Possible Causes of Ileal Injury in Two Models of Microbial Sepsis and Protective Effect of Phytic Acid

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Abstract

Background: Sepsis related-multiple organ dysfunction is associated with ileum injury. We aimed to determine the causes of ileal injury in two models of microbial sepsis resulted from infection with Aeromonas hydrophila or its endotoxin. We also evaluated the protective effect of phytic acid.

Methods: Thin sections of ileum from 60 Swiss male mice in control, bacteria-infected or lipopolysaccharides (LPS) and bacteria-infected or LPS-infected co-administered with phytic acid were subjected to histopathological and TdT-mediated dUTP nick-end labeling (TUNEL) assay for apoptotic cells detection while ultra thin sections were stained with uranyl acetate and lead citrate for cytological changes examination. Also, ileum images were exposed to the image analysis software to determine some related morphometric measures.

Results: Necrosis and apoptosis were observed in ileum injury in both examined sepsis models. The ileum injury was more severe in LPS model. Phytic acid showed the ability to attenuate ileum injury in Aeromonas hydrophila and its endotoxin models of sepsis after four weeks administration where its supplementation significantly minimized the histopathological and cytological complications and morphometric alterations resulted from the injury.

Conclusion: The protective effects of phytic acid may be caused by increased mucous secretion, decreased apoptotic index, attenuating the inflammatory and lymphocytic cells count or increasing the renewal of the crypt cells and villous epithelial cells proliferation.

Keywords ● Phytic acid ● mice ● histopathology ● apoptosis ● morphometry ● ultrastructure

Introduction

Sepsis and its complications are major challenges in clinical medicine.¹ Despite extensive research during the past 20 years and new therapeutic approaches used in clinical settings, the incidence of sepsis and the number of sepsis-related deaths are rising.²,³ Aeromonas hydrophila is the well known strain of Aeromonas. It is a heterotrophic gram-negative bacterium with a lipopolysaccharides (LPS) outer membrane envelope that enters the body of its victim
and travels through the bloodstream to the first available organ, leading to septic shock, multi-organ dysfunction and subsequent death. This multiple organ dysfunction (MODS) is associated with disruption in the protective intestinal mucosal barrier and diarrhea containing blood and mucus. Thus, the integrity of the gastrointestinal tract serves as a critical determinant for clinical outcome in microbial septic.

Phytic acid (inositol hexaphosphate, IP6) is a rice-based product that draws much attention in biomedical research. It constitutes 1-5% of most cereals, nuts, oil seeds, legumes, and grains. IP6 has the ability to enhance the natural disease resistance of the body and affects different pathologic conditions. Proposed mechanisms of its action include gene alteration, enhanced immunity, and antioxidant properties. Moreover, phytic acid has the ability to modulate the selective neutrophil function, and was recently recognized to possess multiple biological functions.

Thus, the present study was performed to determine the causes of ileum injury in two models of microbial sepsis resulted from either Aeromonas hydrophila infection or its endotoxin and the possible protective effect of phytic acid.

Materials and Methods

Animals

Sixty adult male Swiss mice weighing 25-30g were obtained from the Biological Supply Centre, Theodore Bilharz Research Institute (TBRI, Cairo, Egypt) and housed in stainless steel wire cages (8 animals/cage) under pathogen-free conditions. The animals were maintained at 25-28°C on a 12:12hr light/dark cycle and provided with food and water ad libitum. All animal procedures followed the animal research bioethics of TBRI and recommendations for the proper care and use of laboratory animals.

Sepsis Models

2a) - The LPS instillation into the abdominal cavity was the first model to induce sepsis-like symptoms similar to pathophysiological responses in patients with sepsis.

2b) - The bacterial inoculum model was the second model of sepsis in which the pure cultures of bacteria were injected without a carrier.

Bacterial Suspension, LPS, and Phytic Acid

a)- Preparation of Aeromonas hydrophila suspension: Aeromonas hydrophila was grown on a nutrient agar medium at 25°C for 72hours. The cultured strain was inoculated into 150ml of a liquid peptone broth and incubated at 25°C for 24hours with continuous shaking. The harvested bacteria were centrifuged at 6000g for 10 min and the dried pellet was suspended twice in phosphate-buffered saline (PBS; pH 7.4) and frozen at -20°C.

b)- Preparation of bacterial endotoxin suspension (LPS): The LPS was prepared by the phenol-water method. Briefly, 0.5ml PBS was added to the frozen suspension of bacteria and sonicated immediately for 15min until frozen portion thawed. The bacterial debris was removed by centrifugation at 12000g for 10min. The supernatant was collected and stored at -70°C. The water phase (phenol + LPS + nucleic acids) was dialyzed to remove the traces of the phenol and nucleic acids were digested by enzymes and removed by filtration or centrifugation. LPS suspension was prepared using PBS (pH=7.4)

c)- Preparation of phytic acid: The pure phytic acid (IP, 98%) dodecasodium salt (C6H13O6P2Na12), obtained from Sigma Co. USA (P-8810), was dissolved in sterile saline.

Groups

Mice were randomly assigned to the following groups (n=12/group):

Control Group: Intraperitoneally (i.p) injected with PBS (pH 7.4) at similar intervals with other groups.

Bacteria Group: i.p injected with bacterial suspension of Aeromonas hydrophila (0.2 ml=1×10⁸ cells/mouse/week) for two or four weeks.

Phytic acid plus bacterial suspension-treated Group: orally administered phytic acid (40 mg/kg) three times weekly by gastric tube for two or four weeks simultaneously with i.p injection of bacterial suspension (0.2 ml=1×10⁸ cells/mouse/week) for two or four weeks.

Endotoxin Group: i.p injected with the LPS of Aeromonas hydrophila (20 mg/kg/week) once a week for two or four weeks.

Phytic acid plus endotoxin-treated Group: i.p injected with the LPS (20 mg/kg/week) once a week for two or four weeks simultaneously with phytic acid (40 mg/kg) 3 times weekly by gastric tube for two or four weeks.

Histopathological, Histochemical and Morphometric Studies

Histopathological, Histochemical and TUNEL assay preparations: Six animals from each group were sacrificed under mild diethyl ether anesthesia after two and four weeks of treatment. Small segments of ileum were fixed to...
in neutral buffered formalin. Three paraffin sections of 5µm-thick were prepared from each block. One stained with haematoxylin and eosin for histopathological study. The second was stained with periodic acid Schiff (PAS)-Alcian for mucin content determination, and the third mounted onto Super-frost plus slides (Fisher Scientific, Pittsburgh, PA, USA) to detect apoptotic cells using the TdT-mediated dUTP nick-end labeling (TUNEL) assay. The TUNEL assay was performed using a kit (in situ cell death detection kit; Roche Molecular Biochemicals, Germany) according to the protocol provided by the manufacturer. The apoptotic index (the percentage of golden or dark brown stained cells) was determined at 20-random locations within the ileum mucosa for each animal from the villi and the crypt and for six animals from each group using a Leica Qwin 500 image analyzer. All sections were tested blindly by two histopathologists.

Cytological preparations: Specimens of 1 mm²-thick were fixed in 2.5% glutaraldehyde, washed in phosphate buffered solution (pH 7.4) for three hours and then embedded in epoxy resin. Toluidine blue-stained semithin sections were then prepared and ultrathin sections of 60-90 nm were stained with uranyl acetate and lead citrate for ultrastructural (cytological) changes examination.

Morphometric studies preparations: Images of ileum sections stained with haematoxylin and eosin (thin), toluidine blue (semithin), or ultrathin sections captured as tag image format using Scion Image (Scion Image beta 4.0.2, National Institute of Health modified by Scion Corporation-Frederick, MD, USA). The morphometric measurements included the villous height (µm), the crypt depth (µm), the enterocyte height (µm), the lymphocytes' number/mm² in lamina propria and epithelium of the villi (thelio/lymphocytes), the inflammatory cells' number/mm² in villous and muscularis mucosae-villous crypt junction (crypt associated), the microvillous height (µm), the goblet cells' number crypt and villous.

Statistical Analysis
All morphometric data was analyzed using the PC-STAT one way analysis of variance to determine the significance of the treatment effect on each parameter studied in each period. Two way analysis program was used to determine the effect of both time and treatment on each parameter examined. Results were expressed as mean ± standard error (SE). P values were considered as P<0.05: significant, P<0.01: highly significant, and P<0.001: very highly significant.

Results

Histological Studies
The examination of the bacteria infected ileum sections showed large number (62%) of enterocytes with hypertrophied multinuclei, villous atrophy, necrosis and sloughing of the lining epithelium especially at the villous tips with inflammatory cells infiltration in the stroma of the villi after two weeks of the infection. These lesions were much deteriorated in animals given endotoxin after the same period where a complete destruction of the crypt cells and the mucosal layer of the villi with focal aggregation or diffuse infiltration of the inflammatory cells in the muscular layer and lamina propria, were detected, respectively.

However, animals infected with bacteria or endotoxin and treated with phytic acid still suffered from fusion of some villi with focal aggregation or diffuse infiltration of mononuclear leucocytes in the bacteria infection group or ulceration of the lining epithelial layer of mucosa accompanied with diffuse infiltration of the inflammatory cells in the lamina propria in the LPS group.

After four weeks, the pathology became more severe and was associated with lymphoid hyperplasia in the LPS group. Most phytic acid-treated animals (20/24; 83.3%) revealed great improvement in the ileum histological lesions with crypt proliferation but could not completely restore their normal histological structure where serosa and muscularis were thickened and some lymphocyte nodules were still noticed.

Immunohistochemical Studies
Ileum sections of bacteria and LPS-treated animals manifested higher intensity of TUNEL-staining in both crypts and villi after four weeks, compared with control and the phytic acid-treated groups (figures 1a-1h1)

Histochemical Studies
Mucoid substances greatly increased in bacteria and LPS-treated groups compared with the controls after two weeks. Though, this secretion was decreased after treatment with phytic acid at this period.

After four weeks, ileum sections of both bacteria and LPS-treated groups had less amount of mucus compared with the controls or their two-week counterparts. Treatment with phytic acid resulted in mucus hyper-secretion and increase in goblet cells number compared
Protective phytic acid effect on septic injury

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with the control group and epithelial cells proliferation in LPS-treated group.

Cytological Studies

The enterocytes of the control group were united at their uppermost lateral membrane by a well-developed tight junction. The supranuclear region of these enterocytes had several spherical mitochondria and numerous cisternae of rough endoplasmic reticulum. The luminal surface of the enterocytes possessed many closely packed parallel finger-like microvilli. Each microvillous had a filamentous core that was united forming the terminal web. Under this web a clear zone - almost free of cytoplasmic organelles except for some rough endoplasmic reticulum (RER) cisternae- was noticed. These cells were characterized by their mucin granules at their distal ends and stacks of Golgi complex near their basal ends.

A number of enterocytes of the bacteria-infected group lost their microvilli and showed mild destructive changes in cellular organelles including degenerated mitochondria and RER, while the LPS-infected animals had numerous enterocytes with completely deteriorated organelles, several lysosomes or vesicles, and fused clumps of intact microvilli with dissolved cytoskeletal structures especially at the cell base or totally degenerated ones. Most enterocytes had detached lateral plasma membrane in several foci. Moreover, many inflammatory cells and mononuclear leucocytes were seen in the lamina propria of the ileum villi of the LPS-infected mice.

After treatment of the bacteria-infected group with phytic acid, a few number of vacu-
Morphometric Studies

One way analysis: when the ileum measurements of the bacteria-infected or the endotoxin-injected animals were compared with those of the control, the percentage change of crypt depth and villous, enterocytes' and microvillous' heights showed significant decrease after two weeks.

The goblet cells' number in the crypts and villi manifested significant decrease after four weeks in both models. This decrease started from the second week except for goblet cells number in the bacteria-infected animals, which showed a non-significant increase.

The percentage change of the apoptotic index showed significant increase in both models (bacteria and LPS) after two weeks and this increase was reversely correlated with the experimental time course.

Treatment with phytic acid abated the decrease in all the tested measurements at the two experimental time courses. All these values exceeded the control values after four weeks except for the villous and enterocyte heights in the LPS model. Though, after two weeks of phytic acid administration in both models, the number of inflammatory cells in bacteria and LPS models revealed great decline. This decrease was time-dependent.

Treatment with phytic acid also diminished the mean apoptotic index in crypts or villi after four weeks in bacteria or LPS-treated animals, respectively. However, the values in crypts were still higher than the negative control.

Two way analysis: Analysis of the effect of treatment and time interaction on different morphometric indices indicated that both parameters had very highly significant effects ($P<0.001$) on enterocyte height, crypt depth, lymphocytes' number, inflammatory cells' number, goblet cells number, and apoptotic index of the villi. The effect was highly significant ($P<0.01$) on the apoptotic index of the crypt and non-significant on the villous height.

Discussion

The vast majority of bacteria and their endotoxin enter the host through the gastrointestinal tract. The intestinal mucosa presents a barrier that limits absorption of vast array of potential allergens contained within the digesta. Toxic enteric contents like bacteria and endotoxin disrupt this protective intestinal barrier.

Findings of the present study suggest that infection with either bacteria or LPS was characterized by massive influx of inflammatory cells into the villi and the crypts and lymphocytes in the enterocytes or the lamina propria. This flood was greater in the LPS model.

Many studies documented stimulation of vigorous systemic inflammatory response after endotoxin administration. The interaction of LPS with the cellular components may be the cause of the inflammatory cells infiltration. The infiltration and activation of inflammatory cells at the site of injury may cause the release of vigorous reactive oxygen metabolites and various proteases, which may contribute to the tissue damage.

Apoptotic index, however, showed non-significant decrease in 2 weeks. The case was reversed after four weeks where the inflammatory response decreased and the apoptotic percent increased. This decreased incidence of apoptosis after two weeks may reflect a certain strategy to slow down the cell turnover to delay the rapid clearance of the infection.

The bacterial damage affected only the differentiated cells in the villi tips and thus may cause much less damage than LPS, which also destroyed the crypt cells. The lymphoid hyperplasia in the LPS group might be another cause of increased number of lymphocytes in the LPS group compared with bacteria group that could lead to much damage. Moreover, the hypertrophied multinuclei noticed in the enterocytes of bacteria infected mice may reveal increased proliferating rate of the enterocytes that minimize the harmful effect of bacteria compared with LPS model.

Cell necrosis has been established as a hallmark of sepsis-related organ failure in some reports. However, Vitovec and colleagues reported no clinical or histopathological changes in the intestine of neonatal BALB/mice infected with Aeromonas spp.

Loss of epithelial cells from the villi may be
the main reason of the reduction in their height after bacteria or LPS infection. Besides, the damage in the proliferative crypt region may be another reason for the reduction of this height and the appearance of the deformed tips with LPS infection. Moreover, the decreased crypt depth may be allied to its increased cell death rate, severe destruction, or the movement of cells from the crypts up to villous in the absence of replacement by cellular proliferation where crypt transit cells either undergo apoptosis or cease replication. This could give an explanation of the non-significant change of the crypt depth after two weeks in the both models as it is accompanied with a non-significant change of the apoptotic index of the crypts at the same period.

The histopathological changes of ileum mucosa were confirmed by the altered mucin histochemistry and the ultrastructural alterations.

Mucus production was increased despite the significant decrease in the mean goblet cells’ number of the villi after two weeks in the both models, while its amount showed great reduction after four weeks accompanied with decreased mean goblet cells’ number of crypts and villi. This decrease was more pronounced in LPS model.

The increased amount of mucin noticed after two weeks may be a protective mechanism to remove the infection. Though, this protection may be attenuated via certain bacteria or LPS reactions. Decreased mucous content after four weeks of infection compared with two weeks may be resulted from the increasing severity of the histopathological lesions. Also, the obvious decrease of mucus in LPS model compared with bacteria-infected model may explain its stern effects.

Leiper and co-workers, reported that mucin secretion by colon epithelial cell lines after bacterial infection was related to specific bacterial peptides interactions with gastrointestinal mucosa.

On the ultrastructure level, the present study showed that the first-line damage caused by the endotoxin or Aeromonas hydrophila, was related to brush border microvilli. Most enterocytes of the intestinal epithelium of the LPS-infected mice lost their microvilli and had completely destructed organelles, numerous vesicles, several lysosomes, and detached lateral plasma membranes. These changes were mild in the bacteria-sepsis model. The disappearance of luminal microvilli in certain foci may indicate the cytolitic action of LPS or bacteria.

Consistent with our results, Lorenzson and Olsen, reported that Aeromonas hydrophila could lead to shedding of the brush border membranes and decreased villous length with consequent reduction in the surface area absorption in the small intestine. Also, Arai and Nakazawa, explained the reduction of the actin microfilaments in the microvilli on the basis of the rearrangement of cytoskeleton filaments. Moreover, Crouser and colleagues, reported different degrees of mitochondrial injury in bacteria- and LPS-treated animals.

Treatment with phytic acid resulted in a great improvement of the histological lesions in both LPS- and bacteria-infected groups after four weeks with villous epithelial cells’ or crypt proliferation. Though, few lesions were still noticed after two weeks of treatment and were accompanied with focal aggregation or diffuse infiltration of mononuclear leucocytes and inflammatory cells. The number of lymphocytes and inflammatory cells were less prominent compared with their respective positive controls. This decline was time-dependent and was associated with reduced apoptotic index. The lesions in bacteria model showed better amelioration after phytic acid administration.

The ameliorative effect of phytic acid on the histological lesions may be attributed to antioxidative properties, upshot in various cellular functions, slow-down effect of the inflammatory and lymphocytic cells’ influx or its stimulatory effect of crypts and/or villous epithelial cells’ proliferation.

Contrary to our results, Johnson and co-workers, reported an excitatory effect of phytic acid on the inflammatory cells secretion. Also, Shamsuddin and colleagues, found no pathological adverse effects of phytic acid after its administration to F344 or female Sprague Dawley rats for 40 weeks.

Vucenik and others, showed that phytic acid diminished the asbestos-induced oxidative damage in the rat lungs and attributed this effect to iron-chelating activity and antioxidant properties of the compound.

Morphometric analysis showed that after four weeks of treatment with phytic acid, the mean crypt depth and villous and enterocytes’ height showed significantly higher values compared with their respective positive controls. The effect of phytic acid on morphometric parameters was time-dependent. The mitigation in these morphometric measurements may be related to the decrease in the lost epithelial cells resulted from necrosis and/or apoptosis, the increase in the cellular proliferation or the renewal of the crypt transit cells.

Moreover, treatment with phytic acid caused goblet cells hyperplasia with mucus
hyper-secretion after four weeks in both villi and crypts compared with their respective positive controls. The increase in mucous secretion after four weeks may be resulted from better improving effects of phytic acid on the histological lesions after four weeks of treatment and can explain the protective effect of phytic acid on ileum injury in sepsis.

Conclusion

In both sepsis models, necrotic and apoptotic pathways were involved in ileum injury but necrosis may precede and promote programmed cell death.

There was a link between the severity of ileum injury and mucin secretion.

Mucosal barrier dysfunction may be related to the extensive structural disruption of the intestinal microvilli, inflammatory and lymphocyte infiltration, apoptosis and/or necrosis and it was much rigorous after four weeks of the sepsis onset.

The ileum injury was more severe in LPS model.

Phytic acid had the ability to attenuate ileum injury in both sepsis models after four weeks via minimizing the histopathological and cytological complications and morphometric alterations, increasing mucus secretion, decreasing apoptotic index, attenuating the inflammatory and lymphocytic cells' count or increasing the renewal of the crypt cells and villous epithelial cells proliferation.

Conflict of Interest: None declared

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