Apoptosis and cerebral ischemic reperfusion injury developed after haemorrhagic shock: experimental study

Hemorajik şok sonrası gelişen serebral iskemi reperfüzyon yaralanması ve apoptosis: Deneysel çalışma

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BACKGROUND

Apoptosis is a process of programmed cell death that plays a role in some normal and pathological conditions. In this study, we investigated the apoptosis during cerebral ischemic reperfusion injury in response to haemorrhagic shock in a rat model.

METHODS

Thirty-six adult Sprague-Dawley rats were divided into six groups: control, haemorrhagic shock (HS), ischemic reperfusion (IR), 1st hour IR, 3rd hour IR, 6th hour IR and 24th hour IR. Rats were sacrificed by taking blood from intracardiac area after finishing the experiment. The tissues were fixed using neutral buffered 10% formaldehyde solution for histopathological examination. Tissues were stained immunohistochemically with APO 2.7 and positive expression apoptotic cells were counted using a Clemex Vision Lite 3.5 vision analysis system.

RESULTS

There were 2-3 apoptotic cells in the control group (group 1) and this number increased to 8-11 in the haemorrhagic shock group (group 2) ($p<0.05$). Secondary or more serious injury occurs during ischemic reperfusion injury. The number of apoptotic cells increased to 11-14 at the 1st hour (group 3) and it was significant as compared to group 2 ($p<0.05$). The number of apoptotic cells significantly increased to 15-17 by the 3rd hour (group 4) as compared to group 3 ($p<0.05$). While there was no additional increase by the end of the 6th hour (group 5) as compared to group 4, the number of apoptotic cells significantly increased to 18-24 by the end of 24th hour (group 6) as compared to group 5 ($p<0.05$).

CONCLUSION

The majority of injuries to the brain following haemorrhagic shock occur during ischemic reperfusion. We observed that apoptosis increases step by step on the 1st, 3rd and 24th hours after ischemic reperfusion injury.

Key Words: Apoptosis; haemorrhagic shock; rats, Sprague-Dawley; reperfusion injury.

AMAÇ

Apoptosis, normal ve patolojik şartlarda gerçekleşen programlanmış hücre ölümüdür. Biz bu çalışmada, hemorajik şoka uğratılmış sıçan modelinde serebral iskemik reperfüzyon hasarını apoptosis açısından araştırdık.

GEREÇ VE YÖNTEM

Toplam 36 adet Sprague-Dawley cinsi sıçan; kontrol, hemorajik şok (HS), iskemik reperfüzyon (İR) 1. saat, IR 3. saat, İR 6. saat, İR 24. saat olmak üzere 6 gruba ayrıldı. Sıçanlar, deney çalışması bittikten sonra intrakardiyak yolla kan alınarak sakrifiye edildiler. Dokular, histopatolojik çalışma için %10'luk tamponatlı solüsyona alındı. Dokular APO 2.7 ile immünohistokimyasal olarak boyandı ve pozitif ekspresyon gösteren apoptotik hücreler Clemex Vision Lite 3.5 vision analiz sistemi kullanılarak sayıldı.

BULGULAR

Kontrol grubunda (grup 1), 2-3 tane apoptotik hücre vardı ve bu sayı hemorajik şok grubunda (grup 2) 8-11'e çıkıyordu ($p<0.05$). İskemik reperfüzyon esnasında daha ciddi hasarlar oluştu. İR'den sonraki 1. saatte (grup 3) apoptotik hücre sayısı 11-14'dü ve bu sonuç group 2 ile karşılaştırılığında anlamlıydı ($p<0.05$). Apoptotik hücre sayısı 3. saatte (grup 4) 15-17'ye çıktı ve bu sonuç grup 3 ile karşılaştırıldığında anlamlıydı ($p<0.05$). Apoptotik hücre sayısında İR'den sonra 6.saat sonunda (grup 5) ek bir artış meydana gelmezken, 24. saat sonunda (grup 6) 18-24'e çıktı ve bu sonuç grup 5 ile karşılaştırıldığında anlamlıydı ($p<0.05$).

SONUÇ

Hemorajik şoku takiben oluşan beyin hasarlarının çoğu iskemik reperfüzyon esnasında oluşur. Biz bu çalışma sonucuna göre, apoptozisin iskemik reperfüzyon sonrası 1., 3. ve 24. saatlerde adım adım arttığını düşünüyoruz.

Anahtar Sözcükler: Apoptozis; hemorajik şok; sıçan, Sprague-Dawley cinsi; reperfüzyon hasarı.

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A common cause of sudden death after trauma is haemorrhagic shock due to incisive or penetrative injuries of the cranium.[1-3] Initial tissue damage occurs because of ischemia. Reperfusion is intended to correct cell function and remove toxic metabolites. Oxygen molecules which come to the region after reperfusion are responsible for secondary tissue damage.[4] Therefore, the cell membrane loses its permeability and apoptosis and necrosis occur.[5]

However, the apoptotic mechanism is elusive. Generally, apoptosis is started by a physiological signal which triggers the programmed suicide. While the energy sources of the cell do not decrease, it synthesises mainly macromolecules.[6] In addition, it is characterized with physiological and morphological changes in the cell such as increased nuclear density, formation of apoptotic bodies, and DNA fragmentation.[7]

Ischemia and apoptotis is activated during haemorrhagic shock and this process increases after ischemia/reperfusion (IR) injury. In this study, we investigated the cellular mechanism by which IR injury occurred following haemorrhagic shock, and its effects on cell apoptosis.

MATERIALS AND METHODS

The study was conducted in accordance with ethical principles of the Declaration of Helsinki, and permission was secured from local institutional review boards. In this study, six groups of adult, male Sprague-Dawley rats with a weight between 250 and 300 g, with six rats per group were used. Rats were anaesthetised by intraperitoneal administration of 100 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Warner Lambert, Istanbul, Turkey) and 10 mg/kg Xylasine Hydrochloride.

Both the femoral vein and artery were canalised with branule (no: 24) by inguinal incision. The arterial pressures and arterial pulses of the rats were monitored and measured by branule (no: 24) (Datex/Ohmeda S/5, Helsinki, Finland). Systolic blood pressures of the rats decreased to 30-40% of the normal level by taking blood from femoral vein. This process contributed to the formation of haemorrhagic shock. Group 1 was termed as control (C) and group 2 was termed as the haemorrhagic shock (HS) group. Blood was retained and given to the remaining groups (3, 4, 5 and 6) for reperfusion 20 minutes after the procedure. Rats were sacrificed by taking blood with intracardiac punction at the 1st hour, in group 4 at the 3rd hour, in group 5 at the 6th hour and in group 6 at the 24th hour. Brain tissue samples were taken from all groups and suspended in buffered 10% formaldehyde solution.

Histopathology

All specimens were fixed in buffered 10% paraformaldehyde solution for a minimum 24 hours. Tissue samples were taken from bilateral hemispheres and thalamus. They were taken from the same area for all members of groups. Tissue samples were prepared in an autotechnicon and then embedded in paraffin. Two sections (5 μm) were prepared. One section was stained with haematoxylin-eosin, and the excess was used for immunohistochemical staining. Apo 2, 7 (Monoclonal Antibody Apo 2.7 Cat. No: 2087 Immunotech) was used as a primary antibody for immunohistochemical staining.[8] Basal cell layers of squamous epithelium were used as positive control. Stained specimens were investigated by a Nikon Eclipse E400 light microscope. For each specimen, the same area was photographed after staining by using a Nikon Coolpix 5000 photograph attachment. The photograph of Nikon micrometer microscope slide was also taken during the procedure. All photographs were then imported and analyzed using Clemex Vision Lite 3.5 (Clemex Technologies Inc. Guimond, Longueuil, Canada) image analysis program. The length was calibrated by comparing the photograph of the specimen with the photograph of the Nikon micrometer microscope slide, which was taken under the same magnification. The areas (0.1 mm^2) of both hemisphere and thalamus preparations were designated using a Clemex Vision Lite 3.5 Image Analyses program for each samples. Positively stained cells were marked with the same Image Analyses program in a 0.1 mm^2 area. Damaged cells were not evaluated. The marked cells were counted automatically with the same Image Analyses program.

For each case, the sum of numbers in 0.1 mm^2 area of bilateral hemisphere and thalamus was considered as number of apoptosis. The reader was masked to the origin of the specimen (Fig. 1).

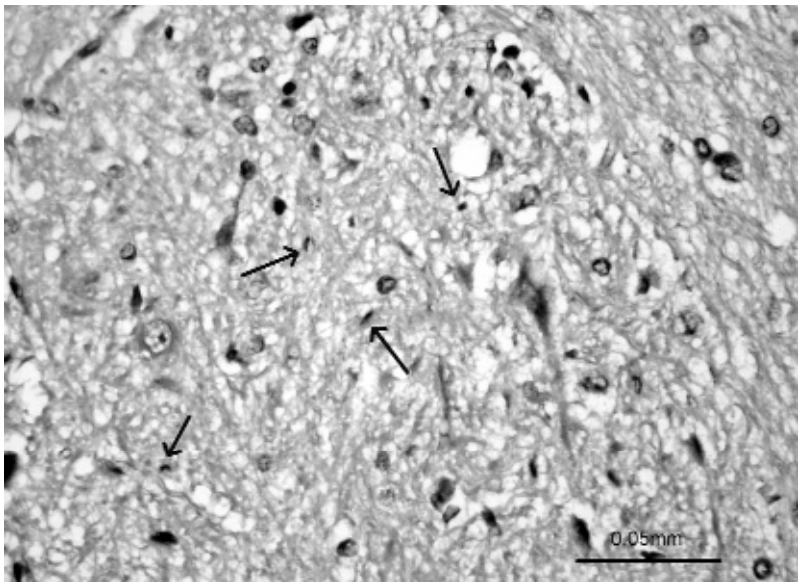

Fig. 1. Apo 2.7 is a specific apoptosis marker, and anti-Apo 2.7 antibody stains cell membranes of reactive cells. Arrows indicate positive cells (0.05 mm).

Statistical analyses were performed using SPSS for Windows (Version 11.0, SPSS Inc., Chicago, Illinois, USA). Apoptotic cell numbers are given as median±standard deviation (Table 1). Statistical analysis of data was performed by using one-way analysis of variance (ANOVA), and homogeneity of the groups was evaluated with the Levene test. Tukey's HSD test was used for intra-group comparisons. A p value of <0.05 was accepted as statistically significant.

RESULTS

There were 2-3 apoptotic cells in the control group (group 1) which increased to 8-11 in the haemorrhagic shock group ($p<0.05$). Secondary or more serious injury occurs during ischemic reperfusion injury. The number of apoptotic cells increased to 11-14 at the 1st hour (group 3) ($p<0.05$) and increased significantly to 15-17 by the 3rd hour (group 4; $p<0.05$). While there was no additional increase by the end of the 6th hour, the number of apoptotic cells increased to 18-24 by the end of the 24th hour of IR (group 6; $p<0.05$).

DISCUSSION

The most important organs affected by haemorrhagic shock are the central nervous system (CNS) organs, heart and kidneys.[9] The CNS can protect its functions until arterial blood pressure decreases till 60-70 mmHg. With progressive hypovolaemia agitation, coma, and then death can occur.[10] In this study, we observed that ischemic reperfusion

Table 1. Minimum-maximum and mean-standard deviation values of of the numbers of apoptotic cells in all groups

Groups	n	Number of apoptotic cells (min-max)	Number of apoptotic cells (mean±s.d.)
Control	6	2-3	2.7±0.5
Haemorrhagic shock	6	8-11	9.3±1.2
IR (1st hour)	6	11-14	13.0±1.2
IR (3rd hour)	6	15-17	15.8±0.7
IR (6th hour)	6	15-18	17.0±1.09
IR (24th hour)	6	18-24	21.1±2.1

IR: Ischemic reperfusion; HS: Hemorrhagic shock.

injury is responsible for the majority of damage following haemorrhagic shock. In addition to this, reperfusion which occurs at the 24th hour causes the most brain injury.

Haemorrhagic shock is a situation where tissues can not take enough blood and oxygen for their normal metabolic activities, and all tissues are effected.[11,12] In the ischemic tissues during haemorrhagic shock, synthesis of ATP decreases because of negative effect on mitochondrial phosphorylation and increase of anaerobic glycolysis cycle. As the ATP synthesis decreases, plasma membrane sodium pump activity decreases, the cell swells and intracellular pH decreases.[13] Nuclear chromatin aggregates, protein synthesis decreases and lipid is stored in the cell. During reperfusion, ischemic tissues come together with high concentration of Ca^{+2} and O_2 molecules. Because of the effect of high intracellular Ca^{+2} concentration, ATPase, phospholipase, protease, endonuclease enzymes are activated and cellular integrity is destroyed.[13]

O_2 molecules join the biochemical reactions and form free oxygen radicals. Free oxygen radicals react on all oxidizable cell structures including lipids, proteins and nucleic acids.[14-17] As cell injury occurs, permeability of the cell membrane increases, accompanied by cell lysis by the lipid peroxidation of membrane. Structural and enzymatic proteins are denatured by free oxygen radicals. They directly attack nucleic acids and cause base hydroxylation, cross-bonding and cell death.[18,19]

Apoptosis is a genetically-programmed cell death which initiates the metabolic and physiological suicide process.[20] Cell injury is expected during ischemic reperfusion injury. Apoptosis during ischemic reperfusion injury has been published in some tissues.[21] Ischemic reperfusion injury and relation with apoptosis had been demonstrated in animal models with radiological and pathological methods.[22,23]

The mechanisms underlying apoptosis are still unclear.[24] Intrinsic and extrinsic factors can induce apoptosis.[25] Harmful stimuli such as radiation and free radicals (which damages DNA and activates TP 53 ways) hinder the receptor binding (FAS and TNF receptors) or secretion of catatonic T cells from granzyms.[26]

Some stimulus (like cytotoxic T cells) activates (enzymatic cascade) directly in the suicide process. Activation of this cascade induces the latent cytoplasmic endonuclease and protease and damage the cell skeleton and nuclear proteins.[27] The damage to the cell skeleton and nuclear chromatin results in an intracellular destroying cascade.[28] Regulator proteins including Bcl-2 and Bcl-xL inhibit cell death, while others like Bax and Bad promote the cell death.[26,29,30] The other proteins affect adaptor proteins or mitochondrial releasing of cytochrome c.[31]

Previous studies have demonstrated the relationship between apoptosis and signal messenger mechanisms.[32] Raff[33] declared that all cells are programmed for death and they have to accept signals from other cells to live. If the signal is interrupted, the cell initiates the suicide process. It has been shown that Ca^{+2}, which is the part of the signal messenger mechanism, activates apoptosis in some cells. When the Ca^{+2} signalling mechanism is blocked, apoptosis does not occur.[34] While the mechanism is unclear, endonuclease enzymes which fragments DNA and is related to Ca^{+2} / Mg^{+2}, may be partly responsible for this process.[35]

CONCLUSION

The increasing number of apoptotic cells in the hours following ischemic-reperfusion injury indicates that treatment should occur immediately in emergency situations. We think that more studies are needed about ischemic-reperfusion injury especially for neural tissues.

REFERENCES

1. Acosta JA, Yang JC, Winchell RJ, Simons RK, Fortlage DA, Hollingsworth-Fridlund P, et al. Lethal injuries and time to death in a level I trauma center. J Am Coll Surg 1998;186:528-33.
2. Demetriades D, Murray J, Charalambides K, Alo K, Velmahos G, Rhee P, et al. Trauma fatalities: time and location of hospital deaths. J Am Coll Surg 2004;198:20-6.
3. Sosin DM, Sacks JJ, Smith SM. Head injury-associated deaths in the United States from 1979 to 1986. JAMA 1989;262:2251-5.
4. Rotstein OD. Modeling the two-hit hypothesis for evaluating strategies to prevent organ injury after shock/resuscitation. J Trauma 2003;54(5 Suppl):S203-6.
5. Power C, Fanning N, Redmond HP. Cellular apoptosis and organ injury in sepsis: a review. Shock 2002;18:197-211.
6. Bellamy CO, Malcomson RD, Harrison DJ, Wyllie AH.

Cell death in health and disease: the biology and regulation of apoptosis. Semin Cancer Biol 1995;6:3-16.
7. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.
8. Avunduk AM, Avunduk MC, Varnell ED, Kaufman HE. The comparison of efficacies of topical corticosteroids and nonsteroidal anti-inflammatory drops on dry eye patients: a clinical and immunocytochemical study. Am J Ophthalmol 2003;136:593-602.
9. Falk JL, O'Brien JF, Kerr R. Fluid resuscitation in traumatic hemorrhagic shock. Crit Care Clin 1992;8:323-40.
10. Smith HO. Shock in the gynecologic patient. In: Rock JA, Thomson JD, editors. Te Linde's operative gynecology. 8th ed. Philadelphia, PA: Lippincott-Raven; 1997. p. 245-61.
11. Shires GT, Barber AE, Illner HP. Current status of resuscitation: solutions including hypertonic saline. Adv Surg 1995;28:133-70.
12. Domsky MF, Wilson RF. Hemodynamic resuscitation. Crit Care Clin 1993;9:715-26.
13. Singer M, De Santis V, Vitale D, Jeffcoate W. Multiorgan failure is an adaptive, endocrine-mediated, metabolic response to overwhelming systemic inflammation. Lancet 2004;364(9433):545-8.
14. Nemeth I, Boda D. Xanthine oxidase activity and blood glutathione redox ratio in infants and children with septic shock syndrome. Intensive Care Med 2001;27:216-21.
15. Till GO, Friedl HP, Ward PA. Lung injury and complement activation: role of neutrophils and xanthine oxidase. Free Radic Biol Med 1991;10:379-86.
16. Zimmerman BJ, Granger DN. Reperfusion injury. Surg Clin North Am 1992;72:65-83.
17. Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 1993;14:303-11.
18. Cristofori L, Tavazzi B, Gambin R, Vagnozzi R, Vivenza C, Amorini AM, et al. Early onset of lipid peroxidation after human traumatic brain injury: a fatal limitation for the free radical scavenger pharmacological therapy? J Investig Med 2001;49:450-8.
19. Kong SE, Blennerhassett LR, Heel KA, McCauley RD, Hall JC. Ischaemia-reperfusion injury to the intestine. Aust N Z J Surg 1998;68:554-61.
20. Vaux DL. Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci U S A 1993;90:786-9.
21. Quadri SM, Segall L, de Perrot M, Han B, Edwards V, Jones N, et al. Caspase inhibition improves ischemia-reperfusion injury after lung transplantation. Am J Transplant 2005;5:292-9.
22. Lee SK, Kim DI, Kim SY, Kim DJ, Lee JE, Kim JH. Reperfusion cellular injury in an animal model of transient ischemia. AJNR Am J Neuroradiol 2004;25:1342-7.
23. Engelhard K, Werner C, Eberspacher E, Pape M, Stegemann U, Kellermann K, et al. Influence of propofol on neuronal damage and apoptotic factors after incomplete cerebral ischemia and reperfusion in rats: a long-term observation. Anesthesiology 2004;101:912-7.
24. Johnson EM Jr, Deckwerth TL. Molecular mechanisms of developmental neuronal death. Annu Rev Neurosci 1993;16:31-46.
25. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.
26. Takahashi M, Saito H, Okuyama T, Miyashita T, Kosuga M, Sumisa F, et al. Overexpression of Bcl-2 protects human hepatoma cells from Fas-antibody-mediated apoptosis. J Hepatol 1999;31:315-22.
27. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 1999;274:1156-63.
28. Mittler R, Lam E. Identification, characterization, and purification of a tobacco endonuclease activity induced upon hypersensitive response cell death. Plant Cell 1995;7:1951-62.
29. Nunez G, Clarke MF. The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol 1994;4:399-403.
30. Hara A, Iwai T, Niwa M, Uematsu T, Yoshimi N, Tanaka T, et al. Immunohistochemical detection of Bax and Bcl-2 proteins in gerbil hippocampus following transient forebrain ischemia. Brain Res 1996;711:249-53.
31. Beidler DR, Ahuja D, Wicha MS, Toogood PL. Inhibition of protein synthesis by didemnin B is not sufficient to induce apoptosis in human mammary carcinoma (MCF7) cells. Biochem Pharmacol 1999;58:1067-74.
32. Lee S, Christakos S, Small MB. Apoptosis and signal transduction: clues to a molecular mechanism. Curr Opin Cell Biol 1993;5:286-91.
33. Raff MC. Social controls on cell survival and cell death. Nature 1992;356(6368):397-400.
34. Bates RC, Buret A, van Helden DF, Horton MA, Burns GF. Apoptosis induced by inhibition of intercellular contact. J Cell Biol 1994;125:403-15.
35. Arends MJ, Morris RG, Wyllie AH. Apoptosis. The role of the endonuclease. Am J Pathol 1990;136:593-608.