Research Article / Araştırma Makalesi

The Effect of Ca-074 (Cathepsın B Inhibitor) on Necrotic and Apoptotic Neuronal Cell Death in Model of Cerebral Ischemia

Katepsin B İnhibitörü olan CA074'ün Serebral İskemi Modelinde Apoptoz ve Hücre Ölümü Üzerine Etkisi

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Abstract: Lysosomes and cathepsins, the most common hydrolytic enzymes in lysosomes, are available in the different models of cell death as necrosis and apoptosis. This study investigated the effect of cathepsin B-selective inhibitor CA-074 on apoptotic and necrotic neuronal cell death. Focal cerebral ischemia which has been formed by occlusion of the three-vessel consisting permanent middle cerebral artery occlusion and temporary bilateral common carotid artery occlusion for 60 minutes was selected as ischemia model. Two sets of rats were used in this study. The rats in the first set were used formeasurement of sulfhydryl groups in the lysosomal membrane, lysosomal integrity, cathepsins B and L activities and caspase-3 activity. The rats in the second set were used as histological study including "hematoxylin and eosin" for the detection of necrotic neuronal deathand "TUNEL" staining for the detection of apoptotic neuronal death. 4 mg/kg CA-074 was administered intravenouslyin the treatment group. CA-074 has substantially reduced levels of cathepsins B and L compared to ischemia and solvent groups (respectively, $p<0.05$ and $p<0.01$). Similarly, CA-074 has reduced increase in caspase-3 activity compared to ischemia and solvent groups ($p<0.05$). While the number of eosinophilic (necrotic) and apoptotic neurons has highly increased in post-ischemic cerebral tissue in middle cerebral artery feeding area ($p<0.001$), CA-074 could only reduce significantly the number of apoptotic neurons ($p<0.05$). CA-074 has reduced apoptotic neuronal death by inhibiting caspase and cathepsin activity. It may be useful that CA074 is used with other therapeutic drugs in stroke patients.
Keywords: İschemia, Cerebral, Rat model, CA074

Özet: Lizozomlarda en yaygın hidrolitik enzimlerden olan lizozomlar ve katepsinler, nekroz ve apoptoz olarak farklı hücre ölümü modellerinde görev almaktadır. Bu çalışma, katepsin B-seçici inhibitörü olan CA-074'ün apoptotik ve nekrotik nöronal hücre ölümü üzerindeki etkisini araştırdı. Bu çalışmada İskemi modeli olarak kalıcı orta serebral arterin tıkanıklığı ve geçici bilateral ana karotid arter tıkanıklığından oluşan üç damarın 60 dakika süreyle oklüzyonu ile oluşturulan fokal serebral iskemi modeli seçilmiştir. Çalışmada iki set sıçan kullanıldı. Birinci setteki ratlar lizozomal membrandaki sülfhidril gruplarının, lizozomal bütünlüğün, katepsin B ve L aktivitelerinin ve kaspaz-3 aktivitesinin ölçülmesinde kullanıldı. İkinci setteki sıçanlar, nekrotik nöronal ölümün saptanması için "hematoksilen ve eozin" ve apoptotik nöronal ölümün saptanması için "TUNEL" boyamasını içeren histolojik çalışma olarak kullanıldı. Tedavi grubuna 4 mg/kg CA-074 intravenöz olarak uygulandı. CA-074, iskemi ve solvent gruplarına kıyasla önemli ölçüde azalmış katepsin B ve L seviyelerine sahiptir (sırasıyla, $p<0.05$ ve $p<0.01$). Benzer şekilde CA-074 kaspaz-3 aktivitesindeki artışı iskemi ve solvent gruplarına göre azaltmıştır ($p<0.05$). Orta serebral arter beslenme alanındaki iskemik sonrası serebral dokuda eozinofilik (nekrotik) ve apoptotik nöronların sayısı oldukça artarken ($p<0.001$), CA-074 sadece apoptotik nöronların sayısını önemli ölçüde azaltabildi ($p<0.05$). CA-074, kaspaz ve katepsin aktivitesini inhibe ederek apoptotik nöronal ölümü azaltmıştır. CA074'ün inme hastalarında diğer terapötik ilaçlarla birlikte kullanılması faydalı olabilir.
Anahtar Kelimeler: İskemi, Beyin, Rat modeli, CA074

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1. Introduction

Cell death is a multifactorial condition including many immunological and biological systems (1). The disruption of the lysosomal membrane integrity causes the release of lysosomal enzymes into the cytosol, this situation leads to the activation of many cascade systems on cell death (2). While cathepsins which are released by complete lysosome rupture lead to direct cell necrosis, partial membrane rupture can cause apoptosis (3). Therefore, the lysosomal cysteine proteases cathepsin B and cathepsin L are key molecules in the studies of cell death.

Cathepsin B is an enzyme relating to the physiological cycle of cell proteins which has a fragmentation capacity for neuronal proteins, nucleic acids, carbohydrates and lipids (4). Cathepsin-B was investigated as both immunohistochemistry and quantitative in brain after experimental ischemia and its effect has been demonstrated (4,5).CA-074 is a selective inhibitor of cathepsin B. In studies performed, it was shown that CA-074 has brain protective effect on global or focal cerebral ischemia (5,6).

In recent times, inhibition of cathepsins emerges as a strategy in the experimental studies. In this project,we aimed to investigate whether inhibition of cathepsins B and L and its induced caspase 3 activation by cathepsin B inhibitor CA-074 has the protective effect on ischemic brain.

2. Materials and Methods

Establishment of surgical procedure and ischemia

The rats were operated and euthanized under anesthesia and were intubated with endotracheal. Controlled breathing was provided using small animal ventilator (small rodent ventilator-Ugo Basile Biological Research Apparatus).Catheter was inserted into left femoral artery in order to measure the blood pressure of rats and collect blood samples during the experiment and was connected to “data acquisition system” with a pressure-sensing system (Biopac, USA). Surgical procedures were performed using a dissecting microscope. Focal cerebral ischemia was performed by the three-vessel occlusion method (7).

Experimental groups

Experiment were performedin two sets of rats. Biochemical analyzes were made in the first set. Cytosolic/lysosomal ratio of cathepsin B and L was measured in permanent infarction area. Total thiol groups in the lysosomal membrane and lysosomal integrity were measured. Caspase-3 was measured in the ischemic brain tissue supplied by the middle cerebral artery. The rats in the first set were divided into 4 groups; pseudo-operation (sham operation), ischemia group, treatment group and solvent group. The rats in the second set were used for histological study. Quantitative analysis of living (intact), ischemic (necrotic) and apoptotic neurons was made in the cerebral infarction area formed by permanent middle cerebral artery occlusion and temporary bilateral common carotid artery occlusion for 1 hour. For this purpose, they were divided into 4 groups similarly to first set.

The preparation of CA-074

CA-074 was dissolved by adding 100 µl (0.1 ml) DMSO. Then 2% CA-074 stock solution was prepared by adding 4.9 ml 0.9% saline solution on it. Drug volume calculated for each animal weight was increased 2-fold (0.7-0.8 mL) by adding serum physiologic andso that the ratio of CA-074 was reduced to 1% in DMSO. 4 mg/kg CA-074 dose was used.

The measurement of membrane thiol groups

the protein concentration of lysosomal suspension was measured by the biuret method. The results of the thiol group were given as nmol/mg proteins by dividing protein.

The calculation of lysosome integrity by measuring free and total β-hexosaminidase activity

Lysosomal integrity was calculated as [1-(free) / total activity] x100. Loss of lysosomal integrity was determined by an increase in percentage of free activity.

The measurement of cathepsin B and cathepsin L activity

Enzyme activity was measured using methylkumarilamid substrate.0.75 ml 8.0 mM L-cysteine, 0.90 ml 0.1% (v/v) Brij 35 solution and 0.10 mL homogenatewere mixed. After 0.02 mM Z-Phe-Arg-7-Amido-4-Methylcoumarin was put it on(Respectively, cathepsin B substrate and cathepsin L substrate, Z-Arg-Arg-Nmec for cathepsin B and Z-Phe-Arg-Nmec for cathepsin L), the change in fluorescence has been followed for 5 minutes at 348 nm excitation and 440 nm emission. The results were given as U/mg protein.

The measurement of caspase-3 activity

Caspase-3 activity was determined using a commercial kit in the supernatant fraction. Caspase-3 activity was given as µmol pNA/mg protein.

Histological determinationof intact and necrotic neurons

Serial sections at 5 µm thickness were taken by preparing paraffin blocks from fixed rat brains. Then hematoxylin and eosin (H & E) stain was applied to sections in order to demonstrate general structure features. Basophilic neurons had a blue-purple cytoplasm and they were defined as intact neuron. Eosinophilic neurons had a pink cytoplasm and they were defined as necrotic neuron (8).

In situ detection of apoptotic neurons in histological examination

Apoptosis was examined in 5µm thick paraffin sections of the experimental and control groups by TUNEL activity (ApopTag Plus Peroxidase Kits; Chemicon International, USA).

The counting of intact, necrotic and apoptotic neurons

Two independent observershave evaluated ten optical field (1600 µm2) using an ocular micrometer with the help of light microscopy on randomly selected ten sections in MCA feeding area in every animal in order to to analyze the basophilic, necrotic and apoptotic cells in post-ischemic cortex(OLYMPUS PM 10 ADS with CameraDP 70).

The statistical analysis

The data were given as mean±standard error. The statistical analysis was made using “SPSS for windows 15.0 and Sigmastat 3.1” software package. The data were normally distributed. Therefore, the comparison of the groups was made using Tukey's multiple comparison test following significant ANOVA results. If p was less than 0.05, it was considered to be statistically significant.

3. Results

Sulfhydryl measurements which are used as an indicator of disruption of cell membrane integrity has shown a significant reduction in ischemia group compared to control group ($p<0.05$).Treatment with CA-074 caused an increase in sulfhydryl groups. However, this increase was not statistically significant ($p>0.05$). In other words, CA-074 treatment was inadequate in protecting the sulfhydryl group compared to ischemia group. Moreover, when CA-074-treated group and solvent-treated group were compared, it was not statistically significant ($p>0.05$) (Figure 1).

Lysosomal integrity showed a significant reduction in ischemia group compared to control group ($p<0.001$). There was a decrease in loss of lysosomal integrity in CA-074-treated group compared to ischemia group ($p<0.05$)(Figure 2).

Cytosolic/lysosomal ratio of cathepsin B was increased 1.91-fold in ischemia group compared to control group. This increase was statistically highly significant ($p<0.001$). CA-074 has reduced significantly cytosolic/lysosomal ratio of cathepsin B which had increased in ischemia group ($p<0.05$). Moreover, there was no difference between treatment group and control group ($p>0.05$, $p=0.61$). A significant decrease was observed in CA-074-treated group compared to solvent-treated group ($p<0.01$). Cytosolic/lysosomal ratio of cathepsin L was increased 2.21-fold in ischemia group compared to control group ($p<0.001$).

Cytosolic/lysosomal ratio of cathepsin L has shown a significant reduction in CA-074-treated group compared to ischemia group and solvent-treated group (respectively, $p<0.01$ and $p<0.05$). There was no difference between ischemia and solvent-treated groups ($p>0.05$). However, there was difference between CA-074-treated group and control group. So, CA-074 could not reduce values of cytosolic/lysosomal ratio of cathepsin L to the control values (Figure 3 A,B).

Caspase-3 was increased 3.8-fold in ischemia group compared to control group and this increase was statistically highly significant ($p<0.001$). Similarly, an increase in caspase-3 was also statistically highly significant in solvent-treated group compared to control group ($p<0.001$). While treatment with CA074 reduced significantly an increase in caspase-3 in ischemia group ($p<0.05$), the same significance was also obtained in a reduction in solvent-treated group ($p<0.05$). Treatment with CA-074 could not reduce measured value of caspase-3 in ischemia to the control level ($p<0.05$)(Figure 4).

Eosinophilic and TUNEL (+) neurons were commonly observed in ischemic center (core) area of cerebral cortex rather than the penumbra strip. These ischemic areas contained intact neurons. The numbers of basophilic (intact), eosinophilic (ischemic) and TUNEL (+) (apoptotic) neurons have been given in Table 1. The number of living neurons decreased significantly 24 hours after permanent middle cerebral artery occlusion($p<0.001$). Similar result was also obtained in brains of rats undergoing solvent and permanent middle cerebral artery occlusion ($p<0.001$).Despite these data, although the number of living neurons increased considerably in CA-074-treated group, this increase was not statistically significant compared to ischemia group ($p>0.05$). Moreover, there was also no statistical differencecompared to solvent-treated group ($p>0.05$). Necrotic neurons with eosinophilic cytoplasmhave beenshown in figurein control, ischemia and CA-074-treated groups(Figure 5 A,B,C,D).

The number of necrotic neurons in infarction area increased significantly in ischemia group undergoing middle cerebral artery occlusion ($p<0.001$). Although the number of necrotic neurons decreased considerably in the group undergoing middle cerebral artery occlusion and treating with CA-074 compared to ischemia group, CA-074 did not reduce significantly the number of necrotic neurons ($p>0.05$).

The number of apoptotic neurons; permanent middle cerebral artery occlusion has caused to increase significantly the number of apoptotic neurons in the infarction area of middle cerebral artery ($p<0.001$). Treatment with CA-07 made a statistically significant decrease in the number of apoptotic neurons in the infarction area compared to ischemia group andsolvent-treated group ($p<0.05$). Apoptotic neurons; control, ischemia and CA-074-treated groups have been shown in figure 6 (Figure 6 A, B, C, D).

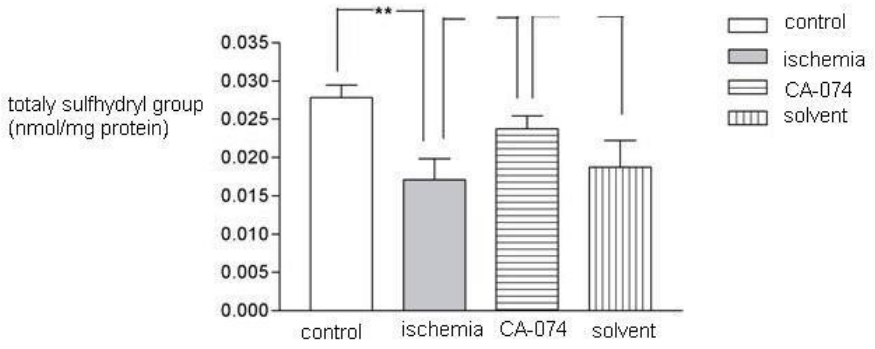

Figure 1. *The measurement of thiol group in cell membrane. While sulfhydryl decreased in ischemia group, CA-074 was inadequate in protecting thiol group. **: p<0.01.*

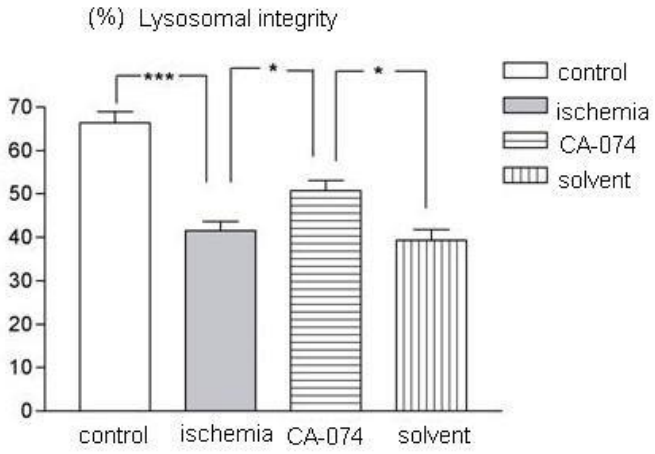

Figure 2. *Lysosomal integrity was found to be decreased in ischemia group. CA-074 has been shown to have a protective effect on lysosomal integrity. ***: p<.0.001, *:p<0.05.*

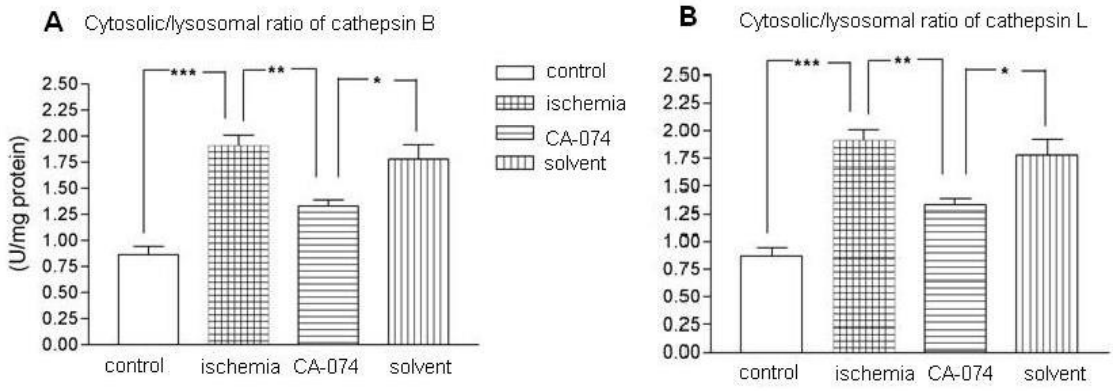

Figure 3. ***A-*** *Cytosolic/lysosomal ratio of cathepsin B. The increase in ischemia was decreased by treatment with CA-074. ***:p<0.001, **:p<0.01, *:p<0.05.* ***B-*** *Cytosolic/lysosomal ratio of cathepsin L. CA-074 has reduced the increase in ischemia. ***:p<0.001, **:p<0.01, *:p<0.05.*

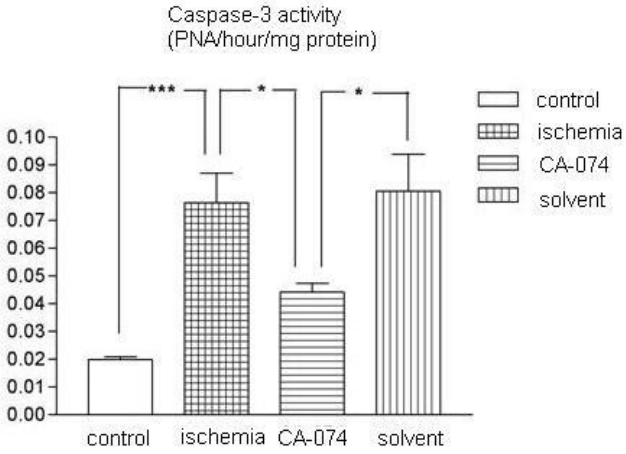

Figure 4. Caspase-3 activity. *Caspase-3 activity was significantly increased in ischemia group. This increased activity was found to be inhibited by CA-074. ***:p<0.001, *:p<0.05.*

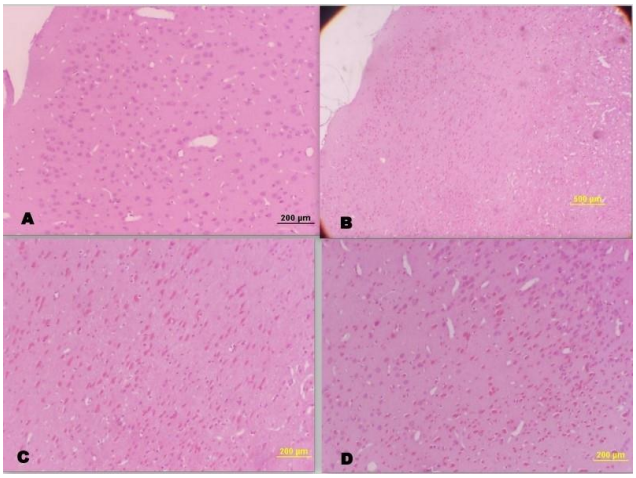

Figure 5.*A-* *Basophilic (intact) neurons are usually observed in the control group. HXE (Bar 200µm).**B-*** *It draws attention that necrotic neurons are numerous and common in ischemia group. HXE (Bar 500µm).**C-*** *The large number of necrotic neurons have been shown in high magnification in ischemia group. HXE (Bar 200µm).**D-*** *Necrotic cells were partially decreased in the group treated with CA-074**.*** *HXE(Bar 200µm).*

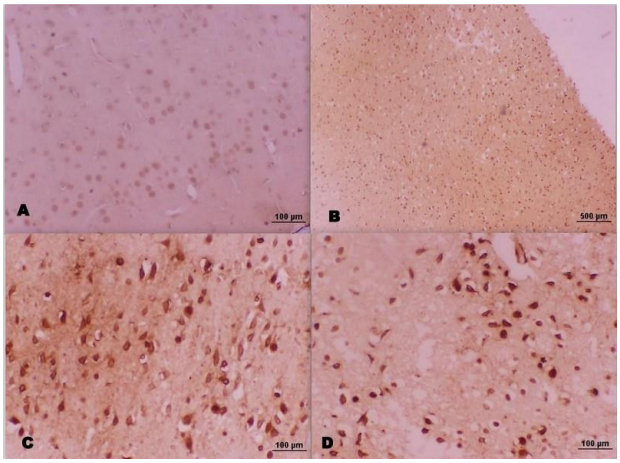

Figure 6.*A-* *The majority of neurons are observed as TUNEL (-) in control group. TUNEL (Bar 100µm).* ***B-*** *It draws attention that apoptotic neurons are numerous and common in ischemia group. TUNEL (Bar 500µm).* ***C-*** *Apoptotic neurons are observed in high magnification in ischemia group. TUNEL (Bar 100µm).* ***D-*** *Treatment group. CA-074 are observed to reduce the number of apoptotic neurons compared to ischemia group. TUNEL (Bar 100µm).*

Table 1. The numbers of intact, necrotic and apoptotic neurons *(1600 μ^2 field). M: Mean, S: Standard.*

	Control (med) ±Std. Std Error	**Ischemia (med) ± Std. Error**	**CA074 (med) ± Std. Error**	**DMSO(med) ± Std. Error**
Intact neuron	880,0±34,35	183.8±46,32	306,0±32,65	230,0±25,30
Necrotic neuron	14,60±2,040	811,6±62,05	630,0±51,09	744,0±50,56
Apoptotic neuron (TUNEL +)	12,40±1,806	634,2±45,58	472,0±50,17	669,8±34,52

4. Discussion

Lysosomes are classically defined as ‘’suicide bags’’ of cells (9). Today, in the modern literature, lysosomes are known to have a key role in cell death with their acidic endosomal/lysosomal components (10). In classical information, cell death has been modeled in two ways; unregulated necrosis and apoptosis (programmed cell death). The recent studies have shown that necrosis has a high regulated mechanism as apoptosis and

specific check points (11,12). Pyroptosis which is the most common form of necrosis requires inflammasome stimulation and caspase-1 activation (13,14). Necroptosis which is another form of necrotic cell death occurs by stimulation of specific receptors such as TNF alpha in the presence of caspase inhibitors (15,16). The recent studies have demonstrated a definition of 'Lysosome-mediated necrosis' which is a different kind of programmed cell necrosis (17,18). Although necrosis and apoptosis have different mechanisms or pathways, they are eventually multifactorial and regulated cascade systems. Acidic endosomal/lysosomal hydrolases especially cathepsins are key molecules in cell death.

Cathepsin B and L are lysosomal cysteine proteases which are present in all mammalian cells and well-defined. The main role of cathepsin B and L is intracellular protein degradation/turnover, antigen presentation, proenzyme activation, hormone maturation and epidermal hemostasis (19,20). The disruption of the lysosomal membrane integrity causes the release of cathepsins B and L into the cytosol, this situation leads the activation of different mechanisms resulting in necrosis. Both histological expression and enzymatic activation of cathepsin B and L have been shown after transient global and focal cerebral ischemia and this was consistent with the neuronal cell death (8,21,22,23,24). CA-074 was developed by Towatari as a specific cathepsin B inhibitor and it prevented delayed cell death observed in pyramidal cells in transient global ischemia, this situation has shown that cathepsin inhibitors can be used in pharmacological treatment of stroke (25).

In our study, cathepsin B and cathepsin L have increased at least two fold in the cerebral infarction area formed in middle cerebral artery feeding area. CA-074 which is known as specific cathepsin B inhibitör has reduced significantly the increase of cathepsin L besides cathepsin B. This observation is also supported with the inhibitory effect of the drug on loss of lysosomal integrity which is the inhibition of calpain-induced lysosomal rupture and the release of cathepsins B and L into the cytosol.

In our study, CA-074 has prevented the apoptotic cell death: it inhibits also e caspase-3 activity besides the inhibition of cathepsin B and L, this situation shows that CA-074 is effective on apoptotic neuronal death. The activation of calpains causes apoptotic neuronal death by triggering directly caspase activation in traumatic or ischemic cerebral damage (26,27). In addition, cathepsins which are released from lysosome having a permeable or ruptured membrane also cause the activation of caspases: Benchoua et al. have shown that the release of cathepsin B caused the activation of caspase-11 and caspase-1 in the early period in infarct tissue formed depending on permanent middle cerebral artery occlusion in mice (5). This situation suggests that cathepsins may play a role in apoptotic cell death and it is called caspase-dependent apoptotic death pathway. While calpain activation causes necrotic and apoptotic neuronal death, caspase activation is only held responsible for apoptotic neuronal death. The interaction of cathepsins and Bcl-2 family proteins with a proapoptotic function also causes apoptotic death of neuronswith mitochondrial pathway by releasing cytochrome c and apoptosis inducing factor (AIF) and "soluble intermembrane mitochondrial proteins (SIMPs)" in the mitochondria (28). In our study, the inhibition of release of cathepsin B and L by CA-074 could suppress mitochondrial apoptosis pathway. Moreover, AIF has been shown to cause caspase-independent cell death in ischemia (29). For example, nitric oxide can directly make apoptotic death by causing mitochondrial dysfunctionand translocating AIF from mitochondria to nucleus in developing oligodendrocytes (30). In recent times, calpain I activation was shown to cause the release of AIF in ischemic neuronal damage (31). However, in our study, CA-074 is unlikely to make its anti-apoptotic effect with caspase-independent pathway.

In our study, both quantitative and histological data have shown that necrotic death has occurred more than apoptotic death.

Treatment with CA-074 before ischemia has failed to achieve a significant increase in the number of living neurons. The blood flow has dropped below the critical level in the middle cerebral artery area with permanent middle cerebral artery occlusion, this situation has caused discontinuation of feeders and energy depletion, irreversible necrotic death. In studied performed, threshold of cerebral blood flow has been reported to beas 4.8-8.4 ml/100 g tissue per minute in ischemic core area and as 14.1-35.0 ml / 100 g tissue per minute in the penumbra (32). This situation also explains why the drug is ineffective on necrotic death. In transient ischemic models, although CA-074 has been reported to reduce cell death especially on hippocampal pyramidal neurons (33,34), data is not available on our hands whether it reduces infarction area. Moreover, it has been reported that penumbra region has formed a narrow space compared to ischemic core area in middle cerebral artery infarction in people, it is not determinative for results of treatment, the primary determinant isthe response of central area of the regionto treatment (35). Although the effective dose of the drug has been reported to be as 4 mg/kg, we believe that it should be tried in high doses. In addition, it has been demonstrated in recent studies that CA-074 Me which is a methyl ester derivative of CA-074 is a more potent inhibitor. This drug could be used in this study, this could be our missing aspect. Therefore, similar studies are needed to be repeated with different doses and derivatives in similar models.

Sulfhydryl groups are reactive structural molecules of proteins and play a role in cell division, blood coagulation, continuation of protein systems and enzymatic activation of antioxidant enzymes such as catalase and superoxide dismutase. However, it plays a role in scavenging oxygen derived free radicals (36). Malondialdehyde and sulphydryl groups are measured as indicators of lipid peroxidation and oxidative stress (37). In our study, lysosomal membrane sulphydryl (S-H) groups were observed to be decreased significantly in rat cerebral infarction formed with three-vessel occlusion model. This decrease depends on destruction of reactive oxygen species and lipid developing secondary to ischemia. Although CA-074 which is not already an antioxidant has reduced considerably the decrease in sulfhydryl group, it could not prevent the decrease in sulfhydryl group of lysosomal in statistically meaningful way.

The selection of permanent ischemic model can be considered as a disadvantage in this study where cathepsin inhibitor was only used. However, most of ischemic strokes in people occur as infarction.

5. Conclusion

CA-074 has reduced the apoptotic neuronal death by inhibiting cathepsins and caspase activities. It may be useful that CA-074 is used with other therapeutic drugs in stroke due to this feature.

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Ethics

Ethics Ethics Committee Approval: The study was approved by Eskisehir Osmangazi University animal testing local Ethical Committee (Approval Date/ Number: 05.11.2007/27).
Informed Consent:
Author Contributions: Idea/concept: EÖ,RD,ZÖ, HÖ, GK, FA, KU., Design: EÖ,RD,ZÖ, HÖ, GK, FA, KU Data Collection: EÖ,RD,ZÖ, HÖ, GK, FA, KU, Data Processing: EÖ,RD,ZÖ, HÖ, GK, FA, KU Analysis/Comment: EÖ,RD,ZÖ, HÖ, GK, FA, KU Literature research/review: EÖ,RD,ZÖ, HÖ, GK, FA, KU., Writing: EÖ. authors discussed the results and contributed to the final manuscript.
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