Original Research

Fetuin-A exerts a protective effect against experimentally induced intestinal ischemia/reperfusion by suppressing autophagic cell death

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Impact statement

The morbidity and mortality rates following intestinal I/R injury are increasing despite the clinical trials of usage of drugs regulating the inflammation and oxidative stress. However, the potential protective effect of fetuin-A against intestinal I/R injury and its defensive or detrimental modulation of autophagic response remains elusive. In this study, fetuin-A had an influence against intestinal I/R injury. As it improved the amplitude of spontaneous jejunal contraction and improved mucosal histopathological disturbances, it could be related to its antioxidant and anti-inflammatory properties in jejunum. Our outstanding observation is that fetuin-A reversed jejunum autophagy disturbance which was characterized by increased beclin-1 and LC3 with decreased p62 expression that could add a new mechanism of action for this hepatokine. These findings suggest that fetuin-A could be a promising potential therapeutic medication against intestinal I/R complications, and targeting autophagy signaling pathways could have a critical role in preventing and/or treating of this condition.

Abstract

Intestinal tissue is highly susceptible to ischemia/reperfusion injury in many hazardous health conditions. The anti-inflammatory and antioxidant glycoprotein fetuin-A showed efficacy in cerebral ischemic injury; however, its protective role against intestinal ischemia/reperfusion remains elusive. Therefore, this study investigated the protective role of fetuin-A supplementation against intestinal structural changes and dysfunction in a rat model of intestinal ischemia/reperfusion. We equally divided 72 male rats into control, sham, ischemia/reperfusion, and fetuin-A-pretreated ischemia/reperfusion (100 mg/kg/day fetuin-A intraperitoneally for three days prior to surgery and a third dose 1 h prior to the experiment) groups. After 2 h of reperfusion, the jejunum was dissected and examined for spontaneous contractility. A jejunal homogenate was used to assess inflammatory and oxidative stress enzymes. Staining of histological sections was carried out with hematoxylin, eosin and Masson's trichrome stain for evaluation. Immunohistochemistry was performed to detect autophagy proteins beclin-1, LC3, and p62. This study found that fetuin-A significantly improved ischemia/reperfusion-induced mucosal injury by reducing the percentage of areas of collagen deposition, increasing the amplitude of spontaneous contraction, decreasing inflammation and oxidative stress, and upregulating p62 expression, which was accompanied by beclin-1 and LC3 downregulation. Our findings suggest that fetuin-A treatment can prevent ischemia/reperfusion-induced jejunal structural and functional changes by increasing antioxidant activity and regulating autophagy disturbances observed in the ischemia/reperfusion rat model. Furthermore, fetuin-A may provide a protective influence against intestinal ischemia/reperfusion complications.

Keywords: Ischemia/reperfusion, fetuin-A, autophagy, inflammation, jejunum, antioxidant

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Introduction

Ischemia/reperfusion (I/R) injury happens on triggering tissue injury with restoration of the blood flow to ischemic tissues or organs. The small intestine is the most susceptible organ to I/R in cases of hemorrhagic shock and trauma. The pathophysiological mechanisms of intestinal I/R injury are highly complex, as intestinal local tissue necrosis stimulates the release of oxygen free radicals and inflammatory mediators and enhances energy metabolism disturbance.[1] Intestinal I/R injury results in a serious clinical problem. Therefore, its prevention has become the target of clinical research. At present, drugs regulating the activity

of macrophage and oxidative stress are implicated in the clinical management of intestinal I/R injury. However, these drugs are not ideal, and their use has not decreased the mortality rate of intestinal I/R.[2]

Autophagy is a self-degradative cellular procedure whereby cells self-digest cytoplasmic components, such as misfolded proteins, aggregated proteins, and damaged organelles, to counteract toxic or damaged products. This aids in organelle recycling and maintains cellular homeostasis in cases of cellular stress.[3]

It has become evident that autophagy is involved in pathophysiological cellular alterations. Recent reports have shown the interplay between oxidative stress and autophagy pathways in multiple diseases.[4] Interestingly, autophagy was found to be enhanced in intestinal mucosa following intestinal I/R injury.[5]

Fetuin-A is a multifunctional glycoprotein that is mainly secreted by hepatocytes. It has been shown that fetuin-A acts as a negative acute-phase protein that increases phagocytosis and regulates neutrophil function.[6] Fetuin-A was also found to inhibit the release of proinflammatory mediators induced by bacterial endotoxins in macrophage cultures.[7] Interestingly, fetuin-A is considered a prognostic biomarker in ischemic stroke patients.[8] Additionally, fetuin-A protects against early cerebral ischemic injury by attenuating the brain inflammatory responses.[7]

Moreover, exogenous fetuin-A was reported to target autophagosomes localized in the cytoplasmic punctate structures in close proximity to the autophagy marker LC3 and can modulate the autophagic response under inflammatory conditions.[9]

Since the potential protective role of fetuin-A in intestinal I/R injury remains unknown, our aim is to investigate the influence and the probable pathophysiological mechanism/s of fetuin-A administration on jejunal mucosal damage and contractile response in an experimental rat model of intestinal I/R.

Materials and methods

Animals

Seventy-two adult male *Rattus norvegicus domestica* (body weight, 240–280 g) were bought from the Laboratory Animal House (College of Veterinary Medicine, Zagazig University, Egypt). The rats were preserved in individual cages at room temperature and maintained under a natural (light-dark) cycle in the animal house at the Faculty Medicine, Zagazig University, with free access to chow and water. The rat experiments were approved by the Institutional Animal Care and Use Committee of Zagazig University (approval no: ZU-IACUC/3/F/5/2020), and all experimental maneuvers were according to the guidelines documented in the Guide for the Care and Use of Laboratory Animals.

Experimental groups

Assuming that mean TNFα in sham group versus I/R group are 257 ± 90 versus 400 ± 340 (pg/100 g tissue), at confidence level 95% and power 80%, total sample size is 72 (18 in every group). Calculated by OpenEpi.[10]

Following an accommodation phase, 72 male rats were distributed in equal groups ($n = 18$), namely, a control (healthy control, did not receive medication or surgical interference), sham-operated vehicle-pretreated group, I/R vehicle-pretreated group, and fetuin-A-pretreated I/R group. The rats in the I/R vehicle-treated group were anesthetized, and laparotomy was performed. Then, ischemia was induced for 1 h, and reperfusion was allowed for 2 h. Rats in the fetuin-A-pretreated I/R group were treated with fetuin-A (100 mg/kg/day intraperitoneally (i.p.))[11] for three days prior to the day of surgery, and a fourth dose was given 1 h prior to the experiment. The surgical procedure was as that for the I/R vehicle-treated group.

This dose was selected based on the work of Li *et al.*[12] who found that such a dose was able to produce a minimal tissue level of 100 mg/mL fetuin-A (assuming even distribution in all tissues, including bone, muscle, blood, and others) that could stimulate the formation of autophagosomes and inhibit the inflammation in animal models of lethal endotoxemia and sepsis. With regard to the safety of the fetuin-A, the dose (100 mg/kg) produced no detected pathological damage to the liver, kidney, or heart of rats.[11]

Additionally, the drug administration protocol followed that of Patel *et al.*[13] who reported that the degree of protection was dependent on the timing of drug administration considering that pretreatment for three days proved to be more effective than a single treatment at the time of surgical intervention in animal model of renal I/R pretreated with erythropoietin. However, we added an additional dose 1 h before surgery to maintain serum fetuin-A levels in rats

Induction of intestinal I/R model

After fasting for 12 h, the rats were anesthetized with xylazine hydrochloride (10 mg/kg, i.p.) and ketamine (75 mg/kg, i.p.) (in the same syringe) under aseptic conditions.[14] A midline laparotomy was done to access intestinal region. The superior mesenteric artery (SMA) was recognized and exposed and reversibly obstructed by a traumatic arterial lock. Then, intestinal ischemia was allowed for 1 h, which was recognized by the complete loss of mesenteric artery pulse and the color of intestine immediately altering pale. Throughout the period of ischemia, body temperature was kept by locating the rats under a heater. The incision was protected by aseptic gauze to avoid bacterial contamination. After 1 h of ischemia, reperfusion was allowed for 2 h, and the incision was closed. The Sham group underwent the same surgical maneuver without SMA closure.[15]

Euthanization of the rats using anesthetic overdose (three times the anesthetic dose)[14] was performed at the end of the reperfusion period. Then jejunum was dissected and cleaned with isotonic saline and then divided into three pieces of 2–3 cm each. One jejunum strip was used to analyze spontaneous contractility. The second strip was fixed in 10% neutral buffered formalin solution for 24–48 h for histological assessment. The third strip was preserved at −80°C for subsequent biochemical examination.

Contractility analysis of isolated jejunum

A 2-cm-long jejunal strip was isolated; its contents were rinsed off using the Tyrode's solution. Then, the strip was mounted vertically in a 5 mL Panlab Multi Chamber Organ Bath (AD Instruments, Australia) continuously perfused with Tyrode's solution and bubbled with 95% O_2/5% CO_2 at 37°C. The pH was kept at 7.40 throughout the experiment with a four-channel data achievement system (PowerLab 4/30, AD Instruments) that was connected to a computer device. Data were saved using the Chart 7.2 program. The constant recording time was up to 30 min. The contractile frequency (per min) and amplitude (mm) were recorded, calculated, and compared.

Tissue homogenate preparation for biochemical analysis

Jejunum tissue was homogenized in ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.4), and then the homogenate was centrifuged for 10 min at $1.500 \times g$. The supernatant was separated and stored at −20°C for analysis. Interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) levels were determined using a corresponding rat enzyme-linked immunoassay kit (Bender MedSystems GmbH, Vienna, Austria), according to Yasuoka *et al.*[16] and Engelberts *et al.*,[17] respectively. Malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity were determined colorimetrically using the corresponding rat assay kits (Elabscience, Inc., China), as per Ohkawa *et al.*[18] and Kakkar *et al.*,[19] respectively.

Histopathological evaluation of jejunal injury

Tissue samples were taken from the jejunum for histopathological evaluation. The samples were fixed in formalin solution and in paraffin. The block was sectioned at a 4–5 μm thickness, and the sections were mounted on glass slides, deparaffinized in xylene, and stained with hematoxylin and eosin stain (H&E). Masson's trichrome stain was used to identify collagen fibers so that the amount of fibrosis in the jejunum sections could be assessed. The stained slides were examined under light microscopy (DM4000; Leica, Wetzlar, Germany).[20]

The histo-pathological assessments were done by two different pathologists in a blinded method. The extent of the intestinal injury was evaluated histopathologically using Chiu's scoring system[21] as follows: normal villus appearance, grade 0; subepithelial Gruenhagen's space (subepithelial spaces/lifting of surface epithelium) and capillary congestion, grade 1; enlarged subepithelial distance and parting of epithelial layer from lamina propria, grade 2; huge epithelial parting and denudation of some villi tips, grade 3; denudation on villi and widened capillary, grade 4; digestion and breakdown of lamina propria with ulceration and hemorrhage, grade 5.

Immunohistochemistry of autophagy markers

The prepared paraffin-embedded intestinal sections (5 μm thick) were heated at 67°C for 45 min. Then, the sections were rehydrated in an arranged series of alcohol and pretreated with an antigen-retrieval technique comprising of heating in a water bath at 90°C for 10 min and cooling for 5 min to recover the antigens. The samples were treated with 3% H_2O_2 for 15 min at 37°C to block endogenous peroxidase activity, and broad binding was blocked with 5% bovine serum albumin at room temperature for 20 min. Finally, an overnight specimen incubation with antibodies against the microtubule-associated protein 1 light chain 3 (LC3) (1:100), beclin-1 (1:100), and p62 (1:200) was done. Then, the jejunal specimens were washed with PBS followed by direct incubation with the secondary antibody (1:500 in PBS) for 30 min. Finally, antibody binding was visualized using a diaminobenzidine kit, and the jejunal specimens were detected under light microscopy. ImageJ software was used to calculate the ratio of the brown-stained area to the total area.[22]

Morphometric analysis

Morphometric analysis was performed by examining three different sections from each animal in all groups using ImageJ software. The villus height was determined by measuring the villi from their base at the level of the crypt's entrance through to their distal tips. Villus width was determined by measuring a villus from one side to the other. Only full finger shaped and well-oriented villi were used (100× magnification). The epithelium length was determined by measuring the jejunal epithelial cells of different villi from the basement membrane to the tip of their microvilli on (400× magnification). Crypt depth was measured from the crypt's base to the nearby villus base (100× magnification). Submucosal thickness was measured at different sites (400× magnification).

Statistical analysis

The collected data were entered to and analyzed by computer using Statistical Package of Social Services, version 25 (SPSS).[23] Shapiro–Wilk test was used to determine the distribution characteristics of variables and variance homogeneity. Normally distributed data were described using mean and SD, while heterogeneous data were described using median and interquartile range (IQR). One-way analysis of variance (ANOVA) and post hoc (LSD) were used to test differences between groups when variances were equal, while Kruskal–Wallis test and Dunn's multiple comparison test were used when equal variances were not present. In all the tests, P value of <0.05 was taken as significant.

Results

Effect of fetuin-A treatment on contractility of isolated jejunum

The spontaneous contractility of the isolated jejunal strips was significantly reduced in both frequency and amplitude in the I/R injury group ($P < 0.005$) when compared with the control and sham groups. Interestingly, fetuin-A

administration significantly increased the amplitude with a non-significant effect on the frequency of the spontaneous jejunal activity when compared with the I/R injury group ($P < 0.005$). However, both the amplitude and frequency of the isolated strips were significantly reduced compared with the control ($P < 0.005$) and sham groups ($P < 0.005$) (Table 1 and Figure 1).

General observations

In control and sham groups, the jejunal loops appear pink in color as in Figure 2(a). But during the period of occlusion, the intestinal region that was deprived of blood flow became pale. When blood flow was restored in the I/R group, the tissue was distinctly congested and dark red in color (Figure 2(b)). The jejunal loops were less congested in

Table 1. Spontaneous basal activity of rat jejunum in the studied groups.

Variables	Control group	Sham group	I/R group	Fetuin-A pretreated I/R group	P
Amplitude (mm)	2.3 (0.1)	2.2 (0.1)	0.9 (0.1)[a,b]	1.9 (0.1)[a,b,c]	<0.005
Frequency (permin.)	17.2 (0.7)	17.0 (0.8)	11.5 (0.5)[a,b]	11.1 (0.1) [a,b]	<0.005

[a]Significant versus control group.
[b]Significant versus sham group
[c]Significant versus I/R group.
P: Significance value of F test; results are presented as mean (SD).
I/R: intestinal ischemia/reperfusion.

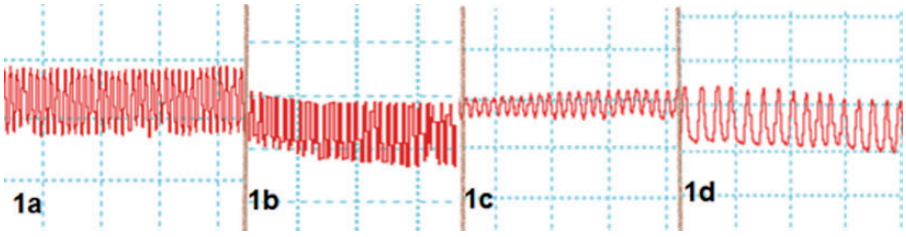

Figure 1. Recorded tracing of spontaneous contractility of isolated jejunal strips in: (a) (control group), (b) (sham group), (c) (I/R group) with significant reduction in the frequency and the amplitude and (d) (Fetuin-A pretreated I/R group) showing significant improvement in the amplitude vs. I/R group. (A color version of this figure is available in the online journal.)

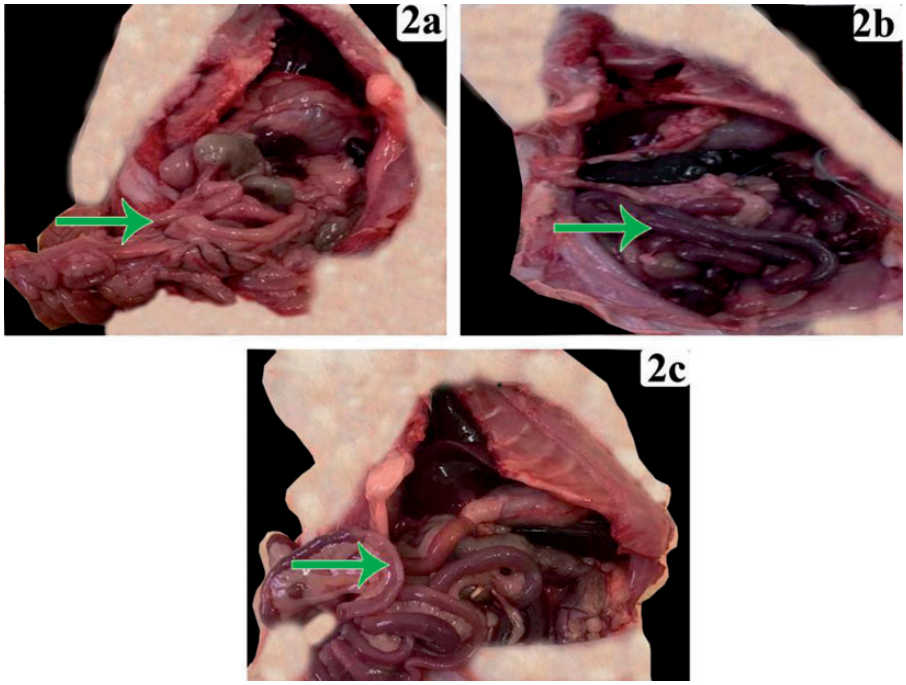

Figure 2. Gross photographs of the jejunum of adult rats from different groups. (a) In the sham/control group: green arrow: normal pinkish-colored jejunal loops. (b) I/R group: green arrow: dark-colored and markedly congested jejunal loops. (c) Fetuin-A-pretreated I/R group: green arrow: dark pink and less congested jejunal loops. (A color version of this figure is available in the online journal.)

the fetuin-A-pretreated I/R group (Figure 2(c)) than in the I/R group, and most regions appeared apparently normal as in control group.

Effect of fetuin-A treatment on oxidative stress and inflammatory markers

I/R injury significantly increased MDA, TNF-α, and IL-1β levels ($P < 0.005$) and significantly decreased SOD levels ($P < 0.005$) in comparison with the control and sham groups. Fetuin-A administration showed a significant decrease in MDA, TNFα, and IL-1β levels ($P < 0.005$) compared with the I/R group, but they were significantly increased when compared with the control group ($P < 0.005$) and sham group ($P < 0.005$). Additionally, fetuin-A treatment significantly increased SOD levels ($P < 0.005$) compared with the I/R group. However, the SOD level was significantly less than those of both groups I ($P < 0.005$) and II ($P < 0.005$) (Table 2). A non-significant difference was detected in all the studied parameters between the control and sham groups ($P > 0.05$).

Histopathological results

There were no detectable histological differences between the control and sham groups. Therefore, they were considered as one group, termed the control group.

Fetuin-A treatment prevented I/R-induced jejunal structure injury

The sham and control groups presented a normal structure under light microscopy in the H&E sections, and the lining of the jejunum showed sequences of permanent circular or semilunar folds consisting of mucosa, submucosa, muscularis externa, and serosa (Figure 3(a)).

The entire mucosa of the small intestine was densely covered by short mucosal outgrowths (villi) that projected into the lumen, which was covered by a simple columnar epithelium of absorptive cells (enterocytes), with many scattered goblet cells. Each villus had a central loose connective tissue that extended from the lamina propria (Figure 3(b)), with circular intestinal glands lined by Paneth cells underlying the intestinal villi. The muscularis mucosa could be seen below the intestinal glands (Figure 3(c)).

The I/R group showed severe mucosal damage in comparison with the sham group. We observed the development of marked mononuclear cellular infiltrations, degeneration of the epithelium lining the villi which was flattened and damaged causing impairment in the villi integrity. Widening in submucosal space with obvious blood vessels can be observed (Figure 3(d) to (f)).

The fetuin-A-pretreated I/R group showed restoration of the normal intestinal mucosa, and the injury was much less severe relative to the I/R group. The epithelial lining with goblet cells was easily seen (Figure 3(g) to (i)).

Morphometric measurements were performed to assess the intestinal injury. There were no histopathological changes in the control group. I/R caused intense injury to the intestinal mucosa, and this was confirmed by a statistically significant decrease in villus length and width, epithelial thickness, and crypt depth as well as a statistically significant increase in submucosal thickness in comparison with the control and sham groups ($P < 0.005$). These damages were remarkably improved in the fetuin-A-pretreated I/R group, which was confirmed by a statistically significant increase in villus length and width, epithelial thickness, and crypt depth as well as a statistically significant decrease in submucosal thickness compared with the I/R group ($P < 0.005$) (Table 3).

Chiu's score was used to assess the intestinal injury after reperfusion and the effect of fetuin-A pretreatment. There were no histopathological changes in the control group and minimal changes in the sham group. I/R caused intense injury to the intestinal mucosa, categorized by ulceration and hemorrhage ($P < 0.005$), compared with the control and sham groups. These damages were remarkably absent in the fetuin-A-pretreated I/R group ($P < 0.005$) compared with the I/R group (Table 4).

Effect of fetuin-A pretreatment on collagen deposition

Masson's trichome staining of the jejunum sections of the control groups showed a normal distribution of collagen fibers in the submucosa. In contrast, fibrotic changes were present in the I/R group, as demonstrated by a significant increase in the submucosal deposition of collagen fibers and indicated by more intense green staining. However, there was a significant decrease in the number of collagen fibers in the submucosa in the sections obtained from the fetuin-A-pretreated I/R group in comparison with those from the I/R group (Figure 4). These results were confirmed both morphometrically and statistically by measuring the percentage area of the green staining which was

Table 2. Biochemical parameters of the studied groups.

Variables	Control group	Sham group	I/R group	Fetuin-A pretreated I/R group	P
MDA (nmol/g protein)	4.1 (0.3)	4.0 (0.4)	6.1 (0.5)[a,b]	4.5 (0.3)[a,b,c]	<0.005
SOD (U/g protein)	38.4 (2.2)	37.1 (3.0)	24.3 (2.9)[a,b]	30.5 (1.6)[a,b,c]	<0.005
TNFα (pg/g tissue)	47.5 (3.1)	48.7 (3.4)	91.0 (5.2)[a,b]	74.5 (5.6)[a,b,c]	<0.005
IL-1β (pg/g tissue)	9.9 (0.8)	10.4 (0.5)	23.4 (2.1)[a,b]	15.3 (1.4)[a,b,c]	<0.005

[a]Significant versus control group.
[b]Significant versus sham group.
[c]Significant versus I/R group.
P: Significance value of F test; results are presented as mean (SD).
I/R: intestinal ischemia/reperfusion.

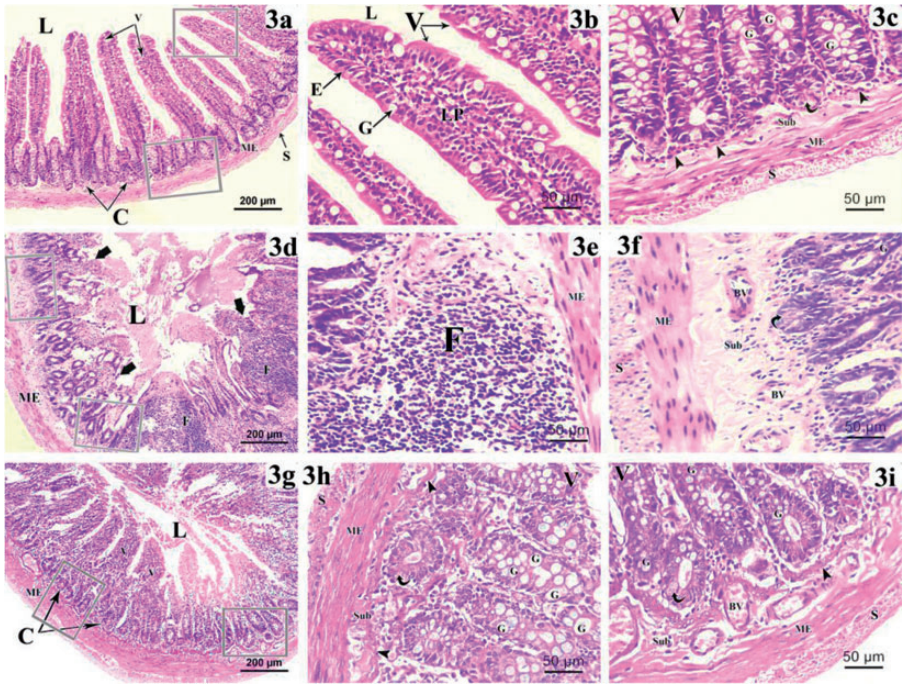

Figure 3. Photomicrographs of H&E sections of rat jejunum from the different groups. (a) In the sham/control group. L: lumen, v: villus, ME: muscularis externa, C: crypts, and S: serosa. (b) Higher magnification of the villi in figure (a). L: lumen, v: villus, G: goblet cell, E: enterocytes, and LP: lamina propria. (c) Higher magnification of the base of villi in figure (a) shows the intestinal glands. In the I/R group. (d) L: lumen, thick arrows: disrupted villi, ME: muscularis externa, and F: cellular infiltration. (e and f) Higher magnification of figure (d) shows intestinal glands. The fetuin-A-pretreated I/R group. (g) L: lumen, v: villus, ME: muscularis externa, and C: crypts. (h and i) Higher magnification of figure (a) shows intestinal glands. Curved arrow: Paneth cells, v: villus, g: goblet cell, arrowheads: muscularis mucosa, Sub: submucosa, ME: muscularis externa, BV: blood vessels, and S: serosa. (a,d,g) Scale bar = 200 μm, 100× magnification; (b,c,e,f,h,i) Scale bar = 50 μm, 400× magnification. (A color version of this figure is available in the online journal.)

Table 3: Quantitative analysis of the epithelial villi thickness, length, width, submucosa thickness and crypts depth in the studied groups

Variables	Control group	Sham group	I/R group	fetuin-A pretreated I/R group	*P*
Epithelial villi Thickness (μm)	26.2 (3.1)	24.8 (3.0)	12.6 (2.5)[a,b]	20.7 (2.8)[a,b,c]	<0.005
Villi length (μm)	405.9 (22.7)	394.9 (34.2)	169.0 (23.4)[a,b]	272.8 (12.3)[a,b,c]	<0.005
Villi width (μm)	64.4 (3.8)	62.1 (2.2)	37.8 (4.2)[a,b]	55.3 (4.4)[a,b,c]	<0.005
Submucosa thickness (μm)	22.9 (2.1)	31.8 (6.5)	104.7 (18.6)[a,b]	90.8 (12.8)[a,b,c]	<0.005
Crypts depth (μm)	74.2 (15.2)	71.6 (7.1)	36.1 (4.3)[a,b]	63.0 (6.7)[a,b,c]	<0.005

I/R = intestinal ischemia/reperfusion; a = significant versus control group; b = significant versus sham group; c = significant versus I/R group; p = significance value of F test; results are presented as mean (SD).

Table 4. Chiu's histo-pathological evaluation scoring of intestine injury in the studied groups.

Chiu's Scoring	Control group	Sham group	I/R group	Fetuin-A pretreated I/R group	*P*
Median (IQR)	0.0 (1.0)	1.0 (1.0)	5.0 (0.3)[a,b]	2.0 (1.0)[a,b,c]	<0.005

[a]Significant versus control group.
[b]Significant versus sham group.
[c]Significant versus I/R group.
P: Significance value of F test.
I/R: intestinal ischemia/reperfusion.

significantly reduced in fetuin-A-pretreated I/R group when compared with I/R group ($P < 0.005$) (Figure 4(d)).

Impact of fetuin-A pretreatment on the immunoexpression of autophagy markers

The immunohistochemical expression of autophagy-associated proteins in the jejunum of the control group showed normal crypt and villi, with weak beclin-1 and LC3 immunoexpression (Figure 5(a) and (d)). Noticeably, elevated beclin-1 and LC3 levels following I/R insult were confirmed by a significant increment in the percentage area of brown staining, indicating beclin-1 and LC3 immunoexpression (Figure 5(b) and (e)), compared with the control group ($P < 0.005$). However, treatment with fetuin-A prior to I/R insult revealed downregulation of beclin-1 and LC3 expression, confirmed by a significant decrease in the percentage area of beclin-1 and LC3

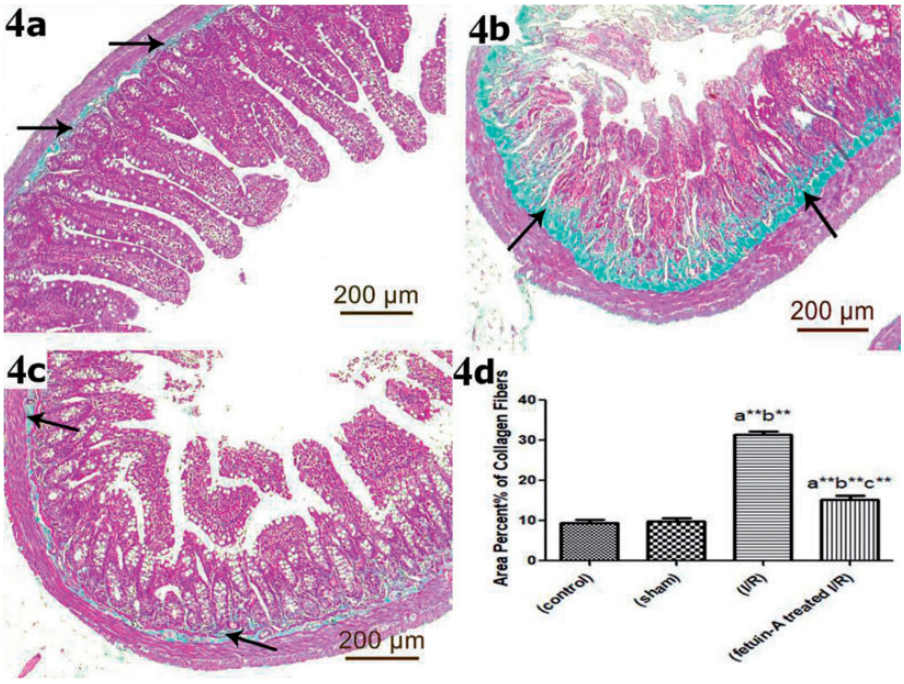

Figure 4. Representative photomicrographs of sections of rat jejunum from the different groups stained with Masson's trichome stain to show collagen fiber distribution. (a) Control group, (b) I/R group, and (c) fetuin-A-pretreated I/R group. Arrow indicates the green staining of the collagen fibers. (d) Bar chart presenting mean (SE) of the percentage area of collagen fibers (200× magnification) of the three experimental groups. Scale bar = 200 μm, 100× magnification. (A color version of this figure is available in the online journal.)

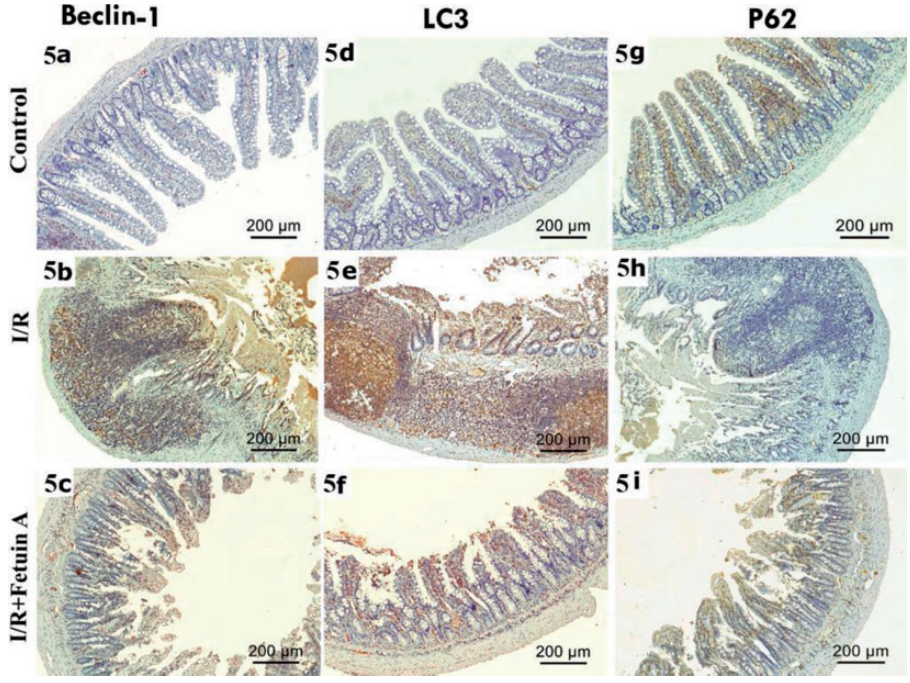

Figure 5. Representative photomicrographs showing the immunoexpression of autophagy-associated proteins beclin-1, LC3, and p62 in rat jejunum from the different groups. Positive immunoexpression is indicated by brown discoloration. Control group showing normal crypt and villi with weak beclin-1 (a) and LC3 (d) immunoexpression. Elevated levels of beclin-1 (b) and LC3 (e) with weak p62 expression (h) were detected in the I/R group. The fetuin-A-pretreated I/R group revealed the downregulation of beclin-1 (c) and LC3 (f) and increased expression of p62 (i) compared with the I/R group. Scale bar = 200 μm, 100× magnification. (A color version of this figure is available in the online journal.)

(Figure 5(c) and (f)) immunoexpression compared with the I/R group. In contrast, p62 detected down-regulation in its level in the I/R group, identified by a significant decrease in the percentage area (Figure 5(h)) when compared with the control group (Figure 5(g)) ($P < 0.05$). However, fetuin-A pretreatment (Figure 5(i)) significantly upregulated p62 expression compared with the I/R group ($P < 0.005$) (Table 5).

Discussion

I/R injury occurs when the reperfusion of ischemic organs or tissues exaggerates their damage[24] Intestinal I/R can be a serious pathological condition that not only leads to local tissue damage but also induces systemic inflammatory reactions and, ultimately, multiple organ dysfunction syndromes with a high mortality rate[25]

As intestinal I/R is mainly observed in SMA occlusion, it could be caused by atherosclerosis, hypervolemia, and cardiac diseases (including chronic heart failure, recent myocardial infarction, and valvular diseases). Moreover, intra-abdominal malignancy and inflammatory bowel disease (IBD) have been implicated in its pathogenesis.[26] As fetuin-A deficiency has an adverse impact on cardiovascular system; diffuse extraosseous calcifications (including vessels and cardiac valves) have been detected in fetuin-A knockout mice.[27] Furthermore, decreased serum fetuin-A levels were independently associated with disease activity in patients with IBD.[28]

Despite the ability of fetuin-A to protect against cerebral ischemic injury,[7] the potential clinical advantage of using fetuin-A in ischemic intestinal injury, as well as its role as anti-inflammatory, antioxidant, and autophagy modulator was not previously studied in this animal model; therefore, a model of intestinal I/R injury was established to explore the possible protective effects of fetuin-A against mesenteric ischemia in adult male rats to avoid sex and hormonal variability that occur in female rats, as well as changing from one rat to another depending on its estrus cycle phase. Additionally, clinical reports showed that men are more susceptible to intestinal IR injury than women given those female sex hormones reportedly preserve endothelial integrity and reduce inflammation.[29] The current study simulated 1 h of ischemia by clamping the SMA, and reperfusion was allowed for 2 h thereafter.

The main findings obtained from the present work were that administration of fetuin-A during intestinal ischemia/reperfusion condition (1) improved the amplitude of spontaneous jejunal contraction, (2) improved mucosal histopathological disturbances in this animal model, (3) reversed jejunum autophagy disturbance that was characterized by increased beclin-1 and LC3 with decreased p62 expression.

Notably, the fetuin-A-pretreated I/R group showed significantly restored normal intestinal mucosa and a decreased severity of injury compared to the I/R group, as showed by a significant increase in the villus length and width, epithelial thickness, crypt depth, and decreased submucosal thickness. Additionally, pretreatment with fetuin-A was concomitant with a significant decrease in Chiu's score, indicated by the absence of ulceration and hemorrhage observed in the I/R group, and a significant reduction in the percentage area of collagen deposition when compared with the I/R group. These structural enhancements were associated with a significant improvement in the amplitude but not frequency of spontaneous jejunal contractility change when compared to the I/R group. These effects could be explained by the ability of fetuin-A to significantly increase the levels of intestinal tissue SOD, an enzyme that has the ability to overwhelm oxygen-free radicals. It was reported that the higher the SOD level, the stronger its ability to destroy these harmful free radicals.[30]

Additionally, we found that fetuin-A downregulated tissue MDA, TNF-α, and IL-1β levels. Furthermore, our results were in line with those of Asadi *et al*.[31] who noticed that fetuin enhanced total antioxidant capacity levels and also prevented the formation of MDA in ovarian tissue. These findings are in agreement with the observations of Sarıözkan *et al*.,[32] who indicated that supplementation of the medium used to freeze sperm with fetuin or a combination of fetuin and hyaluronan decreased ROS and MDA levels.

In accordance with our results, Yen *et al*.[33] reported that fetuin-A played a vital role in regulating inflammatory responses, as its administration might be beneficial in protecting against systemic inflammation.[34] In preeclampsia, a decreased concentration of fetuin-A may reflects a systemic inflammatory condition.[35]

Because the intestine has a high oxygen requirement, it is extremely sensitive to released oxygen-free radicals.[36] It was found that I/R injury was accompanied with excess oxygen-free radicals, which caused cell membrane lipid peroxidation, membrane damage, inhibition of mitochondrial activity, and augmented membrane permeability.[37] Furthermore, Chen *et al*.[38] mentioned that the generation of an extreme amount of reactive oxygen species (ROS) in injured cells and tissues during I/R lesion triggered different signaling pathways, promoted inflammatory reactions, and damaged the function of the intestinal mucosal barrier. Our results regarding the state of oxidant/antioxidant imbalance in I/R control group were in line with the results of Mohamed and Almallah[39] and Tassopoulos *et al*.[40] who demonstrated that the MDA level is considered a consistent

Table 5. Area percentage (%) of positive reaction in the autophagy markers in the studied groups.

Variables	Control group	Sham group	I/R group	Fetuin-A pretreated I/R group	*P*
Beclin-1	6.5 (1.3)	7.0 (0.8)	27.8 (1.3)[a,b]	13.2 (1.3)[a,b,c]	<0.005
LC3	7.9 (1.4)	8.9 (1.7)	16.8 (1.6)[a,b]	11.9 (1.7)[a,b,c]	<0.005
P62	32.3 (1.6)	31.3 (1.5)	15.7 (1.8)[a,b]	26.9 (2.2)[a,b,c]	<0.005

[a]Significant versus control group.
[b]Significant versus sham group.
[c]Significant versus I/R group.
P: Significance value of F test; results are presented as mean (SD).
I/R: intestinal ischemia/reperfusion.

indicator of an I/R incident, and it increases with the severity of the injury.[41]

Moreover, Jin *et al.*[2] reported that this oxidative stress process interacts closely with inflammation, and Soares *et al.*[42] noticed that neutrophils were activated during the process of reperfusion and that they could immigrate through the endothelial wall into the tissue parenchyma, releasing cytotoxic mediators, such as TNF-α, ILs, and nitric oxide. These inflammatory mediators damage the mucosal epithelium and stimulate the production of extracellular matrix ROS and cytotoxic mediators that trigger the inflammatory reaction cascade characterizing I/R insult.[43]

Wehner *et al.*[44] indicated that the released inflammatory cytokines induced an inhibition in small intestine motility and intestinal obstruction. Furthermore, IL-1β plays an essential role in decreased gastrointestinal smooth muscle contractility in Th1 cytokine-dominant colitis by downregulating CPI-17 expression, which is a phosphorylation-dependent myosin phosphatase inhibitory protein.[45] The previous observations could explain our intestinal motility results, as there was a significant decrease in the amplitude and frequency of spontaneous jejunal contractile activity in the I/R group compared with the control and sham groups. The results of Luo *et al.*[46] were in agreement with ours. We could also conclude that the anti-oxidative stress and the anti-inflammatory effects of fetuin-A not only improved intestinal motility but also restored intestinal mucosal integrity. It was noticed that, the reduction of serum TNF-α, protected intestinal barrier integrity from ischemic injury, and reduced postoperative local inflammatory response which alleviated adhesion formation in septic rats[47]

It is strongly suggested that calcium ion is involved in the pathogenesis of intestinal I/R injury and agents that prevent the intracellular calcium influx that occurs during the reperfusion period, could attenuate this injury.[48] Interestingly, fetuin-A has the capacity of binding Ca^{2+}, inhibiting intracellular Ca^{2+}-dependent damaging enzymes such as proteases and polyamine oxidase, which explains its short-term protection against cerebral ischemic injury.[9]

Autophagy involves the degradation and recycling of damaged or worn-out organelles within the cell via autophagosome formation, a double-membrane structure that engulfs cytoplasmic material and decomposes it via lysosomal activity. An efficient autophagy process is essential for cell survival against different stresses. Evidence has indicated that impaired autophagosome processing may also be responsible for unfavorable cell death and impaired function.[49]

Cumulative data have demonstrated that autophagy shares in the pathological process of I/R injury in neuronal tissue.[36] Interestingly, autophagy dysregulation has been engaged in the pathogenesis of different intestinal illnesses, including IBD.[50] However, little is recognized about its role in intestinal injury induced by I/R and the influence of oxidative stress on autophagy flux, which requires further investigation.[51]

Our results revealed a significant increase in beclin-1 and LC3 with a significant decrease in p62 immunoexpression in the I/R group comparing it with the sham and control groups. These findings were significantly reversed in the fetuin-A-pretreated I/R group and agreed with the findings of Li *et al.*,[5] who noticed that autophagy was significantly increased in intestinal mucosa following intestinal I/R and that autophagy inhibition weakened intestinal injury induced by I/R by stimulating mTOR signaling, which functions as a serine/threonine protein kinase that regulates cell growth, proliferation, motility, survival, protein synthesis, and autophagy. The excess autophagic protein expression observed in the I/R group could be explained by the oxidative stress condition in this group, which was in line with Wang *et al.*,[51] who showed that the ROS-induced increase in beclin-1 and LC3 autophagy execution proteins could be a major cause of neuronal cell death. However, they also reported the increased expression of p62, which contradicted our findings.

Beclin 1 and LC3 are central regulatory proteins of autophagy that act during the initiation stage by forming the autophagosome. Beclin-1 overexpression increases LC3-II expression, accentuates autophagosome formation, and impairs autophagosome clearance by inhibiting autophagy-lysosomal gene transcription.[51] Baseline autophagy is essential for the maintaining intestinal homeostasis and the function of the intestinal defensive barrier. However, it was established that autophagy overactivation might exaggerate IBD by inducing autophagic cell death, leading to intestinal barrier disruption and the excessive production of proinflammatory cytokines.[52]

Our results contradicted those of Li *et al.*,[53] who showed that reduced autophagy was in charge of I/R-induced intestinal injury. This discrepancy could be related to genetic, species, and/or environmental differences.

Regarding the effect of fetuin-A on autophagy proteins, Kim *et al.*[54] reported the increased expression of p62 during autophagy inhibition, which correlated with our results. Additionally, it appeared to be upregulated in both LC3- and beclin 1-depleted cells.[55] They also reported that p62 was responsible for the inhibition of H_2O_2-induced autophagy by sunitinib, a multitargeted receptor tyrosine kinase inhibitor, which has been demonstrated as an effective autophagy mediator. p62 revealed an unknown link between the activation of adenosine monophosphate-activated protein kinase and the inhibition of autophagy.

Our results were in accordance with the findings of Liu *et al.*,[23] who stated that autophagy inhibition in shikonin-treated liver I/R animals was characterized by a significant decrease in beclin-1 and LC3 expression with increase in p62 expression. Additionally, Liu *et al.*[56] stated that pharmacological and genetic downregulation of autophagy can increase the level of p62 in several cell lines.

Autophagy inhibition has been confirmed to act as a potential treatment for IBD via the regulation of cellular metabolism and modulation of inflammatory reactions.[51] In our study, we consistently observed another mechanism by which fetuin-A could prevent I/R intestinal insult: inhibition of the autophagy process.

Conclusions

The present study revealed that fetuin-A supplementation could prevent I/R injury-induced jejunal structural and functional disturbances via its anti-inflammatory, antioxidant, and autophagy modulating properties. Moreover, our findings demonstrate, for the first time, the potential for fetuin-A as an effective therapeutic modality in patients at risk of ischemic intestinal insults. This study highlights the need for targeting autophagy and the development of drugs that can mitigate the exaggerated autophagic response for the clinical management of patients with syndromes of acute ischemic injury. Clinical trials that support the efficacy of using fetuin-A for the treatment of intestinal I/R injury, as well as clinical and basic researches that study potential sex differences in its effects, are certainly warranted.

AUTHORS' CONTRIBUTIONS

All authors contributed in research design and interpretation of the studies. NFE-M, AEA, WMRA, and NHH conducted experiments. WMRA, AEA, NHH Performed data analysis. NFE-M, AEA, NHH, HE contributed to review and writing of the manuscript.

All individuals contributed in this research are included in the list of authors,

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