellularly and converted from the cytoplasmic form of LC3. LC3B, on the other hand, is the membrane-bound cleavage product of LC3 [30,31]. Finally, Beclin 1 participates in autophagosome formation [29].

In this study on CDV-infected dogs with acute neurological signs, we hypothesized that expression levels of autophagic markers in brain tissues should be higher than that of apoptotic markers. We also hoped to confirm the concurrent occurrence of autophagy and apoptosis with a transition from autophagy at the beginning to apoptosis at the end. Several studies show that CDV induces apoptosis and autophagy. However, to our knowledge, no study evaluates apoptosis and autophagy together and the dominancy of such forms of cell death through determining the expression levels of caspases (Cas-3, Cas-8, Cas-9), Bax, Bcl-2, LC3B, and Beclin-1 via real-time PCR.

MATERIALS AND METHODS

Animals

The study material consisted of ten dogs (four males and six females) of different breeds, weighing 8±2 kg, ageing between 3-5 months as displayed in Table 1. The group of CDV-positive dogs (n= 5) consisted of Kangal crossbreed dogs (two males and three females) with involuntary contractions of limbs and facial muscles which were tested positive for the CDV antigen (Ag) with a rapid test kit (catalogue no. RG1303DD, BioNote, Korea). Since the owners refused the treatment, the CDV-positive dogs were euthanized. Rottweiler (n=2) and Golden Retriever (n=3) dogs (two males and three females) constituted the control group (n= 5) with negative results for CDV test kits, which died due to trauma. In this study, we examined the left-over brain tissues (cortex and cerebellum) of dogs that we used in our previous study [30]. For this very reason, the records of the animals used in the previous study were ad verbum taken. Brain tissues of dogs were dissected and frozen at -80 °C for later investigation using the RT-PCR method. For all experiments, we investigated the same region of the brain tissues. Atatürk University Faculty of Veterinary Medicine Ethics Committee approved the study for compliance with ethical rules (protocol no. 2020/09).

Table 1. Ages of dogs with CDV and control dogs

Animal	C1	C2	C3	C4	C5	CDV1	CDV2	CDV3	CDV4	CDV5
Age	3 m	3.5 m	5 m	3 m	4 m	3.5 m	3 m	5 m	4 m	4 m

m: months, C: control

Detection of CDV

CDV mRNA was detected with Real-Time PCR (RT-PCR, ROTOR-GENE Q 5plex HRM, Qiagen, Germany). The specific primers of CDV were used as described previously [33] (Table 2). The RT-PCR procedure was realized according to the previous study [32].

Table 2. Sequences of the RT-PCR primers

Primers	**Sequences (5'-3')**	**Fragment length (bp)**
CDV-F	TGCGGTCTTACATTTGCATC	669
CDV-R	ACTCCAGAGCAATGGGTAGGG	
Cas3-F	TTC ATT ATT CAG GCC TGC CGA GG	83
Cas3-R	TTC TGA CAG GCC ATG TCA TCC TCA	
Cas8-F	ACA AGG GCA TCA TCT ATG GCT CTG A	70
Cas8-R	CCA GTG AAG TAA GAG GTC AGC TCA T	
Cas9-F	TCA GTG ACG TCT GTG TTC AGG AGA	97
Cas9-R	TTG TTG ATG ATG AGG CAG TAG CCG	
Bax-F	TTC CGA GTG GCA GCT GAG ATG TTT	79
Bax-R	TGC TGG CAA AGT AGA AGA GGG CAA	
Bcl-2-F	CAT GCC AAG AGG GAA ACA CCA GAA	76
Bcl-2-R	GTG CTT TGC ATT CTT GGA TGA GGG	
LC3B-F	GTC GCA CCT TCG AAC AAA GA	190
LC3B-R	AGC TGT AAG CGC CTC CTA AT	
Beclin1-F	TTG GTC ACC AGG TGG TGT GA	199
Beclin1-R	CAA CAG TGT ACC TCT GGG CA	
GAPDH-F	TCCATGACCACTTCGGCATC	310
GAPDH-R	TCCGATGCCTGCTTCACTAC	

Total RNA isolation and cDNA synthesis

Total RNA extraction was realized with QIAzol Lysis Reagent (Cat. No. 79306; Qiagen, Germany) from the brains of dogs following the product guidance. Following total RNA isolation, the RNA concentration was ascertained via a NanoDrop (Epoch

Microplate Spectrophotometer, USA). QuantiTect Reverse Transcription (Cat. No. 330411; Qiagen, Germany) was used for cDNA synthesis consistent with the product guidance.

Real-Time-PCR

The mRNA transcript levels of Cas-3, Cas-8, Cas-9, Bax, Bcl-2, LC3B, and Beclin-1 were determined with the RT-PCR in the brain of dogs. GAPDH gene was used for normalization. The primer sequences were designed explicitly for *Canis lupus familiaris* (Table 2) [10]. The Ct/Cq values in gene expression level were evaluated based on the 2-ΔΔCT method [34-36].

Statistical analysis

A one-way analysis of variance (IBM SPSS 20) was used to determine statistical differences in the expressions of Cas-3, Cas-8, Cas-9, Bax, Bcl-2, LC3B, and Beclin-1 of both groups. Relative mRNA fold change graphics were generated with GraphPad Prism software, version 7.0 (GraphPad Software Inc., CA, USA). The differences were regarded to be significant for $p<0.001$. $p<0.01$, and $p<0.05$.

RESULTS

Clinical findings of dogs with CDV

In the clinical examination of the dogs, mild fever, crusty discharge and involuntary contractions in the whole body, especially in the facial and leg muscles were detected. In the anamnesis, the dogs with CDV generally have been reported as having bilateral ocular discharge for the last 4-5 days, initiation of neurological findings for the previous 2 days and gradually worsening of such findings. Since the owners refused the treatment, the CDV-infected dogs were euthanized.

Detection of CDV in brain tissue

The RT-PCR method was employed to detect CDV in infected and non-infected brain tissues of dogs. While the brain tissues of infected dogs were tested as CDV positive, those of non-infected ones were tested negative for CDV (Table 3).

Table 3. Detection of CDV in serial dilutions (10-fold) by RT-PCR

Dilutions	10^0	10^1	10^2	10^3	10^4
Real-time PCR[a]	P (5/5)	P (5/5)	P (5/5)	P (5/5)	P (5/5)
Ct values[b]	20.2 ± 0.3	21.2 ± 0.2	25.9 ± 0.6	33.5 ± 0.4	36.9 ± 0.8

a: results of the 5 analyses. b: mean values and standard errors of the 3 measurements, P: positive

Detection of apoptotic and autophagic markers

When apoptotic markers were examined, the expression levels of Cas-3, Cas-8, Cas-9, and Bax were slightly up-regulated in the CDV infected brain tissues compared to the control group ($p < 0.05$). The Bcl-2 expression level was slightly down-regulated in the CDV infected brain tissues in comparison to the control group ($p < 0.05$) (Figs. 1A-1E). The expression levels of Beclin-1 and LC3B, autophagy markers, were remarkably up-regulated in the CDV-infected brain tissues in contrast to the control group ($p < 0.001$) (Figs. 2A and 2B). Comparing the expression levels of the markers of the two death mechanisms, autophagy markers were found to be highly activated compared to the apoptosis markers.

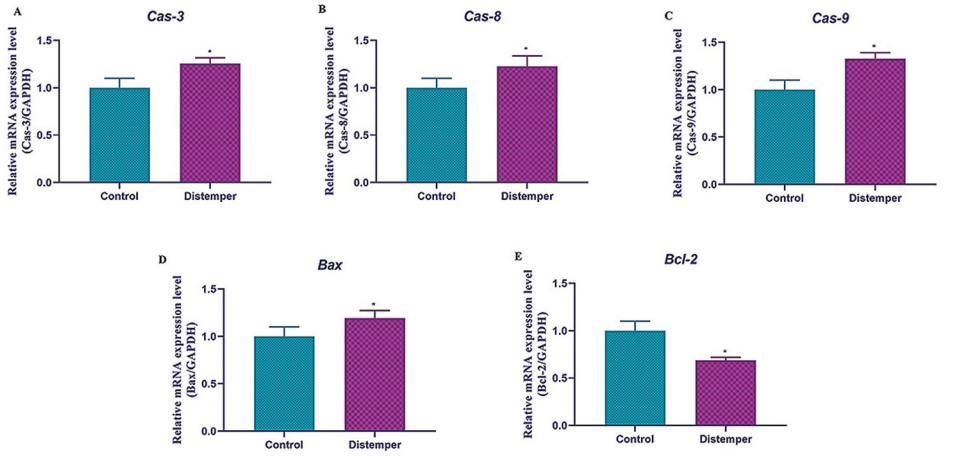


Figure 1. The mRNA transcript levels of Cas-3, Cas-8, Cas-9, Bax, and Bcl-2 in the brain of dogs. Values illustrate the mean ± SE of 3 independent experiments for each sample. One-Way ANOVA is used to analyse statistical significance (*p<0.05, **p<0.01, ***p<0.001). **A)** Depict the relative mRNA expression levels of Cas-3. **B)** Illustrate the relative mRNA expression levels of Cas-8. **C)** Illustrate the relative mRNA expression levels of Cas-9. **D)** Show the relative mRNA expression levels of Bax. E) Show the relative mRNA expression levels of Bcl-2.

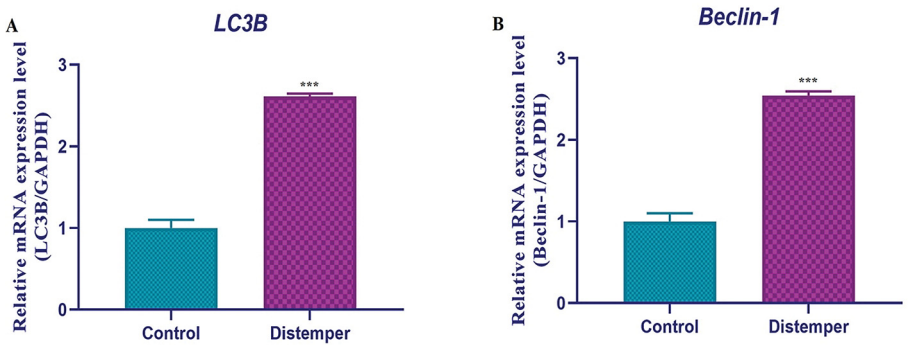


Figure 2. The mRNA transcript levels of LC3B *and* Beclin-1 in the brain of dogs. Values denote the mean ± SE of 3 independent experiments for each sample. One-Way ANOVA is used to analyse statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). A) Illustrate the relative mRNA expression levels of LC3B. B) Illustrate the relative mRNA expression levels of Beclin-1.

DISCUSSION

In this study, we examined the brain tissues of CDV-infected dogs with acute neurological signs. We hypothesized that expression levels of autophagic markers in brain tissues should be higher than that of apoptotic markers given that (1) viral proteins may disrupt the programmed cell death/apoptosis, (2) autophagy was reported to be more dominant in dogs with acute CDV infection and (3) autophagy was found to be activated by morbilliviruses expeditiously. In this context, we investigated the activation of apoptosis and autophagy in CDV-infected brain tissues of dogs, by identifying Cas-3, Cas-8, Cas-9, Bax, Bcl-2, LC3B, and Beclin-1 expressions with real-time PCR. We did not prefer to run the histopathological investigation since demyelination is a landmark of CDV infection, so this investigation would not add to the study. We also hoped to confirm the concurrent occurrence of autophagy and apoptosis with a transition from autophagy at the beginning to apoptosis at the end as reported in the studies of Boya et al.[23], Canu et al.[22], and Gonzalez-Polo et al.[24].

We found that CDV induced both mechanisms and in support of our hypothesis, autophagy was more active than apoptosis in the brain tissues of CDV-infected dogs with acute neurological signs. This result showed the concurrent occurrence of autophagy and apoptosis. Therefore, it also separately complies with the previous reports on the presence of autophagy-only ([14,15]) or apoptosis-only ([10,11]) degradation processes in CDV infection.

The first component of our hypothesis was that viral proteins might disrupt apoptosis. O'Brien has reported that viral proteins can affect the apoptosis pathway by interacting with cell components related to cell death [37]. It has been known that viral proteins prevent apoptosis, hereby the death of the host cell, in order to maximise and facilitate viral replication and a persistent infection. It was also reported that other forms of

cell death can only occur when apoptosis is inhibited [38]. In line with these reports, the expression levels of autophagic markers were significantly up-regulated than that of apoptotic markers. This stance was also supported by the slightly down-regulation of Bcl-2 as it is known as an anti-apoptotic protein. However, this issue is highly complicated given that both mechanisms are interconnected, concurrently occurring with a transition from autophagy at the beginning to apoptosis at the end. The fact that we found the apoptotic markers, Cas-3, Cas-8, Cas-9 and Bax slightly up-regulated could refer to the onset of such transition. It was also reported that Bcl-2 binds to Beclin-1, which prevents the pre-autophagosomal assembly, resulting in inhibition of autophagy [39]. We assert that the down-regulation of Bcl-2 occurs through this binding. As Bcl-2 binds to Beclin-1, this prevents autophagy and decreases the presence of anti-apoptotic protein in the medium, leading to apoptosis. This assertion may illuminate the transition claimed by Boya et al.[23], Canu et al.[22] and Gonzalez-Polo et al.[24]. This transition could have been demonstrated better if the dogs of this study were left to die naturally or be exposed to euthanasia in a few days later. However, it should also be noted that in the natural death scenario, glucocorticoid-based treatment would greatly alter the expected results.

The other pillars of our hypothesis were that autophagy was more dominant in acute CDV infection and activated by morbilliviruses expeditiously. We would like to explain these pillars with respect to the clinical findings, so that we can strive to illuminate the so-called controversial mechanism behind the demyelination. Neurological signs can be greatly various in canine distemper. The symptoms would reflect the viral spread and lesions in the CNS [40]. So, we assert that autophagy primarily caused the viral spread which then led to lesions in the brain. Furthermore, Kabak et al. detected a strong correlation between demyelination intensity and the number of LC3-positive cells and claimed that the acute phase of CDV infection induces autophagy [15]. In line with these reports, we also found that all dogs with CDV had an acute onset of neurological signs with rapid clinical deterioration. Therefore, it can be inferred that (1) autophagic activity in brain tissue is high in such cases where the neurological findings are progressive, (2) which then causes the brain tissue to be significantly affected by provoking viral spread. (3) Furthermore, the rapid deterioration and viral spread could be due to the weak immune response of the dogs [41] as the study material consisted of dogs which were 3-5 months old (Table 1).

Our results showed that in line with our hypothesis, the expression levels of autophagy markers were higher than that of apoptotic markers in CDV infected brain tissues of dogs. Thus, we concluded that both autophagy and apoptosis concurrently play a role in the pathogenesis of demyelination in canine distemper, but autophagy was more dominant and significant. Therefore, understanding the role of autophagy in viral pathogenesis may be helpful to create new therapeutic strategies for CDV infection by developing new antiviral medicines.

CONCLUSION

In the current study, the cell death mechanisms of apoptosis and autophagy were examined by detecting the expression levels of Cas-3, Cas-8, Cas-9, Bax, Bcl-2, LC3B and Beclin-1 in the brain tissues of dogs naturally-infected with CDV. Overall, we found that CDV induced both mechanisms. However, autophagy was more activated than apoptosis in the brain tissues of CDV-infected dogs with acute neurological signs.

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Authors' contributions

All authors contributed to the planning, designing and analyses of the experiments, data collection, quality control, and writing of this manuscript. SÖ performed the statistical analysis. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

the owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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PROCENA AKTIVNOSTI APOPTOZE I AUTOFAGIJE U MOZGU PASA PRIRODNO INFICIRANIH VIRUSOM ŠTENEĆAKA NA OSNOVU PROMENA U MARKERIMA APOPTOZE I AUTOFAGIJE

Şükrü DEĞİRMENÇAY, Selçuk ÖZDEMİR

U ovom radu ispitivana je aktivacija apoptoze i autofagije u CDV inficiranim moždanim tkivima pasa sa akutnim neurološkim znacima, određivanjem Cas-3, Cas-8, Cas-9, Bak, Bcl-2, LC3B i Beclin-1 ekspresije pomo'u real-time PCR. Nivoi ekspresije Beclin-1 i LC3B, markera autofagije, bili su značajno povećani u poređenju sa kontrolnom grupom ($p < 0,001$). Nivoi ekspresije apoptotskih markera Cas-3, Cas-8, Cas-9 i Bak bili su blago povišeni, ali je Bcl-2 bio blago niže regulisan za razliku od kontrolne grupe ($p < 0,05$). Dakle, markeri autofagije su bili značajno više aktivirani od apoptotskih markera kod pasa sa akutnim neurološkim znacima. U zaključku, autofagija učestvuje u patogenezi demijelinizacije kod pasa obolelih od štenećaka. Poznavanje ovoga može biti od pomoći za kreiranje novih terapijskih strategija, kao što su novi efikasni antivirusni lekovi.