Intestinal Epithelial Barrier Dysfunction After Hemorrhagic Shock in a Rat Model

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Abstract:

Introduction: Hemorrhagic shock is a frequent complication in trauma patients, after gastrointestinal bleeding and major surgery. Hemorrhagic shock is associated with end organ damage, caused by hypoperfusion and local and systemic inflammation. The intestine is one of the first organs affected by hemorrhagic shock. An early event in intestinal damage is gut wall integrity loss, including the opening or breakdown of tight junctions. In this study, the sequence of events in the gut after hemorrhagic shock which causing tight junction loss were studied. **Aim of the study:** The aim of this study was to investigate the effect of the hemorrhagic shock on intestinal epithelial barrier function in a rat model. **Material and Methods:** This study was carried out on 40 male Wistar albino rats 4-6 months old with body weight (250-300g). Rats were divided randomly into four main groups: **Group 1 (Control group):** It consisted of 10 normal healthy rats served as control and sacrificed without intervention. **Group 2 (30 minutes group):** Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock. Rats were sacrificed (30 minutes) after the onset of hemorrhagic shock. **Group 3 (60 minutes group):** Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock. Rats were sacrificed (60 minutes) after the onset of hemorrhagic shock. **Group 4 (90 minutes group):** Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock and sacrificed (90 minutes) after the hemorrhagic shock. At the end of the study: Blood samples were collected for measurement of serum tumor necrosis factor alpha (TNF-α) and serum lipopolysaccharide (LPS). Distal segments of the ileum were excised immediately for histopathological study and measuring Zonula Occludens (ZO-1) gene expression. **Results:** TNF-α serum levels were significantly increased in all groups of hemorrhagic shock in comparison to their levels in the control group. Serum LPS levels measured in hemorrhagic groups showed significant increase in 60 and 90 min. groups when compared to the control group, while 30 min. group showed no significant change when compared to the control group. Histopathological study of the ileum segment revealed extension of the subepithelial space, focal mucosal infarction, a massive lifting down sides of the villi, some villus tips are denuded and epithelial cells are severely injured. PCR study of ZO-1 showed significant increase in ZO-1 gene expression in all groups of hemorrhagic shock as compared with the control group. **Conclusion:** This study gives more insight into the sequence of events in the gut after hemorrhagic shock. Hemorrhagic shock leads to intestinal tight junction integrity loss. This is followed by bacterial translocation and systemic inflammation.

Keywords: hemorrhagic shock, small intestine, epithelial barrier function, zonula occluden-1.

Introduction:

Traumatic death remains a major public health issue. Injury occurs unexpectedly and often affects the most productive segment of society, and constitutes the leading cause of death among middle age group especially in the developing countries, where death rate due to infection is also high. Every day about 16.00 of people die worldwide from injuries, and for every person who dies, several

thousands more are injured, many of them with permanent sequelae. [1] Overall, it is estimated that 16% of the world's burden of disease can be attributed to injury. [2]

Hemorrhage is the second leading cause of death in trauma patients, exceeded only by deaths caused by traumatic brain injuries. If the shock state becomes severe, the survival rate is less than 50%, which highlights the need for early detection and proper management of hemorrhagic shock. With organized trauma care systems. Early death from hemorrhage is now much less common, but the sequelae of hemorrhagic shock in the form of infection, organ failure, and prolonged illness is encountered with much greater frequency.[3]

Hemorrhagic shock is a clinical condition of reduced organ perfusion, which leads to an inadequate delivery of oxygen and nutrients necessary for normal tissue, cellular function, and it is frequently caused by trauma, gastrointestinal bleeding and surgery. When compensatory mechanisms like tachycardia and increased stroke volume fail to restore the consequences of blood loss, hemorrhagic shock can lead to serious organ damage and even death.[4]

The hallmark of compensated shock is maldistribution of blood flow and tissue oxygenation. The blood flow to the vital organs such as the heart and brain is preserved on the expense of blood flow to less vital organs particularly the renal and splanchnic perfusion.[5] This reduction is due in part to the selective effect of endogenous vasoconstrictors on the splanchnic vascular bed, and also to the relatively high demand for oxygen by the heart and the brain.[6]

The intestinal tract is highly susceptible to hypoperfusion because of its greater level of critical oxygen delivery and counter-current microcirculation of the villi.[7] There is increasing evidence that persistent occult gastrointestinal hypoperfusion within the first 24 hours after injury leads to functional and structural changes in gut mucosa, with loss of gut barrier function leading to translocation of endotoxins and bacteria into the circulation, which plays an important role in initiation of systemic inflammatory response syndrome (SIRS) with subsequent multiple organ failure which is a major cause of late death in trauma patients.[8] This has been referred to as the gut hypothesis of sepsis and multiple organ dysfunction syndrome (MODS).[9]

Hypovolemia from hemorrhage results in intestinal ischemia and subsequent apoptosis of epithelial cells.[10] This results in epithelial shedding and loss of the attached mucin. Together the mucin and epithelial tight junctions form the mucosal barrier that is responsible for keeping the intestinal contents, including pancreatic digestive enzymes and digested food particles, compartmentalized in the intestine's lumen. Failure of the barrier as a result of ischemia allows the contents of the intestine to penetrate into the wall of the intestine and contribute to further intestinal damage.[11]

The primary function of the intestine is the digestion and uptake of nutrients and water and electrolyte exchange.[12] To that end, the intestine is equipped with an epithelial lining that is characterized by a pattern of densely packed invaginations termed crypts, which, in the small intestine, give rise to long, finger-like protrusions known as villi. The different cell types of the epithelial lining all originate from multipotent stem cells, which reside deep in the intestinal crypts. These highly proliferating cells allow renewal of the villus epithelium every 4-5 days.[13]

The intestinal epithelium forms a second crucial physical barrier against the penetration of microorganisms. It consists of a cohesive monolayer of columnar epithelial cells, which are tightly sealed

by junctional complexes to prevent translocation of pathogens through the paracellular pathway.[14] Intestinal epithelial cells adhere to one another through junctional complexes located at the lateral membrane. An interepithelial junction consists of four components: tight junctions, adherens junctions, desmosomes and gap junctions. Tight junctions and adherens junctions are collectively referred to as the apical junctional complex (AJC), which is the most apical intercellular junction. Both are associated with the actin cytoskeleton of epithelial cells. The AJC regulates cell-cell adhesion, paracellular permeability and cell polarity.[15] Tight junctions, or zonula occludens (ZO), represent the most important component of an intercellular junctional complex. They consist of protein complexes that connect the apical lateral membrane of two epithelial cells. Tight junctions are attached to actin and myosin filaments by interaction with intercellular proteins. Adherens junctions, or zonula adherens, are a region of the plasma membrane where cadherin molecules and actin filaments are densely associated.[16]

A large body of evidence underlines the importance of intestinal barrier function. First, studies showing that mice deficient in MUC2, which is the structural component of the mucus layer, have an impaired mucus barrier function. These mice develop spontaneous chronic large bowel inflammation, and exhibit increased susceptibility to infections caused by commensal and pathogenic microbiota. Apart from reduced mucus quantity, also decreased quality of the mucus layer as a consequence of decreased oligomerization and glycosylation is associated with the development of intestinal inflammation.[17] Second, disruption of either epithelial cells or the tight junctions has been linked to a variety of diseases, including inflammatory bowel disease and celiac disease. Moreover, recent studies showed that barrier function loss in patients with decreased intestinal perfusion following (major) surgery, trauma and shock is associated with the development of complications, including sepsis and multiple organ failure.[18,19]

In conclusion, maintenance of any of the intestinal barriers is of the most importance, since penetration of commensal and pathogenic bacteria towards the lamina propria and the circulation induce severe systemic inflammation which potentially leads to multiple organ failure and death.[20]

One of the frequently observed events that can potentially harm the intestinal barrier, intestinal ischemia-reperfusion (IR). To some extent intestinal IR can occur as part of normal physiology. In exercising healthy individuals, redistribution of blood flow to the vital organs and muscles can lead to significant intestinal hypoperfusion.[21] Based on etiological background, intestinal ischemia is divided into a chronic and an acute form. Chronic gastrointestinal ischemia (CGI) is most commonly due to atherosclerotic disease. Acute intestinal ischemia is a consequence of a rapid reduction in intestinal blood flow, caused by severe hypoperfusion (non-occlusive mesenteric ischemia (NOMI)) of mesenteric vessels or occlusion (occlusive disease). [22]

Acute intestinal ischemia is a potentially fatal clinical emergency with an overall mortality of 60% to 80%. Mortality rates are especially high when ischemia progresses towards bowel necrosis, leading to a severe inflammatory response, sepsis and shock. It is remarkable that although medical practice has evolved considerably, the mortality rates of intestinal ischemia did not improve over the past 70 years. The first and most important cause is the continued difficulty to recognize intestinal ischemia at an early stage, which is the key to reducing high morbidity and mortality.[23]

This diagnostic delay is due to the nonspecific clinical presentation and the lack of early, non-

invasive diagnostic markers for intestinal ischemia. The second major reason for intestinal ischemia related high morbidity and mortality is the paucity in preventive and/or therapeutic options. Obviously, rapid reperfusion after intestinal ischemia is of major importance, but reperfusion can paradoxically also contribute to tissue injury and severe inflammation.[24]

Hemorrhagic shock is a frequent clinical manifestation caused by trauma, gastrointestinal bleeding and major surgery. This is particularly important, since intestinal epithelial wounding occurs regularly as a result of physiologic shedding of apoptotic cells or minor trauma, inflammation and ischemia. Moreover, intestinal ischemia is an important mechanism of intestinal barrier integrity loss during major surgery, trauma and shock, which accounts for significant morbidity and mortality.[25]

Intestinal epithelial barrier integrity can be assessed by evaluating structure and function of the tight junctions (TJs). The morphological structure of the TJs can be examined in intestinal mucosal biopsies using freeze fracture, and transmission electron microscopy.[26] Furthermore, expression of TJs and its associated proteins in tissue samples and cell cultures can be evaluated at the protein level by immunohistochemistry, immunocytochemistry and Western blotting, and at gene level by means of quantitative polymerase chain reaction.[27]

The key feature of short intestinal ischemia is the appearance of subepithelial spaces. These spaces have been described previously in animal studies and are classically called the spaces of Gruenhagen. The subepithelial spaces are the consequence of active contraction of the basement membrane and detachment of the upper, ischemically damaged, epithelial cells from its basal membrane. The villus tip cells are more susceptible to ischemia due to countercurrent flow in the villi, and due to the more pro-apoptotic characteristics that enterocytes obtain as they migrate towards the tip of the villus.[28]

Directly after ischemia, leakage of cytosolic proteins from damaged epithelial cells at the villus tips into the subepithelial spaces can be observed. This explains the rapid appearance of markers for intestinal epithelial damage in the circulation, directly after ischemia. [29]

This results in prolonged exposure of lamina propria immune cells to pathogen associated molecular patterns (PAMPs: microbiota and their pro-inflammatory products) and damage associated molecular patterns (DAMPs: mainly cytosolic proteins leaking from damaged cells) which both elicit inflammation.[30] Consequently, increased mRNA expression of pro-inflammatory cytokines TNF-α and interleukins, occurs indicating release of these cytokines from the gut.[31] TNF- α expression is increased in response to hypoperfusion and nutrient deprivation, this increase is associated with mucosal atrophy and loss of epithelial barrier function.[32]

Because a large amount of microbes colonize in the gut, blood from the intestine contains not only products of digestion but also microbial products. Therefore, the liver, the initial site of filtration of gut-derived products, is susceptible to the exposure to the microbial products from the gut, such as lipopolysaccharide (LPS). [33] Lipopolysaccharide (LPS), glycolipids derived from the outer membrane of gram-negative bacteria, is a potent activator of immune responses: very tiny amount of LPS can induce the manifestations of septic shock in human.[34]

In normal conditions, translocation of the microbial products from the gut to extra intestinal space, including systemic circulation, is effectively prevented by our defense mechanisms: the barrier function of the gut and cleansing, detoxifying function of the liver.[35] However, disruption of these

defense mechanisms can lead bacterial translocation to extra intestinal space and aberrant activation of immune system, which can trigger harmful sepsis or chronic inflammation. [36]

Bacterial translocation is defined as the migration of bacteria or bacterial products from the gut to the extra intestinal space.[37] Because gut barrier system by intestinal epithelial cells prevent translocation of large amounts of bacteria and bacterial products from the gut, only very small amount of them can reach the liver in a healthy state.[104] However, this effective gut barrier function can be disrupted by various pathological conditions and this disruption leads to bacterial translocation.[38]

Previous studies suggested that LPS significantly induces the mRNA expression of inflammation some components, including TNF- α, caspase-1, pro-IL-1β and pro-IL-18 via NF κ B activation.[39,40]

Material and Methods:

I. Experimental animals:

This study was carried out on 40 male Wistar albino rat four –six months old with body weight (250-300g). The rats were fed on standard diet with free access to tap water. Animals were maintained under controlled conditions of temperature and humidity with regular 12 hrs light dark cycle. All procedures involving the animals were conducted in accordance with the protocol of animal care approved by the Ethics Committee, Faculty of Medicine, Alexandria University.

II. Non-lethal hemorrhagic shock:

A non-lethal hemorrhagic shock model was used for this experiment. After anaesthesia with ether, the femoral artery was dissected after shaving. Hemorrhagic shock was induced by withdrawal of 2.1 ml blood per 100 gram of body weight. Rats were sacrificed by decapitation at several time points (30, 60, 90 minutes) after shock. Control rats were sacrificed without intervention. [41]

III. Experimental design:

Rats were divided randomly into four main groups:

Group 1 (Control group): It consisted of 10 normal healthy rats served as control and sacrificed without intervention.

Group 2 (30 minutes group): Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock. Rats were sacrificed (30minutes) after induction of the hemorrhagic shock.

Group 3 (60 minutes group): Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock. Rats were sacrificed (60minutes) after induction of the hemorrhagic shock.

Group 4 (90 minutes group): Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock and sacrificed (90 minutes) after induction of the hemorrhagic shock.

IV. Blood and Tissues Sampling

Blood collection and serum preparation:

The rats were anaesthetized with ether inhalation, the anterior abdomen was incised, liver was exposed, blood samples were collected from abdominal aorta into a clean dry non- heparinized

Wassermann tubes for separation of serum. The serum was separated by centrifugation at 3000 rpm for 15 minutes and was aliquoted into 2 samples then stored at – 80°C until assayed for measuring the following parameters:

1. Serum tumor necrosis factor-α (TNF-α) by ELISA Kit. [42]
2. Determination of rat serum lipopolysaccharide (LPS) concentration using enzyme linked immunosorbant technique (ELISA).[43]

Intestinal tissues preparation:

After the abdomen was opened as described above distal segment of the ileum was excised immediately and rinsed with ice-cold saline solution. Parts of ileum segment of each rat were fixed in 10% neutral buffered formalin for histopathological study.

The remaining part of the ileum was homogenized in 0.9% saline, then centrifuged at 400xg for 15minutes and the supernatants were decanted and stored at -80°C until used for: PCR for gene expression of intestinal Zonula Occludens-1(ZO-1).[44]

V. Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[45] Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. The distributions of quantitative variables were tested for normality. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparisons between more than two populations were analyzed F-test (ANOVA) to be used and Post Hoc test (TUKEY). For abnormally distributed data, comparison between more than two groups using Kruskal Wallis test and pair wise comparison was assessed using Mann-Whitney test. Significance of the obtained results was judged at the 5% level.[46]

Results:

Histopathological assessment of the intestinal tissues: (Table I, Figures 1, 2):

Control rats showed a normal intestinal structure. Intact villous mucosal surface epithelium, with average number of goblet cells and mucin content, mucosal, submucosa, muscle and serosal layers were unremarkable and inflammatory cellular infiltrate was not detected. (Fig 2 A).

Rats sacrificed 30 minutes after shock showed a mild extension of the subepithelial space with focal loss of the surface epithelium and inflammatory cells (neutrophils) were seen scattered in lamina propria. (Fig 2 B).

More focal loss of epithelium and mucosal infarction (loss of variable amounts of lamina propria) were noticed in rat sacrificed 60 minutes after hemorrhagic shock, and more inflammatory cells (neutrophils) were seen within the lamina propria. (Fig 2 C).

However, most extensive injury was seen 90 minutes after hemorrhagic shock. There was extensive loss of the surface epithelium, focal mucosal infarction, a massive lifting down sides of the villi, some villus tips are denuded and epithelial cells are severely injured. Inflammatory cells (neutrophils) are seen within the lamina propria and extending to the submucosa. (Figure 2 D).

According to the intestinal ischemia grading system there was significant difference between all groups in comparison to the control where $P<0.001$.

Table (I): Comparison between the studied groups according to grading of the intestinal Ischemia

Grading of the intestinal Ischemia	Control (n =10)		30 min (n =10)		60 min (n =10)		90 min (n =10)		χ^2	^{MC}p
	No.	%	No.	%	No.	%	No.	%		
0	10	100.0	7	70.0	0	0.0	0	0.0	44.840*	<0.001*
1	0	0.0	3	30.0	8	80.0	1	10.0		
2	0	0.0	0	0.0	2	20.0	9	90.0		

χ^2: Chi square test MC: Monte Carlo test *: Statistically significant at $p \leq 0.05$

0: No acute inflammatory cellular infiltrate

1: scattered inflammatory cells (neutrophils)

2: more inflammatory cells (neutrophils) are seen within the lamina propria

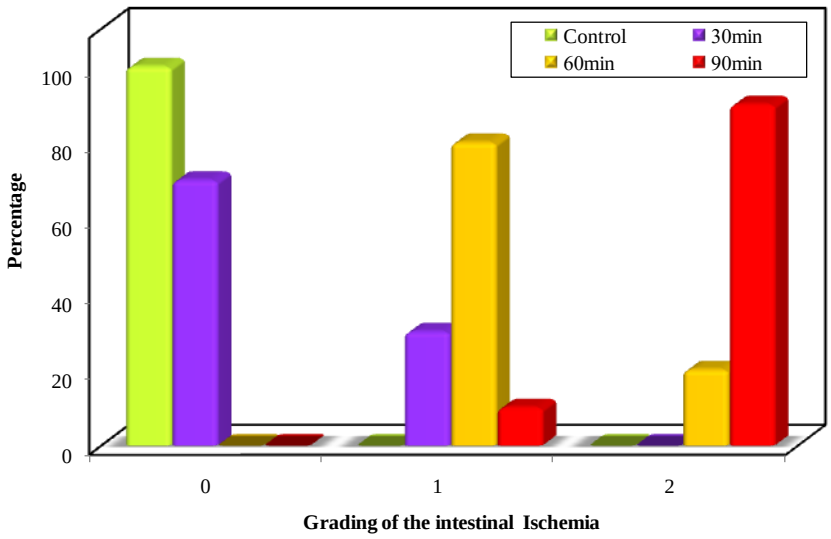

Figure (1): Comparison between the studied groups according to grading of the intestinal Ischemia

PCR for gene expression of intestinal Zonula Occludens-1(ZO-1) in different studied groups (Table I1, Fig 3-6):

All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized with a UV transilluminator. Semi-quantitation was performed using a gel documentation system (BioDO, Analyser). Bands were photographed with a Kodak DC120 digital camera.

The mean values for the ZO-1 cDNA band intensities were 1.46 ± 0.13, 2.16 ± 0.17, 3.93 ± 0.13 and 6.86 ± 0.17 in the control, 30 min, 60 min and 90 min groups respectively.

A significant difference was detected among the studied groups of hemorrhagic shock as compared to the control one where P<0.001.

A significant difference was detected in 90 min group as compared to 60 min and 30 min group where P<0.001, also 60 min group showed significant difference in comparison with 30 min group where P<0.001.

The percentage of changes increase dramatically in relation to the time of the hemorrhagic shock to reach 369.9% in the 90 minutes shock group.

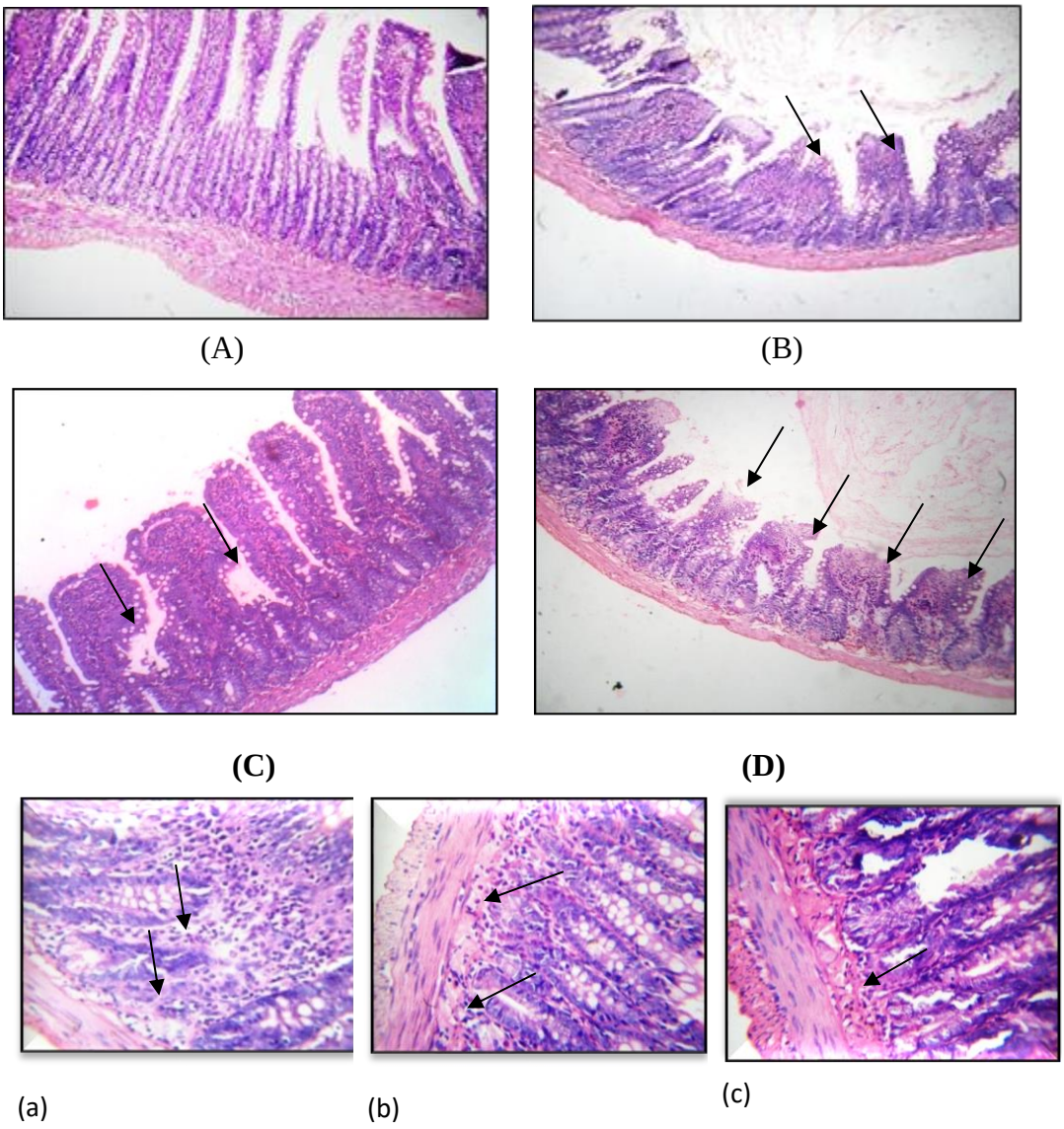

Fig. (2): H/E staining of intestinal sections (H/E × 100) (A): Control rats show intact villi, (B): Rats sacrificed 30 min. after H/S show a mild extension of sub epithelial space, (C): Rats sacrificed after 60 min show loss of epithelium and mucosal infarction, (D): Rats sacrificed 90 min after H/S show extensive loss of epithelium and massive lifting down side of the villi. High power view (H/E × 400) showing neutrophilic infiltrate in (a) mucosa, (b) sub mucosa, (c) intralamnia propria.

Table (II): Comparison between the studied groups concerning Intestinal ZO-1 PCR

	Control (n =10)	30 min (n =10)	60 min (n =10)	90 min (n =10)	F	p
Intestinal ZO						
Range	1.27 – 1.65	1.90 – 2.38	3.70 – 4.12	6.52 – 7.05	2509.331*	<0.001*
Mean	1.46	2.16	3.93	6.86		
± SD.	0.13	0.17	0.13	0.17		
% of change		↑47.9	↑169.2	↑369.9		
$p_{Con.}$		<0.001*	<0.001*	<0.001*		
Sig. bet. grps	p_1 <0.001* , p_2 <0.001* , p_3<0.001*					

F: F test (ANOVA)
p_{Con}: p value for Post Hoc Test (Tukey) for comparing between Group I with each other groups
p_1 : p value for Post Hoc Test (Tukey) for comparing between group II and group III
p_2 : p value for Post Hoc Test (Tukey) for comparing between group II and group IV
p_3 : p value for Post Hoc Test (Tukey) for comparing between group III and group IV
*: Statistically significant at $p \leq 0.05$

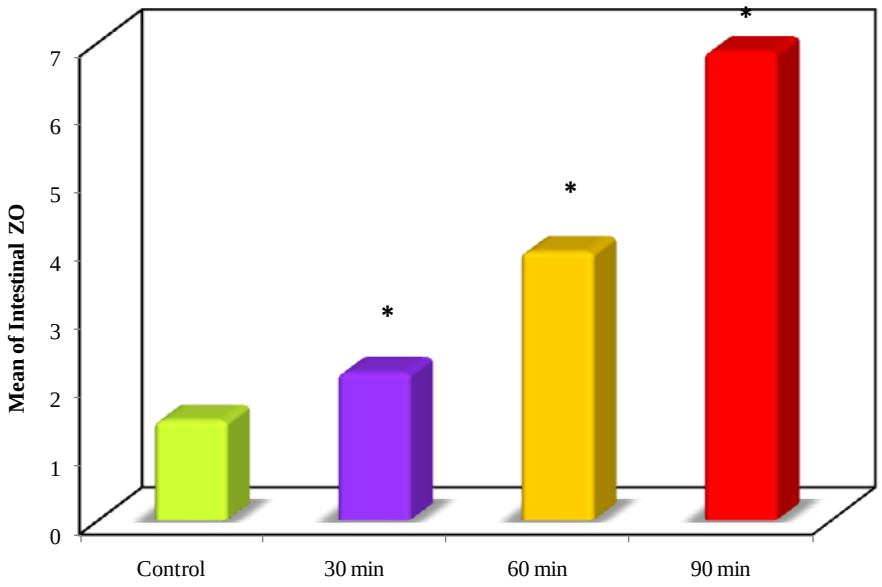

Figure (3): Comparison between the studied groups concerning Intestinal ZO-1 PCR

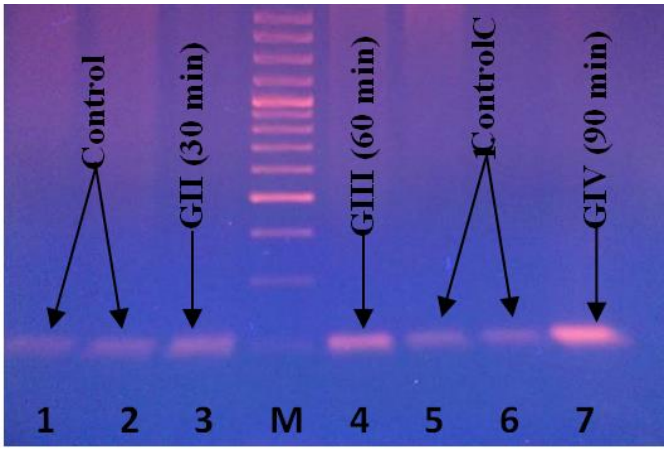

Figure 4: Agarose gel electrophoresis profiles showing PCR products of intestinal ZO gene.

Lane 1, 2, 5, 6: Group I (control), Lane 3 Group II (30 min), Lane 4: Group III (60 min), Lane 7: Group IV (90 min), M: DNA size marker 50 – 1000 bp.

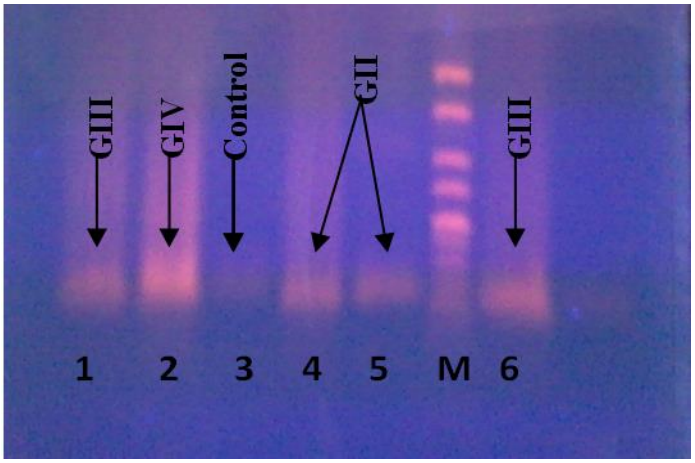

Figure 5: Agarose gel electrophoresis profiles showing PCR products of intestinal ZO-1 gene. Lane 3: Group1 (control), Lane 4, 5 Group II: 30 min, Lane 1, 6 Group III (60 min), Lane 2 Group IV (90 min), M: DNA size marker 50 – 1000 bp

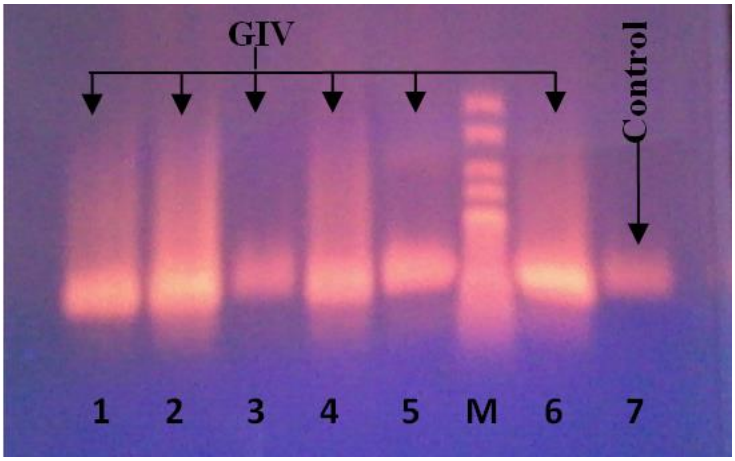

Figure 6: Agarose gel electrophoresis profiles showing PCR products of intestinal ZO-1 gene. Lane 7: Group I (control), Lane 1-6: Group IV (90 min group), M: DNA size marker 50 – 1000 bp.

Serum TNFα level (pg/ml) of the different studied groups (Table III, Fig 7):

To study systemic inflammation, the plasma concentration of TNF-α was measured by performing an ELISA and the mean values for TNFα level were 11.62(pg/ml) ± 3.42, 11.98(pg/ml) ± 1.73, 18.08(pg/ml) ± 2.06 and 23.71(pg/ml) ± 6.52 for the control, 30, 60 and 90 minutes groups respectively. TNF-α concentration is significantly increased 60 and 90 minutes after hemorrhagic shock as compared to control rats ($P<0.001$).

Rats sacrificed 30 minutes after hemorrhagic shock revealed no significant difference as compared

with the control group.
TNFα serum level after 90 minutes of hemorrhagic shock (group IV) was the highest one with significant difference (P<0.001) and the percentage of change reach 104.04%.
A significant difference was detected in 90 min group as compared to 60 min and 30 min group where P<0.045, P<0.001, also 60 min group showed significant difference in comparison with 30 min group where P<0.001.

Table (III): Comparison between the studied groups concerning serum levels of TNF alpha (pg/ml)

	Control (n =10)	30 min (n =10)	60 min (n =10)	90 min (n =10)	$^{KW}\chi^2$	p
TNF α (pg/ml)						
Range	8.70 – 18.70	9.60 – 15.0	14.90 – 21.10	12.70 – 31.70	25.694*	<0.001*
Mean	11.62	11.98	18.08	23.71		
±SD.	3.24	1.73	2.06	6.52		
% of change		↑3.10	↑55.59	↑104.04		
$p_{Con.}$		0.290	0.001*	0.001*		
Sig. bet. grps	p_1 <0.001*, p_2= 0.001*, p_3= 0.045*					

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
p_{Con}: p value for Mann Whitney test for comparing between Group I with each other groups
p_1 : p value for Mann Whitney test for comparing between group II and group III
p_2 : p value for Mann Whitney test for comparing between group II and group IV
p_3 : p value for Mann Whitney test for comparing between group III and group IV
*: Statistically significant at $p \leq 0.05$

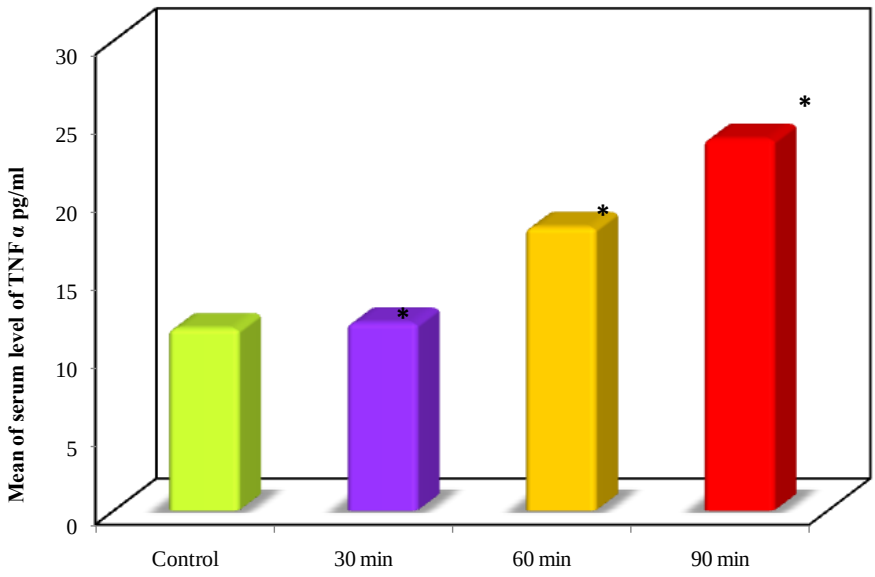

Figure (7): Comparison between the studied groups concerning serum levels of TNF- alpha

(pg/ml)

Serum lipopolysaccharide (LPS) level (ng/ml) of the different studied groups (Table IV, Fig 8):
To evaluate changes in intestinal barrier function, the intestinal permeability was investigated indirectly through measurement of the serum lipopolysaccharide as indicator of bacteria and or bacterial products leakage from the gut to the circulation as a result of damaged intestinal barrier after hemorrhagic shock.

The mean values of the serum LPS concentration for the different studied groups were 116.7(ng/ml) ±21.68, 125.6(ng/ml) ±16.79, 131.4(ng/ml) ±13.34 and 147.3(ng/ml) ± 15.35for the four groups respectively.

There was a significant increase in the LPS level in all groups as compared with the control one P<0.001 and this was in accordance with the time of the hemorrhagic shock, high significant in LPS was detected 90 minutes after the shock P<0.001.

A significant difference was detected in 90 min group as compared to 60 min and 30 min group where P<0.001, P<0.003, also 60 min group showed significant difference in comparison with 30 min group where P<0.026.

Table (IV): Comparison between the studied groups concerning serum levels of LPS (mg/ml)

	Control (n =10)	**30 min (n =10)**	**60 min (n =10)**	**90 min (n =10)**	**F**	**p**
LPS(mg/ml)						
Range	83.50 – 144.0	92.50 – 145.0	115.50 - 157.50	138.50 - 154.50	6.842*	0.001*
Mean	116.7	125.60	131.45	147.14		
± SD.	21.68	16.79	13.34	15.35		
% of change		↑7.63	↑12.64	↑26.08		
$p_{Con.}$		0.026*	0.003*	0.001*		
Sig. bet. grps	p_1= 0.833 , p_2 = 0.018*, p_3 = 0.125					

F: F test (ANOVA)
p_{Con}: p value for Post Hoc Test (Tukey) for comparing between Group I with each other groups
p_1 : p value for Post Hoc Test (Tukey) for comparing between group II and group III
p_2 : p value for Post Hoc Test (Tukey) for comparing between group II and group IV
p_3 : p value for Post Hoc Test (Tukey) for comparing between group III and group IV
*: Statistically significant at $p \leq 0.05$

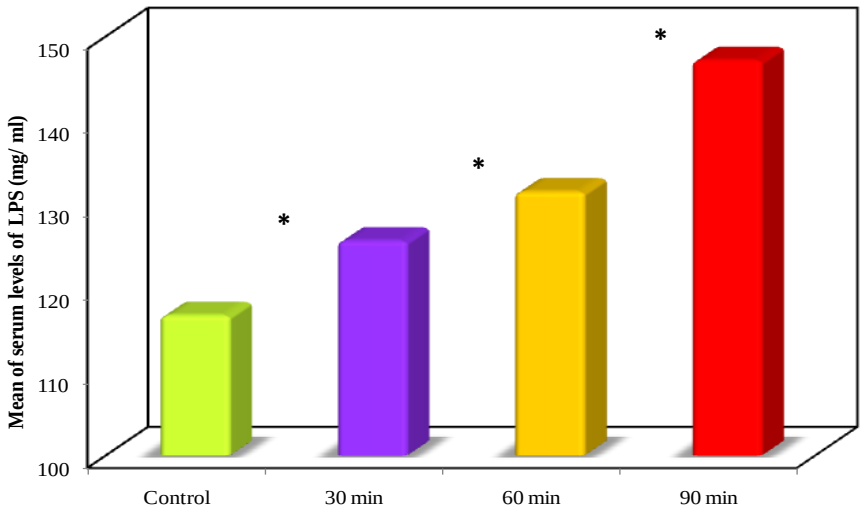

Figure (8): Comparison between the studied groups concerning serum levels of LPS (mg/ml)

Discussion:

Shock is an acute circulatory failure caused by the action of various strong pathogenic factors on the body, which is characterized by circulation disorder, hypoperfusion of important organs and cell function metabolic disorders, thereby causing systemic critical pathological process. Shock is a common clinical Intensive disease, and if not treated timely, it is often life-threatening. (47)

Hemorrhagic shock (HS) is a frequent clinical manifestation in trauma patients. Previous studies have demonstrated that the intestinal barrier function is compromised after hemorrhagic shock.(48, 49) Hemorrhagic shock is induced by massive blood loss after trauma or surgery, which leads to severe reduction in circulating blood volume and tissue hypoperfusion. HS is characterized by a series of physiologic compensatory adjustments to maintain the blood-supply of vital organs like heart and brain aimed at saving the life of the organism. At the same time, the blood supply of the intestine is greatly reduced, and with resuscitation, a reperfusion injury is introduced to cause obligatory fluid sequestration and enhance intestine-derived endotoxemia and exaggerated systemic inflammatory response. (50)

The present study was conducted to explore the effect of hemorrhagic shock on intestinal epithelial barrier function, also to evaluate the role of gut-liver axis in translocation of bacteria from the intestine after tight junction loss to the liver and its effect on the liver functions.

It is hypothesized that the gut is one of the first organs affected by hemorrhagic shock. (51) In the present work data of haematoxylin/eosin staining support this hypothesis. Thirty minutes after shock villi are damaged. Intestinal injury further increases 60 and 90 minutes after shock, at this time villus tips are denuded and epithelial cells are severely injured. We conclude that hemorrhagic shock causes intestinal injury early.

These findings go in line with Zhang et al (52) who investigated direct peritoneal resuscitation on intestinal ischemia-reperfusion injury in rats with hemorrhagic shock, they found that hemorrhagic shock resulted in a significant increase in intestinal injury proportionally to the time of HS. Despite

that they used ischemia reperfusion injury, hemorrhagic shock model was used in this study, and the net results were similar, which support the hypothesis of the early gut injury after hemorrhagic shock.

On the other hand the results of this work are in sharp contrast with previous studies, which showed that after short intestinal ischemia (30 minutes), the epithelial lining remained intact, [53,54] data of this work revealed that this previously observed ability of intestine to persist intestinal IR-induced inflammation is abolished by exposure to prolonged ischemia.

A major function of the gut is to maintain a physical barrier to prevent absorption of toxin, antigen, and microorganisms. However, the gut is highly vulnerable to ischemic insult during the hemorrhagic shock.

One of the early events in the loss of gut wall integrity is tight junction loss. [55] Tight junction is regarded as the most important foundation for maintaining the structure of intestinal mucosal mechanical barrier. ZO-1 protein is one of tight joint structural proteins that found in the surface of the cytoplasmic membrane. [56]

Results of polymerase chain reaction of the ZO-1 gene expression showed significant increase among the studied groups of hemorrhagic shock (30, 60, 90 minutes) as compared to the control one where $P<0.001$. At functional level, these results are in line with those of Xiaoguang Lu et al [57] expression of ZO-1 protein is significantly down regulated, after HS , indicating that HS leads to the minute structure damages of the intestinal tissue.

In the same way in 2013 Demehri FR et al, [58] observed that total parenteral nutrition as treatment in hemorrhagic shock is associated with loss of intestinal epithelial barrier function and increased rate of infectious complications, A mouse model of total parenteral nutrition when compared to the control one revealed that total parenteral nutrition induced changes that result in a pro-inflammatory state within the intestinal mucosa, leading to villous atrophy, an increase in ZO-1 disruption and barrier dysfunction.

As regard the results of the serum TNF-α: The data of the present work showed that systemic TNF-α levels are significantly increased in group 60 and 90 minutes after hemorrhagic shock. This observation suggests that intestinal hypoperfusion (ischemia) plays a role in local and systemic inflammation, caused by hemorrhagic shock. In turn, sepsis can cause damage to other organs. In this manner, the gut could be involved in the induction of injury to other organs during hemorrhagic shock.

The above findings are running in parallel with Zhang et al [59] where they investigated the effect of hemorrhagic shock in murine model and reported that hemorrhagic shock induces a surge of inflammatory markers, including tumor necrosis factor-α (TNF-α) and interlukin-6, which have been shown to be a reliable early indicator of morbidity and mortality in a variety of clinical conditions. [60] Assessment of intestinal barrier permeability to large antigenic molecules such as bacterial endotoxins lipopolysaccharide (LPS) and dietary proteins is becoming important in the understanding of the pathogenesis of gastrointestinal and autoimmune diseases.

Scientific evidence indicates that many gastrointestinal and autoimmune disorders are accompanied by an increased translocation of endotoxins and other bacterial toxins from aerobic

and anaerobic bacteria through the gut wall. [61]

In the present study, results regarding serum levels of lipopolysaccharide showed that significant increase in the all groups of the hemorrhagic shock (group 30, 60 and 90 minutes group), the highest significant increase in LPS was detected 90 min after the HS with $P<0.001$. The reason therefore could be that at this time point epithelial cell injury and tight junction losses are both present, resulting in an increase of intestinal permeability.

These data are in agreement with a previous work which demonstrated that bacterial translocation to mesenteric lymph nodes due to gut injury, increase intestinal permeability occurred after the resuscitation in a rat model of hemorrhagic shock/ resuscitation. [62]

Recent study has demonstrated that LPS level increase significantly after hemorrhagic shock and it leads to enhance the innate immune system for more production of tumor necrosis factor and sever inflammatory response. [63]

Conclusions:

From the results of the present study, it could be concluded that prolonged intestinal ischemia results in extended loss of intestinal barrier integrity with exposure to both intraluminal pathogens and intracellular components leaking from damaged enterocytes. The subsequent inflammatory response is characterized by increase in cytokines production and bacterial and/or bacterial products translocation. These data allow future translational studies to investigate targeted therapy to reduce intestinal IR-associated high morbidity and mortality.

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