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World J Gastroenterol 2005;11(43):6757-6764
World Journal of Gastroenterology ISSN 1007-9327

• *BASIC RESEARCH* •

Effect of bombesin and neurotensin on gut barrier function in partially hepatectomized rats

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Received: 2005-03-28 Accepted: 2005-04-26

Abstract

AIM: To investigate the effect of regulatory peptides bombesin (BBS) and neurotensin (NT) on intestinal barrier function in partially hepatectomized rats.

METHODS: Ninety male Wistar rats were randomly divided into five groups: I (*n* = 10): controls, II (*n* = 20): sham operated, III (*n* = 20): partial hepatectomy 70% (PHx), IV (*n* = 20): PHx+BBS (30 μg/kg/d), V (*n* = 20): PHx+NT (300 μg/kg/d). Groups IV and V were treated for 8 days before PHx and 48 h post surgery. At the end of the experiment, on day 10, intestinal barrier function was assessed by measuring endotoxin concentrations in portal and aortic blood. Tissue sections of the terminal ileum were examined histologically and villus density, mucosal thickness, mitotic activity and apoptosis in crypts were assessed. In addition, ileal mucosa was analyzed for DNA and protein content and microbiological analysis was performed in cecal contents. To estimate intestinal oxidative stress, lipid peroxidation was determined on tissue homogenates from terminal ileum.

RESULTS: BBS or NT administration significantly reduced portal and systemic endotoxemia observed 48 h after partial hepatectomy. In hepatectomized rats (group III), a trend towards induction of mucosal atrophy was observed, demonstrated by the reduction of villus density, mucosal thickness, protein content and significant reduction of DNA, while these alterations were reversed by regulatory peptides administration. This trophic effect of BBS and NT was accompanied by induction of mitoses above control levels and a significant reduction of apoptosis in intestinal crypts. Intestinal lipid peroxidation was found significantly lower in PHx group and regulatory peptides exerted an antioxidant action, further decreasing this parameter of oxidative stress. The bacterial population of *E. coli* and aerobic Gram (+) cocci was increased in cecal content of hepatectomized rats, while this parameter was not affected by the administration of BBS or NT.

CONCLUSION: Gut regulatory peptides BBS and NT improve intestinal barrier function and reduce endotoxemia in experimental partial hepatectomy. This effect is, at least in part, mediated by their trophic, antiapoptotic, mitogenic, and antioxidant effect on the intestinal epithelium. This observation might be of potential value in patients undergoing liver resection.

Key words: Hepatectomy; Rats; Bombesin; Neurotensin; Intestinal barrier; Apoptosis; Oxidative stress

Assimakopoulos SF, Alexandris IH, Scopa CD, Mylonas PG, Thomopoulos KC, Georgiou CD, Nikolopoulou VN, Vagianos CE. Effect of bombesin and neurotensin on gut barrier function in partially hepatectomized rats. *World J Gastroenterol* 2005; 11(43): 6757-6764
http://www.wjgnet.com/1007-9327/11/6757.asp

INTRODUCTION

Despite current advances in the safety of hepatic resection and perioperative care, which have led to a significant reduction of perioperative mortality, septic events remain still an important problem complicating the outcome of hepatectomized patients[1]. It has been previously shown that enteric bacteria and endotoxins passing through the intestinal barrier to extraintestinal sites and the systemic circulation result in intra-abdominal abscess formation, pulmonary infections, sepsis and multiple organ dysfunction in patients and rats after liver resection[1,2]. A

compromised gut barrier function promotes the escape of enteric bacteria and endotoxins into portal circulation, while the reduction of the functional reticuloendothelial volume after hepatectomies permits their systemic spread[3].

Bombesin (BBS), a tetradecapeptide originally isolated from the skin of the European frog *Bombina bombina* is analogous to gastrin-releasing peptide found in mammalians[4]. BBS stimulates the release of various gut hormones and peptides e.g, gastrin, cholecystokinine, insulin, glucagons, somatostatin, motilin, pancreatic polypeptide, neurotensin and exerts a trophic effect on intestinal and gastric mucosa and pancreas, while it stimulates intestinal motility[5,6]. It has been previously shown that BBS improves intestinal integrity in experimental models of gut barrier dysfunction, such as after elemental diets, methotrexate administration, chemically induced colitis and burns[5,7-9].

Neurotensin (NT), a tridecapeptide originally isolated from the bovine hypothalamus, is additionally found in the gut mucosal endocrine cells (N cells), especially in the ileum[10]. NT stimulates pancreaticobiliary secretions, intestinal blood flow and colonic motility, while it inhibits small intestinal and gastric motility. It is a potent trophic agent for small and large intestine, gastric mucosa and pancreas[11-14]. NT has been shown to prevent intestinal atrophy induced by feeding rats an elemental diet, restores mucosal ulceration after radiation therapy and enhances intestinal regeneration after small bowel resection[7,15,16].

We have recently shown that BBS and NT improve intestinal barrier function in experimental obstructive jaundice by exerting mitogenic, antiapoptotic, and antioxidant effects on the intestinal epithelium[17]. The present study was undertaken to investigate the effect of exogenous administration of BBS and NT on gut barrier function after partial hepatectomy in rats.

MATERIALS AND METHODS

Animals

Ninety male albino Wistar rats, weighing 250-320 g, were used. They were housed in stainless-steel cages, three rats per cage, under controlled temperature (23 °C) and humidity conditions, with 12-h dark/light cycles, and maintained on standard laboratory diet with tap water *ad libitum* throughout the experiment, except for an overnight fast before surgery.

The experiments were carried out according to the guidelines set forth by the Ethics Committee of Patras University Hospital, Patras, Greece.

Experimental design

Animals were divided randomly into five groups: Group I (n = 10): non-operated controls, group II (n = 20): sham operated, group III (n = 20): partial hepatectomy (70%), group IV (n = 20): partial hepatectomy and BBS administration, group V (n = 20): partial hepatectomy and NT administration.

Starting on d 0, the animals of groups IV and V were treated daily with BBS (10 μg/kg, subcutaneously, thrice a day) and NT (300 μg/kg, intraperitoneally, once a day), respectively, while the animals of groups I, II, and III were divided to receive daily either three subcutaneous or one intraperitoneal injection of 0.5 mL normal saline. Previous pilot studies showed that the way of saline administration does not affect the results. On the 8th day, animals from groups III, IV, and V underwent laparotomy and partial hepatectomy (almost 70%) as described by Higgins and Andersson[18], while animals in group II underwent laparotomy and mobilization of the liver. The abdominal incision was closed in two layers with chromic 4-0 cat gut and 4-0 silk. All surgical procedures were performed under strict sterile conditions, using light ether anesthesia. Administration of BBS, NT, and normal saline was continued for 48 h after surgery. On the 10th d, all animals were operated (group I) or reoperated (groups II, III, IV, and V), again under strict sterile conditions. Samples were obtained according to the experimental protocol, after which the rats were killed by exsanguination.

Peptides preparation

A stock solution of BBS (Sigma Chemical Co, St. Louis, MO, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 1% (w/v) bovine serum albumin, so that the amount of BBS needed for each injection to be contained in a volume of 0.1 mL. This solution was divided into equal aliquots of 0.1 mL that were stored in plastic tubes at -20 °C. At the time of administration, in order to prolong absorption, each aliquot was mixed with 0.4 mL of a solution of 8% (w/v) hydrolyzed gelatin (Sigma Chemical Co, St. Louis, MO, USA). A final volume of 0.5 mL, containing 10 μg BBS/kg body weight, was injected subcutaneously thrice daily.

A stock solution of NT (Sigma Chemical Co, St. Louis, MO, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 0.1% (w/v) bovine serum albumin, so that the dose of NT needed for each injection to be contained in a volume of 0.1 mL. This solution was divided into equal aliquots of 0.1 mL that were stored in glass vials at -20 °C. At the time of administration, each aliquot was further diluted with 0.4 mL sterile saline to a final volume of 0.5 mL and was given intraperitoneally as a bolus injection containing 300 μg NT/kg body weight.

Endotoxin measurements

For the determination of endotoxin concentrations, a laparotomy was performed in all groups, the portal vein and the abdominal aorta were punctured and samples of 1 and 2 mL of blood were obtained, respectively. Endotoxin concentration was determined by the quantitative chromogenic Limulus amebocyte lysate test according to the manufacturer's instructions (QCL-1 000, BioWhittaker, Walkersville, USA) and expressed in EU/mL.

Measurements of mucosal DNA and protein

DNA and protein content in the terminal ileal mucosa were determined in all animals. A 1-cm long sample of the terminal ileum was excised, opened by longitudinal incision and washed with cold normal saline. Using a clean glass slide the mucosa was removed and homogenized in 1 mL NaOH 1 N, by means of a polytron homogenizer. The protein was measured according to Lowry's method[19] using a commercial kit (Sigma Diagnostics, Deisenhofen, Germany) and the DNA was determined according to a modified Barton technique[20].

Histological evaluation

For histological examination, tissue samples from the terminal ileum were obtained from all animals. The ileal samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. In each ileal specimen, several histologic features were evaluated and recorded. These features included architectural distortion, villous blunting, surface and crypt epithelial injury, presence and cell type of inflammation of the lamina propria, surface and cryptal intraepithelial infiltration, lamina propria fibrosis and granulation tissue formation. Ileal mucosal morphometric characteristics were studied by measurements of villus density, defined as the number of villi per centimeter (V/cm), and villus height (Vh) in micrometers (μm). Villus height was measured with a micrometer eyepiece affixed to a Reichert–Jung light microscope and at least 20 well-preserved villi were estimated in each sample. In addition, the number of mitoses and apoptotic bodies per crypt were also counted. Apoptotic bodies of the cryptal epithelium were identified and tallied using a morphometric analysis, which has been described in detail elsewhere[21]. Apoptotic bodies were defined as rounded vacuoles with fragments of karyorrhectic nuclear debris and were differentiated from small isolated fragments of nuclear chromatin and intraepithelial neutrophils. Apoptotic bodies and mitoses were counted in all architecturally successive crypts included in the specimen, regardless of crypt orientation, and their total number was divided by the number of the crypts. The number of apoptotic bodies per crypt is referred to as the apoptotic body count (ABC).

Cecal bacterial population

Collection of cecal contents for microbiological analysis, was performed by the following technique: the ascending colon was ligated below the hepatic flexure, and a 21-gauge needle mounted on a 5 mL syringe was introduced into the cecum through the ileocecal valve, after puncturing the terminal ileum. Two milliliters of sterile saline was infused in the cecum, after which the needle was withdrawn and the terminal ileum was ligated. The cecal content was manually mollified for 2 min and after a good mixture was achieved, 1 mL of colonic content was removed; serial tenfold dilutions were performed and 0.001 mL of each sample was inoculated into 5% blood and McConkey's agar plates for recovery of aerobic bacteria and Gram-negative/nonspore-forming agar plates for anaerobes. After 24 and 48 h of incubation at 37 °C, for aerobic and anaerobic cultures respectively, colonies were identified and counted. Quantitative culture results were expressed as the number of colony-forming units (CFU) per milliliter or per gram of the cecal samples, calculated from the dilutions of colonic content. *E coli* was identified by Gram stain and a standard biochemical identification system (API-20E, Biomérieux, Marcy-l'Etoile, France, according to the analytical profile index).

Intestinal lipid peroxidation

A 1-cm long tissue sample of the terminal ileum of each animal was excised, washed in 9 g/L of NaCl and was homogenized in a porcelain mortar in liquid nitrogen. Intestinal homogenates were processed for the determination of lipid peroxidation, according to a modified [2-thiobarbituric acid (TBA)]-based method, as reported previously[22]. Lipid peroxidation was expressed in pmoles malondialdehyde (MDA)/mg total protein.

Statistical analysis

The results are expressed as mean (SD). Comparisons among multiple groups were performed using the one-way ANOVA, followed by Bonferroni's *post hoc* test, when variances across groups were equal or by Dunnett's T3 *post hoc* test, when variances were not equal. Variance equality was tested by Levene statistical analysis. Differences were considered significant, when $P<0.05$.

RESULTS

Portal and aortic endotoxin concentrations

Hepatectomized animals (group III) presented significantly elevated endotoxin concentrations in portal and aortic blood compared with groups I and II ($P<0.001$, respectively). Treatment with BBS or NT led to significantly lower endotoxin values both in portal vein ($P<0.001$ *vs* group III, respectively) and aorta ($P<0.001$ and $P<0.01$ *vs* group III, respectively) (Figure 1).

Mucosal DNA and protein

DNA content of the intestinal mucosa was significantly decreased in partially hepatectomized rats as compared to controls ($P<0.01$) and increased in BBS and NT treated rats ($P<0.05$ and $P<0.01$ *vs* group III, respectively)

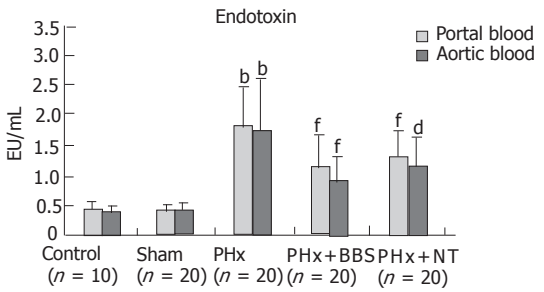

Figure 1 Portal and aortic endotoxin concentrations. Values are mean±SD. [b]$P<0.001$ *vs* sham, [d]$P<0.01$ and [f]$P<0.001$ *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin.

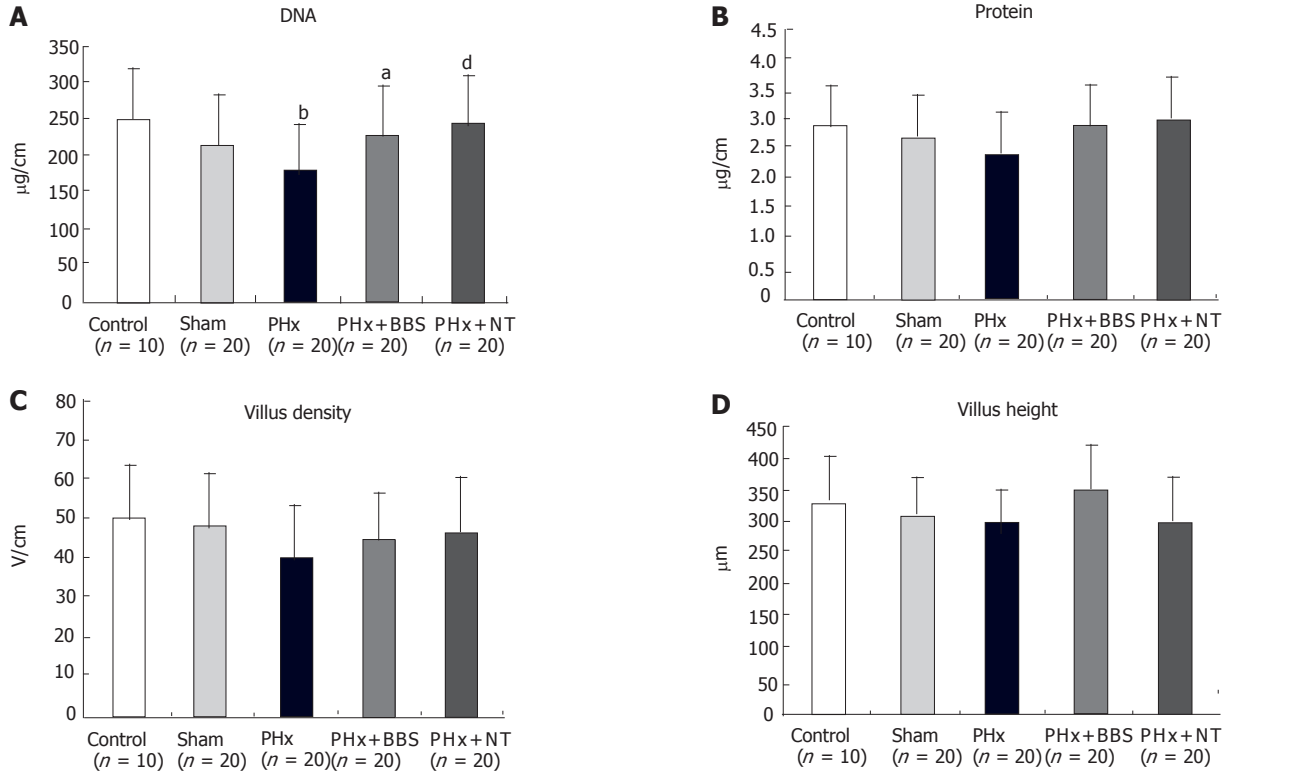

Figure 2 Indices of ileal mucosa trophic state. Values are mean±SD. [b]*P*<0.01 *vs* control, [a]*P*<0.05 and [d]*P*<0.01 *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin(**A-D**).

(Figure 2A). Protein content was also decreased in hepatectomized rats and increased to normal levels after peptides administration, but these differences did not reach statistical significant levels (Figure 2B).

Intestinal morphology

Overall, the ileal architecture remained intact and epithelial continuity was retained, in all specimens studied. The ileal biopsies from group III demonstrated a reduction in the number of villi per centimeter (Figure 2C) and in villus height (Figure 2D), which were increased towards control values in rats treated with BBS or NT; however, the differences among groups were not significant. Intestinal crypt mitotic activity was reduced in hepatectomized rats, though not to significant levels, and increased above normal levels after BBS or NT administration (Figure 3A). Crypt epithelial apoptosis was present in all intestinal samples evaluated. The ABC in ileal specimens from control group was significantly lower compared with group III ($P<0.001$) (Figure 3B). After BBS or NT administration the ABC was significantly reduced ($P<0.05$ and $P<0.01$, compared to PHx, respectively).

Cecal bacterial population

Table 1 provides the quantitative culture results of the cecal contents for aerobic and anaerobic bacteria. In partially hepatectomized rats (group III) there was a significant increase in Gram (+) cocci and *E coli* cecal count as compared with group I ($P<0.05$, respectively). Neurotensin presented a trend towards reduction of aerobic and anaerobic bacterial populations, which reached significant levels for Gram (+) anaerobic bacteria ($P<0.001$ *vs* group III).

Table 1 Bacterial cecal population (CFU×10^5/mL)

Groups	*n*	Aerobic bacteria		Anaerobic bacteria	
		Gram (+) cocci	*E coli*	Gram (+)	Gram (–)
Control	10	6.70 (1.62)	1.04 (0.88)	9 (2.74)	4.64 (1.99)
Sham	20	9.45 (4.92)	1.60 (2.07)	13.34 (5.62)	6.5 (4.35)
PHx	20	11.09 (5.71)[a]	6.43 (6.38)[a]	13.17 (3.88)	5.48 (4.57)
PHx+BBS	20	11.70 (6.41)	6.31 (6.20)	10 (4.29)	4.45 (2.86)
PHx+NT	20	6.76 (4.31)	2.77 (3.92)	6.76 (5.64)[b]	3.08 (4.48)

Data expressed as mean (SD). [a]*P*<0.05 *vs* control, [b]*P*<0.001 *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin.

Intestinal lipid peroxidation

Liver resection resulted in decreased intestinal lipid peroxidation ($P<0.001$ *vs* group II), while administration of BBS or NT led to further significant decrease of this index of oxidative stress ($P<0.01$ and $P<0.001$ *vs* group III, respectively, Figure 4).

DISCUSSION

An intact intestinal barrier function effectively separates potentially harmful intraluminal elements such as bacteria and endotoxins from extraintestinal tissues and the systemic circulation. Major liver resection has been shown to compromise the anatomic and functional integrity of the gut barrier resulting in the translocation of indigenous bacteria and endotoxins to remote organs and

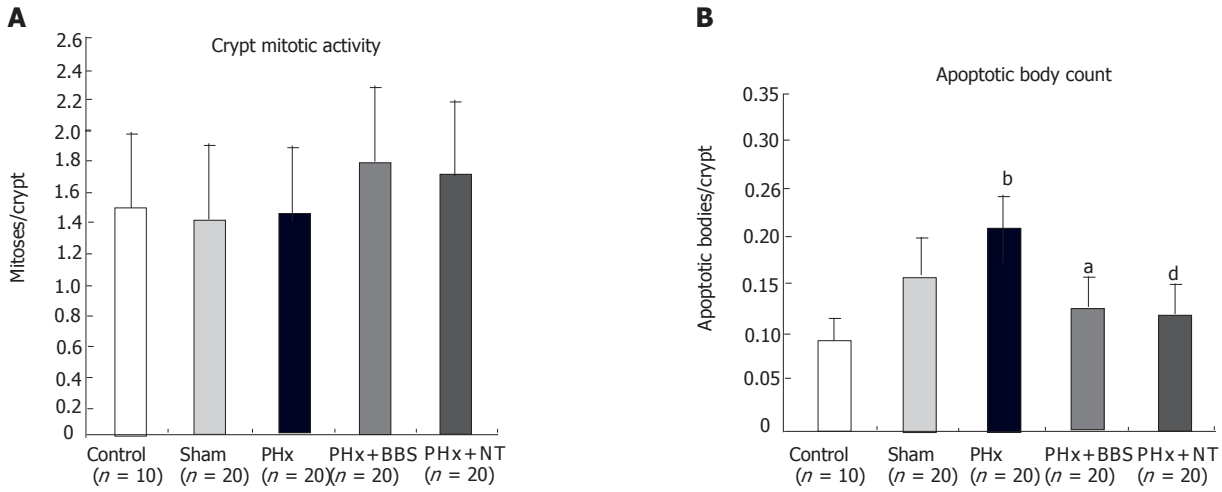

Figure 3 Crypt epithelial cell proliferation and apoptosis. Values are mean±SD. $^{b}P<0.001$ *vs* control, $^{a}P<0.05$ and $^{d}P<0.01$ *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin(**A**, **B**).

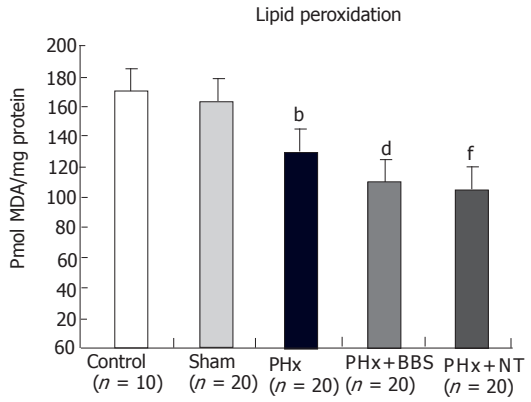

Figure 4 Lipid peroxidation in the intestine. Values are mean±SD. $^{b}P<0.001$ *vs* sham, $^{d}P<0.01$ and $^{f}P<0.001$ *vs* PHx. PHx, partial hepatectomy; BBS, bombesin; NT, neurotensin.

tissues[3,23]. Systemic endotoxemia plays a pivotal role in the development of septic complications and dysfunction of remote organs after liver resections through activation of a systemic inflammatory response, which is associated with structural and functional deleterious effects on vital organs[24]. Failure of the gut barrier leads to bacterial and endotoxin translocation and the pathogenesis of the so-called "gut-derived sepsis"[25]. However, the mechanisms implicated in intestinal barrier failure after liver resection have not been fully elucidated.

Physical injury to the intestinal mucosa is one of the mechanisms postulated to promote bacterial translocation. In our study, the ileal architecture remained intact and epithelial continuity was retained in hepatectomized rats. Previous experimental studies have shown that 70% hepatectomy induces trophic changes on the distal ileum after 30 d, causing atrophy of the ileum wall and a drop in villus thickness[26]. The present study shows that 48 h after partial liver resection there is a trend for decreased villous density and mucosal thickness that does not reach statistical significant levels. Diminished bile production and secretion to the duodenum, after partial hepatectomy, deprives partly the gut from its trophic action thus promoting mucosal atrophy. On the other hand, the plethora of enterotrophic growth factors and cytokines that are elaborated after liver resection exert the opposite effect[27]. The combinatory action of these factors seems to produce a balanced adaptive intestinal response, 48 h after partial hepatectomy.

The balance between cell proliferation and death in intestinal crypts is crucial for epithelial homeostasis because the total of epithelial cells lining villi originate from stem cells located in the proliferation zone of the crypt. Apoptosis was the only histologic parameter that altered significantly in the present study. Definite evidence of apoptosis is seen in the crypt, where apoptotic cells are seen once in every 5^{th}-10^{th} crypt section[28]. Occasional apoptotic bodies found in the rapidly proliferating epithelial cells of the normal gastrointestinal mucosa, help to maintain a steady-state in cellular populations[29]. Control animals presented this basal level of apoptotic activity known as spontaneous apoptosis, which serves to remove either occasionally overproduced stem cells or stem cells with minor DNA damage caused by external factors. Two-thirds hepatectomy resulted in a significant increase of apoptosis in the crypt, which may explain the trend for induction of mucosal atrophy without alterations in the mitotic activity of the intestinal epithelium. In addition, increase of the apoptotic process in the intestinal epithelium may partly contribute to the decrease of mucosal DNA content. Apoptotic cells have a lower DNA content than the G0/G1 value and this hypo-diploid DNA is a hallmark to show apoptosis[30].

Apoptosis is a morphologically distinct, gene-directed, form of cell death that contributes to both physiological and pathological processes[31]. Although in gastrointestinal epithelium it has been associated with several conditions[17,21,32], the biological significance of intestinal apoptosis after liver resection is not clear till

date. The responsible mechanism could reflect primary immunologic events following hepatectomy (apoptosis has been shown to be induced by a variety of triggers, including proinflammatory cytokines such as TNF, IL-1, and IL-6, or by cytotoxic T lymphocytes that act through either granzyme B or Fas receptor pathways) or a direct action of bacterial toxins[31,33,34].

Another factor associated with bacterial translocation is intestinal flora disturbances. In our study hepatectomized rats presented a significant increase of cecal aerobic bacteria [*E coli* and Gram (+) cocci]. Diminished bile production and disturbances of interdigestive motility may have led to bacterial overgrowth. Bile salts have a constraining role on the indigenous microflora, while secretory *IgA* contained in bile modulates the local immunological milieu[35]. In addition, delayed intestinal transit time in experimental liver resection seem to be implicated in intestinal bacterial overgrowth and increased bacterial translocation[36]. A correlation between cecal overgrowth of a specific organism and bacterial translocation of the same organism is well documented[37]. Overgrowth of *E coli* detected in our study and diminished biliary *IgA* may lead to increased attachment of this bacterial strain to the intestinal mucosa, predisposing to bacterial translocation and previous studies have shown that *E coli* is usually cultured from the mesenteric lymph nodes of hepatectomized rats[2].

Another parameter we have addressed in our study is intestinal oxidative stress estimated by quantification of lipid peroxidation, which is a common indicator of oxidative stress. Our data show that 48 h after partial hepatectomy decreased levels of lipid peroxidation were measured in the intestine. This may be attributed to liver regeneration and release of several growth factors and hormones that may influence intestinal oxidative stress[27]. In addition, previous studies demonstrate evidence for activation of the intestinal cytosolic antioxidant enzyme glutathione *S*-transferase after hepatectomy[38]. Another explanation might be that the removal of a large portion of the liver could result in a decrease of oxidants originating from the liver, therefore lowering lipid peroxidation. The liver and gut are considered as an anatomic and functional unit with inseparable and interdependent functions and from this point of view liver regeneration under low oxidative stress[39] may be associated with similar oxidant alterations in the intestine. A question that rose is if low oxidative stress is compatible with increased apoptosis. Whether a cell will enter or not the apoptotic process is determined by a variety of stimuli and intrinsic pathways and it seems that apoptosis of enterocytes after partial hepatectomy is not mediated by oxidative stress.

The present study has further investigated the role of gut regulatory peptides BBS and NT on gut barrier function in experimental partial hepatectomy. To the best of our knowledge, our results demonstrate for the first time that administration of BBS or NT improves gut barrier function after partial hepatectomy, leading to significantly lower portal and aortic endotoxin concentrations. Treatment with these factors reversed the decrease of intestinal morphometric characteristics and preserved mucosal DNA and protein content to control levels. Although these alterations were statistically significant only for DNA, they show a trend for induction of a trophic effect on the intestinal mucosa. This effect was accompanied by the increase of cell proliferation beyond control levels and inhibition of programmed cell death in intestinal crypts. The mitogenic effect of BBS and NT may be a direct receptor-mediated effect, since intestinal epithelial cells express receptors for both peptides[40-42] and it is known that BBS and NT are potent cellular growth factors whose binding to their receptors activates a mitogenic signal to the nucleus[43]. An indirect mechanism may be related to a further reduction of intestinal lipid peroxidation since oxidative state is involved in the modulation of cell proliferation and death[44]. The antiapoptotic effect of BBS and NT may be mediated by the reduction of endotoxemia and amelioration of the subsequent systemic inflammatory response, characterized by the release of numerous cytokines and proinflammatory mediators, such as tumor necrosis factor-alpha, which may activate the apoptotic process. Another explanation of their antiapoptotic action may be provided through induction of vasodilation[45,46], which improves intestinal microcirculation thus preventing enterocytes hypoxia, energy depletion and activation of apoptotic pathways.

Apart from their antiapoptotic action on the intestinal epithelium, BBS and NT exerted an antioxidant effect as well, further decreasing intestinal lipid peroxidation. The compromise of the intestinal barrier function, after partial hepatectomy, shows that intestinal compensatory response, which may be partly expressed by low oxidative stress, is relatively insufficient to overwhelm the noxious effect of hepatectomy on the intestinal epithelium. From this point of view, the additional decrease of intestinal oxidative stress achieved by peptides treatment may contribute to the enhancement of intestinal barrier. The results of this study are consistent with our previous work demonstrating the antioxidant effect of BBS and NT on the intestinal epithelium of bile duct ligated rats[17]. Possible explanations of their antioxidant action is the reduction of systemic endotoxemia, which is associated with generation of oxygen free radicals via a xanthine oxidase depended pathway, improvement of oxygen supply to enterocytes through vasodilation, or receptor-mediated activation of intracellular antioxidant pathways. It has been shown that binding of regulatory peptides to their specific G-protein coupled receptors is followed by a pivotal activation of protein kinase C[43]. Increased cellular PKC activity promotes the mitochondrial translocation and organization in a catalytically active form of a representative isoform of glutathione *S*-transferases, which are an important antioxidant enzyme system[47].

In conclusion, the present study shows that the gut regulatory peptides BBS and NT improve the intestinal barrier function and reduce endotoxemia, in experimental partial hepatectomy. This effect is, at least in part, mediated by their antiapoptotic, mitogenic and antioxidant effect on the intestinal epithelium. Although laboratory results

should not be easily extrapolated to the clinical situation, we feel that BBS and NT merit consideration, as potential therapeutic agents, for the improvement of gut barrier function in cases of liver resection.

ACKNOWLEDGMENT

The authors wish to express their thanks to Dr I Spiliopoulou, Department of Microbiology of Patras University Medical School, for her excellent work in microbiological analyses.

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