

# Histopathological investigation of the effects of a combination of parenteral nutrition and hunger on rabbit splenic tissue

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## Abstract

**Aim:** Parenteral nutrition (PN) is a life-saving practice when the use of the gastrointestinal tract is not possible. However, PN can cause a variety of complications despite its many benefits. In this study, we examined the histopathological effects of PN combined with hunger in the splenic tissue of rabbits.

**Materials and Methods:** The rabbits were divided into four groups. The hunger + PN (PNH) group was completely starved and received the daily energy requirement with PN via an intravenous central catheter. The second group received enteral nutrition + PN (PNEN), wherein half of the required daily calories were provided through enteral nutrition, and the other half, through PN. A third group comprised semi-hungry (SH) rabbits that were provided only half the required daily calories by oral feeding. They did not receive PN. The last group was the control group. After 10 days, the rabbits were euthanized, and splenic tissue samples were collected from all the groups and examined histopathologically.

**Results:** The histopathological examination for the PNH group revealed damage in the form of germinal center effacement, reduction of white pulp, congestion of red pulp, and decreased cellularity in the periarteriolar lymphoid sheath. Immunohistochemical examination revealed a significant increase in the apoptotic activity of the splenic tissues of this group. The corresponding findings were mild in the PNEN group, and no signs of damage were found in the other two groups.

**Conclusion:** PN combined with hunger appears to cause damaging effects on splenic tissue in rabbits. Adding enteral nutrition to PN may reduce these effects.

**Keywords:** Parenteral nutrition; hunger; spleen; histopathology; apoptosis

## INTRODUCTION

Good treatment outcomes are possible with good nutritional conditions during disease management; conversely, poor nutritional conditions lead to poor treatment outcomes (1). It is quite difficult to ensure successful treatment in conditions where the patient's gastrointestinal system cannot be used (1, 2). In such cases, all nutrients, fluids, electrolytes, vitamins, and other nutritional substances needed by the body are provided parenterally via parenteral nutrition (PN). The clinical use of PN has led to significant success in the treatment of many diseases (1). However, the rise in PN administration use has also led to some reportedly negative effects during

PN implementation (2). They include cholestatic effects on the liver, cardiotoxicity, impaired kidney function, and negative effects on the vascular endothelium (3-6).

The spleen is an important organ as it plays a role in humoral immunity and hematopoiesis in mammals. It is quite basic in histological terms, comprising mainly red and white pulp structures. It filters out existing antigenic particles and aged or abnormal blood cells found in the circulating blood (7).

The aim of this study was to investigate the histopathological changes in splenic tissue due to the administration of PN after a total cessation of oral feeding in rabbits.

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## MATERIALS and METHODS

Before the commencement of the study, the required approval was obtained from Inonu University's Experimental Animal Ethics Committee (No: 2020/16-11). Four groups containing the same numbers of male and female rabbits were designed for this study (n=56). This study used a free web-based randomization program to assign each rabbit to a particular group.

The following groups were created. Group 1 or the PNH group comprised hungry rabbits that received a full dose of PN. Group 2 or the PN-enteral nutrition (PNEN) group was fed enterally with half a dose of rabbit feed and half a dose of PN. Group 3 or the semi-hungry (SH) group was provided rabbit feed containing half of the total daily calorie requirement. Group 4 served as the control group.

This research followed the oral nutrition and PN protocols of our previous study (4). All the rabbits had free access to water throughout the experiment. The oral and enteral feeding protocols of all the rabbits for the course of this research are shown in Table 1. The relative humidity of the room housing the rabbits was set to a comfortable value (45%–50%), and the average temperature was 20–22°C. A 12-h day-and-night cycle was implemented.

A central catheter was inserted in the internal jugular vein of each rabbit in the PNH and PNEN groups under sterile conditions. For the venous cutdown procedure, 25–35 mg/kg of ketamine (10% Alfamine; Ata Fen Ata Veterinary Materials Co., Ltd., Izmir, Turkey) and 3–7 mg/kg of xylazine (2% Alfazyn; Alfasan International, Woerden, The Netherlands) were both administered intramuscularly. A polyethylene catheter (20 G Cut DownCatheter, UPS Medical Instruments Co., Ltd., Balgat, Ankara, Turkey) was placed up to 2-cm-long in a supine position inside the internal jugular vein. The proximal end of the catheter was directed away from the vein insertion site to the back through a subdermal tunnel. Intravenous PN was administered to the PNH and PNEN groups for 10 d (Table 1). Each rabbit in the PNH group was completely starved and administered a full dose of PN intravenously within 24 h. The number of PN calories and PN dose given to each rabbit in this group was 204 kcal•kg<sup>-1</sup>•d<sup>-1</sup> and 230 mL•kg<sup>-1</sup>•d<sup>-1</sup>, respectively (Table 1). Half of daily calorie needs of each rabbit in the PNEN group were met with oral nutrition, and the remainder, with intravenously administered PN. TV-01 rabbit pellet feed (DSA Agrifood Products TS Inc., Kirikkale, Turkey) was used as the oral feed (composition: 18.75% protein, 6.38% cellulose, 2.28% fat, 6.2% ash, 2.45% minerals, 0.98% lysine, 0.36% methionine, and 12% starch; calorie count: 240kcal/100g). The PN formula was administered intravenously to the rabbits. It contained 10% (w/v) amino acids (Primene®, E.Baxter, Istanbul, Turkey), trace elements (Addamel® N, Fresenius Kabi, Uppsala, Sweden), 20% (w/v) lipids (Lipofundin® MCT/LCT 20%, B. Braun Melsungen, Germany), and 30% glucose (Dextrose 30% Polyflex®, Polypharma Pharmaceuticals San.Tic.A. S., Tekirdag, Turkey) (Table 1). Oral rabbit pellet feed (TV-01 Rabbit

Pellet Feed, Kirikkale, Turkey; calories: 102 kcal•kg<sup>-1</sup>•d<sup>-1</sup>) was given orally to each rabbit in the SH group. A fourth group, which was fed a diet providing 204 kcal/kg/day, served as a control. All the groups were provided with water ad libitum. At the end of the 10-d study period, all the rabbits were weighed, anesthetized, and euthanized, and their spleen tissue samples were collected.

### Histopathological Methods

#### Collection and preparation of spleen tissue samples

The animals were euthanized, their abdominal cavities were opened, and the spleen of each animal was removed and rinsed with physiological saline. One sample of size 2–3 cm in diameter was taken from each spleen and placed in 10% formalin solution for 24 h. A 3–4-mm-thick section from each sample was taken, passed through tissue processing, and embedded in a paraffin block. Moreover, 3–5-µm-thick sections were taken for hematoxylin and eosin (H&E) and Masson's trichrome staining. Routine H&E and Masson's trichrome staining procedures were performed on the sections. For the histopathological grading, we scored the changes in the H&E sections with a light microscope (Nikon Eclipse E200, Nikon, Japan) with 4×, 10×, 20×, and 40× objectives.

#### Immunohistochemical Methods

After the tissue sections were collected, they were deparaffinized and fixed on adhesive slides. Citrate buffer (pH = 7.6, Thermo Scientific, Fremont, CA) and an antigen retriever (Retriever 2100, Aptum, Southampton, UK) were applied in a pressure cooker for 15 min at 121°C. After cooling the sections, the citrate buffer was removed and saline with phosphate buffer was added. The slides were washed using the saline with phosphate buffer at every stage except protein blocking and primary antibody application. The following steps were implemented at room temperature, and the HRP kit (Thermo Scientific, Kalamazoo, Michigan, USA) was used for immunohistochemical painting according to the manufacturer's protocol. First, 3% hydrogen peroxide was added for 10 min. Second, the protein-V blocking solution was added for 5 min and followed by incubation of the Caspase-3 primary antibody (Anti-Caspase-3 antibody [ABM1C12] ab208161, Abcam, Cambridge, UK; dilution rate = 1:300) at room temperature for 1 h. Third, the glass slides were incubated for 10 min in biotinylated goat anti-polyvalent secondary antibody. Streptavidin peroxidase was added and incubated for 10 min. AEC chromogen (Thermo Scientific, Kalamazoo, Michigan, USA) was added to the slides and incubated for 15 min. Finally, the slides were washed with distilled water, counter-stained with Mayer's hematoxylin solution, and mounted.

#### Scoring and histopathologic evaluations of stained spleen tissue sections

The following histopathological changes were observed: germinal center effacement, reduction of the white pulp, congestion of the red pulp, and decreased cellularity in the periarteriolar lymphoid sheath (8,9). Two different scoring methods were used for the evaluation: the first

evaluated severity, and the other evaluated extensiveness. For the first method, the absence of lesions, mild changes, moderate changes, and severe changes were scored as 0, 1, 2 and 3, respectively (8). With regard to extensiveness, the absence of lesions, changes up to 33%, changes between 33% and 66%, and changes exceeding 66% to the section were scored as 0, 1, 2, and 3, respectively (10). We multiplied the two different evaluation results to arrive at a score for each parameter. Thereafter, we summed up all the scores and calculated the average by dividing the value by three. The final answer was the histopathological score for each animal. We also searched for the presence of fibrosis in the Masson's trichrome sections.

Evaluation of apoptosis was performed in the immunohistochemically stained sections using Caspase-3. Ten areas of lymphoid tissue magnified 400× were evaluated for each rabbit. One hundred cells were counted in every evaluated field. The positive cells

among them were counted too. The ratio of positive cells to the total counted cells was calculated to arrive at the apoptotic score for each rabbit, expressed as Caspase-3-positive cells per 1000 lymphoid cells (8).

### Statistical Analysis

The normality of the data was evaluated by the Shapiro–Wilk test. Normally distributed data were summarized as the mean and standard deviation. The homogeneity of the group variances was assessed with the Levene test. Since the variances of the groups were found to be heterogeneous, the Welch and Tamhane T2 post-hoc tests were used for comparisons. Distributions of the non-normal data were presented by median, minimum, and maximum values. For these data, group comparisons were performed with the Kruskal–Wallis test, and the Conover method was used for pairwise comparisons. The two-sided significance level was considered to be 0.05 for all the tests.

**Table 1. PN solution formula used in this work (total liquid volume 230 ml/kg/d and total calories 204 kcal/kg/d)**

Ingredient	Volume (ml)		Calories (kcal)		Calories Percentage (%)	
	PNH group	PNEN group	PNH group	PNEN group	PNH group	PNEN group
20% medium long-chain fat emulsion (1)	38	19	72.2	36.1	35	17.4
10% compound aminoacids	88	44	34.8	17.4	17	8.9
30% glucose	80	40	97	48.5	48	24
3% sodium chloride	13	6.5				
10% Potassium chloride	3	1				
10% calcium glukonate	3	1				
Water-soluble vitamins (2)	1/2 ampoule	1/4 ampoule				
Fat- Soluble vitamins (3)	1/2 ampoule	1/4 ampoule				
Trace elements (4)	1	0.5				
<b>Total</b>	<b>230</b>	<b>115</b>	<b>204</b>	<b>102</b>		

PNH group: Full dose Parenteral Nutrition along with hunger PNEN group: Enteral feeding along with half a dose of Parenteral Nutrition  
 Note (1) medium/long-chain fat emulsion (250ml) composition: soybean oil 25 gr, medium chain triglycerides 25gr and lecithin 3gr Linoleic acid 13gr,  $\alpha$ -Linolenic acid 1.5 g. 3 g egg phospholipids.  
 (2) Water-soluble vitamins composition: vitamin B 1 0.5mg, vitamin B 2 0.7mg, nicotinamide 8mg, vitamin B 6 0.2mg, panthotehnic acid 3mg, vitamin C 20mg, biotin 12 $\mu$ g, folic acid 80 $\mu$ g, vitamin B 100 $\mu$ g. (3) Fat soluble vitamins composition: vitamin A 50 $\mu$ g (165IU), vitamin D 20.25 $\mu$ g (10IU), vitamin E 0.455 $\mu$ g (0.5IU) and vitamin K 7.5 $\mu$ g.(4) Trace elements : Chromic chloride 6 H<sub>2</sub>O 5.33  $\mu$ g/ml, Copper chloride 2 H<sub>2</sub>O 0.34 mg/ml, Ferric chloride 6 H<sub>2</sub>O 0.54 mg/ml, Manganese chloride 4 H<sub>2</sub>O 99.0  $\mu$ g/ml, Potassium iodide 16.6  $\mu$ g/ml, Sodium fluoride 0.21 mg/ml, Sodium molybdate 2 H<sub>2</sub>O 4.85  $\mu$ g/ml, Sodium selenite anhydrous 6.90  $\mu$ g/ml, Zinc chloride 1.36 mg/ml

## RESULTS

### Weight changes

Table 2 shows the results of the statistical analysis of the weight changes in the four groups. The difference in weight change between the two PN groups and the control group was not statistically significant. The SH group showed a statistically significant decrease in weight.

Groups	Weight before PN (g)	Weight after PN (g)	p value
Control (n=14)	3390.28±107.52	3392.98±119.29	0.931
SH (n=14)	3349.54±155.59	3183.84±124.05	<0.001
PNH (n=14)	3408.92±106.22	3392.93±85.73	0.226
PNEN (n=14)	3374.64±108.58	3367.07±137.65	0.631

### Fibrosis

We searched for the presence of fibrosis in Masson's trichrome sections in the spleen tissues of all groups. However, fibrosis was not detected.

### Analysis of the histopathological scores

The histopathological evaluation revealed germinal center effacement and reduction of the white pulp, congestion of the red pulp, and decreased cellularity in the periarteriolar lymphoid sheath (Table 3 and Figs. 1A, 2 and 3). The histopathological score of the splenic tissues was the highest for the PNH group [7(4–33.9)] (Table 3 and Figs. 1A and 2). In comparison, this score was lower for the PNEN group but higher than those of the control and SH groups [2.17 (1–3), 0.33 (0–0.66), and 0.33 (0–0.66), respectively] (Table 3 and Figs. 1A and 3). All the results mentioned in the paragraph were found to be statistically significant. The scores of the control and SH groups were similar and these groups scored the lowest values (Table 3).

Table 3. Statistical analysis of the histopathologic scores of all four groups

Groups	Median Pathologic Score (min-max)	p-value
PNH (n=14)	7 (4.33-9) <sup>a</sup>	<0.001
PNEN (n=14)	2.17 (1-3) <sup>b</sup>	<0.001
SH (n=14)	0.33 (0-0.66) <sup>c</sup>	<0.001
Control (n=14)	0.33 (0-0.66) <sup>c</sup>	<0.001

\* The difference between the groups with different superscript letters are statistically significant

The evaluation results of the Caspase-3 expression in the splenic tissues of the groups are shown in Table 4 and Figs. 1B, 4, and 5. The PNH group exhibited the highest Caspase-3 expression in the splenic tissues (0.249±0.022) (Table 4, Figs 1B, 4). The PNEN group showed lower levels compared to the PNH group but exceeded those of the control and SH groups [0.074±0.003, 0.057±0.004, and 0.056±0.004, respectively] (Table 4 and Figs. 1B and 5). These results were found to be statistically significant. The control and SH groups showed the lowest Caspase-3 expression, and these levels were similar for these two groups (Table 4).

Table 4. Statistical analysis of the Caspase-3 expression in the spleen tissues of the four groups.

Groups	Caspase-3 expression Mean±SD	p-value
PNH (n=14)	0.249±0.022 <sup>a</sup>	<0.001
PNEN (n=14)	0.074±0.003 <sup>b</sup>	<0.001
SH (n=14)	0.057±0.004 <sup>c</sup>	<0.001
Control (n=14)	0.056±0.004 <sup>c</sup>	<0.001

\* The difference between the groups with different superscript letters are statistically significant

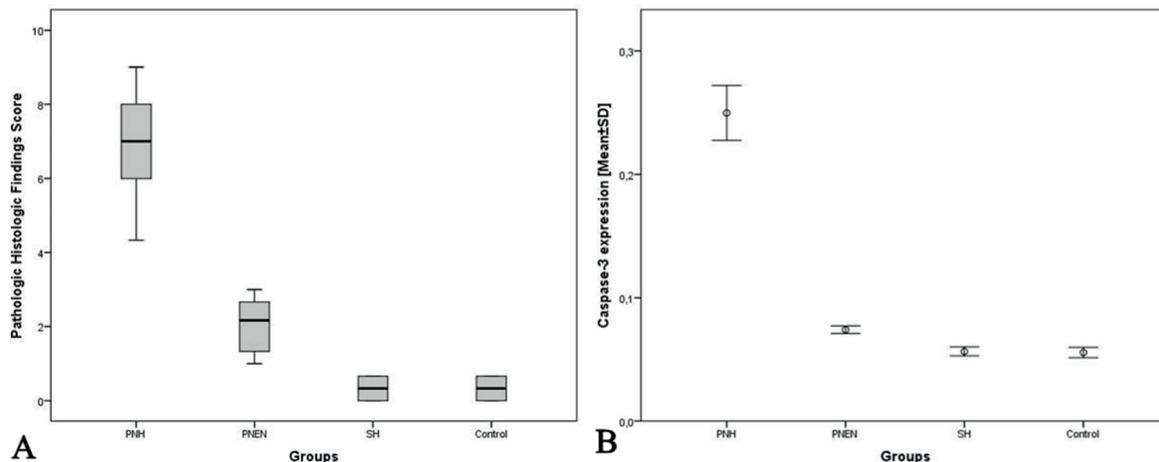
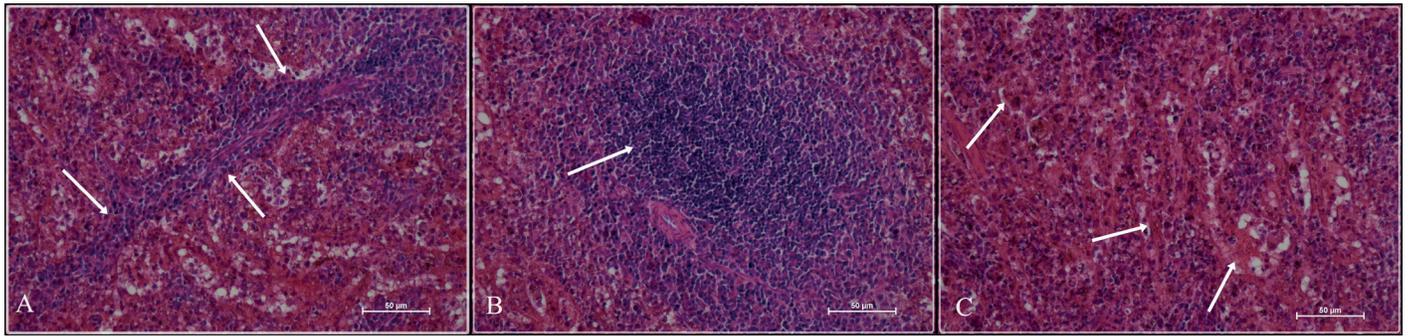
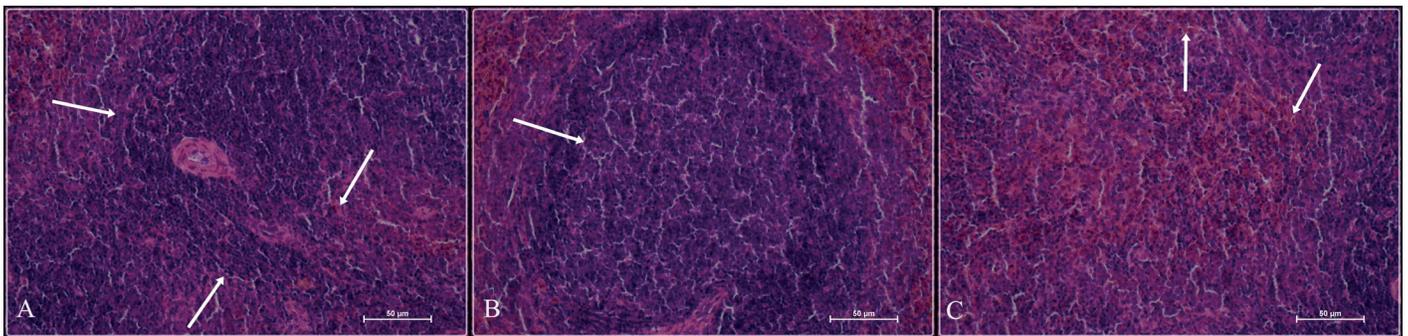


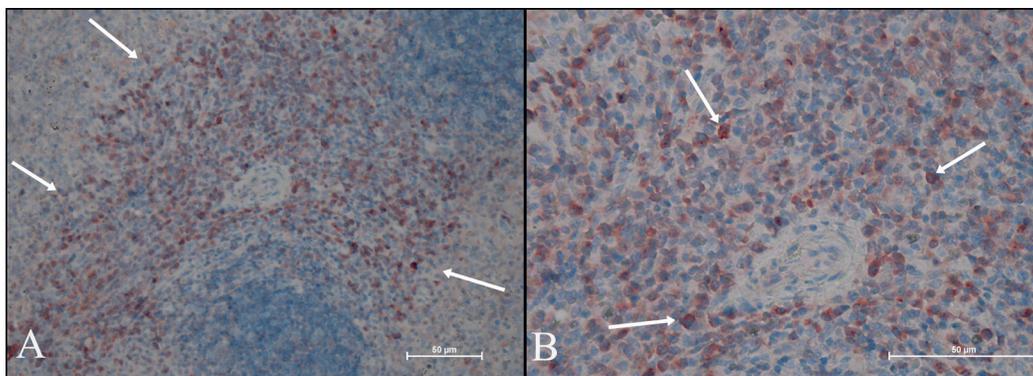
Figure 1. Histopathological and Caspase-3 expression evaluation results of the four groups. A) Histopathological evaluation results. B) Caspase-3 expression evaluation results



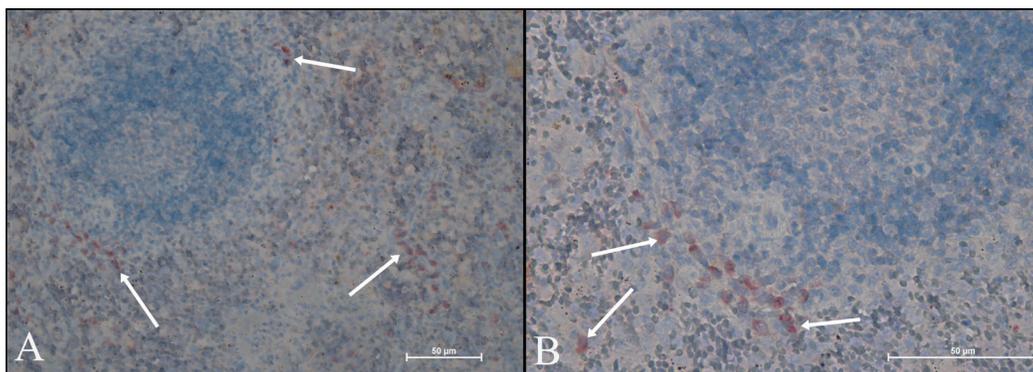
**Figure 2.** Images of splenic tissue from the PNH group (H&E staining). A) Decreased cellularity in periarteriolar lymphoid tissue (H&E staining, 200× magnification). B) Germinal center effacement (H&E staining, 200× magnification). C) High degree of congestion (H&E staining, 200× magnification)



**Figure 3.** Images of splenic tissue from the PNEN group (H&E staining). A) Periarteriolar lymphoid tissue showing high cellularity (H&E staining, 200× magnification). B) Absence of effacement in the germinal center (H&E staining, 200× magnification). C) Mild degree of congestion (white arrows, H&E staining, 200× magnification)



**Figure 4.** A) Caspase-3 staining in a wider area in the PNH group (200× magnification). B) Intensive staining in the cytoplasm of many cells (400× magnification)



**Figure 5.** A) Caspase-3 staining over a much smaller area in the PNEN group (200× magnification). B) Caspase-3-positive in a much lower number of cells compared to those in the PNH group (white arrows, 400× magnification)

## DISCUSSION

Previous works analyzing the negative effects of PN focused on the liver, vascular endothelium, small intestinal epithelium, and kidney function (2,3,5,6,11,12). These studies examined the substances in the PN composition but failed to describe the effects of PN combined with hunger. Gurunluoglu et al. claimed that the cardiotoxic effects of PN could be devastating when combined with hunger. They showed that these destructive effects did not occur in the enteral-fed group provided with PN. They reported that the combination of PN and hunger leads to oxidative stress and hyperglycemia, and both are involved in this process (4). They speculated that low insulin levels may play an important role in pathogenesis (4). However, this study did not include blood sample analyses.

In this study, we investigated the effects of PN on rabbit splenic tissue and found that PN leads to negative effects on this tissue, namely germinal center effacement, reduction of the white pulp, congestion of the red pulp, and decreased cellularity in the periarteriolar lymphoid sheath. These effects occurred minimally when enteral nutrition was provided with PN. Past studies reported that Caspase-3 expression indicates apoptotic activity in tissue (13,14). Thus, we investigated Caspase-3 expression in this study and found that PN combined with hunger significantly increases apoptotic activity in the lymphoid tissue of the spleen.

Only a handful studies have examined the effects of PN on splenic tissue. These studies were primarily associated with PN content. For example, Li et al. showed that including lipids in the composition of PN created sepsis in rats (15). They found that cytokines, which increase inflammation in the splenic tissue, were secreted to a lesser amount in another group that was administered fish oil (15). Zoli et al. evaluated the splenic function of 20 patients with intestinal failure and long-term administration of PN. They found that when patients were administered PN over the long term, their spleen function deteriorated significantly, and simultaneously, the activity of tuftsin, which is released from the spleen and necessary for immunomodulation, decreased significantly (16). In another experimental study, Fell et al. changed the lipid content in the PN and found that fat-laden macrophages and systemic inflammation developed in spleen tissue in the group administered PN with soybean oil. They also noted that these negative consequences did not occur in the group that was administered PN with fish oil (17). Tian et al. conducted an experimental study and found that administering PN rich in fish oil content to rats with sepsis dramatically reduced the secretion of Sphingosin Kinase 1 in the splenic tissue, thereby leading to an inflammatory response (18).

Different lipid emulsions can be used in PN compositions (19). One such emulsion is Lipofundin 20%. It contains 20% medium-chain triglycerides (MCTs). As it is produced from soybeans, it has moderate phytosterol content. Lipofundin consists of palmitic acid, arachidonic acid, oleic acid,

$\alpha$ -linolenic acid, stearic acid, and linoleic acid. Another lipid emulsion used in PN compositions is Intralipid 20%. It is made from soybean oil, phytosterols, and  $\alpha$ -tocopherol. It contains higher amounts of phytosterol and market-available lipid emulsions. It also contains oleic acid, linoleic acid, palmitic acid,  $\alpha$ -linolenic acid, and stearic acid (20,21). Omegaven is produced from fish oil. It does not contain phytosterol. Omegaven consists of eicosapentaenoic acid, linoleic acid, docosahexaenoic acid,  $\alpha$ -linolenic acid, and glyceroleol (22). SMOF lipid 20% is produced from soybeans and contains MCTs, fish oil, phytosterol, and  $\alpha$ -tocopherol. It contains lower amounts of phytosterols than other lipids. SMOF lipid 20% also consists of lipids, eicosapentaenoic acid,  $\alpha$ -linolenic acid, linoleic acid, palmitic acid, oleic acid, docosahexaenoic acid, stearic acid, and arachidonic acid (20,21).

In our study, we used Lipofundin 20% for all the groups that received the PN. We believe that the damage caused to the splenic tissue by PN administration depends to a greater extent on the combination of PN with hunger than the lipid content in the PN. This is because despite using the same lipid content, we found that the rabbits in the PNH group developed more damage in their splenic tissue than those in the PNEN group.

Our study also has some limitations. We used experimental animals, and thus, the findings of this study may not be exactly replicable in humans. Our work did not include a group that was completely starved (i.e., no nutrition in any way). Our study also does not explain which components in the PN were responsible for the negative effects on the splenic tissue. However, the data and findings of our research show that continuing enteral nutrition has an important role in reducing spleen damage caused by PN administration. A very small amount of enteral nutrition during PN administration can likely help lower the splenic damage that was observed in this study.

## CONCLUSION

PN combined with hunger can have damaging effects on splenic tissue, such as germinal center effacement, reduction of the white pulp, congestion of the red pulp, decreased cellularity in the periarteriolar lymphoid sheath, and increased apoptosis in the lymphoid cells. The addition of enteral nutrition to PN may reduce these destructive effects. Additional studies are required to confirm the results of this work.

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