

Bacteria induced extrinsic and intrinsic apoptotic pathways in the rat gastrointestinal system.

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Abstract

Apoptosis is a key factor in the death of organ-specific cells. Developing a clear understanding of the effect that bacterial translocation has on initiating apoptotic pathways that induce multiple organ dysfunction syndrome (MODS) is essential for developing effective treatment modalities. Translocation does not occur naturally and apoptotic pathways remain unclear. The aim of this study is to investigate the effect of bacterial translocation on apoptotic pathways in the spleen, small intestine, colon, liver, and mesenteric lymph nodes (MLN) of rats. We randomly divided 12 healthy Wistar-Albino rats into two groups and induced bacterial translocation in the experiment group by clamping the superior mesenteric arteries (SMA) for comparison of test results to the control group. Samples from both groups were collected under sterile conditions and an inoculation procedure was performed. Caspase 3, 8, 9 and p53 were evaluated by immunohistochemistry, and cell expressions were counted. *Klebsiella* and *E. coli* colonies were observed in the bacteria cultures associated with the experiment group. Bacterial translocation-activated caspase 8 and 3 were found in all tissues of the experiment group; however, activated p53 was identified only in the colon and liver and activated caspase 9 was seen in small intestine, mesenteric lymph nodes and liver. We concluded that translocated bacteria stimulated extrinsic and intrinsic signaling pathway in gastrointestinal systems.

Keywords: Apoptosis, Bacteria translocation, Caspases, P53, Pathway, Sepsis.

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Introduction

Bacterial translocation (BT) is the passage of microflora through the lamina propria to local mesenteric lymph nodes (MLN) and to other organs, such as the liver and spleen [1]. Translocation does not occur under normal conditions. In conditions such as burns; starvation; intestine obstruction; surgical trauma; anesthesia; obstruction of the gall bladder; and shock, bacterial translocation impairs intestinal barrier function and the mucosal structure in the GI tract [1,2]. Following translocation, enteric bacteria pass into the systemic circulation and spread to the entire body, which may cause sepsis; shock; multiorgan failure; and death [1-3]. Multiorgan failure is a chief concern due to the associated mortality. Multiple organ failure is often caused by Multiple Organ Dysfunction Syndrome (MODS). MODS is most commonly found in patients hospitalized in intensive care units [2-4]. Apoptosis also is an important player in the death of organ-specific cells

and in multiorgan failure. In the presence of bacterial translocation, particularly in sepsis, the retardation and delay in neutrophil apoptosis deteriorates the immune response and worsen the cascade that underpins MODS [1-3]. There are two major pathways in apoptosis: Extrinsic and intrinsic pathway (Figure 1).

Extrinsic pathway

The extrinsic apoptosis pathways were classified as type I and type II. In type I, cells' capacity to activate direct caspase 8 apoptosis pathways was via death receptors (TNF-R, FAS, DR3, DR4, DR5, DR6) [5]. Activated caspase 8 initiates a caspase cascade. Cleavage of caspases 3, 6 and 7 leads to the characteristic morphological and biochemical features of apoptosis [6]. In type II, the signal coming from the activated receptor needs to be amplified via mitochondria-dependent apoptotic pathways. In this case, caspase 8 is activated later,

Bid is cleaved by caspase 8 and in its truncated form (tBid) translocates to the mitochondria to induce the release of cytochrome c into the cytosol [7]. Cytosolic cytochrome c binds to monomeric Apaf-1, which then triggers the activation of the initiator procaspase 9 [8,9]. Activated caspase 9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase 3, caspase 7, and caspase 6 [9]. In this type II response, Bid is the key protein that facilitates the cross-talk between the extrinsic and intrinsic pathways. In so-called Type I cells stimulation of the extrinsic pathway is sufficient to drive apoptosis, whereas in Type II cells required further amplification by Bid for commitment to apoptosis.

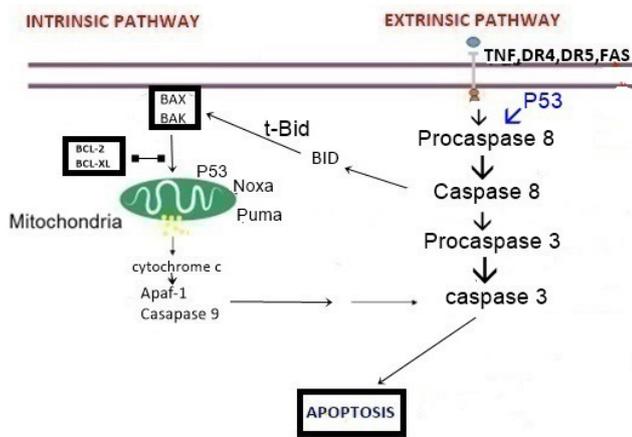


Figure 1. The Picture depicts the intrinsic and extrinsic pathways of apoptosis.

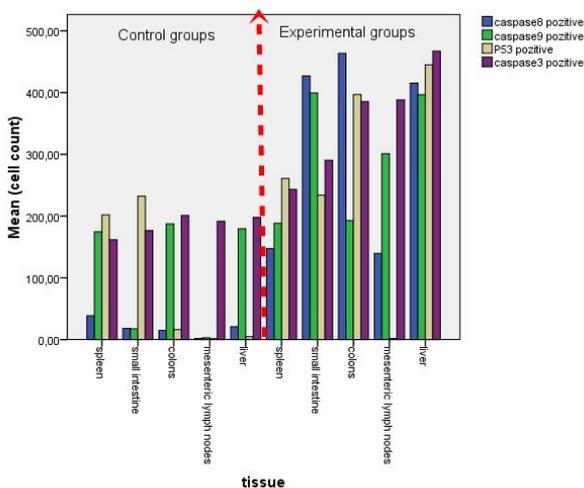


Figure 2. Positive cell number for caspase 3, 8, 9 and p53 in spleen, small intestine, large intestine, mesenteric lymph nodes and livers. Immunoperoxidase- hematoxylin.

Intrinsic pathway

In the intrinsic or mitochondrial pathway, The Bcl-2 family of proteins have either pro-or anti-apoptotic activities and regulate apoptosis by controlling mitochondrial permeability. In response to many types of stress or damage the pro-apoptotic Bcl-2-associated X protein (Bax) or Bcl-2 antagonist/

killer-1 (Bak) are activated and they promote the release of cytochrome c. The association of cytochrome c with apaf-1 recruit procaspase 9, cleavage of activated caspase 9, and the activation of caspase 3 which terminate the cell death [10]. In the literature, there are few studies showing the induction of apoptosis through the investigation of bacterial translocation. More significantly, the pathways of apoptosis were not clear in previous similar studies. In this study our aim was to show the apoptotic pathways in gastrointestinal system. In order to do caspase 3, 8, 9 and p53 expressions were investigated immunohistochemically.

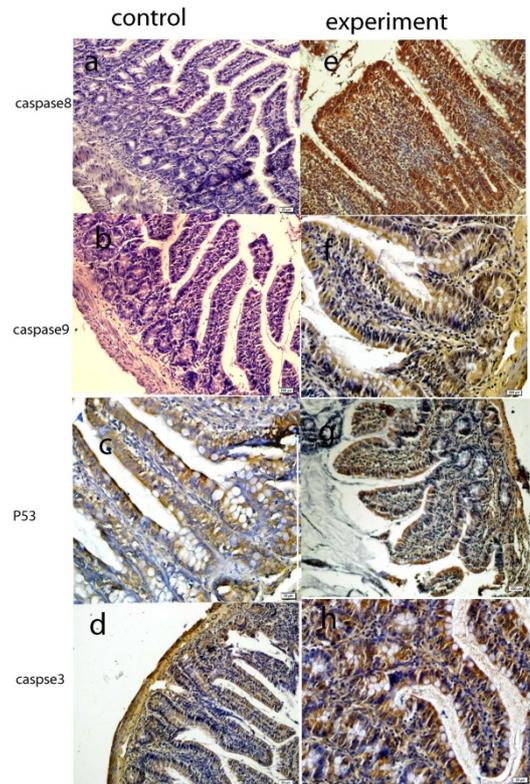


Figure 3. The expression and localization of caspase 3, 8 and 9 were detected by immunohistochemistry in small intestine. Control groups; (a, b, c, d) were compare with experimental groups; (e, f, g, h). Immunoperoxidase- hematoxylin.

Materials and Methods

Ethics

This experiment was conducted according to the Turkish “Experiments on Animals Act” and was approved by the Institutional Animal Ethics Committee of the Central Animal Laboratory of Pamukkale University (01.31.2012-PAU.HDEK-2012/007).

Animals

Twelve healthy Wistar-Albino rats were used in this study. The mean weight of the rats was 230 g (200-250 g) and the age of the rats was 20 weeks. Care for the animal care use was provided in an Experimental Animal Laboratory in Pamukkale

University. The rats were humanely treated and divided by four rats per cage in a well-ventilated, 12 h dark and light cycle room at the temperature of 24°C throughout the experiment.

Groups

The rats were randomly divided into two groups. Group I was assigned as the control and Group II was assigned as the experiment group.

Interventions and surgical procedures

The rats were anesthetized with intraperitoneal ketamine (50 ml/kg). Following the skin shave, the skin was cleaned by povidone iodide and median laparotomies were performed under sterile conditions. The superior mesenteric artery (SMA) was clamped on the rats in the experimental group and ischemia was produced. Forty-eight hours after the experiment, samples of liver, spleen, small intestine, and colon were taken into sterile tubes. These samples were homogenized by adding 1 ml saline. The inoculation was performed on blood agar and eosine methylene blue (EMB) agar, and colonies were counted on the plaques. The blood samples of 0.5 ml were taken under sterile conditions and 5 ml brain-heart infusion was placed into Broth medium. The blood cultures were incubated for 7 days at 37°C and subcultured in blood agar and EMB agar mediums and the production was evaluated.

Apoptosis procedure and histology

Mesenteric lymph nodes, liver, spleen, small intestine, and colon tissues were left in 10% formaldehyde for 72 h. The fixed tissues were processed with light microscopy procedure and 5 µm sections from the paraffin-embedded tissues were taken onto positive loaded slides.

Antibodies and staining procedure

Endogenous peroxidase activity was blocked in 3% hydrogen peroxidase for 10 min, and the sections were incubated with saponin to facilitate binding of the primary antibody to the antigenic areas. Epitopes were stabilized by application of serum blocking solution (goat serum; Lot# 20570999; Zymed Laboratories Inc., South San Francisco, CA, USA) for 60 min at room temperature. Sections were incubated in phosphate-buffered saline (PBS) at room temperature for 60 min using the following primary antibodies: caspase 8 (1:200 sc-5263; Lot# H0911 Santa Cruz Biotechnology), caspase 9 (1:100, Ab-4 Lot# H0911 Neomarker Biotechnology), caspase 3 (1:200, Sc-1226 Lot# F0206, Santa Cruz Biotechnology), p53 (1:200, sc-1311; Lot# D1911, Santa Cruz Biotechnology). In the next step, secondary antibodies were applied to tissue slides: anti-rabbit IgG and avidin-biotin-complex-peroxidase (ABC; Lot# 903532A; Invitrogen, Carlsbad, CA, USA). Diaminobenzidine (DAB; Lot# 100M16942; Sigma-Aldrich, St. Louis, MO, USA) was used as the chromogen. In the following step, the slides were counterstained with hematoxylin for 1 min, dehydrated in graded ethanol, and mounted in conventional medium. The immunoperoxidase reaction was determined to

be negative (-) or positive (+). The findings were observed and photographed under OLYMPUS BX51 microscope.

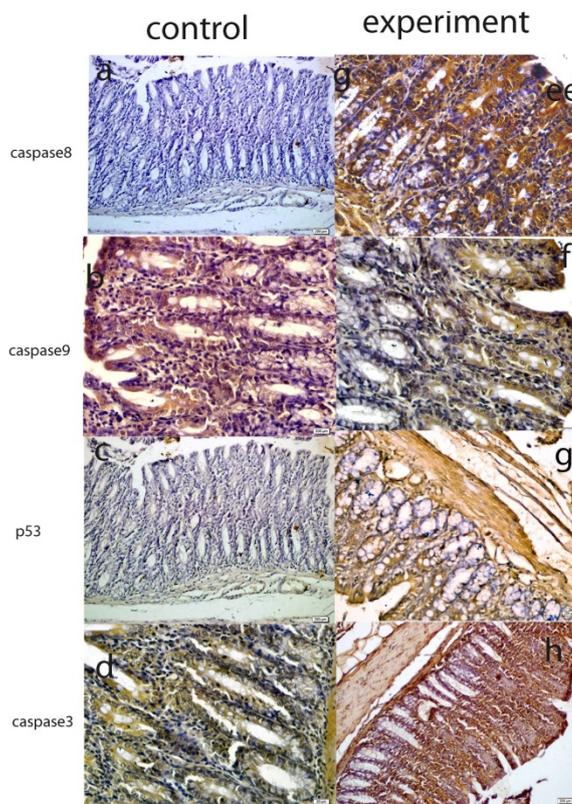


Figure 4. The expression and localization of caspase 3, 8 and 9 were detected by immunohistochemistry in colon. Control groups; (a, b, c, d) were comparing with experiment groups; (e, f, g, h). Caspase 3, 8, 9 and p53 were positive in the experimental groups. Caspase 9 and caspase 3 were positive in the control groups (b, d). Immunoperoxidase-hematoxylin.

Statistical analysis

In each slide, five areas and 100 cells/areas were evaluated under 40X magnification. The percentages of the positive cells within these areas were determined at different times by two investigators blinded to the source of the samples, and the mean was calculated. The data were analysed in an SPSS 17 program. In addition, P<0.05 was estimated as significant for the non-parametric Mann-Whitney U test.

Results

Microbiological results

The significant reproduction was observed in six rats where translocation had been applied. Three of the six were found in the liver, five in the mesenteric lymph nodes, and two in the spleen. *Klebsiella* and *E. coli* reproduction were observed, throughout.

Histological results

Histological results are summarized in Table 1. We also analyzed positive cell number for caspase 3, 8, 9 and p53 in

spleen, small intestine, colon, mesenteric lymph nodes and livers in Figure 2 and Table 2. As can be seen in the results for the small intestines in Table 1, while caspase 8 and caspase 9 were negative in the control group, these two proteins were positive in the experimental group. The same results were also found for p53 expression in the experimental and the control groups; however, caspase 3 expression was increased in the experimental groups (Figure 3). In the colon, in the control group, expressions of caspase 8, 9, and p53 were negative

while in the experiment groups they were positive. P53 expression was different from the results of the small intestine. Similarly, caspase 3 expression was increased in the experiment group when compared with the control group (Figure 4). Likewise, the liver also showed increased expression of caspase 3, 8, and 9 in the experiment group when compared with the control group; however, while the p53 expression was negative in control group, the results had changed to a positive in the experiment group (Figure 5).

Table 1. The localization and expression of caspase 3, 8, 9 and p53 in spleen, liver, small intestine and MNL.

		C	EXP	C	EXP	C	EXP	C	EXP
		Caspase 8		Caspase 9		P53		Caspase 3	
spleen	White pulp	-	+	-	+	+	+	+	++
	Red pulp	+	++	+	++	+	+	+	+
small intestine	Epithelium	-	+++	-	++	++	++	++	+++
	Connective tissue	-	+++	-	++	++	++	++	+++
	Muscle tissue		+++	-	++	++	++	++	+++
colons	Epithelium	-	+++	-	++	-	+++	++	+++
	Connective tissue cells	-	+++	-	++	-	+++	-	+
	Muscle tissue		+++	-	++		+++	++	+++
MNL	Lenf nodules	-	++	-	++	-	-	-	+++
	Medulla	-	+	-	+	-	-	-	+
liver	Hepatocyte cytoplasm	+	+++	++	+++	-	+++	++	+++
	Hepatocyte nuclei	-	+++	-	-	-	-	-	+++

Table 2. Positive cell number for caspase 3, 8, 9 and p53 in spleen, small intestine, colon, mesnteric lymph nodes and livers.

Tissue		Caspase 8 positive cell number		Caspase 9 positive cell number		p53 Positive cell number		Caspase 3 positive cell number	
spleen	Mean	39	148	175	188	202	261	162	243
	Std. Deviation	14	12	14	9	55	6	7	10
small intestine	Mean	18	427	18	399	232	234	177	290
	Std. Deviation	3	33	3	7	11	3	6	14
colons	Mean	15	464	187	193	16	397	201	385
	Std. Deviation	3	41	9	9	3	9	4	10
mesenteric lymph nodes	Mean	2	140	3	301	2	2	192	388
	Std. Deviation	1	19	2	8	1	1	11	12
liver	Mean	21	415	179	397	5	445	198	467
	Std. Deviation	3	7	27	5	2	8	7	24

Another important finding across all organ tissues from the experimental group was a positive expression of caspase 8 and caspase 3 in the nuclei and cytoplasm. In MLN tissue groups, while caspase 8, 9, and 3 expressions were negative in the control groups, those proteins were positive in the experiment

groups; and p53 expression was negative, too. (Figure 6). In the spleen, caspase 8, 9, 3, and p53 were expressed positively in both the experiment and control groups, but the expression was more elevated in the experimental group (Figure 7). According to the cell number for expression, caspase 8 and

caspace 3 were increased significantly ($p < 0.05$) in all experimental tissue groups when compared to the control tissue groups. Caspace 9 was high significantly ($p < 0.05$) in the small intestine, MLN, and liver, but not in the spleen and colon. Although p53 was positive, the expression was poor in almost all tissues, but a significant difference was observed in colon and liver tissue specimens for the experiment group ($p < 0.05$) (Table 2 and Figure 2).

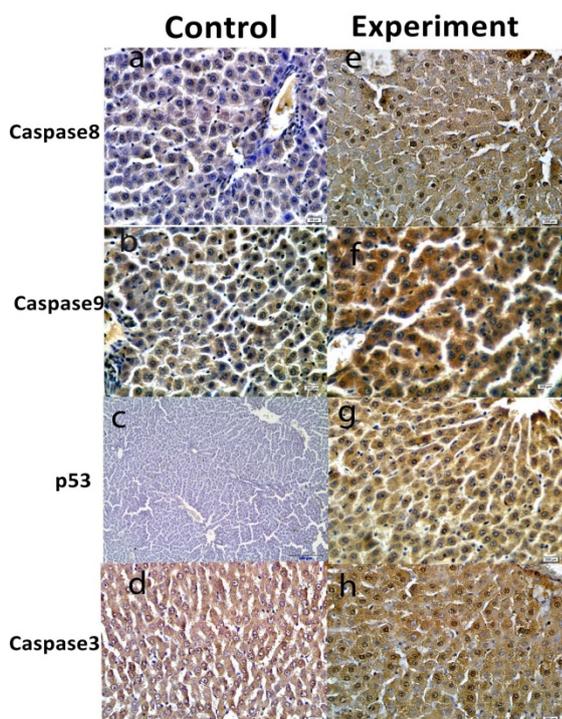


Figure 5. The expression and localization of caspase 3, 8 and 9 were detected by immunohistochemistry in liver. Control groups; (a, b, c, d) were compare with experiment groups; (e, f, g, h). Immunoperoxidase- hematoxylin.

Discussion

We investigated the effects of bacterial translocation caused by mesenteric artery obstruction on apoptosis in the small intestine, colon, mesenteric lymph nodes, liver, and spleen tissues using both inferential and descriptive statistical measures. Significant reproduction was observed in applied bacterial translocation for 6 rats in the experiment group. The most common translocated microorganisms were *E. coli* and *Klebsiella* spp. Markers of apoptosis: caspase 3, 8 and 9 were activated in small intestine, MLN, Liver samples from the experiment group, but p53 activation was seen only in colon and liver tissue samples. Bacteria generally induce apoptosis with the secretion of protein synthesis inhibitors, the production of pore proteins, and the activation of death machinery molecules such as lipopolysaccharides (LPS), or other super antigens [11-13]. LPS which is component of the outer wall of gram-negative bacteria such as *E. coli* binds to LBP, a soluble acute-phase protein, LPS+LPB can induced expression of CD14, TNF-related apoptosis inducing ligand, and TLR14 (the toll like receptor for LPS) thus stimulating

nuclear transcription factor kappa B (NF- κ B) which results in the release of many types of inflammatory factors and cellular toxic substances. Consequently, LPS impairs intestinal barrier function by activating chemical mediators, i.e. eicosanoids, interleukin-1, interleukin- 6, TNF α , superoxide, and nitric oxide. Impaired intestinal barrier leads to multiple organ failure (Figure 8) [14-21].

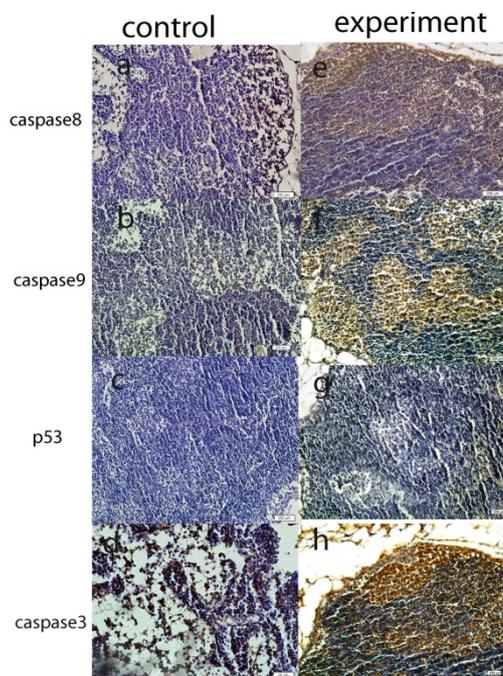


Figure 6. The expression and localization of caspase 3, 8 and 9 were detected by immunohistochemistry in mesenteric lenf nodes (MLN). Control groups; (a, b, c, d) were compare with experiment groups; (e, f, g, h). Immunoperoxidase- hematoxylin.

In our study the expression of caspase 8 and caspase 3 were increased in all experimental tissue groups when compared to the control tissue groups ($p = 0.004$). The caspase 9 expression was intense and the increase in positive cell in the small intestine, MLN, and in liver was statistically significant ($p = 0.004$), but it was not in the spleen ($p = 0.065$) and colon ($p = 0.336$). Although p53 was positive, the expression was poor in almost all tissues, but a significant difference was observed in colon and liver tissue specimens for the experiment group ($p < 0.05$) (Table 2 and Figure 2). In experimental sepsis models there was a correlation between increase of apoptosis in paranchmal tissue and increase of TNF α level in serum [22]. We argue further that increased caspase 8 and 9 expressions can be linked to tumor necrosis factor-alpha (TNF α) due to the inflammatory factors that would accompany TNF α induce apoptosis. TNF α binds to membrane-bound death receptors, such as TNF- receptor 1 (TNF-R1). TNF-R activates the caspase 8 and then pro-apoptotic BCL-2 family proteins are activated, leading to changes in the integrity of the mitochondrial membrane so that pro-apoptotic Bcl 2 family members Bax and Bak can induce the release of cytochrome c and active caspases 9 and 3 (Figure 1) [7-11]. In our study, there is a significant increase of p53 positive cell in liver while there was no p53 activation in small intestine and MLN

suggesting that apoptotic pathways in liver are different from small intestine and MLN ($p=0.004$). We think that apoptotic pathway in small intestine and in MLN might be due to TNF alpha.

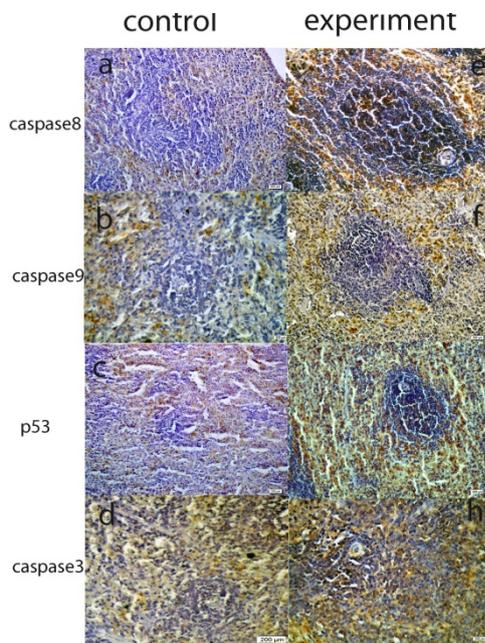


Figure 7. The expression and localization of caspase 3, 8 and 9 were detected by immunohistochemistry in spleen. Control; (a, b, c, d), experiment; (e, f, g, h) Immunoperoxidase-hematoxylin.

increased in experiment group compared to control ($p=0.004$) however, in colon caspase 8 and p53 positive cell number was increased significantly between experiment and control ($p=0.004$). On the other hand, there was no significance in the number of caspase 9 positive cells ($p=0.336$).

Our findings obtained from the small intestine, MLN and liver demonstrate that activation of caspase 8 may be related to an increased TNF α expression. However, we view this connection only appears plausible because the mesenteric lymph nodes and remained negative in both the control and experiment groups in small intestine p53 expression was similar in both the control and experiment groups. On the other hand, in the small intestine and MLN groups the caspase 9 increase without an accompanying change in p53 expression may indicate crosstalk between intrinsic and extrinsic pathways. In addition to that in liver caspase 8, 9 and p53 increase suggests that there is a cross talk between intrinsic and extrinsic pathways and there is also a p53 activation. On the other hand, there is a non-significant caspase 9 increase and there is only significant caspase 8 increase suggests that there is only Type I extrinsic pathway activation in spleen. Our results indicate that translocates bacteria created by hypoxia in the tissue due to clamping of the SMA activated the extrinsic apoptosis pathway type II in the small intestine and mesenteric lymph nodes, extrinsic apoptosis pathway type II and p53 activation in liver, extrinsic apoptosis pathway type I and p53 activation (intrinsic apoptotic pathway) in colon and extrinsic apoptosis pathway type I in spleen.

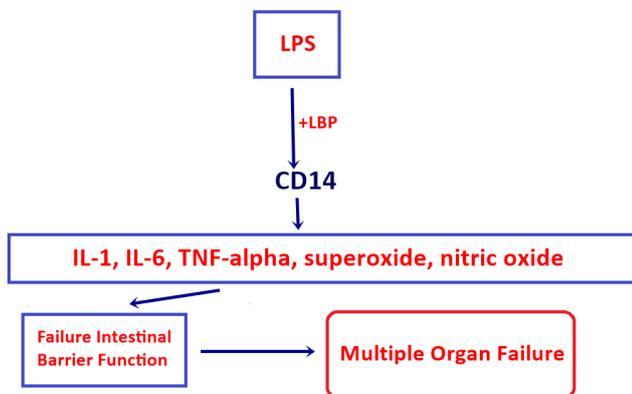


Figure 8. LPS, TLR and cytokine relationship.

p53 expression that had been negative in liver and colon in the control group changed to a positive expression in the experiment group in our study. In spleen, an increased p53 expression was observed in the experiment group when compared with the control group. However, this increase was not statistically significant ($p=0.574$). Intrinsic (mitochondrial) pathway are often used by p53; however, at the same time, this protein can activate the extrinsic apoptotic pathway through the induction of genes by encoding three trans-membranic proteins: Fas, DR5, and PERP [23,24]. In other studies, p53-dependent Fas mRNA induction has been demonstrated in the spleen, thymus, kidney, and lung [25,26]. Figure 1 shows the p53 mediated extrinsic pathway [26,27]. Statistically, in liver caspase 8, caspase 9 and p53 positive cell number was

Conclusions

In this study we detected both extrinsic and intrinsic apoptotic pathways in the gastrointestinal systems (GIS) of the experimental group rats. Despite numerous intensive treatment modalities, an effective method for treating MODS has not yet been found. This study is important because it expands the current body of knowledge on the effect of bacterial translocation on apoptotic pathways; however, further exploration of apoptosis and cell survival mechanisms will facilitate development of new, more effective strategies for MODS treatment. We believe that the prevention of apoptosis in the intestinal epithelium is the most effective, leading edge intervention to pursue for MODS treatment in the future.

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