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# Ultrastructural Changes and CD14 Expression in the Liver in the Short Bowel Syndrome

## Kısa Bağırsak Sendromunda Karaciğerde Ultrastrüktürel Değişiklikler ve CD14 Ekspresyonu

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## ABSTRACT

**Objective:** Our aim was to explore the effects of short bowel syndrome (SBS) on CD14 mRNA expression in the liver and examine ultrastructural and biochemical changes in a rat model of total parenteral nutrition (TPN) independent SBS.

**Material and Methods:** Male Wistar Albino rats, weighing 180 to 220 g (n=37) were randomly divided into two groups as sham operated (n=16) and short bowel syndrome (n=21). Division and re-anastomosis of the bowel 15 cm proximal to the ileocecal junction was performed in sham operated animals. 75% of the small intestine was resected in the short bowel syndrome group. All animals were pair-fed. CD14 mRNA expression was evaluated semiquantitatively via RT-PCR. Concomitantly, liver function test were performed and ultrastructural changes in the liver were evaluated.

**Results:** There was no statistical significant difference regarding liver function tests. There were no significant differences for inflammation, fibrosis and steatosis on histological examination of liver. Cholestasis was more prominent in the SBS group ($p<0.05$). There were marked ultrastructural changes on electron microscopy. Microvilli canaliculus length, presence of luminal granular biliary material and luminal length of canaliculus were increased in the SBS group ($p<0.05$). CD14 expression was significantly increased in the liver in the SBS group ($p<0.05$). The correlation between the ultrastructural changes and CD14 mRNA expression was also prominent ($p<0.05$)

**Conclusion:** Cholestasis was demonstrated ultrastructurally in this rat model of TPN independent SBS. Increased expression of CD14 mRNA in the SBS group supports that these changes might occur because of portal venous endotoxemia.

**Key Words:** Cholestasis, Endotoxemia, Hepatic failure, PCR, Short bowel syndrome

## ÖZ

**Amaç:** Sıçanlarda kısa bağırsak sendromunun (KBS) total parenteral beslenmeden (TPB) bağımsız karaciğer üzerindeki ultrastrüktürel ve biyokimyasal değişiklikler ve CD14 mRNA ekspresyonu üzerindeki etkilerinin araştırılması amaçlandı.

**Gereç ve Yöntemler:** Erkek 180-220 g ağırlığındaki (n=37) Wistar Albino sıçan sham (n=16), KBS (n=21) grubu olmak üzere rastgele iki gruba ayrıldı. Sham grubunda ileoçekal valvin 15 cm proksimalinden bağırsak ayrıldı ve tekrar anastomoz edildi. KBS grubunda ise bağırsakların %75'i çıkarıldı. Tüm hayvanlara eş besleme yapıldı. CD14 mRNA ekspresyonu semikantitatif olarak RT- PCR ile değerlendirildi. Eş zamanlı olarak karaciğer fonksiyon testleri ölçüldü ve karaciğerdeki ultrastrüktürel değişiklikler incelendi.

**Bulgular:** Karaciğer fonksiyon testlerinde istatistiksel olarak anlamlı fark saptanmadı. Karaciğerin histolojik incelenmesinde inflamasyon, fibroz ve steatoz açısından anlamlı fark bulunmamakla beraber Kolestaz KBS grubunda daha belirgindi ($p<0,05$). Elektron mikroskobide belirgin ultrastrüktürel değişiklikler izlendi; microvillus kanalikül uzunluğu, luminal granüler materyal varlığı ve kanalikül uzunluğunun arttığı görüldü ($p<0,05$). Karaciğer CD14 mRNA ekspresyonu KBS grupta anlamlı olarak arttı ($p<0,05$).Ultrastrüktürel değişiklikler ile CD14 mRNA ekspresyonu arasındaki korelasyon belirgin olarak gözlendi ($p<0,05$).

**Sonuç:** TPN'den bağımsız KBS modelinde kolestaz ultrastrüktürel olarak gösterildi. KBS grubunda artmış CD14 mRNA ekspresyonu, portal venöz endotoksemi nedeniyle bu değişikliklerin oluşabileceğini desteklemektedir.

**Anahtar Sözcükler:** Kolestaz, Endotoksemi, Karaciğer yetmezliği, PCR, Kısa bağırsak sendromu

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## INTRODUCTION

Short bowel syndrome (SBS) is a disorder in which inadequate digestion and malabsorption occur due to massive surgical removal of small intestines causing a short transit time (1-4). The reason of mortality and morbidity in SBS is liver failure rather than malabsorption or malnutrition. Long-term parenteral nutrition is live-saving in patients after massive small intestine removal.

To the best of our knowledge, total parenteral nutrition (TPN) is the main reason of liver failure in SBS patients (1,5). However, although all of the patients receive TPN, not all of them develop cholestasis or liver failure. Besides, there is no correlation between the period of parenteral nutrition and severity and extent of cholestasis (6).

The gastrointestinal system has a mucosal barrier that prevents the entry of intraluminal bacteria and toxins. This barrier can be damaged as a result of intestinal stasis (7). Impairments in intestinal motility and small intestinal microflora cause bacterial overgrowth, which in turn causes destruction of the barrier. Organs like mesenteric lymph nodes, blood, spleen and liver are invaded by this microflora. Furthermore, weakening of the intestinal immunity causes a decrease in intestinal immunoglobulin A (IgA) and increase in the production of hepatotoxic cytokines. It is well known that bacterial endotoxins and cytokines have harmful effects on the liver (1,8,9). Lipopolysaccharide endotoxin of gram-negative microorganisms and peptidoglycan polysaccharide of gram-positive microorganisms cause IL-1, IL-6 and TNF-α secretion from Kupffer cells. These cytokines are the reason for acute and chronic hepatic inflammation and fibrosis. Lipopolysaccharide-lipopolysaccharide binding protein complex has a very high affinity for CD14 receptors on Kupffer cells. It is important to show CD14 expression in hepatic inflammation and fibrosis (6,9-12).

Therefore; the authors hypothesized that the adaptation process in SBS causes gastrointestinal dysmotility and intestinal stasis, which lead to cholestasis and liver damage. This particular study aims to demonstrate the ultrastructural changes and specific tissue markers of liver damage in a rat model of SBS.

## MATERIAL and METHODS

Ethical approval was received from local ethical committee (Ethics No 04.07.2005/ 75-1942) and the institutional and national guide for the care and use of laboratory animals was followed during the study. The study complied with the guidelines for human studies and animal welfare regulations. Wistar Albino rats, weighing 180-220g were used in the study and were randomly divided into two groups as the SBS (n=12) and sham groups (n=14).

The intestines between a point 5cm distal to the Treitz ligament and 10cm proximal to the ileocecal valve were resected after ligation of mesenteric vessels by preserving vascular arcades in the SBS group. A total of seventy-five percent of the small bowel was resected with this procedure and end-to-end anastomoses were performed using 5/0 silk sutures. Transection and re-anastomosis without resection of the ileum 15cm proximal to the ileocecal valve was performed in the sham group using the same materials. All of the animals received tap water in the first postoperative 24 hours. Eight grams of standard laboratory rat food and tap water were given in the postoperative first day and food was increased to 22g for the following days. The rats were weighed daily during the first week and then weekly. Portal venous blood sampling was performed seven weeks later. Liver biopsy was also performed seven weeks later for histopathological, electron microscopic and mRNA examination.

Portal venous blood plasma was studied with the Cobas 6000-501 autoanalyzer and alkaline phosphatase (ALP), total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured.

The liver samples preserved in 10% formaldehyde were stained by Hematoxylin-Eosin, Periodic Acid Schiff, Trichrome Masson stains and evaluated for inflammation, fibrosis and fatty degeneration (Table I). Cholestasis was recorded as percentile. Liver samples preserved in 2% gluteraldehyde were stained by toluidine blue. Cholestasis and liver injury were examined ultrastructurally by examining the increase in microvillus length, the presence of intraluminal granular biliary material, hyperplasia of microfilaments and the size of canalicular lumen and pericanalicular lumen (Table II).

Total RNA isolation from frozen tissues was performed using the RNeasy Mini Kit (QIAGEN, Germany). The RNA isolation procedure was started on a standard amount (30 mg) of tissue samples after mixing with 300µL of RTL solution (QIAGEN, Germany). The rest of the RNA isolation protocol was carried out as described by the Producer Company. In the final step, total RNA captured on silica beads was eluted by adding 50µL of RNase-free water into spin-columns. The quantitation and purity of isolated RNAs were analyzed spectrophotometrically.

Complementary DNA synthesis was performed using the random hexamers priming protocol. Reverse transcription reactions were set up based on the protocol proposed by the manufacturer including 10µL of RNA for each sample and cDNA synthesis was performed at 37°C for 1h using MMLV-RT (RevertAid, Fermentas, Ontario, Canada). Three microliter of finalized RT reaction mixture was used as template DNA in PCR amplifications. PCR amplifications were performed in 30µL reaction mixture including 1.25U of Taq polymerase (Fermentas, Ontario, Canada), 3µL of 10X Taq Buffer, 2.4µL of MgCl2, 0.5µL of dNTP, 0.16µM (0.5µL) of each primer (CD14-F

5'-TGG AGC ACG TAC CTA AAG GG-3'; CD14-R 5'-GAG CTG TGG CTA TGA CTA CGC-3') and 19.85µL of deionized water. Thermal cycling was performed as initial denaturation for 10m at 95°C flowed by 35 cycles of annealing at 58°C for 45sec, elongation at 72°C for 60sec and denaturation at 95°C for 30sec. DNA products (70bp) were visualized by UV-transillumination after the run in 1.5% agarose gel containing ethidium bromide. The obtained bands were scanned together with control DNA strands (Fermentas, Ontario, Canada) in Image Analysis Software (Gel Logic 100 Imaging System, Kodak, USA). The ratios of surfaces areas of control DNA and obtained DNA were determined and the numbers derived were recorded. The mean values of these data were represented as mean±standard deviation.

**Table I:** Evaluation of liver samples for inflammation, fibrosis and fatty degeneration.

| |
|---|
| **Inflammation** |
| 0 = no inflammation |
| 1 = inflammation in one/two bile duct or epithelial area |
| 2 = epithelial hyperplasia and 3-5 bile duct inflammation |
| 3 = epithelial hyperplasia and 5 or more bile duct inflammation |
| **Fibrosis** |
| 0 = no fibrosis |
| 1 = limited proliferation in portal canals |
| 2 = dilated portal canals and increased fibrosis |
| 3 = bridging fibrosis between portal areas |
| **Fatty Degeneration** |
| 0 = no fatty degeneration |
| 1 = 25% fatty degeneration |
| 2 = 25-50% fatty degeneration |
| 3 = 50-100% fatty degeneration |

**Table II:** Ultrastructural evaluation of liver samples for cholestasis and liver injury.

| |
|---|
| **Microvillus Length** |
| 0 = normal |
| 1 = minimal increase |
| 2 = significant increase |
| **Presence of Intraluminal Granular Biliary Material** |
| 0 = absent |
| 1 = present |
| **Size of Canalicular Lumen** |
| 0 = normal |
| 1 = minimal dilatation |
| 2 = mild dilatation |
| 3 = significant dilatation |
| **Size of Pericanalicular Lumen** |
| 0 = normal |
| 1 = minimal increase |
| 2 = mild increase |
| 3 = significant increase |
| **Microfilament Hyperplasia** |
| 0 = normal |
| 1 = minimal increase |
| 2 = mild increase |
| 3 = significant increase |

Changes in weight, biochemical examinations and semi-quantitative RT-PCR study were compared by using one-way ANOVA and Student's t test. The data obtained from light and electron microscopic examination were compared by using the chi square test (Microsoft Excel, Analyze-it 2.09). Values of $p<0.05$ were considered statistically significant.

## RESULTS

Thirty-seven rats were included in the study. Twelve in the SBS group and 14 in the sham group survived the experimental period. Among the 11 dead rats, the reason of death was intestinal leakage in six, hemorrhage in three and intestinal ischemia in two. The weight loss of the rats in the SBS group was significantly more than the rats in the sham group after first week. Although they had weight gain in due course, they could not catch up with the rats in the sham group which mimics a TPN-independent SBS model ($p<0.05$) (Figure 1).

There was no statistically significant difference between the sham and SBS groups regarding liver function parameters (ALP, total bilirubin, AST, ALT) that were obtained by using melted plasma samples following removal from -80°C ($p=0.49$). The histopathological examination of liver tissue of the SBS group revealed 41% cholestasis whereas there was no cholestasis in the sham group (in every magnification) ($p<0.05$) (Figure 2A, B). There were no significant differences regarding other histopathological parameters such as inflammation, fibrosis, and fatty degeneration.

Regarding ultrastructural changes; 77% of the sham group had normal microvillus length, 58% of the SBS group had a minimal increase and 17% had a significant increase in microvillus canalicular length. There was no luminal granular biliary material in the sham group whereas it was found in 25% of the SBS group. The canalicular lumen was normal in 85% of the sham group whereas there was mild dilatation in 8% and minimal dilatation in 67% of the SBS group. Therefore, as regards the ultrastructural changes, the microvillus length, luminal granular biliary material and the canalicular lumen were significantly increased in the SBS group when compared to the sham group ($p<0.05$) (Figure 3A-C). These are accepted to be ultrastructural changes seen in cholestasis.

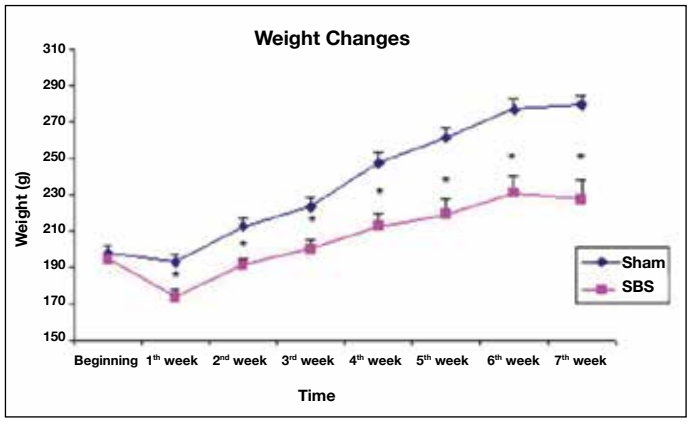


**Figure 1:** Schematic presentation of weight changes of rats in the sham group and the SBS group (*p<0.05).

RNA was evaluated spectrophotometrically using 260 and 280nm wavelength after isolation. Bands obtained by RT-PCR amplification are shown in Figure 4A, B. The results of semiquantitative ratios of area to β-actin were 36.96 ± 0.43 in the SBS group and 35.55 ± 0.30 in sham group. This indicated that CD14 expression was significantly increased in the SBS group when compared to the sham group (p<0.05) (Figure 5).

## DISCUSSION

All daily nutritional requirements of the patients with SBS is met only by TPN in the early postoperative period and partly by

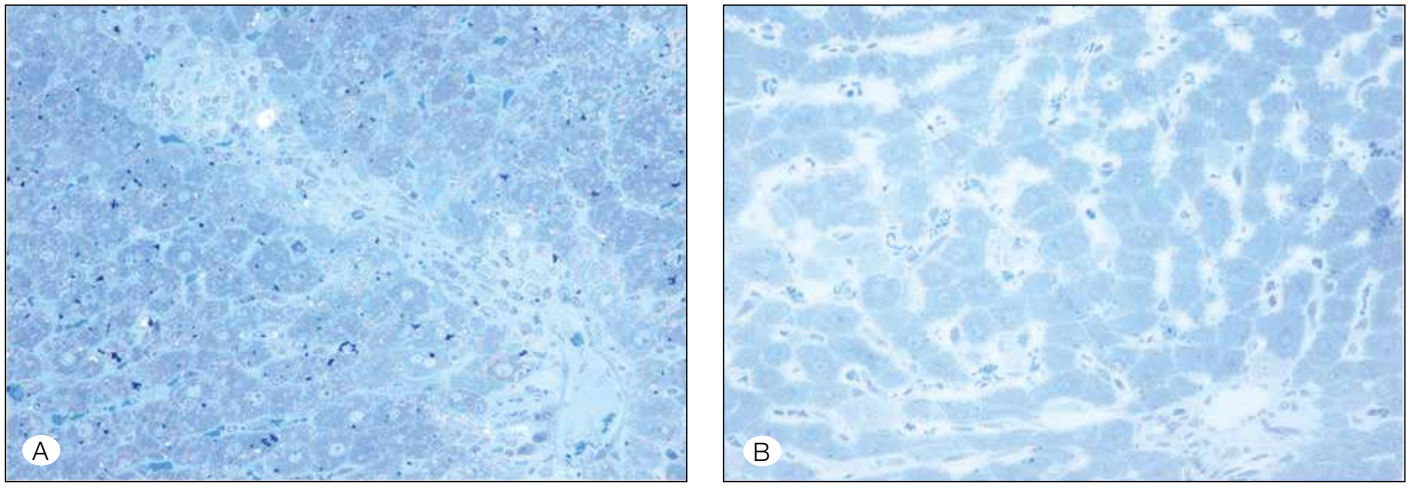


**Figure 2: A)** Tissue sample of a rat in the sham group showing no trace of specific pathology (Toluidine blue, x100 magnification). **B)** Cholestasis affecting 15% of the parenchyma in SBS group (Toluidine blue, x100 magnification).

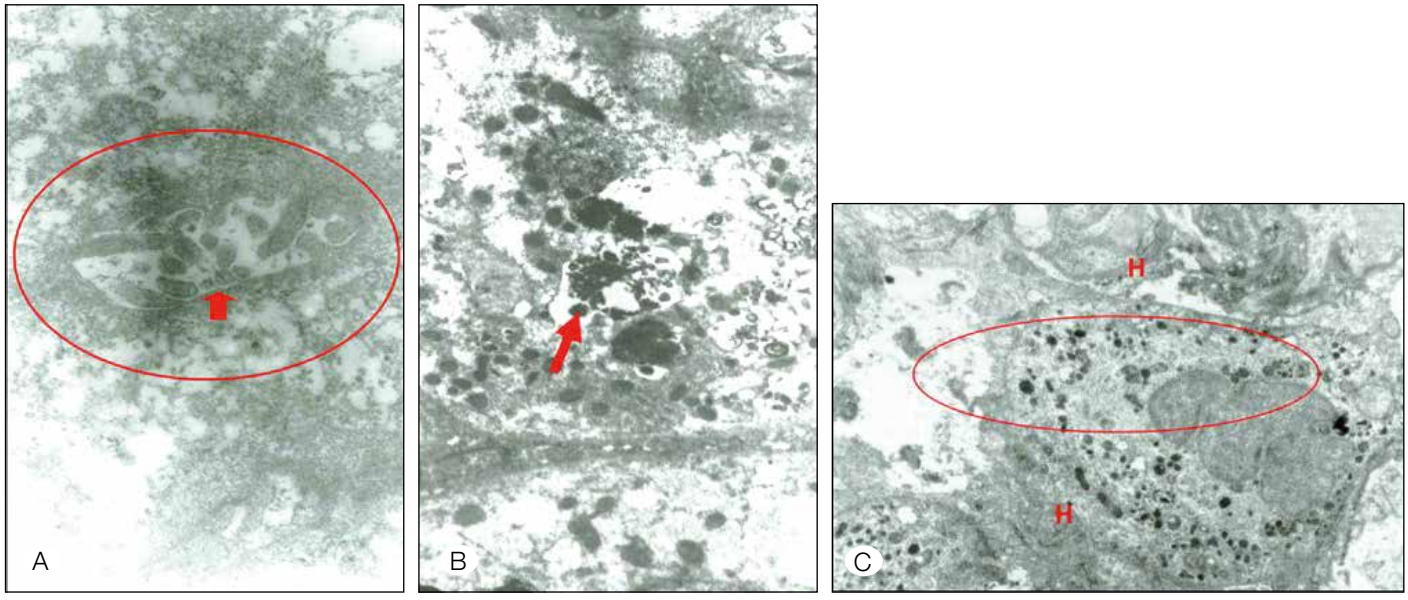


**Figure 3: A)** Minimal dilatation of bile canaliculus, increased number of microvillus and elongation in some of them (arrow) can be seen in the sample from the SBS group. No luminal granular biliary material was detected. (Uranyl acetate x20000 magnification). **B)** Electron microscopic examination of the sample of the SBS group showing intracytoplasmic glycogen and rough granular dense electron deposit (arrow) between mitochondria in a hepatocyte. (Uranyl acetate x4400 magnification). **C)** Minimal dilatation (frame) of canaliculus between the hepatocytes (H) in the liver tissue of the SBS group. (Uranyl acetate x3000 magnification).

TPN in late postoperative period after the beginning of enteral feeding. The main reason of liver injury in SBS is considered to be TPN. However, it is not true to consider parenteral nutrition as the only reason of cholestasis and liver failure in patients with intestinal resection, since not all of the patients followed by TPN develop cholestasis and there is no direct relation between the period of TPN use and cholestasis prevalence and severity. Besides, cholestasis may occur in early periods TPN use, may be self-limiting and may not recur although parenteral nutrition continues (6).

In this rat model of TPN-independent SBS, it has been demonstrated that ultrastructural changes concomitant with liver injury may develop. Microvillus canalicular length, luminal granular biliary material and canalicular lumen size, which are the electron microscopic markers of cholestasis, were significantly increased in the SBS group when compared to the sham group. Electron microscopic changes are the earliest signs of cholestasis. It can be clarified by the results that ultrastructural differentiation begins after the postoperative seventh week. Hua et al. carried out a study on newborn piglets where they resected 75% of the intestines and followed the piglets up by TPN and enteral feeding. They concluded that the SBS group had biochemical and histological cholestasis. Ultrastructural examination showed dilatation of bile canaliculus with bile plugging, microvillus flattening and disappearance, but there were no abnormalities of the pericanalicular zone. According to Hua et al., intestinal failure-associated liver disease is a multifactorial condition and premature birth, disruption of the enterohepatic circulation of bile acids by resection of the ileum, intestinal stasis, bacterial overgrowth, and early and/or prolonged parenteral protein, fat, and/or energy intake may be the causes of this condition (13). It can therefore be concluded that, SBS itself can cause some ultrastructural changes which can lead to liver injury later independent from TPN.

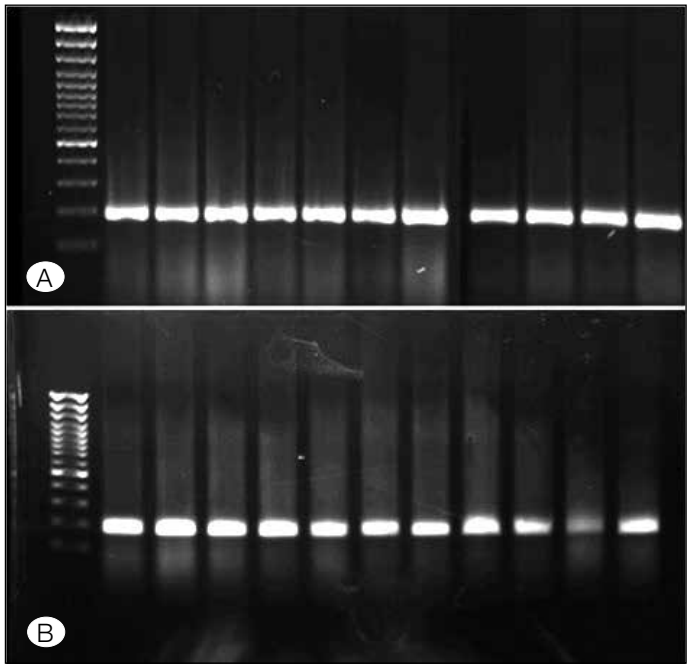


**Figure 4: A)** CD14 expression of liver in the SBS group. **B)** CD14 expression of liver in the sham group.

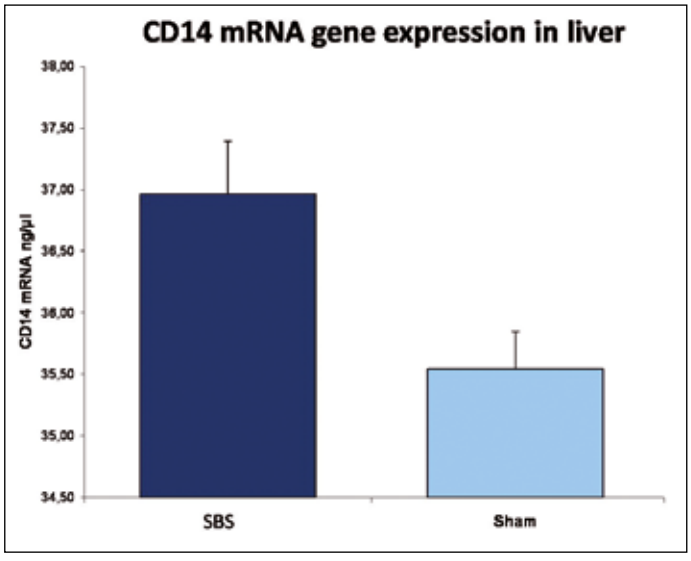


**Figure 5:** The increase in CD14 expression of SBS group was statistically significant when compared to the sham group (p=0.013).

The cholestasis occurring with intestinal insufficiency may result in histopathological changes ranging from hepatic steatosis to biliary cirrhosis. Hepatic steatosis is more frequently seen in adults and may develop without inflammation, cholestasis or hepatic cellular necrosis. Hepatosteatosis is rarely seen in children. Centrilobular cholestasis is characterized by portal inflammation and fatty degeneration with or without necrosis. Advanced liver disease is composed of portal fibrosis (100%), pericellular fibrosis (95%) and bile duct proliferation (86%). The most apparent features of advanced liver disease are pigmented Kupffer cells (81%) and portal bridging (86%) (14). Cholestasis was significantly demonstrated in the SBS group when compared to the sham group ($p<0.05$) and fibrosis was almost significantly more in the SBS group than in the sham group ($p=0.08$) in the presented study. These findings can be interpreted as the manifestation of ultrastructural changes into light microscopic histopathological changes. Fibrosis was only seen in one rat in the sham group whereas it was seen in five rats of the SBS group in which two of them had prominent fibrosis. Their difference is statistically significant when a comparison is carried out according to the presence or absence of fibrosis. However the results were also almost statistically significant when compared according to the staging system described in the methodology.

Regarding the biochemical results of this study, the reason of not detecting difference is that these parameters increase in the late period of liver injury and could not be detected in the stage of intracellular changes. Future prolonged evaluation of these parameters could possibly demonstrate some changes. Following the bacterial overgrowth as a result of low intestinal motility, the lipopolysaccharide endotoxins of gram negative

microorganisms and peptidoglycan polysaccharide endotoxins of gram-negative and gram-positive microorganisms penetrate through the intestines to the liver as a result of the mucosal injury and increased mucosal permeability and translocate through lymphatic and/or mesenteric venous system (6,9). The Kupffer cells, which are the macrophages in the liver, are activated by endotoxins and secrete proinflammatory cytokines such as TNF-α and IL-1β. This occurs by the stimulation of macrophages after lipopolysaccharide-lipopolysaccharide binding complexes bind to the CD14 receptors and stimulate macrophages (6,9,12,15). Mogilner et al. performed bowel transaction and reanastomosis in the sham group and small bowel resection in 75% of the SBS group. There was 20% bacterial translocation in the mesenteric lymph nodes and portal blood whereas no translocation could be shown in the liver and peripheral blood in the rats in the sham group. However in the SBS group the bacterial translocation was 100% in mesenteric lymph node, 60% in portal blood, 100% in liver and 40% in the peripheral blood (16). Aprahamian et al. stated that serum IL-6 levels were much higher in the SBS group when compared to the sham group. IL-6 and TNF-α mRNA levels were found to be increased in that TPN-independent study (17). The Kupffer cells have relatively less CD14 expression base when compared to peripheral blood monocytes (18-20). CD14 expression of Kupffer cells is upregulated by multistimulation of polysaccharide by means of an increase in functional receptor number (21-23). Upregulation of CD14 expression in liver cells occurs in many conditions of liver injury such as alcoholic and cholestatic liver injury (24).

Bacteria translocate through the epithelium after a long duration of intestinal stasis, and release endotoxins. Endotoxin binds to CD14 receptors on hepatic macrophages which then release inflammatory cytokines such as IL- 1, IL-6, TNF and these cause hepatic injury (25). The data reported in this experimental study suggest that CD14 expression is significantly increased in the SBS group, indicating that proinflammatory cytokines are activated and SBS causes liver injury independent from TPN.

## CONCLUSION

This unique study of TPN independent SBS in a rat model demonstrated that SBS can lead to liver injury by itself, apart from any other parameter. Further and prolonged studies are needed to show the reflection of these ultrastructural changes in the liver on the biochemical and histologic parameters in SBS.

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