Effects of Anti-NGF on Apoptosis in Rats with Experimentally Induced Sepsis Model

Deneysel Sepsis Modeli Oluşturulan Ratlarda Anti-NGF Uygulamasının Apopitoz Üzerindeki Etkileri

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ABSTRACT Objective: This study aimed to investigate the effects of anti-NGF administration on apoptosis in rats with experimentally induced sepsis model, and to examine the possible role of nerve growth factor (NGF) in pathogenesis and treatment of sepsis. **Material and Methods:** Thirty three Wistar Albino rats were randomly allocated into four groups. After sparing the control group, the remaining three groups were given intraperitoneal *E. coli* lipopolysaccaride to induce sepsis. Early anti-NGF group (Group EA-NGF, n=9) was injected with intraperitoneal anti-NGF one hour before LPS injection, and late anti-NGF group (Group LA-NGF, n=9) after sepsis was induced. Baselines, 2, 12, 24, and 72nd hours were analyzed to determine NGF levels. The rats were sacrificed on the 3rd day, and tissue samples separated from the small intestines, liver and lung. HE, bcl-2 and bax staining were used to evaluate apoptosis. **Results:** Apoptosis increased most markedly in the intestine tissue, as well as in the liver and lung tissue, and this increase was most evident in the early anti-NGF group. Bcl-2 staining in the subjects was less apparent while bax staining was more intensely detected in the sepsis groups, with a significant difference between the sepsis and early anti-NGF groups. Bax staining in the EA-NGF group was more clearly observed than that in LA-NGF group. NGF levels were found to increase in the 24th hour in sepsis group (Group S) and decrease in the second hour in Group EA-NGF. **Conclusion:** It can be concluded that anti-NGF administration in the early period of sepsis increases apoptosis at least to the same extent with sepsis, and it decreases apoptosis when administered in the later period of sepsis.

Key Words: Sepsis; 7S nerve growth factor protein, mouse; apoptosis

ÖZET Amaç: Bu çalışmada deneysel sepsis modeli oluşturulan ratlarda, anti-NGF uygulanmasının apopitoz üzerine etkilerini değerlendirerek Nerve growth faktörün (NGF) sepsis patogenez ve tedavisindeki olası rolünün araştırılması amaçlandı. **Gereç ve Yöntemler:** Otuz üç tane Wistar-Albino cinsi rat rastgele dört gruba ayrıldı. Kontrol grubu ayrıldıktan sonra diğer üç gruba *E. coli* lipopolisakkaridi intraperitoneal olarak uygulanarak sepsis kliniği oluşturuldu. Farklı olarak erken anti-NGF grup (Grup EA-NGF, n=9) LPS uygulanmadan 1 saat önce ve geç anti-NGF grup (Grup LA-NGF, n=9)'ye sepsis kliniği oluşturulduktan sonra anti-NGF intraperitoneal olarak uygulandı. Deneklerden bazal, 2, 12, 24 ve 72. saatlerde kan örnekleri alınarak serum NGF düzeyleri ölçüldü. Deneyin üçüncü gününde sakrifiye edilen ratların ince barsak, karaciğer ve akciğer dokuları ayrılarak hematoksilen eozin (H/E) ve immunohistokimyasal olarak bcl-2 ve bax boyaması ile apopitoz değerlendirildi. **Bulgular:** Çalışmamızda en belirgin barsak dokusunda olmak üzere karaciğer ve akciğer dokusunda apopitozun arttığı ve bu artışın en belirgin olarak erken dönemde anti-NGF uygulanan grupta olduğunu saptadık. Sepsis ve EA-NGF grubunda daha anlamlı olmak üzere sepsis gelişen deneklerde bcl-2 boyanmasının kontrole göre daha az olduğunu ve bax boyanmasının ise daha fazla olduğunu saptadık. Erken dönemde anti-NGF uygulanan grupta bax boyanmasının geç dönemde anti-NGF uygulanan gruba göre daha fazla olduğunu saptadık. NGF düzeylerinin sepsis grubunda (Grup S)'de 24. saatte artarken Grup EA-NGF'de ikinci saatte azaldığını saptadık. **Sonuç:** Sepsisin erken döneminde uygulanan anti-NGF'nin apopitozu en az sepsis kadar arttırdığı, geç dönemde uygulanan anti-NGF'nin ise apopitozu azalttığı kanaatine varılabilir.

Anahtar Kelimeler: Sepsis; 7S sinir büyüme faktör proteini, fare; apoptoz

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Sepsis, which is defined as the uncontrolled and systemic inflammatory response of the host to infection, and related organ dysfunctions are the leading cause of mortality in intensive care units except for coronary intensive care.[1,2]

Apoptosis is a type of programmed cell death, which is genetically controlled and it ensures harmless elimination of cells that are not necessary for the organism, that have completed their biological mission or that have been injured.[3,4] Buchman et. al. showed how apoptosis was triggered in an experimental model of sepsis induced by lipopolysaccaride (LPS) for the first time in 1992.[5,6] Apoptosis which is seen in physiological, adaptive and pathological events activates proteases called caspase through intra- and extracellular signals. Cellular death receptors activate caspases via adaptor proteins and intracellular signals via mitochondria. Stimulations increase the permeability of the external mitochondrial membrane. The permeability of the external mitochondrial membrane is enabled by some proteins, the most important of which is bcl-2 group of proteins. Some bcl-2 group proteins have a pro-apoptotic effect, while others have an anti-apoptotic effect. [5-7] Those with a pro-apoptotic effect (Bax) induce the release of cytochrome C from mitochondria to cytoplasm. Those with an anti-apoptotic effect (Bcl-2), on the other hand, inhibit the release of cytochrome C. The balance between pro- and anti-apoptotic members determines the choice between life and death. It is seen that overexpression of anti-apoptotic members inhibits apoptosis, whereas over expression of pro-apoptotic members causes cell death.[5-7]

Neurotropins are the polypeptide-structured growth factor family that is required for the development and sustenance of the vertebrate nervous system. Nerve growth factor (NGF) has been reported to have effects on inflammation and apoptosis, apart from the nerve system. Its role in inflammation is not clear and varies depending on the type and stage of inflammation.[8] NGF was found to affect apoptosis particularly in neuronal and immune cells and to be responsible for the halving of sympathetic and sensory neurons during development. Exogenous NGF administration increases number of NGF sensitive neurons, while anti-NGF administration leads to cell death in neurons.[9]

The present study aimed to evaluate the effects of anti-NGF administration on apoptosis in rats with an experimentally induced sepsis model and to explore the possible role of NGF in the pathogenesis and treatment of sepsis.

MATERIAL AND METHODS

After the approval of the Faculty Ethic Committee was obtained, Wistar-Albino (male, n= 33) rats weighing between 200 and 250 grams were included in the study. Ethical provisions of the ''European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes'' were observed and care was taken to use minimum number of subjects in the study. The subjects were kept in an air-conditioned room with 12-hour artificial lighting, and fed on standard rat pellet and tap water. Anesthesia was induced in all subjects using intramuscular ketamin HCl (Ketalar ®, Eczacıbaşı, Istanbul 90 mg/kg) and xylazine (Romphun ® Bayer Istanbul 10 mg/kg).

The subjects were marked and randomly divided into four groups. Control group (Group C, n=6) was injected with intraperitoneal 1 ml 0.9% NaCl. Sepsis group (Group S, n=9) was given 1 mg (1 ml) intraperitoneal *E. coli* lipopolysaccaride (LPS) (0111:B4 LPS25, Chemicon USA) to induce an experimental sepsis model.[10] About 8-10 hours after LPS injection, sepsis symptoms reported by Bostanoğlu et. al. (the subjects snuggled together to sustain their body temperatures, their mobility declined and their motions slowed down, piloerection, crusting and bleeding in the eye developed) were observed. Early anti-NGF group (Group EA-NGF, n=9) was given 500 μg/kg intraperitoneal anti-NGF (ab10515, Novus, USA) one hour before LPS injection. Late anti-NGF group (Group LA-NGF, n=9) was given 500 μg/kg anti-NGF antibody immediately after (at the 10th hour) sepsis became apparent following LPS injection.[11] Subjects which did not show sepsis symptoms within 12 hours

were excluded from the study. They were replaced with new ones to reach a total number of 33 subjects.

Small intestine, liver and lung tissues of the rats, which were sacrificed on the third day of the experiment were separated, fixated in formol, and stored. After burying the tissue samples in paraffin, cross sections with 4 μm thickness were prepared and staining procedure was started. In hematoxylin eosin (H/E) staining, as hematoxylin stains chromatin, apoptotic cells were evaluated under light microscopy (Olympus MX50, Japan) based on nucleus morphology. Morphologic changes like shrinking of the cell or cytoplasm, condensation of chromatin and its accumulation in the periphery of the nucleus membrane, shrinking and partitioning of the nucleus were interpreted as apoptosis. Scoring was made depending on the amount of apoptotic cells in an area of one hundred cells (Score 0: less than 1%; 1: 1-5%; 2: 6-25%; 3: 26-50%; 4: more than 50%).[12] Monoclonal bcl-2 (3195-100, Biovision, USA) and monoclonal bax (SC-7480, Santa Cruz Biotechnology, USA) antibodies were employed in immunohistochemical staining. The preparations were evaluated according to the number of stained cells under light microscopy (Olympus MX50, Japan) (Score 0: no staining; 1: 1-5%; 2:6-15%; 3: 16-100% staining). [13]

In order to determine serum NGF levels, blood samples were collected from the subjects, before the intervention, and at the 2^{nd}, 12^{th}, 24^{th} and 72^{nd} hours after the intervention. Before and immediately after the collection of blood samples, the subjects were injected with subcutaneous 1 ml 0.9% NaCl. The blood samples were centrifuged at 3500 rpm for 5 minutes to separate sera, which were then stored at -20°C. Serum NGF levels were evaluated using E_{max} Immunoassay method (G7630, Promega, Madison, USA).

Statistical Analysis

Statistical Package for Social Sciences (SPSS, Inc. Chicago IL) 15.0 software was used for statistical evaluation. The data obtained were considered as mean ± standard deviation. Repeated measures ANOVA and chi-square test was used in the comparisons between groups and $p<0.05$ was considered significant.

RESULTS

The rate of accumulation of apoptotic cells in the liver, lung and intestine tissues with H/E staining was found elevated in all groups with experimentally induced sepsis model, relative to the control group ($p< 0.05$), and the increase was seen to be more significant in the sepsis and early anti-NGF groups ($p< 0.001$). A comparison between the early anti-NGF group and the sepsis group showed that the increase in apoptosis in liver and intestine tissues was higher in the former (Figure 1).

When the early anti-NGF group was compared with the late anti-NGF group significantly increased, apoptosis was seen in all tissues, the most marked increase being in the intestine tissue ($p< 0.05$) (Table 1).

Immunohistochemical staining showed that the rate of staining with bcl-2 in the liver tissue decreased in all groups with induced sepsis, when compared to the control group ($p< 0.001$). Staining with bcl-2 in the lung tissue was found to be lesser in all groups ($p< 0.05$), with a more marked decrease in the sepsis group ($p< 0.001$) relative to the control group. Less staining with Bcl-2 in the intestine tissue was observed in all study groups ($p< 0.05$), particularly in the sepsis and early anti-NGF groups ($p<0.001$), in comparison to the control group (Figure 1).

Rate of staining with bax in the lung and intestine tissues of all sepsis-induced groups was significantly greater when compared to the control group ($p< 0.001$) (Figures 2, 3). All groups with induced sepsis ($p< 0.05$), particularly the sepsis and early anti-NGF groups ($p< 0.001$), showed an increase in staining with bax in the liver tissue (Figure 4). A comparison of early anti-NGF group with the late anti-NGF group revealed that the rate of staining with bax increased in all tissues of the former ($p< 0.05$) (Table 2).

Serum NGF levels increased significantly on the 24^{th} hour in the group with induced sepsis, relative to the control group. Serum NGF levels in the

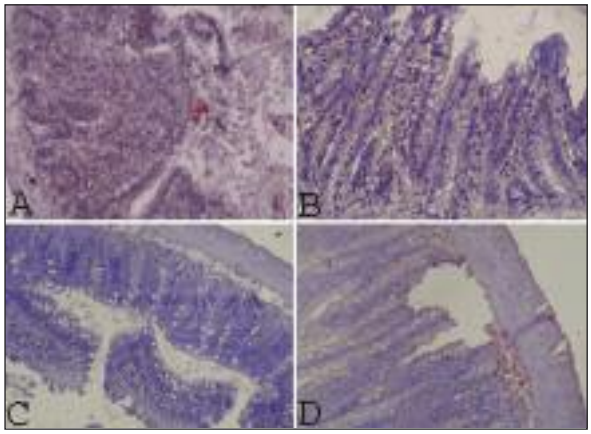

FIGURE 1: Bcl-2 staining in the intestine tissue of the groups. (A: Group C, B: Group S, C: Group EA-NGF, D: Group LA-NGF).

TABLE 1: Scoring means with H/E staining.

	Liver	Lung	Intestine
Group C	0.33 ± 0.51	0.16 ± 0.40	0.33 ± 0.51
Group S	1.54 ± 0.52*	2.18 ± 0.60*	1.90 ± 0.70*
GroupEA-NGF	2.37 ± 0.51*#	2.62 ± 0.51*	2.87 ± 0.35*#
Group LA-NGF	1.37 ± 0.51*+	1.62 ± 0.51*+	1.37 ± 0.33*+

* p<0.05 Group S, EA-NGF, LA-NGF vs Group C
# p<0.05 Group EA-NGF vs Group S
+ p<0.05 Group EA-NGF vs Group LA-NGF

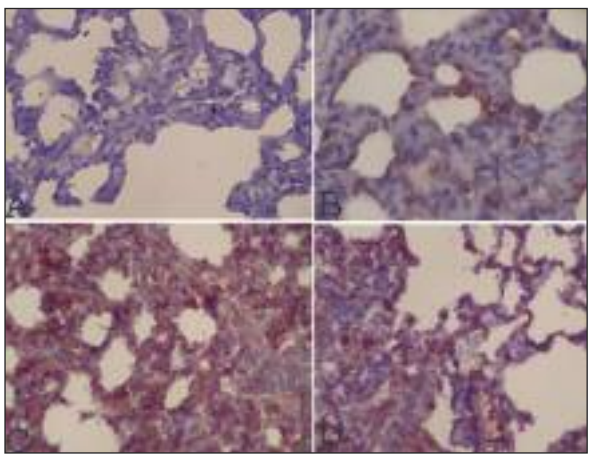

FIGURE 2: Bax staining in the lung tissues of the groups.
(A: Group C, B: Group S, C: Group EA-NGF, D: Group LA-NGF).

early anti-NGF group decreased significantly at the 2nd hour when compared to other groups. There was no marked difference between the groups in the following periods. Serum NGF levels were observed to be close to baseline values throughout the study (Figure 5).

DISCUSSION

There is a balance between cell proliferation and apoptosis in the tissues and that is how the continuity of tissue volume is ensured. Apoptosis is the cell death mechanism charged with the removal of physiologically and pathologically unwanted, injured or potentially neoplastic cells.[14] It is emphasized that lymphocyte apoptosis observed in sepsis takes the lead among immunosuppression mechanisms, and that this is not a compensatory, but a primary response.[15]

In a study where patients diagnosed as sepsis were examined in the postmortem period, Hotchkiss et. al.; and in the experimentally induced bacteremic shock model (monkey), Efron et. al. established that apoptosis, which is evaluated by dif-

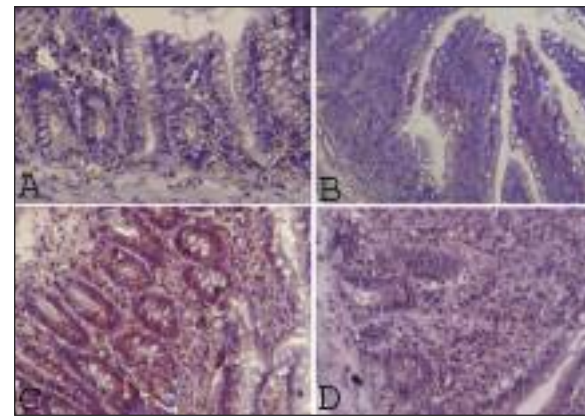

FIGURE 3: Bax staining in the intestine tissues of the groups
(A: Group C, B: Group S, C: Group EA-NGF, D: Group LA-NGF).

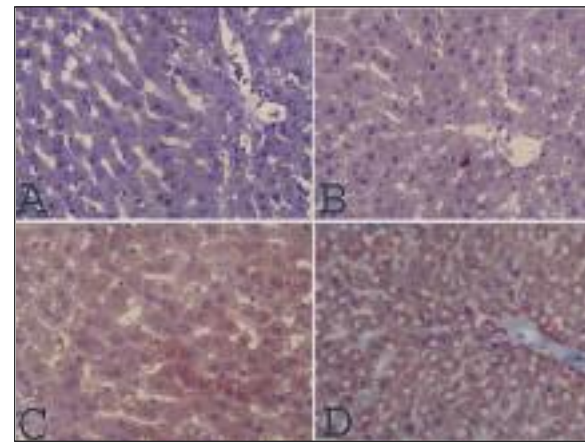

FIGURE 4: Bax staining in the liver tissues of the groups
(A: Group C, B: Group S, C: Group EA-NGF, D: Group LA-NGF).

TABLE 2: Scoring means with immunohistochemical staining.

		Group C	Group S	Group EA-NGF	Group LA-NGF
Liver	Bcl-2	2.16 ± 0.75	0.27 ± 0.46*	0.25 ± 0.46*	0.25 ± 0.46*
	Bax	0.33 ± 0.51	1.90 ± 0.70*	2.50 ± 0.53*#	1.50 ± 0.53*
Lung	Bcl-2	1.66 ± 0.81	0.36 ± 0.50*	0.37 ± 0.51*	0.50 ± 0.53*
	Bax	0.33 ± 0.51	2.09 ± 0.70*	2.75 ± 0.46*#	1.87 ± 0.64*
Intestine	Bcl-2	1.83 ± 0.75	0.54 ± 0.52*	0.37 ± 0.51*	0.62 ± 0.51*
	Bax	0.33 ± 0.51	2.54 ± 0.52*	3.0 ± 0.00*#	2.12 ± 0.64*

* p<0.05 Group S, EA-NGF, LA-NGF vs Group C
# p<0.05 Group EA-NGF vs Group LA-NGF

ferent methods, increased significantly in subjects with induced sepsis.[12,16] Apoptosis was also found significantly elevated in subjects in whom an experimental sepsis model was induced by cecal ligation and perforation (CLP) and lipopolysaccaride (LPS) administration methods.[17,18] Subjects with induced sepsis were observed to have lower bcl-2, which increased after the prevention of apoptosis, and decreased bax. Furthermore, apoptosis was shown to decline dramatically in transgenic subjects in whom over-expression of bcl-2 was ensured.[19-21]

In a study where biopsies obtained from the erythema formed on the skin of volunteers by UV radiation subjected to immunohistochemical staining with anti-NGF, NGF-positive melanocytes and keratinocytes were found to decrease in subjects who were subjected to UV radiation.[22] It was established in another study which evaluated the effect of NGF on apoptosis formed by aging in the peritoneal mast cell culture of rats that NGF prevented apoptosis dose-dependently and administration of antibodies against NGF completely blocked its apoptosis preventing effect.[23] Additionally, it is shown that, NGF due to acetylcholinesterase increase associated with increased apoptosis, and endoplasmic reticulum is associated with suppression of caspase -12 activity to prevent apoptosis.[24,25,]

With H/E staining in sepsis groups we found that apoptosis increased in the liver and lung tissues, however it was more marked in the intestine tissue, and that the most remarkable increase was in early anti-NGF group. It was observed that anti-apoptotic bcl-2 staining was less marked while

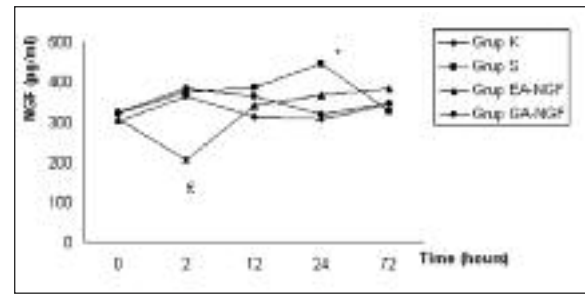

FIGURE 5: Serum NGF levels of the groups.
*: p< 0.05 When the 24th hour vs baseline and the 72nd hour in Group S, Group S is vs Group C at the 24th hour
£: p< 0.05 When the 2nd hour vs other periods in Group EA-NGF, Group EA-NGF vs other groups at the 2nd hour

apoptotic bax staining was more pronounced in the subjects with induced sepsis, and significantly so in the sepsis and early anti-NGF groups with respect to the control group. We established that bax staining in the early anti-NGF group was more discernible than that in the late anti-NGF group.

In addition to apoptosis, NGF has been shown to play a role in inflammatory events in several studies. Increased expression of NGF and its TrkA receptor were immunochemically shown in the inflammation occured with LPS seeded to the monocyte cultures prepared from healthy male donors. In this study, the expression of NGF and its were increased with application of LPS in a dose-dependent-manner and a decrease in the expression of TrkA receptor was detected with use of antibodies against NGF.[26] Additionally a study in which cystitis was producet by turbentine reported that mRNA expression of NGF increased in the first two hours, and this inflammation could be antigonized with TrkA IgG. [27]

In experimentally created defects of the corneal epithelium in dogs, increased NGF content was showed immunohistochemically in the corneal epithelium and lacrimal gland.[28] It was shown that application of anti-NGF antibody caused reactivation of ocular HSV-1 in rabbits with latent herpes simplex virus (HSV-1) infection.[29] The damage was assessed in a study four weeks after colitis produced with sulfonic acid and 2-3 times increase in the severity of experimental inflammation due to pretreatment with anti-NGF and anti-NT-3 was shown; all of those suggested that NGF had an anti-inflammatory role instead of an inflammatory role.[30]

The increase in NGF levels at the 24th hour in the groups with induced sepsis supports our hypothesis that NGF is an effective mediator in sepsis. Decreased serum NGF level in the EA-NGF group at the 2nd hour shows that the administered anti-NGF dose is effective, while restoration of NGF levels after the 2nd hour demonstrates that this effect is short-lived. There was no significant change in NGF levels of the late anti-NGF group. It was determined that anti-NGF dose administered at a later stage could not reduce the NGF level which started to increase in that period however enabled NGF to remain at the baseline level by preventing its further increase. It was found that apoptosis in the late anti-NGF group, where NGF levels remained close to baseline values throughout the experiment, increased to a lesser extent than it did in the other groups, however it showed a significant decrease with respect to the EA-NGF group. In conclusion, anti-NGF administration in the early period of sepsis increases apoptosis at least to the same extent with sepsis, and conversely anti-NGF administered in the later period of sepsis decreased apoptosis with respect to other groups.

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