

University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Nutrition and Health Sciences -- Faculty
Publications

Nutrition and Health Sciences, Department of

2006

Intestinal Mucosal Alterations in Rats With Carbon Tetrachloride-Induced Cirrhosis: Changes in Glycosylation and Luminal Bacteria

Sathish Kumar Natarajan
Christian Medical College, snatarajan2@unl.edu

Prabhu Ramamoorthy

Simmy Thomas

Jayasree Basivireddy

Gagandeep Kang

See next page for additional authors

Follow this and additional works at: <https://digitalcommons.unl.edu/nutritionfacpub>

Part of the [Human and Clinical Nutrition Commons](#), [Molecular, Genetic, and Biochemical Nutrition Commons](#), and the [Other Nutrition Commons](#)

Natarajan, Sathish Kumar; Ramamoorthy, Prabhu; Thomas, Simmy; Basivireddy, Jayasree; Kang, Gagandeep; Ramachandran, Anup; Pulimood, Anna B.; and Balasubramanian, K. A., "Intestinal Mucosal Alterations in Rats With Carbon Tetrachloride-Induced Cirrhosis: Changes in Glycosylation and Luminal Bacteria" (2006). *Nutrition and Health Sciences -- Faculty Publications*. 160.
<https://digitalcommons.unl.edu/nutritionfacpub/160>

This Article is brought to you for free and open access by the Nutrition and Health Sciences, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Nutrition and Health Sciences -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Sathish Kumar Natarajan, Prabhu Ramamoorthy, Simmy Thomas, Jayasree Basivireddy, Gagandeep Kang, Anup Ramachandran, Anna B. Pulimood, and K. A. Balasubramanian

Intestinal Mucosal Alterations in Rats With Carbon Tetrachloride-Induced Cirrhosis: Changes in Glycosylation and Luminal Bacteria

Sathish Kumar Natarajan, Prabhu Ramamoorthy, Simmy Thomas, Jayasree Basivireddy, Gagandeep Kang, Anup Ramachandran, Anna B Pulimood, and K.A. Balasubramanian

Spontaneous bacterial peritonitis is a major cause of mortality after liver cirrhosis. Altered permeability of the mucosa and deficiencies in host immune defenses through bacterial translocation from the intestine due to intestinal bacterial overgrowth have been implicated in the development of this complication. Molecular mechanisms underlying the process are not well known. In order to understand mechanisms involved in translocation of bacteria, this study explored the role of oxidative stress in mediating changes in intestinal mucosal glycosylation and luminal bacterial content during cirrhosis. CCl₄-induced cirrhosis in rats led to prolonged oxidative stress in the intestine, accompanied by increased sugar content of both intestinal brush border and surfactant layers. This was accompanied by changes in bacterial flora in the gut, which showed increased hydrophobicity and adherence to the mucosa. Inhibition of xanthine oxidase using sodium tungstate or antioxidant supplementation using vitamin E reversed the oxidative stress, changes in brush border membrane sugar content, and bacterial adherence. In conclusion, oxidative stress in the intestine during cirrhosis alters mucosal glycosylation, accompanied by an increased hydrophobicity of luminal bacteria, enabling increased bacterial adherence onto epithelial cells. This might facilitate translocation across the mucosa, resulting in complications such as spontaneous bacterial peritonitis. (HEPATOLOGY 2006;43:837-846.)

Liver cirrhosis is a pathological condition that reflects irreversible chronic injury of the hepatic parenchyma in association with extensive fibrosis. Bacterial infection is responsible for up to one quarter of the deaths of patients with chronic liver disease.¹ Spontaneous bacterial peritonitis (SBP) is a common and serious infection developing in patients with cirrhosis, which is thought to appear as a consequence of impaired defense mechanisms against infection.² Clinical and experimental evidence indicate that translocation of bacteria from the

intestinal lumen to the bloodstream is directly involved in the pathogenesis of SBP.³ The gastrointestinal tract is affected during cirrhosis, and mucosal abnormalities secondary to portal hypertension may exist.⁴ A number of mechanisms have been suggested to promote bacterial translocation from the intestine, including intestinal bacterial overgrowth, altered permeability of the intestinal mucosa, and deficiencies in host immune defenses.⁵⁻⁷ Although SBP may be caused predominantly by enteric organisms,² the factors that favor bacterial translocation in cirrhosis are not completely understood. Attachment of luminal bacteria on the mucosal surface, which facilitates translocation, is mediated by sugars present on cellular glycoproteins and glycolipids.⁸ Changes in glycosylation on the surface of intestinal epithelial cells can thus lead to increased bacterial adherence. Reactive oxygen species can modulate glycosylation on the cell surface⁹ and also alter the surface viscosity of the mucus,¹⁰ both of which may facilitate bacterial binding. Earlier studies from our laboratory have shown that oxidative stress in the intestinal mucosa after surgical manipulation results in altered glycosylation of the mucosal membranes and bacterial adherence.¹¹ Oxidative stress has also been demonstrated in the

Abbreviations: SBP, spontaneous bacterial peritonitis; BBM, brush border membrane; cfu, colony-forming units; PBS, phosphate-buffered saline.

From The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Vellore, India.

Received June 13, 2005; accepted January 10, 2006.

Supported by The Wellcome Trust, London, Indian Council of Medical Research, Govt. of India.

Address reprint requests to: K.A. Balasubramanian, Ph.D., Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Vellore-632004, India. E-mail: wubalu@hotmail.com; wellcome@cmcvellore.ac.in; fax: (91) 0416 2232035.

Copyright © 2006 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21097

Potential conflict of interest: Nothing to report.

intestinal mucosa after carbon tetrachloride (CCl₄)-induced liver cirrhosis,¹² where the activity of xanthine oxidase, an important source of free radicals in the small intestine,¹³ was also elevated. These data led to the hypothesis that oxidative stress in the intestine during liver cirrhosis, produced by activation of xanthine oxidase, might alter glycosylation patterns on the intestinal mucosa and influence bacterial binding. To test this hypothesis, this study aims to examine xanthine oxidase and oxidative stress in the intestine during development of cirrhosis and determine its influence on bacterial interaction with the intestine. The role of oxidative stress in influencing bacterial interactions was confirmed by use of xanthine oxidase inhibitors and anti-oxidant therapy with vitamin E.

Materials and Methods

Adenosine diphosphate, dimethyl sulfoxide, MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide), 1,1', 3,3'-tetramethoxy propane, Tris (hydroxymethyl) aminomethane (Tris), N- [2-hydroxyethyl] piperazine-n'- [2—ethanesulfonic acid], thiobarbituric acid, dithio-bis- (2-nitrobenzoic acid), 2,4-dinitrophenyl hydrazine, ArsenazoIII, succinic acid, iodo-nitro tetrazolium salt, para nitro phenyl phosphate, xanthine, xanthine oxidase, glucose oxidase, 1,1',3,3' tetramethoxy propane, and hydroxy proline were obtained from Sigma Chemical Co. (St. Louis, MO). Carbon tetrachloride, vitamin E, and sodium tungstate were obtained from Qualigen Fine Chemicals, Ltd. Mumbai, India, Loba Chemicals, Mumbai, India, and The British Drug Houses, London, respectively. Polyethylene glycol 4000 was obtained from Fluka AG. Switzerland. ¹⁴C-labeled glucose was obtained from Bhaba Atomic Research Center, Bombay, India. Millipore membranes (0.45 μm) were obtained from Millipore, India. All other chemicals and solvents used were of analytical grade.

Animals. Adult Wistar rats of both the sexes (125-150 g), exposed to a daily 12-hour light/dark cycle and fed water and rat chow *ad libitum*, were used for this study. Rats were divided into seven groups (I-VII); each group comprised six animals at each of the different time points: group I, control; group II, phenobarbitone controls; group III, sodium tungstate control; group IV, tocopherol control; group V, CCl₄ treatment alone, group VI, sodium tungstate + CCl₄; group VII, tocopherol + CCl₄. This study was approved by The Animal Experimentation Ethics Committee of the institution.

Induction of Liver Cirrhosis in Rats. Cirrhosis was induced by administering CCl₄ intragastrically. The initial dose of CCl₄ was 40 μL/rat, and subsequent doses were adjusted based on the change in body weight as

described.¹⁴ Control animals received phenobarbitone alone.¹⁵ Animals were killed at 1, 2, 3, 4, and 5 months after CCl₄ treatment.

Sodium Tungstate Treatment and Vitamin E Supplementation. Sodium tungstate (0.7 g/kg body weight, in the drinking water) and tocopherol acetate (300 mg/kg diet mixed with rat chow), were coadministered with CCl₄ from the end of the 2nd month for a period of 1 month. Control animals received sodium tungstate alone or tocopherol acetate alone for 1 month. For all studies with inhibitors, animals were killed after 3 months of CCl₄ treatment (the time point when maximal changes were evident), and the effect of sodium tungstate co-treatment and vitamin E supplementation was compared with CCl₄ alone.

Histology and Measurement of Hydroxyproline and Serum Parameters. Liver tissue was fixed in 10% buffered formalin and processed. Four-micron sections were cut and stained with hematoxylin-eosin and observed under a light microscope. Hepatic hydroxy proline content was measured as described¹⁶ and expressed as micrograms hydroxyproline per grams wet weight of liver tissue. Serum was used for the assay of alanine aminotransferase, aspartate aminotransferase,¹⁷ alkaline phosphatase,¹⁸ total bilirubin,¹⁹ and total protein.²⁰

Intestinal Mitochondrial Preparation and Assessment of Function. The whole intestine from both control and rats with cirrhosis was divided into two portions. Scraped intestinal mucosa from the duodenum and proximal part of the jejunum were used for preparation of mitochondria by differential centrifugation as described.²¹ Mitochondrial function was assessed by oxygen uptake,²² swelling,²³ and MTT reduction.²⁴

Isolation of Surfactant, Brush Border Membranes and Measurement of D-Glucose Uptake and Carbohydrate Content. The distal part of the jejunum and the ileum of control and CCl₄-treated rats was used for the isolation of brush border membranes (BBM) and surfactant as described earlier.^{25,26} Purity of the isolated BBM was checked by enrichment of the marker enzyme alkaline phosphatase. Isolated BBM were assessed for their ability to transport glucose by uptake measurements carried out using the rapid filtration technique, at room temperature as described.²⁷ Hexoses, fucose,²⁸ sialic acid,²⁹ and hexosamine content³⁰ of surfactant and BBM were determined as described and expressed as nanomoles per milligram protein.

Oxidative Stress Parameters. Intestinal homogenate and BBM were used for assessment of oxidative stress parameters. Malonaldehyde,³¹ conjugated diene,³² and protein carbonyl content³³ were measured as described and expressed as nanomoles per milligram protein.

Enzyme Assays. Alkaline phosphatase activity in the BBM was assayed using p-nitrophenyl phosphate as substrate.¹⁸ Xanthine oxidase activity in the intestinal homogenate was measured, as described.³⁴

Bacterial Counts and Harvesting of *Escherichia coli* Strains From Cecal Contents and Cecal Mucosa.

Cecal mucosa and cecal contents were harvested from the sacrificed animals under sterile conditions. The number of aerobic and anaerobic bacteria in the cecum were counted after serial dilution in saline and calculated as colony-forming units (cfu) per gram contents. The media used for aerobic count included blood agar and MacConkey agar. For anaerobic count, neomycin blood agar, 5% sheep blood agar without nutrient agar base, Veillonella agar, and Rogosa SL agar medium were used. *Escherichia coli* strains, identified using standard microbiological techniques, were isolated from specimens grown on McConkey's media. Pools of five isolates of *E. coli* from each of the different groups were catalogued and frozen in cryoprotective media. These groups of isolates were used for adherence studies. Cecal tissue was washed gently in sterile saline to remove non-adherent bacteria, and approximately 1 cm² of the cecal tissue was cut. The cecal mucosa was then homogenized in 1 mL sterile saline, and the homogenate was subjected to quantitative culture analysis as described previously. The number of adherent bacteria present in the cecal mucosa was expressed as percentage difference compared with control, calculated from cfu per square centimeter tissue.

Bacterial Adherence. The assay was carried out as previously described,³⁵ using HEP-2 cell monolayers grown overnight on 10 spot multitest slides (ICN Biomedicals, Aurora, OH). Forty microliters of the overnight bacterial culture of *E. coli* from each group (5×10^5 cfu grown in Luria broth) was added to 0.5 mL minimum essential medium containing 2% fetal calf serum and 1% methyl-alpha-mannoside; 50 μ L of this was overlaid onto each test spot. The slides were then incubated at 37°C with 5% carbon dioxide for 3 hours, washed 3 with minimum essential medium, fixed with 70% ethanol, and stained with 10% Giemsa stain. The slides were examined under the oil immersion lens of a light microscope.

Hydrophobicity Assay. Cell surface hydrophobicity was measured by bacterial adherence to hexadecane.³⁶ *E. coli* were grown in Luria broth to approximately mid log phase, collected by centrifugation, and washed twice in phosphate-buffered saline (PBS), pH 7.0. Washed cells were resuspended in 1 mL PBS, and optical density at 620 nm was measured. This measurement served as the control (C_o). Subsequently, 200 μ L hexadecane was added, and the mixture was vortexed for 1 minute. After the phases were allowed to separate, the optical density of the

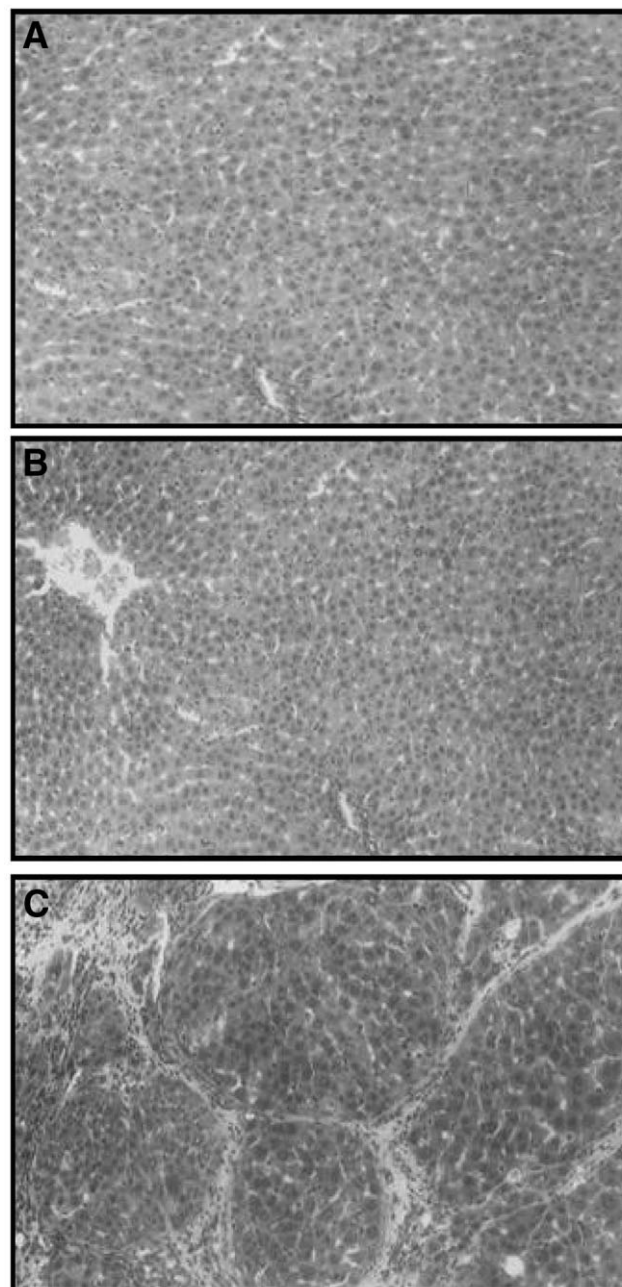


Fig. 1. Light microscopy of liver from controls (A), phenobarbitone control (B), and CCl₄-treated rats (C) after 3 months. Original magnification $\times 30$.

lower aqueous phase was measured (C_H). The percentage hydrocarbon adherence was determined by the following formula: $[(C_o - C_H)/C_o] \times 100$.

In Vitro Bacterial Adherence Assay. Bacterial adhesion to surfactant or BBM was carried out using poly L-lysine-coated microtiter plates. To each well on the plate, surfactant or BBM corresponding to 30 μ g protein was added and allowed to stand at room temperature for 20 minutes; unbound material was removed and washed twice with PBS. This resulted in coating of approximately

Table 1. Serum Markers for Liver Damage in Control and CCL₄-Treated Rats After 1, 3, and 5 Months

	Control	Carbon Tetrachloride Treatment		
		1 month	3 months	5 months
Alanine aminotransferase (IU/L)	140 ± 2	155 ± 5	621 ± 56*	679 ± 62*
Aspartate aminotransferase (IU/L)	133 ± 2	146 ± 4	715 ± 68*	741 ± 50*
Alkaline phosphatase (IU/L)	85 ± 10	95 ± 10	401 ± 52*	392 ± 15*
Total bilirubin (μmol/L)	16.5 ± 4	18.5 ± 4	61 ± 4*	86 ± 5*
Total protein (g/dL)	9.1 ± 0.23	9.1 ± 0.23	3.12 ± 0.13*	3.1 ± 0.2*
Hepatic hydroxyproline content (μg/g wet weight of liver tissue)	201 ± 11	212 ± 11	950 ± 88*	1,060 ± 120*

**P* < .05 when compared with control.

40% to 50% of the surfactant and 20% to 30% of BBM of the added material in each well. Subsequently, 0.1 mL *E. coli* isolated from cecal content of normal rats corresponding to 3×10^6 bacteria was added to each coated well and allowed to stand for 30 minutes at room temperature. The material was removed from the wells and unbound bacteria quantitated by subculturing in MacConkey plates. The number of colonies formed was counted and the percentage of bound bacteria calculated as follows:

Total No. of Bacteria – unbound bacteria = bound bacteria.

Bound bacteria/total no. of bacteria = % of bacteria bound to the sample.

E. coli bound to surfactant and BBM in the wells was also quantitated using crystal violet staining.³⁷

Statistical Analysis. Data are expressed as means ± SD. Statistical analysis was performed with the non-parametric Mann-Whitney test. A *P* value of less than .05 was taken to indicate statistical significance. Statistical calculations were performed using SPSS software for Windows (version 9.0; SPSS Inc., Chicago, IL).

Results

Liver cirrhosis was established by histology and serum markers of liver injury in animals treated with CCL₄. Intra-gastric administration of CCL₄ showed micronodular cirrhosis with extensive fibrosis after 3 month of treatment (Fig. 1). Serum markers for liver injury such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin were also found to be increased significantly in rats treated with CCL₄ by 3 months after treatment (Table 1), accompanied by an increase in liver hydroxy proline content (an indicator of fibrosis) (Table 1). Serum enzymes and liver hydroxy proline stayed elevated even at 5 months, indicating that in this model frank cirrhosis was established by 3 months and sustained at 5 months.

Our earlier work had shown a significant increase in xanthine oxidase activity in the intestine at the 3-month

point in rats with cirrhosis. Because this enzyme is an important source of free radicals in the intestine, we carried out a time course study examining enzyme activity before and after development of frank cirrhosis. As seen in Fig. 2A, xanthine oxidase activity starts to increase be-

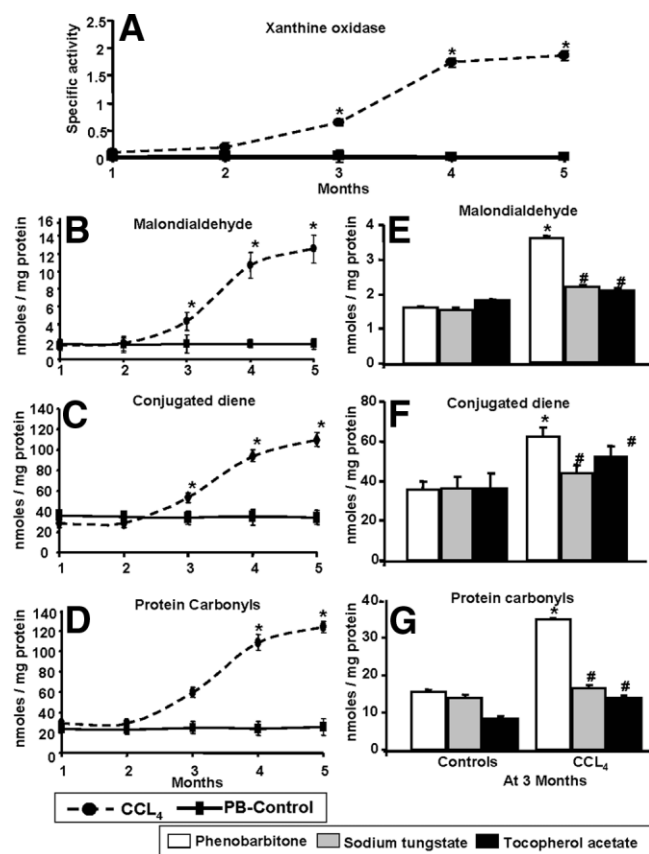


Fig. 2. Activity of xanthine oxidase (A) and oxidative stress parameters; malonaldehyde (B), conjugated diene (C), and protein carbonyl content (D) in intestinal homogenate from rats treated with CCL₄ for 1, 2, 3, 4, and 5 months, compared with phenobarbitone-treated controls. Malonaldehyde (E), conjugated diene (F), and protein carbonyl content (G) in intestinal homogenate from rats treated with CCL₄, CCL₄ + sodium tungstate, and CCL₄ + vitamin E, compared with phenobarbitone, sodium tungstate, and vitamin E-treated controls. The assays were done as described in the text. Each value represents mean ± SD from six separate experiments. **P* < .05, when compared with control, #*P* < .05, when compared with CCL₄-treated rats.

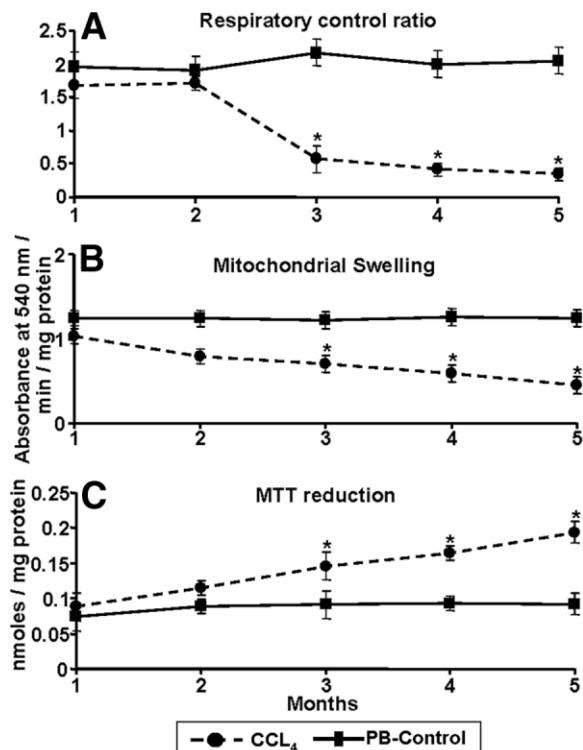


Fig. 3. Mitochondrial functional parameters; respiratory control ratio (A), mitochondrial swelling (B), and MTT reduction (C) from rats treated with CCl₄ for 1, 2, 3, 4, and 5 months, compared with phenobarbitone-treated controls. The assays were done as described in the text. Each value represents mean \pm SD from six separate experiments. * $P < .05$, when compared with control.

tween 2 and 3 months of CCl₄ treatment, peaks at 4 months, and stays elevated until 5 months. Oxidative stress parameters in the mucosal homogenate during this period showed a gradual increase in malondialdehyde, conjugated diene, and protein carbonyls between 3 and 5 months after treatment (Fig. 2B-D). To confirm the role of xanthine oxidase and oxidative stress, and also establish a cause-and-effect relationship, experiments were repeated with xanthine oxidase-deficient animals or long-term antioxidant therapy with vitamin E. These experiments were performed 3 months after CCl₄ treatment, the point when maximal changes were evident. As seen in Fig. 2E-G, both sodium tungstate treatment (to inhibit xanthine oxidase) and vitamin E reversed the oxidative stress in the intestinal mucosa. No xanthine oxidase activity could be detected in animals treated with sodium tungstate (data not shown). Another important source and target of free radicals in the cell are the mitochondria. There was functional impairment of intestinal mitochondria in rats with cirrhosis, with a decreased respiratory control ratio, increased superoxide production (measured by MTT reduction), and mitochondrial swelling by 3 months after treatment, which continued until 5 months

(Fig. 3). Mitochondrial lipid peroxidation was also evident, with increases in malondialdehyde, conjugated diene, and protein carbonyl levels starting at 2 months and continuing until 5 months (data not shown).

The BBM are important functional components of the intestine and in direct contact with luminal contents. A decrease in activity of the BBM enzyme alkaline phosphatase was seen in rats with cirrhosis by 3 months' treatment (Fig. 4A), whereas the activity of sucrase and maltase was unaltered (data not shown). Oxidative stress markers such as malondialdehyde, conjugated diene, and protein carbonyls were increased in BBM of rats with cirrhosis by 3 months of treatment and stayed elevated at 5 months (Fig. 4B-D). The decrease in alkaline phosphatase activity as well as oxidative stress in the intestinal BBM after 3 months CCl₄ treatment were reversed by the sodium

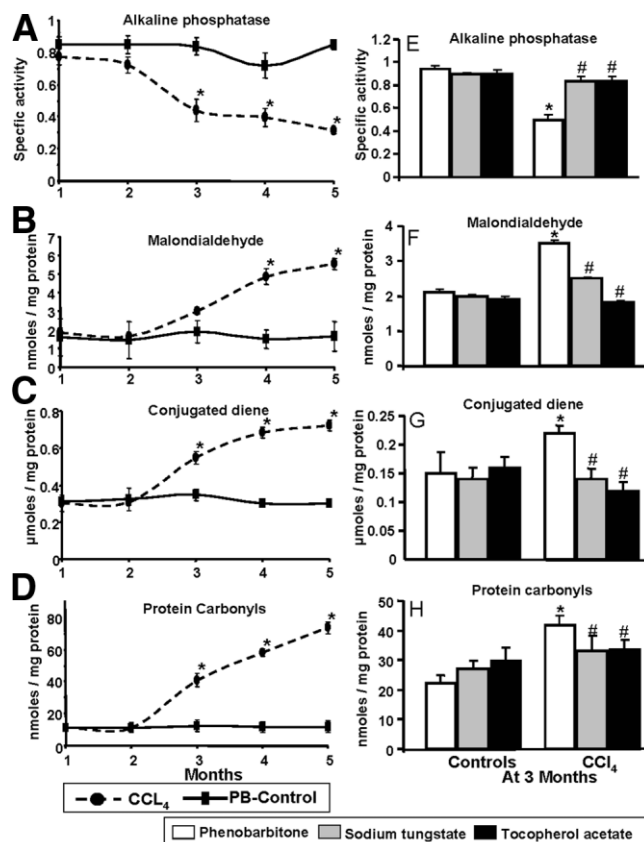


Fig. 4. Alkaline phosphatase activity (A) and oxidative stress parameters; malondialdehyde (B), conjugated diene (C), and protein carbonyl content (D) in BBM isolated from rats treated with CCl₄ for 1, 2, 3, 4, and 5 months, compared with phenobarbitone-treated controls. Alkaline phosphatase activity (E), malondialdehyde (F), conjugated diene (G), and protein carbonyl content (H) in BBM isolated from rats treated with CCl₄, CCl₄ + sodium tungstate, and CCl₄ + vitamin E, compared with phenobarbitone, sodium tungstate, and vitamin E-treated controls. The assays were done as described in the text. Each value represents mean \pm SD from six separate experiments. * $P < .05$, when compared with control, # $P < .05$, when compared with CCl₄-treated rats.

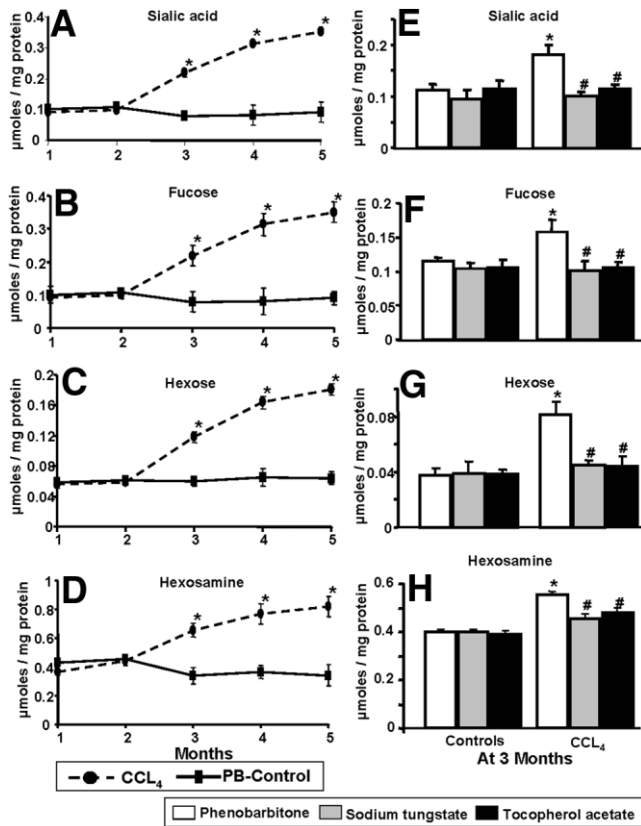


Fig. 5. Sugar composition of BBM; Sialic acid (A), fucose (B), hexose (C), and hexosamine (D) from rats treated with CCl₄ for 1, 2, 3, 4, and 5 months, compared with phenobarbitone-treated controls. Sialic acid (D), fucose (E), and hexose (F) from rats treated with CCl₄, CCl₄ + sodium tungstate, and CCl₄ + Vitamin E, compared with phenobarbitone, sodium tungstate, and vitamin E-treated controls. The assays were done as described in the text. Each value represents mean \pm SD from six separate experiments. * P < .05, when compared with control, # P < .05, when compared with CCl₄-treated rats.

tungstate treatment or vitamin E supplementation (Fig. 4E-H).

Functional capacity of the BBM was also affected, with a significant decrease in glucose transport by 3 months after CCl₄ treatment, continuing until 5 months (¹⁴C D-glucose uptake at 20 seconds, expressed as pmoles/mg protein in controls vs. CCl₄-treated rats for 1, 3, and 5 months were $310 \pm 23^*$ vs. $316 \pm 15^*$, 244 ± 22 , and $228 \pm 10^*$, respectively, * P < .05). Animals fed phenobarbitone alone were similar to the controls in all parameters examined.

Bacterial binding to the epithelium is mediated by receptors that are sugar specific, and these play a crucial role in bacterial translocation. Bacterial binding thus can be modulated by the sugar content of the BBM and surfactant layer, which covers the intestinal mucosa. Figure 5 shows the sugar content of BBM isolated from control and CCl₄ treated rats at various points. An increase in sialic acid, fucose, hexose, and hexosamine

is evident in rats with cirrhosis beginning at 3 months and extending to 5 months of treatment. Similar changes also were present in the surfactant layer (data not shown). Treatment with either sodium tungstate (to inhibit xanthine oxidase) or vitamin E reversed the sugar changes in the BBM after 3 months' CCl₄ treatment (Fig. 5).

One of the major complications of liver cirrhosis is SBP, and it has been suggested that gut is the major source of bacteria in SBP. In our study, ascites was seen in 20%, 60%, and 100% of the animal treated with CCl₄ by 3, 4, and 5 months, respectively, whereas infection of ascitic

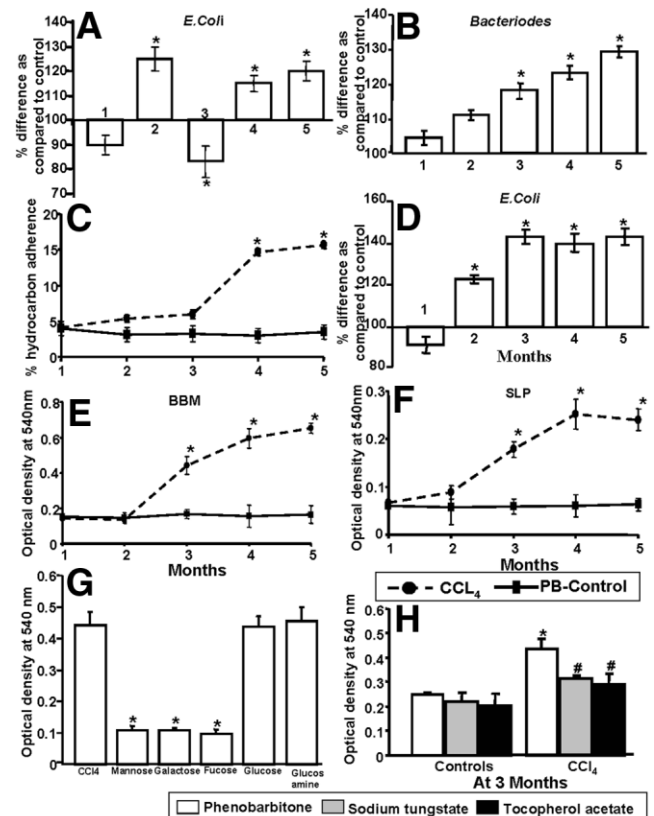


Fig. 6. Bacterial counts in cecal contents; *E. coli* (A), *bacteroides* (B), percentage hydrocarbon adherence of *E. coli* (C), and number of adherent *E. coli* per cm² of cecal mucosa (D) from rats treated with CCl₄ for 1, 2, 3, 4, and 5 months, expressed as percent difference compared with their respective phenobarbitone-treated controls. * P < .05, when compared with control. Binding of normal cecal *E. coli* to mucosa with cirrhosis: Crystal violet staining to detect bacteria bound to BBM (E) and surfactant (F) isolated from cirrhotic animals at 1, 2, 3, 4, and 5 months, compared with phenobarbitone-treated controls. (G) Crystal violet staining to demonstrate the inhibitory effect of various sugars on the binding of normal cecal *E. coli* onto surfactant isolated from animals with cirrhosis at 3 months. (H) Crystal violet staining of normal cecal *E. coli* to mucosa from rats with cirrhosis treated with CCl₄, CCl₄ + sodium tungstate, and CCl₄ + vitamin E, compared with phenobarbitone, sodium tungstate, and vitamin E-treated controls. The assays were done as described in the text. Each value represents mean \pm SD from six separate experiments. * P < .05, when compared with control, # P < .05, when compared with CCl₄-treated rats.

fluid was only seen in 60% of the animals by 5 months. This indicates that translocation of bacteria is a late event. Further studies were then performed to evaluate intestinal flora during development of cirrhosis. Cecal contents isolated from control animals showed both aerobic and anaerobic bacteria. Liver cirrhosis induced changes in the aerobic bacteria such as *E. coli* and anaerobes such as *Bacterioides*, with a significant increase in numbers after development of cirrhosis (Fig. 6A-B). Bacterial adherence can be facilitated by changes in the cell surface hydrophobicity, and this was measured by bacterial adherence to hexadecane. As seen in Fig. 6C, bacterial hydrophobicity was increased significantly in *E. coli* isolated 4 months after CCl₄ treatment, a characteristic that continued until 5 months. The commensal bacteria in the intestine from rats with cirrhosis were also more adherent to the mucosa; *E. coli* counts increased over time in the cecal tissue of CCl₄-treated animals when compared with controls (Fig. 6D). Analysis of the aggregative adherence pattern of *E. coli* isolated from animals with cirrhosis to HEp-2 cells in culture showed a three- to fourfold increase when compared with controls (data not shown). This paralleled the increase in hydrophobicity, as would be expected.³⁸

To determine whether the alteration in sugar content on the intestinal mucosa could influence bacterial binding, we then performed *in vitro* assays to study the interaction of normal *E. coli* with surfactant or BBM isolated from rats with cirrhosis coated onto microtiter plates. Bacterial adherence was quantitated by two methods: measuring bound bacteria by dye-binding and unbound bacteria by sub-culturing the supernatant from the wells. Bacterial binding occurred only with BBM (Fig. 6E) or surfactant (Fig. 6F) isolated from animals with cirrhosis, as seen by increase in optical density of crystal violet staining. Similar results were obtained by sub-culturing of the unbound bacteria (data not shown). The binding of *E. coli* with the BBM from animals with cirrhosis at 3 months was significantly inhibited by inhibition of xanthine oxidase (sodium tungstate treatment) or vitamin E supplementation (Fig. 6H), indicating the importance of oxidative stress in this process. To further characterize the specificity of binding, bacteria pretreated with different sugars were allowed to bind to surfactant isolated from rats with cirrhosis after 3 months of treatment. Bacterial binding to surfactant was inhibited 80% to 90% in the presence of mannose, galactose, and fucose, whereas this inhibition was not seen in the presence of glucose or glucosamine (Fig. 6G).

Discussion

Complications such as portal hypertension and spontaneous bacterial peritonitis (SBP) are major causes of

death after liver cirrhosis.¹ Gastrointestinal functions such as motility are affected during cirrhosis, and mucosal abnormalities secondary to portal hypertension exist.^{39,40} In the current study, although ascites was seen in animals even at 3 months when frank cirrhosis was evident, infection of ascitic fluid was seen only in the 5th month. Complications such as SBP occur long after development of frank cirrhosis, and bacterial overgrowth due to compromised intestinal motility may facilitate translocation in SBP.^{41,42} A number of factors may play a role in mediating bacterial translocation across the intestine during liver cirrhosis. These include (1) damage to the intestinal mucosa; (2) changes on the surfactant and brush border membranes lining the lumen of the gut, making them amenable to bacterial adhesion; and (3) changes in the luminal gut flora, which might alter adherence to the mucosa. This study examines each of these factors during the course of development of liver cirrhosis.

We demonstrated earlier that liver cirrhosis results in significant oxidative stress in the intestine.¹² Important sources of free radicals in the intestinal epithelium are the enzyme xanthine oxidase in the mucosa and enterocyte mitochondria. We examined xanthine oxidase activity in the intestinal mucosa and found that the increase in activity evident by 2 months of treatment is more pronounced by 3 months, when the serum enzyme profile indicated liver damage and frank cirrhosis was histologically evident. However, changes such as lipid peroxidation, which are the consequence of oxidative stress, were obvious only by 3 months, and then progressively increased up to 5 months. This indicates that the initial oxidative stress results in tissue damage, which persists even after establishment of changes in cirrhosis. The importance of xanthine oxidase activation and the resultant oxidative stress was confirmed by the experiments with xanthine oxidase deficient or vitamin E-supplemented animals. At the subcellular level, mitochondrial function is affected early, by 2 months. This indicates that the mitochondria are one of the early targets for damage in cirrhosis, because mitochondrial lipid peroxidation precedes the damage at the cellular level.

The second factor that could influence bacterial adhesion is the intestinal BBM, which is the first line of defense against bacterial translocation across the mucosal barrier. In the current study, oxidative stress resulted in damage of BBM as indicated by lipid peroxidation, which also compromised glucose transport by 3 months of treatment with CCl₄ and persisted till 5 months. Thus, oxidative stress initiated by activation of xanthine oxidase and mitochondrial dysfunction might be an important event leading to early mucosal damage, though frank liver cirrhosis is evident only after 3 months and the data from the

xanthine oxidase-deficient and vitamin E-supplemented animals, which showed protection against oxidative damage in the BBM reiterate the role of oxidative stress in the process.

Bacterial adherence is accomplished by specific adhesins on the outer surface of bacteria that attach to receptors containing sugars such as sialic acid, hexose, fucose, and amino sugars on the surface of the epithelial cell.^{43,44} Studies have shown a role for sialic acid and sugars present on mucosal surfaces as receptors for microorganisms.⁹ We find a significant increase in sialic acid, fucose, hexose, and hexosamine in surfactant and BBM in rats only at 3 months after initiation of CCl₄ treatment, which continued to increase until 5 months. These changes could be the result of the oxidative stress, because free radicals can modulate the activity of glycosyltransferase or glycosidases, which might in turn alter glycosylation pattern.^{8,10} Free radicals also can affect surface viscosity of the mucus as shown in the gastric mucosa,⁴⁵ and this may facilitate bacterial binding. The role of oxidative stress in increasing sugar content of BBM was also confirmed by studies with xanthine oxidase-deficient animals and vitamin E supplementation, which offered significant protection against this.

The data indicate intestinal mucosal damage seen during cirrhosis is associated with changes in mucosal surface glycosylation, which might facilitate bacterial adherence. Do changes in the gut bacterial population occur in cirrhosis? Under normal conditions, bacteria present in the lumen of the gastrointestinal tract remain relatively free of contact with mucosal epithelial cells as a result of a highly evolved mucosal defense system.⁴⁶ However, small intestinal bacterial overgrowth, depression of hepatic monocyte macrophage functions, and reduction of serum and ascitic fluid complement levels may contribute to increased incidence of bacterial infections in cirrhosis.⁶ Studies have shown in rats with cirrhosis, bacterial translocation results from intestinal overgrowth and severe damage to gut permeability,⁴⁰ and our data indicate a gradual increase in the population of *Bacteroides* in the intestine from 1 to 5 months during development of cirrhosis. However, *E. coli* populations showed an interesting trend: within a month after initiation of CCl₄ treatment, a decrease in numbers occurred in the gut as well as on the mucosa. This is probably due to the direct toxic effect of the compound. By 2 months, the gut flora adapt to the insult and the population increases, overtaking the initial numbers due to rapid proliferation. Thus, numbers increase both in the cecum and in

the mucosa. By 3 months, a decrease occurs in the cecal *E. coli* population, accompanied by an increase in numbers bound to the mucosa. As mentioned earlier, a significant increase in sialic acid, fucose, hexose, and hexosamine in surfactant and BBM was seen only at 3 months after initiation of CCl₄ treatment. It is possible that the population pattern of *E. coli* seen at 3 months could be due to an increased binding of cecal bacteria to the mucosa, resulting in lower numbers in the cecum. By 4 months, bacterial overgrowth is established, probably due to decreased intestinal motility. This is evident in the increase in numbers for both cecal bacteria and bacteria bound to the mucosa. This condition then persists at 5 months, by which time ascites is also present, and a significant percentage of animals have infected ascitic fluid indicating bacterial translocation.

The importance of these changes on both bacteria as well as the mucosa was then confirmed by the next series of experiments, which examined bacterial adherence in more detail. The *in vitro* experiments using microtiter plates coated with surfactant and BBM isolated from rats with cirrhosis indicate that these surfaces are more amenable to bacterial adherence when compared with control and confirm that the changes in sugar content of the mucosa affect bacterial binding. We have earlier reported that surgical stress in the small intestine produces similar changes, with increased bacterial adherence to mucosa from surgically stressed animals.¹¹ In addition to the changes in the mucosa, adherence is facilitated by the increased hydrophobicity seen in bacteria from rats with cirrhosis. The role of this bacterial factor in increasing adherence was confirmed by the *in vitro* experiments where bacteria from rats with cirrhosis showed increased adherence to cells in culture as well. The fact that the increased *in vitro* bacterial adherence was reversed by inhibition of xanthine oxidase or vitamin E supplementation confirms the role of oxidative stress in these intestinal alterations during liver cirrhosis. Bacterial adherence was also sugar specific, because galactose, fucose, and mannose were able to inhibit this, whereas glucose and glucosamine did not have any effect.

In conclusion, this study has shown that oxidative stress in the intestine during liver cirrhosis has far-reaching consequences during later stages of the disease. The persistent cellular and subcellular damage results in alterations in the gut flora, with increased bacterial adherence and increased bacterial hydrophobicity. This is accompanied by alteration in the glycosylation pattern in the intestinal mucosa. These changes, which occur later in the disease, might influence bacterial adherence onto the ep-

ithelial cells and facilitate translocation across the mucosa, resulting in complications such as SBP.

Acknowledgment: The Wellcome Trust Research Laboratory is supported by the Wellcome Trust, London. The financial assistance from the Indian Council of Medical Research Government of India, is gratefully acknowledged. G. Jaya Kumar and V. Shiva Kumar are also acknowledged for their help with experimentation. S.K.N is a Senior Research Fellow of the Indian Council of Medical Research.

References

- Wyke RJ. Problems of bacterial infection in patients with liver disease. *Gut* 1987;28:623-641.
- Chang CS, Yang SS, Kao CH, Yeh HZ, Chen GH. Small intestinal bacterial overgrowth versus antimicrobial capacity in patients with spontaneous bacterial peritonitis. *Scand J Gastroenterol* 2001;36:92-96.
- Yeh DC, Wu CC, Ho WM, Cheng SB, Lu IY, Liu TJ, et al. Bacterial translocation after cirrhotic liver resection: a clinical investigation of 181 patients. *J Surg Res* 2003;111:209-214.
- Llover JM, Bartoli R, Planas R, Cabre E, Jimenez M, Urban A, et al. Bacterial translocation in cirrhotic rats. Its role in the development of spontaneous bacterial peritonitis. *Gut* 1994;35:1648-1652.
- Di Leo V, Venturi C, Baragiotta A, Martinez D, Floreani A. Gastrointestinal and intestinal permeability in primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 2003;15:967-973.
- Fromkes JJ, Thomas FB, Kraut EH. Activation of the alternative complement pathway in ascitic fluid during spontaneous bacterial peritonitis. *J Clin Gastroenterol* 1982;4:347-350.
- Guarner C, Runyon BA, Young S, Heck M, Sheikh MY. Intestinal bacterial overgrowth and bacterial translocation in cirrhotic rats with ascites. *J Hepatol* 1997;26:1372-1378.
- Prabhu R, Balasubramanian KA. Altered glycosylation of surfactant and brush border membrane of the small intestine in response to surgical manipulation. *J Surg Res* 2004;117:272-282.
- Delmotte P, Degroote S, Lafitte JJ, Lamblin G, Perini JM, Roussel P. Tumor necrosis factor alpha increases the expression of glycosyltransferases and sulfotransferases responsible for the biosynthesis of sialylated and/or sulfated Lewis x epitopes in the human bronchial mucosa. *J Biol Chem* 2002;277:424-431.
- Tatsumi Y, Kodama T, Kashima K, Ohkuma S, Kuriyama K. Inhibition of gastric glucosamine synthetase activity by oxygen radicals: a possible cause of decreased mucosal protective capacity. *Jpn J Pharmacol* 1992;58:391-398.
- Thomas S, Kang G, Balasubramanian KA. Surgical manipulation of the intestine results in quantitative and qualitative alterations in luminal *Escherichia coli*. *Ann Surg* 2004;240:248-254.
- Ramachandran A, Prabhu R, Thomas S, Reddy JB, Pulimood A, Balasubramanian KA. Intestinal mucosal alterations in experimental cirrhosis in the rat: role of oxygen free radicals. *HEPATOLOGY* 2002;35:622-629.
- Anup R, Susama P, Balasubramanian KA. Role of xanthine oxidase in small bowel mucosal dysfunction after surgical stress. *Br J Surg* 2000;87:1094-1101.
- Ramachandran A, Balasubramanian KA. Intestinal dysfunction in liver cirrhosis: its role in spontaneous bacterial peritonitis. *J Gastroenterol Hepatol* 2001;16:607-612.
- Proctor E, Chatamra K. High yield micronodular cirrhosis in the rat. *Gastroenterology* 1982;83:1183-1190.
- Jamall IS, Finelli VN, Que Hee SS. A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal Biochem* 1981;112:70-75.
- Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957;28:56-63.
- Dorai DT, Bachhawat BK. Purification and properties of brain alkaline phosphatase. *J Neurochem* 1977;29:503-512.
- Shihabi ZK, Scaro J. A modified Malloy-Evelyn procedure for total bilirubin in microsamples. *Am J Med Technol* 1977;43:1004-1007.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-275.
- Masola B, Evered DF. Preparation of rat enterocyte mitochondria. *Biochem J* 1984;218:441-447.
- Madesh M, Ramachandran A, Balasubramanian KA. Nitric oxide prevents anoxia-induced apoptosis in colonic HT29 cells. *Arch Biochem Biophys* 1999;366:240-248.
- Takeyama N, Matsuo N, Tanaka T. Oxidative damage to mitochondria is mediated by the Ca(2+)-dependent inner-membrane permeability transition. *Biochem J* 1993;294 (Pt 3):719-725.
- Madesh M, Bhaskar L, Balasubramanian KA. Enterocyte viability and mitochondrial function after graded intestinal ischemia and reperfusion in rats. *Mol Cell Biochem* 1997;167:81-87.
- Prabhu R, Balasubramanian KA. A novel method of preparation of small intestinal brush border membrane vesicles by polyethylene glycol precipitation. *Anal Biochem* 2001;289:157-161.
- Eliakim R, DeSchryver-Keckskemeti K, Noguee L, Stenson WF, Alpers DH. Isolation and characterization of a small intestinal surfactant-like particle containing alkaline phosphatase and other digestive enzymes. *J Biol Chem* 1989;264:20614-20619.
- Tiruppathi C, Miyamoto Y, Ganapathy V, Leibach FH. Fatty acid-induced alterations in transport systems of the small intestinal brush-border membrane. *Biochem Pharmacol* 1988;37:1399-1405.
- Djordjic V, Mandic L. A spectrophotometric method for simultaneous determination of protein-bound hexoses and fucose with a mixture of L-cysteine and phenol. *Anal Biochem* 1990;188:222-227.
- Saifer A, Gerstenfeld S. Photometric determination of sialic acids in serum and cerebrospinal fluid with the thiobarbituric acid method. *Clin Chim Acta* 1962;7:467-475.
- Winzler RJ. Determination of serum glycoproteins. In: Click D, ed. *Methods of Biochemical Analysis*. Volume 2. New York: Interscience Publishers, 1955:292-294.
- Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351-358.
- Chan HW, Levett G. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoates. *Lipids* 1977;12:99-104.
- Sohal RS, Agarwal S, Dubey A, Orr WC. Protein oxidative damage is associated with life expectancy of houseflies. *Proc Natl Acad Sci U S A* 1993;90:7255-7259.
- Parks DA, Williams TK, Beckman JS. Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: a reevaluation. *Am J Physiol* 1988;254:G768-774.
- Kang G, Mathan MM, Mathan VI. Evaluation of a simplified HEP-2 cell adherence assay for *Escherichia coli* isolated from south Indian children with acute diarrhea and controls. *J Clin Microbiol* 1995;33:2204-2205.
- Swiatlo E, Champlin FR, Holman SC, Wilson WW, Watt JM. Contribution of choline-binding proteins to cell surface properties of *Streptococcus pneumoniae*. *Infect Immun* 2002;70:412-415.
- Schaeffer A, Amundsen SK, Jones J. Effect of carbohydrates on adherence of *Escherichia coli* to human urinary tract epithelial cells. *Infect Immun* 1980;30:531.
- Czeczulin JR, Balepur S, Hicks S, Phillips A, Hall R, Kothary MH, et al. Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli*. *Infect Immun* 1997;65:4135-4145.
- Madrid AM, Cumsille F, Deflippi C. Altered small bowel motility in patients with liver cirrhosis depends on severity of liver disease. *Dig Dis Sci* 1997;42:738-742.

40. Perez-Paramo M, Munoz J, Albillos A, Freile I, Portero F, Santos M, et al. Effect of propranolol on the factors promoting bacterial translocation in cirrhotic rats with ascites. *HEPATOLOGY* 2000;31:43-48.
41. Gunnarsdottir SA, Sadik R, Shev S, Simren M, Sjovall H, Stotzer PO, et al. Small intestinal motility disturbances and bacterial overgrowth in patients with liver cirrhosis and portal hypertension. *Am J Gastroenterol* 2003;98:1362-1370.
42. Madrid AM, Hurtado C, Venegas M, Cumsille F, Defilippi C. Long-term treatment with cisapride and antibiotics in liver cirrhosis: effect on small intestinal motility, bacterial overgrowth, and liver function. *Am J Gastroenterol* 2001;96:1251-1255.
43. Grange P, Mouricout M. Transferrin associated with the porcine intestinal mucosa is a receptor specific for K88ab fimbriae of *Escherichia coli*. *Infect Immun* 1996;64:606-610.
44. Helander A, Hansson GC, Svennerholm AM. Binding of human enterotoxigenic *Escherichia coli* expressing coli surface antigen 6 to rabbit intestinal enterocytes and glycoproteins. *Adv Exp Med Biol* 1997;412:257-258.
45. Mojzis J, Hegedusova R, Mirossay L. Role of mucus in ischemia/reperfusion-induced gastric mucosal injury in rats. *Physiol Res* 2000;49:441-446.
46. Alexander JW, Boyce ST, Babcock GF, Gianotti L, Peck MD, Dunn DL, et al. The process of microbial translocation. *Ann Surg* 1990;212:496-510; discussion 511-492.