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ORIGINAL ARTICLE

Females Are More Resistant to Ischemia-Reperfusion-induced Intestinal Injury Than Males

A Human Study

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Background and Objective: Sex differences in responses to intestinal ischemia-reperfusion (IR) have been recognized in animal studies. We aimed to investigate sexual dimorphism in human small intestinal mucosal responses to IR.

Methods: In 16 patients (8 men and 8 women) undergoing pancreaticoduodenectomy, an isolated part of jejunum was subjected to IR. In each patient, intestinal tissue and blood was collected directly after 45 minutes of ischemia without reperfusion (45I-0R), after 30 minutes of reperfusion (45I-30R), and after 120 minutes of reperfusion (45I-120R), as well as a control sample not exposed to IR, to assess epithelial damage, unfolded protein response (UPR) activation, and inflammation.

Results: More extensive intestinal epithelial damage was observed in males compared to females. Intestinal fatty acid binding protein (I-FABP) arteriovenous (V-A) concentrations differences were significantly higher in males compared to females at 45I-0R (159.0 [41.0–570.5] ng/mL vs 46.9 [0.3–149.9] ng/mL). Male intestine showed significantly higher levels of UPR activation than female intestine, as well as higher number of apoptotic Paneth cells per crypt at 45I-30R (16.4% [7.1–32.1] vs 10.6% [0.0–25.4]). The inflammatory response in male intestine was significantly higher compared to females, with a higher influx of neutrophils per villus at 45I-30R (4.9 [3.1–12.0] vs 3.3 [0.2–4.5]) and a higher gene expression of TNF-α and IL-10 at 45I-120R.

Conclusion: The human female small intestine seems less susceptible to IR-induced tissue injury than the male small intestine. Recognition of such differences could lead to the development of novel therapeutic strategies to reduce IR-associated morbidity and mortality.

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The authors report no conflicts of interest.

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Sex disparities regarding pathogenic mechanisms, age of onset, progression, and therapy suitability are common in human disease.[1] Sex differences in ischemia-reperfusion (IR) injury have, in particular, been investigated in animal studies, and provided evidence for sex differences in responses to IR in the heart,[2] brain,[3] kidney,[4] liver,[5] and intestine.[6,7] Beneficial effects of the female hormone estrogen and/or deleterious effects of the male hormone testosterone are held responsible.[8–13] For example, estradiol-treated male rats displayed less myocardial necrosis and inflammatory responses in a model for cardiac ischemia when compared to non-treated male rats.[9] As an example of the negative effects of male hormones, orchidectomy in male rats prior to intestinal IR resulted in reduced IR-induced intestinal mucosal damage in comparison with nonorchidectomized males.[10]

Even though the small intestine contains functional estrogen receptors and therefore is a potential estrogen-responsive organ[14,15] the impact of sex on the pathogenesis of human IR-induced intestinal epithelial injury, mucosal barrier dysfunction, and inflammation remains unclear. Human intestinal IR is an important clinical problem. Intestinal IR-associated morbidity and mortality rates remain extraordinarily high with reported mortality rates of 60 to 80%.[16] Previous studies from our group demonstrated that 45 minutes of human intestinal ischemia followed by reperfusion led to epithelial cell damage, inflammatory responses, and Paneth cell apoptosis.[17,18] Intestinal IR is also a potent inducer of endoplasmic reticulum (ER) stress, which leads to the activation of the unfolded protein response (UPR) via 3 ER stress sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE-1), and activating transcription factor 6 (ATF6).[18,19] The UPR, among others, is initially directed at cell survival, by inducing a translational block to reduce protein load to the ER and activating programs that increase protein folding capacity of the cell to cope with ER stress.[20,21] Prolonged or extensive ER stress will eventually lead to apoptosis.[22] Several animal studies revealed that ER stress responses are higher in males compared with females, and that this may contribute to the observed sex differences in myocardial apoptosis, kidney injury, and hypertension.[23–25]

Although the consequences of IR in patients participating in our previous studies were consistent, we observed that some patients had considerably less intestinal IR-induced damage than others. In this study, we aimed to investigate whether such differences in disease susceptibility were sex-related. This is important since recognition of potential sex differences and elucidation of the underlying pathophysiological mechanisms is expected to be beneficial for treating both males and females with personalized

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therapies, and for the development of novel therapeutic approaches to reduce IR-associated morbidity and mortality.

METHODS

Ethics

The study was approved by the Medical Ethical Committee of Maastricht University Medical Center+ and was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent of all patients was obtained.

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Patients and Experimental Procedures

Patients

To investigate sex-related differences in human jejunal IR, 16 patients (8 males, 8 females) undergoing pancreaticoduodenectomy for pancreatic head tumors were included in this study after obtaining informed consent. Patients with underlying intestinal diseases were excluded.

Experimental Procedures

The experimental protocol using a model of human small intestinal IR was conducted as previously described.[26,27] In short, the model takes advantage of the fact that during pancreaticoduodenectomy, a variable length of healthy jejunum is routinely resected in continuity with the head of the pancreas and duodenum as part of the standard surgical procedure. Of all enrolled patients, the terminal 6 cm of this jejunal segment was isolated and subjected to 45 minutes of ischemia by placing 2 atraumatic vascular clamps across the mesentery. Meanwhile, surgery proceeded as planned. After ischemia, one-third of the isolated jejunum was resected using a linear cutting stapler (GIA[tm], Covidien, Mansfield, MA). Next, the mesenteric clamps were removed to allow reperfusion, as confirmed by regaining of normal pink color and restoration of gut motility. Another segment of the isolated jejunum was resected similarly after 30 minutes of reperfusion and after 120 minutes of reperfusion. Simultaneously, a small part of jejunum not exposed to IR, was resected and served as internal control tissue.

Tissue and Blood Collection

Jejunal tissue samples were collected directly after 45 minutes of ischemia without reperfusion (45I-0R), after 45 minutes of ischemia followed by 30 minutes of reperfusion (45I-30R), and after 45 minutes of ischemia followed by 120 minutes of reperfusion (45I-120R). Directly after collecting the latter, a control sample was taken as well, resulting in a total of 4 tissue samples for every male (n = 8) and every female (n = 8). All samples were immediately snap-frozen for quantitative polymerase chain reaction (qPCR) or formalin-fixed for histology and immunohistochemistry. Arterial blood was sampled and collected in EDTA tubes before ischemia (control), at 45I-0R, 45I-30R, and 45I-120R. Simultaneously with each respective arterial blood sample, blood was drawn from the venule draining the isolated jejunal segment by direct puncture, resulting in 4 arterial blood samples and 4 venous blood samples for every male (n = 8) and every female (n = 8). All blood samples were centrifuged at 3500 rpm, 4 °C for 15 minutes. Plasma was immediately stored in aliquots at −80°C until analysis.

Histology and Immunohistochemistry

Tissue specimens were processed and stained with hematoxylin and eosin (HE) as described.[27] Paraffin-embedded sections were double-stained for cytokeratin 18 clone M30 (Peviva, Bromma, Sweden) and lysozyme to visualize to visualize apoptosis and Paneth cells, respectively, as described.[18] Neutrophil influx was assessed by staining sections with rabbit anti-human myeloperoxidase (MPO staining; DakoCytomation, Glostrup, Denmark) as described.[27] All stainings were counterstained with hematoxylin. Sections were photographed using a Nikon eclipse E800 microscope equipped with a Nikon digital camera DXM1200F. The number of lysozyme-positive crypt cells (Paneth cells), lysozyme-positive M30-positive crypts cells (apoptotic Paneth cells) and MPO-positive cells (neutrophils), were quantified in a blinded way by 2 independent observers in 5 representative microscopic fields per section (200× magnification).

RNA Isolation, qPCR, and XBP1 Splicing Assay

RNA was isolated from snap-frozen tissue samples followed by cDNA synthesis and qPCR as previously described,[27] to analyze gene expression of interleukin-1 (*IL-1*), interleukin-6 (*IL-6*), interleukin-10 (*IL-10*), tumor necrosis factor alpha (*TNF-α*), C/EBP homologous protein (*CHOP*), growth arrest and DNA damage-inducible protein 34 (*GADD34*) and binding immunoglobulin protein (*BiP*), using *RPLP0* and *CYPA* as reference genes. XBP1 splicing was assessed as described by Grootjans et al.[18] Primer sequences for qPCR are shown in Table 1.

Plasma I-FABP and Cytokines

Plasma concentrations of I-FABP, IL-6, and TNF-α were measured using in-house developed enzyme-linked immunosorbent assay (ELISA). Plates (Greiner Bio-One, Kremsmunster, Austria) were coated overnight with rabbit anti-human I-FABP, or monoclonal antibodies 5E1 (IL-6) or 61E71 (TNF-α). Purified human I-FABP, human recombinant IL-6, and TNF-α were used as standard. After blocking, samples and standard dilution series were incubated for 1 to 2 hours. Biotinylated rabbit anti-human I-FABP and IL-6 were used as detection antibodies. For TNF-α, rabbit anti-human TNF-α was used as detection antibody, followed by incubation with peroxidase-labeled polyclonal goat anti-rabbit antibody (Jackson ImmunoReserach Europe, Suffolk, UK). Finally, 3,3’,5,5’-tetramethyl-benzidine (TMB) was used as a substrate and the reaction

TABLE 1. Human Oligonucleotide Primer Sequences Used for qPCR Analysis

Gene Symbol	Forward Primer Sequence	Reverse Primer Sequence
CYPA	5′-CTCGAATAAGTTTGACTTGTGTTT-3′	5′-CTAGGCATGGGAGGGAACA-3′
RPLP0	5′-GCAATGTTGCCAGTGTCTG-3′	5′-GCCTTGACCTTTTCAGCAA-3′
IL1	5′- CTGAGCTCGCCAGTGAAATG-3′	5′- TTTAGGGCCATCAGCTTCAAA-3′
IL6	5′- TCCAGGAGCCCAGCTATGAA-3′	5′- GAGCAGCCCCAGGGAGAA-3′
IL10	5′- GGCGCTGTCATCGATTTCTT-3′	5′- TGGAGCTTATTAAAGGCATTCTTCA-3′
TNFA	5′-TCAATCGGCCCGACTATCTC-3′	5′-CAGGGCAATGATCCCAAAGT-3′
XBP1	5′-GGAGTTAAGACAGCGCTTGGGGA-3′	5′-TGTTCTGGAGGGGTGACAACTGGG-3′
CHOP	5′-GGAGCATCAGTCCCCCACTT-3′	5′-TGTGGGATTGAGGGTCACATC-3′
GADD34	5′-CCCAGAAACCCCTACTCATGATC-3′	5′-GCCCAGACAGCCAGGAAAT-3′
BIP	5′-CTGCTGTATCCTCTTCACCAGTTG-3′	5′-TGACATTGAAGACTTCAAAGCTAAGA-3′

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was stopped by adding 1 M H_2SO_4. Samples were analyzed spectrophotometrically (450 nm) using an automated ELISA reader. The detection limits for the assays were 25 pg/mL, 31.3 pg/mL, and 78 pg/mL respectively. Samples were run in duplicate and a variability of 15% between sample duplicates was accepted. Plasma IL10 levels were measured using DuoSet ELISA (RnD Systems, Minneapolis, MN) according to manufacturer's protocol, with a lower detection limit of 62.6 pg/mL. Arteriovenous concentration differences across the intestinal segment were calculated by subtracting arterial plasma concentrations from venous plasma concentrations.

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Statistics

Statistical analysis was performed using Prism 6.0 for Windows (GraphPad Software Inc, San Diego, CA). All data are presented as median and range as none of the parameters showed a normal distribution, according to the Shapiro–Wilk test. Data were analyzed using 2-way ANOVA with time points and sex as factors, with Sidak post hoc test for multiple comparisons to identify the difference between males and females at the respective time points. Within group effects were tested with 1-way ANOVA followed by Dunnett post hoc test for multiple comparisons versus control. A P value below 0.05 was considered statistically significant.

RESULTS

Patients Characteristics and Sample Collection

A total of 16 patients (8 males, 8 females) were included in this study. Male and female patients were comparable regarding age (70.5 [65.3–79.0] vs 68.5 [55.8–80.5] respectively, $P > 0.05$) and BMI (22.05 kg/m^2 [20.63–29.95] vs 22.10 kg/m^2 [20.7–23.6] respectively, $P > 0.05$). The procedure was ended early in 2 males and 1 female, resulting in missing blood and tissue samples at 45I-120R. Moreover, blood sampling was unsuccessful at time point 45I-120R in 1 male and 1 female patient. One control tissue sample of a male patient was formalin-fixed only, hence no snap-frozen tissue was available.

Jejunal IR Results in More Extensive Epithelial Damage and Higher Plasma I-FABP Levels in Males Compared to Females

In control small intestine, an intact epithelial lining was visualized by HE staining in both males and females (Fig. 1A and E, respectively). In males, 45I-0R was associated with epithelial damage at the villus tips (Fig. 1B), which worsened at 45I-30R resulting in shedding of damaged enterocytes (Fig. 1C). At 45I-120R, the villi were denudated in 83.3% of the male patients (Fig. 1D). In contrast, in female intestinal IR, 45I-0R was particularly associated with the development of subepithelial spaces, a pathophysiological phenomenon of intestinal IR that we previously linked to short periods of intestinal ischemia of 15 and 30 minutes[28] (Fig. 1F). Although epithelial shedding of villus tips was also observed at 45I-30R (Fig. 1G), the epithelial lining appeared to be mostly intact in 86% of the female patients at 45I-120R (Fig. 1H), in contrast to what was observed in male small intestine.

To provide further evidence for this apparent sex difference, we quantified epithelial injury following intestinal IR in the human intestine by measurement of plasma levels of I-FABP, a protein abundantly present in the cytoplasm of mature enterocytes, which is released into the circulation upon epithelial damage. Significantly higher AV-concentration differences across the isolated intestinal segment were measured in plasma of males than of females exposed to intestinal ischemia, at 45I-0R (159.0 ng/mL [41.0–570.5] vs 46.9 ng/mL [0.3–149.9], Fig. 1I, $P < 0.05$]), and 45I-30R (82.5 ng/mL [43.3–97.2] vs 16.5 ng/mL [12.9–42.5]). AV-concentration differences were not significant at 45I-30R (85.0 ng/mL [13.1–752.3] vs 20.2 [10.3–61.7], $P = 0.12$) and 45I-120R (10.1 ng/mL [6.4–32.1] vs 3.7 [0.0–23.4], Fig. 1I, $P > 0.99$). These results thereby provide further evidence that the female jejunum is less susceptible to IR-induced enterocyte damage than the male jejunum directly after ischemia.

Intestinal IR in Males Is Associated With Increased Levels of UPR Activation Compared to Females

To explore UPR activation in the human small intestine, XBP1 mRNA splicing and mRNA expression levels of ER stress sensor BiP, and pro-apoptotic factors CHOP and GADD34 were assessed. Splice products of *XBP1* mRNA were observed in only minor quantities directly after ischemia in both males and females, but were clearly present after 45I-30R (Fig. 2A, representative subset of 2 male and 2 female patients). Quantification of the XBP1 s/XBP1u ratios showed significantly higher ratios in males at 45I-30R compared to females (4.1 [2.0–7.5] vs 2.1 [0.9–3.8] respectively, Fig. 2B, $P < 0.001$), suggesting more pronounced ER stress in males. XBP1s/XBP1u ratios were not different between males and females at the other time points, 45I-0R and 45I-120R. At these time points, XBP1s/XBP1u ratios were not different from control within groups.

CHOP mRNA expression was significantly increased at 45I-30R and 45I-120R when compared to control in the male gut (3.9 [0.5–5.1] and 2.2 [1.0–4.6] vs 1.0 [0.5–1.6]), $P < 0.0001$ and $P < 0.01$, respectively), and at 45I-30R in the female gut (2.4 [0.9–5.0] vs 1.1 [0.7–1.5], $P < 0.01$), Fig. 2C). No significant differences were observed for CHOP mRNA expression between males and females at the different time points.

GADD34 mRNA levels were significantly upregulated both at 45I-30R and 45I-120R when compared to control only in males (8.7 [0.3–16.5] and 4.3 [1.7–14.5] vs 1.0 [0.6–1.4], $P < 0.0001$ and $P < 0.01$, respectively, Fig. 2D) and not in females. A higher GADD34 mRNA expression was observed in males compared to females at 45I-30R (8.7 [0.3–16.5] vs 3.9 [0.2–8.1], Fig. 2D, $P < 0.01$). Furthermore, BiP mRNA expression was significantly upregulated at 45I-120R when compared to control in both the male and female gut (3.8 [0.9–5.1] vs 1.1 [0.3–1.4], and 3.0 [1.3–4.9] vs 1.3 [0.9–1.6], Fig. 2E, both $P < 0.0001$). Nevertheless, no significant differences were observed for *BIP* mRNA expression between males and females, which suggests that the activation of the IRE-1 rather than the PERK pathway might be sex-driven.

Paneth Cells in the Female Intestine Are More Resistant to IR-induced Apoptosis Compared to the Male Intestine

Paneth cells are very susceptible to ER stress and sustained ER stress can lead to apoptosis. In control tissue, lysozyme staining showed presence of Paneth cells deep in the crypts, and Paneth cell apoptosis was absent in both the male and female small intestine (Fig. 3A and E, respectively). Paneth cell apoptosis increased significantly in males and females at 45I-30R when compared to control (16.4% [7.1–32.1] and 10.6% [0.0–25.4] vs 0% [0.0–0.8] and 0% [0.0–0.0], Fig. 3A, C, E, G, I, both $P < 0.0001$). Both the male and female intestine showed significant Paneth cell apoptosis, and in line with the higher levels of UPR activation in the male intestine, as demonstrated by increased XBP1 splicing, the male small intestine also displayed considerably more Paneth cell apoptosis at 45I-30R, compared to the female small intestine (16.4% [7.1–32.1] vs 10.6% [0.0–25.4], Fig. 3I, $P < 0.05$). Paneth cell apoptosis was not significantly different between males and females at the other time points, 45I-0R and 45I-120R. At these time points, Paneth cell apoptosis was also not different from control within groups.

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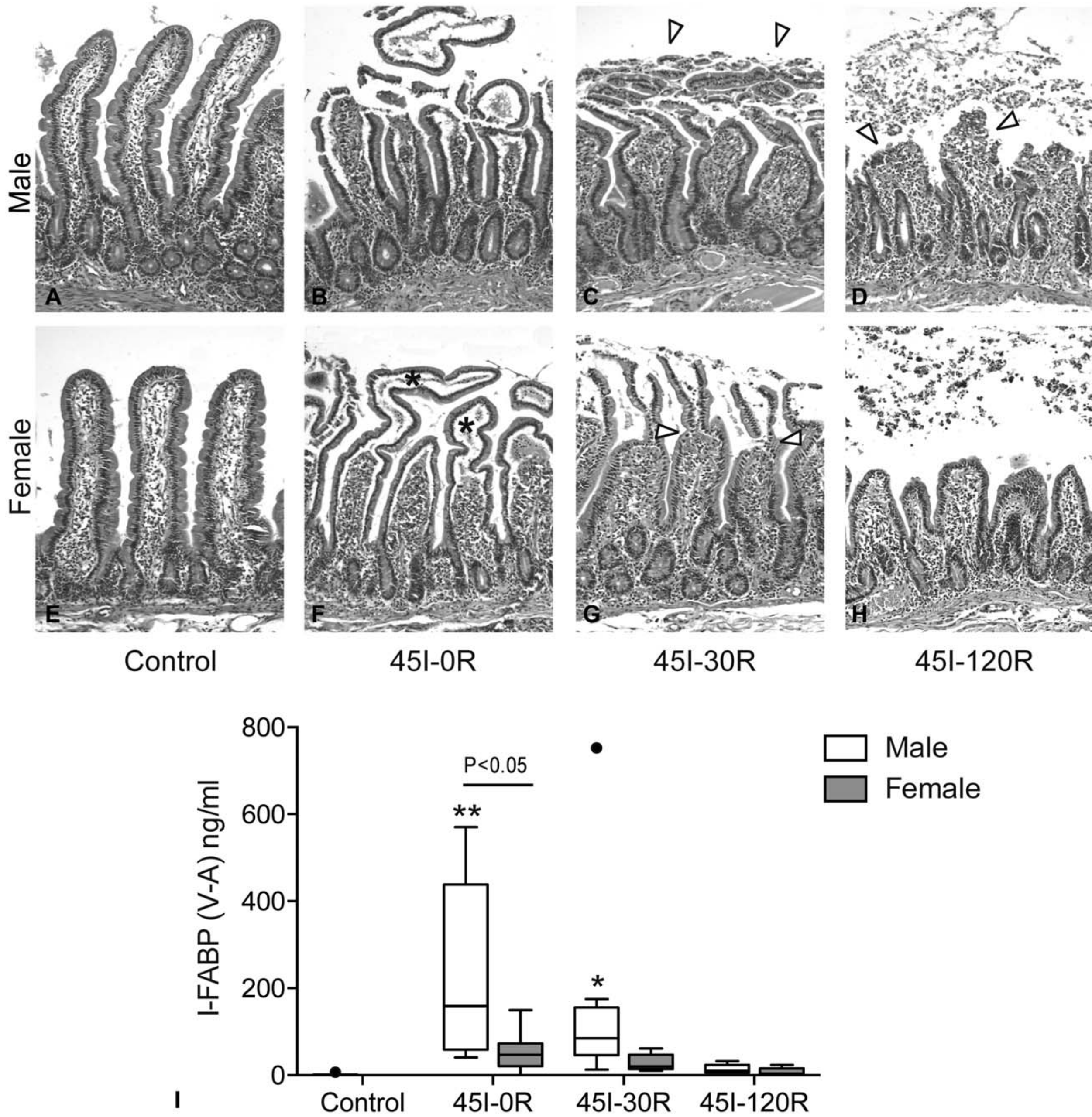

FIGURE 1. More extensive epithelial damage and higher plasma I-FABP levels in IR-injured male small intestine compared to female small intestine. (A) HE staining showed an intact epithelial lining in male jejunal control tissue. (B) 45I-0R resulted in severe epithelial damage with disruption of the epithelial lining. (C) 45I-30R resulted in shedding of damaged cells toward the lumen (arrows). (D) Denudation of the basement membrane was observed at 45I-120R (arrows). (E) An intact epithelial lining was observed in female jejunal control tissue. (F) At 45I-0R, subepithelial spaces (asterisks) were observed, indicating basal membrane retraction. (G) IR-damaged cells were pinched off into the lumen at 45I-30R (arrow), while no obvious disruption of the epithelial lining could be observed, thereby limiting exposure of the lamina propria to luminal content. (H) Although the epithelial lining has a slightly irregular appearance, it appeared fully intact at 45I-120R. The histological features are representative for the tissue samples studied. (I) Plasma I-FABP arteriovenous concentration differences are significantly higher in males after jejunal exposure to 45I-0R compared to females. HE. Control: males n = 8, females n = 8; 45I-0R: males n = 8, females n = 8; 45I-30R: males n = 8, females n = 8; 45I-120R: males n = 6, females n = 7. IFABP. Control: males n = 8, females n = 8. 45I-0R: males n = 8, females n = 8. 45I-30R: males n = 8, females n = 8. 45I-120R: males n = 5, females n = 6. Magnification ×200. I indicates ischemia; R, reperfusion; I-FABP, intestinal fatty acid-binding protein; V-A, arteriovenous concentration differences. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control, • indicates outlier.

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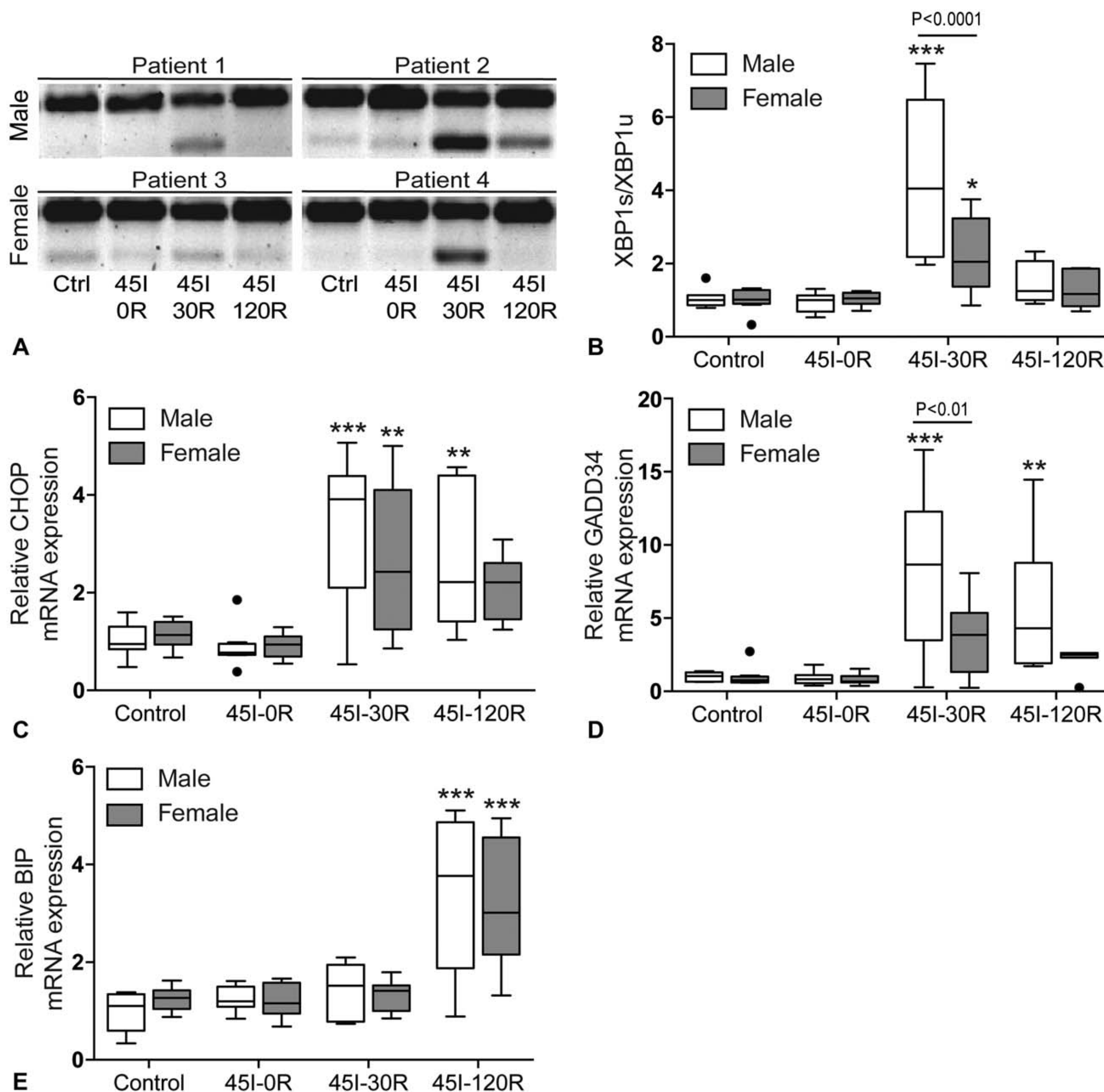

FIGURE 2. More severe ER stress after jejunal IR in males compared to females. (A) Upper electrophoresis bands represent unspliced XBP1 mRNA; lower bands represent spliced XBP1 mRNA. Splice products of XBP1 mRNA were only observed in minor quantities at 45I-0R in either males or females, but were clearly present after 45I-30R. The electrophoresis bands are representative for the tissue samples studied. (B) 45I-30R resulted in enhanced XBP1 splicing in both males and females when compared to control. However, males displayed a significantly higher XBP1s/XBP1u ratio, when compared to females at the same time point. (C, D) Relative CHOP and GADD34 mRNA expression was significantly enhanced at 45I-30R and 45I-120R compared to control in male intestine, which was less evident in female intestine. Pro-apoptotic ER stress marker GADD34 showed a significantly higher relative mRNA expression in males compared to females at 45I-30R. (E) BiP mRNA expression was significantly upregulated in both the male and female jejunum at 45I-120R compared to control, although no expression difference was observed between the 2 groups. Control: males n = 7, females n = 8. 45I-0R: males n = 8, females n = 8. 45I-30R: males n = 8, females n = 8. 45I-120R: males n = 6, females n = 7. XBP1u = unspliced X-box binding protein-1. XBP1s indicates spliced X-box binding protein-1; CHOP, C/EBP homologous protein; GADD34, growth arrest and DNA damage-inducible protein 34; BiP, binding immunoglobulin protein. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control, $^{***}P < 0.0001$ compared to control. • indicates outlier; I, ischemia; R, reperfusion.

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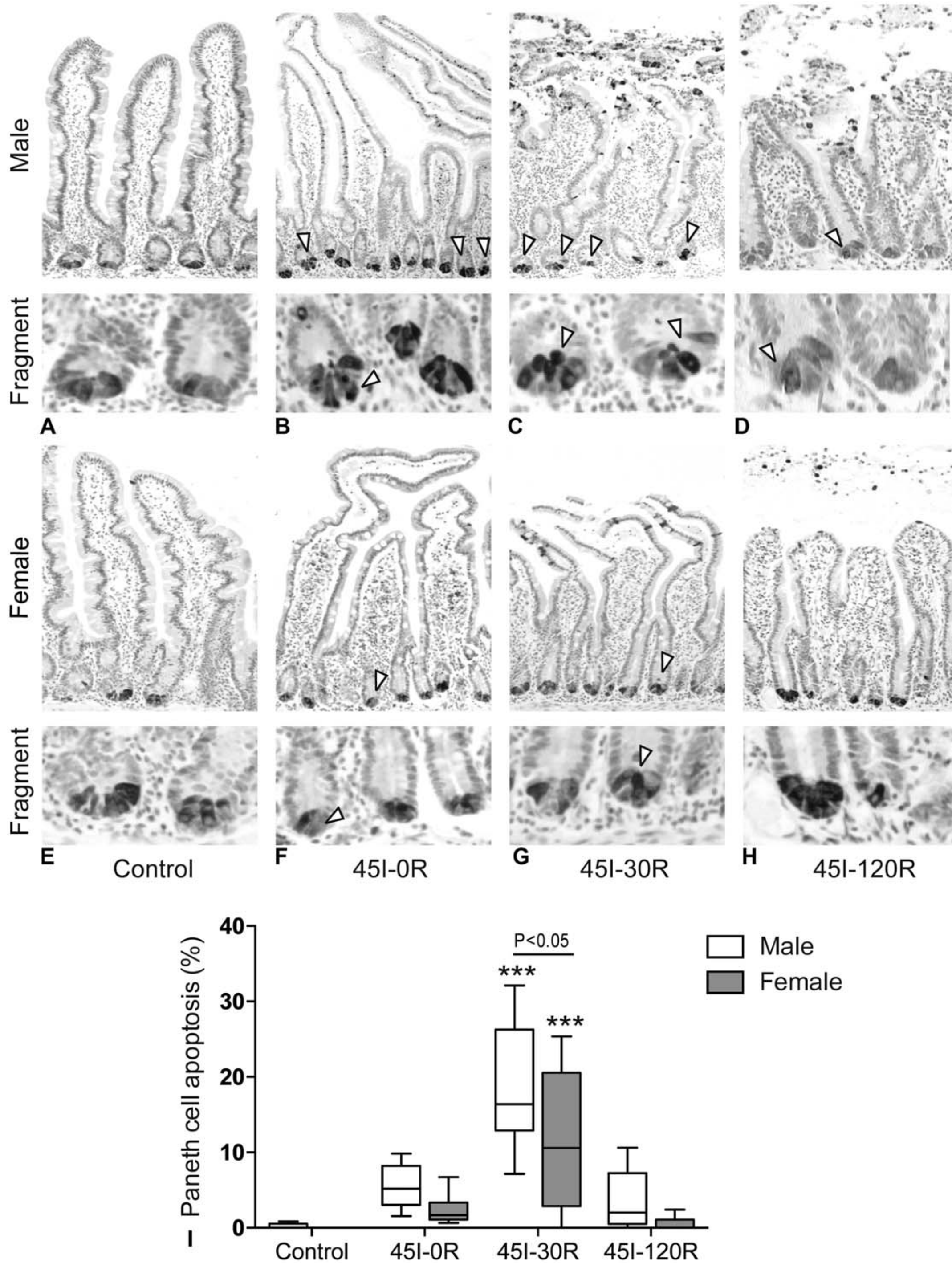

FIGURE 3. More Paneth cell apoptosis in male jejunum compared to less Paneth cell apoptosis in female jejunum. (A) Paneth cells (lysozyme, blue staining) are visible in the crypts of male control jejunum. (B, C) Paneth cell apoptosis is clearly present in male jejunal crypts at 45I-0R and 45I-30R (blue-brown double staining, arrowheads). (D) At 45I-120R, Paneth cell apoptosis was still present, but to a lesser extent (blue-brown double staining, arrowheads). (E) Female control jejunum displayed the presence of nonapoptotic Paneth cells. (F, G) Only minor Paneth cell apoptosis was observed in female jejunal crypts at 45I-0R and 45I-30R (blue-brown double staining, arrowheads). (H) At 45I-120R, hardly any apoptotic Paneth cells were observed. Fragments: Magnification of jejunal crypts with healthy and apoptotic Paneth cells. The histological features are representative for the tissue samples studied. (I) Quantification of the number of apoptotic Paneth cells showed a significant increase in Paneth cell apoptosis in both sexes at 45I-30R when compared to control. However, significantly more Paneth cell apoptosis was present in male small intestine exposed to 45I-30R compared to female small intestine. Control: males n = 8, females n = 8; 45I-0R: males n = 8, females n = 8; 45I-30R: males n = 8, females n = 8; 45I-120R: males n = 6, females n = 7. Magnification upper panels ×200. $^{***}P < 0.0001$ compared to control. • indicates outlier; I, ischemia; R, reperfusion.

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IR-induced Inflammation Is More Pronounced in the Male Intestine Than in the Female Intestine

Intestinal IR led to neutrophil infiltration in both male and female small intestine, which was only significant in males at 45I-0R and 45I-30R versus control (3.0% [1.2–5.8] and 4.9% [3.1–12.0] vs 1.5% [0.6–2.0]) (Fig. 4A, B). Neutrophil influx was significantly more abundant in male intestinal villi compared to female intestinal villi after exposure to 45I-30R (Fig. 4B, 4.9% [3.1–12.0] vs 3.3 [0.2–4.5], $P < 0.01$). Neutrophil influx was not different between males and females at 45I-0R and 45I-120R.

Although relative mRNA expression of pro-inflammatory cytokine *IL-1* was not significantly different between the 2 groups (data not shown), relative TNF-α mRNA expression was significantly higher in males compared to females at 45I-30R (1.2 [0.1–1.9] vs 0.5 [0.3–1.2], $P < 0.05$) and at 45I-120R (1.0 [0.4–1.7] vs 0.4 [0.1–1.5], $P < 0.05$, Fig. 4C).

In addition, relative IL-10 mRNA was significantly higher in males compared to females at 45I-120R (1.3 [0.0–1.7] vs 0.2 [0.0–0.4], Fig. 4D, $P = 0.001$), indicative for a more activated inflammatory response in males. IL-6 mRNA levels were not significantly upregulated during IR in either males or females compared to their respective controls or between the 2 groups (Fig. 4E). IL-6 protein AV-concentration differences however were significantly increased in males at 45I-30R and 45I-120R compared to control (41.2 pg/mL and 58.7 pg/mL vs 0.0 pg/mL respectively, Fig. 4F, $P < 0.05$ and $P < 0.01$, respectively). In females, AV-concentration differences of IL-6 were only significantly upregulated at 45I-120R (0.0 vs 60.0 pg/mL, Fig. 4F, $P < 0.01$). A trend toward higher IL-6 protein expression level in males compared to females was observed (overall sex effect $P = 0.05$; post hoc comparisons not significant). Plasma TNF-α and IL-10 protein levels were not detectable in both males and females (data not shown). These results may implicate that reperfusion times were too short for the generation of these proteins in the intestinal mucosa.

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DISCUSSION

It is well known that sexual dimorphism exists in trauma, shock and sepsis, cardiovascular diseases and cerebral damage after stroke.[29–31] Data on the influences of male or female sex on intestinal IR are scarce and only described in animal studies.[29–32] In this study, we compared the differences between the susceptibility of the human female and male small intestine to 45 minutes of ischemia followed by 0, 30, and 120 minutes of reperfusion. Our results demonstrate a clear sexual dimorphism in human small intestinal mucosal responses to ischemia and reperfusion, with females displaying less epithelial damage, less I-FABP release, less UPR activation, and less inflammatory responses.

Our data, demonstrating the female small intestine to be more resistant to IR injury, are supported by clinical studies suggesting that women have an immunological advantage and decreased in-hospital mortality after abdominal surgery when compared to men.[33,34] Furthermore, it was demonstrated that despite comparable age and comparable surgical procedures being performed, a smaller number of female patients required intensive care after surgery and females who were admitted to the intensive care unit demonstrated a significantly lower incidence of severe sepsis or septic shock.[35] The exact mechanisms accounting for the differential response to intestinal IR in men and women remain to be determined. Sex differences have mainly been attributed to the effect of sex hormones. The deleterious effects of testosterone and the beneficial effects of estrogen on IR-induced injury in murine hearts, liver and kidneys and rat liver and small intestine have previously been demonstrated.[10,36–42] Estrogen has been shown to have anti-apoptotic and anti-inflammatory properties and strong anti-oxidative actions, thereby protecting tissues from oxidative damage.[1,13,43–45] Testosterone was demonstrated to have a pro-apoptotic effect.[46] Based on age, the majority of the women included in our study were postmenopausal. Nevertheless, the low levels of estrogen still present in the population studied here could be enough to exert a protective effect. At the same time, testosterone could be held accountable for the higher disease susceptibility in males. Due to sampling limitations, we were not able to measure hormone levels in the blood of our patients in order to study the abovementioned hormonal effects in more detail. Nonetheless, other mechanisms responsible for the feminine intestinal resistance to IR could certainly be present.

It is known that female rats have a higher antioxidant capacity in a model of renal IR,[4] and higher autophagy and anti-apoptotic activity compared to male rats in a model of cardiac ischemia, suggesting that female rats have stronger adaptation and repair abilities in response to nutrient and oxygen deprivation.[2,13] Additionally, it was shown that female rat intestinal segments produced more nitrogen oxide in a model of intestinal hypoxia, than male rat intestinal segments, resulting in improved tissue perfusion and less inflammation.[41,47,48] Leptin, secreted by adipocytes, and its receptors have been found to be present in intestinal mucosa of rats, where it exerted protective effects in response to intestinal IR injury via anti-inflammatory and antioxidative processes.[49] It could be hypothesized that females exhibit more leptin activity, since females generally exhibit a higher percentage of body fat compared to males, resulting in mitigated intestinal injury in females after IR. These events and processes observed in rodents might also be present in human intestine upon IR exposure. On the other hand, the BMIs of our patients were not significantly different between the males and females, rendering the leptin-theory less likely as possible explanation for the observed sex differences in our study.

In the intestinal mucosa, a wide variety of cytokines are produced locally by the epithelium, the fibroblasts and immune cells present in the lamina propria. The epithelium itself may provide early signals for the acute mucosal inflammatory response via the release of proinflammatory cytokines and is demonstrated to be an important early source of IL-6 production or release in response to epithelial damage.[50] Our data demonstrate a significant increase of the pro-inflammatory IL-6 plasma protein levels after 30 and 120 minutes of reperfusion in males and after 120 minutes of reperfusion in females compared to control, while the IL-6 mRNA levels in both groups were not significantly upregulated during IR. In our study mRNA levels were measured in whole tissue, rather than only in epithelial cells, possibly explaining why IL-6 mRNA levels were not upregulated during IR.

In a previous study, it was demonstrated that small intestinal IR induces ER stress, resulting in activation of the unfolded protein response and subsequent XPB1 splicing.[18] This XBP1 splicing was shown to be strongly correlated with Paneth cell apoptosis, which might be explained by the high secretory activity of these cells and the subsequent susceptibility to ER stress. In line with these results, our data demonstrate presence of ER stress and Paneth cell apoptosis in both males and females, especially after short reperfusion. Interestingly, male small intestine displayed a higher amount of ER stress with significantly more XBP1 splicing and a higher number of apoptotic Paneth cells per crypt than female small intestine, rendering them more prone to decreased production of antimicrobial proteins and subsequent bacterial translocation and systemic inflammation.[18] These phenomena support the recent observations that differential ER stress responses in males and females may contribute to the sex differences in susceptibility to organ injury.[23–25]

In conclusion, we showed that the human female small intestine appears to be better protected from IR-induced epithelial

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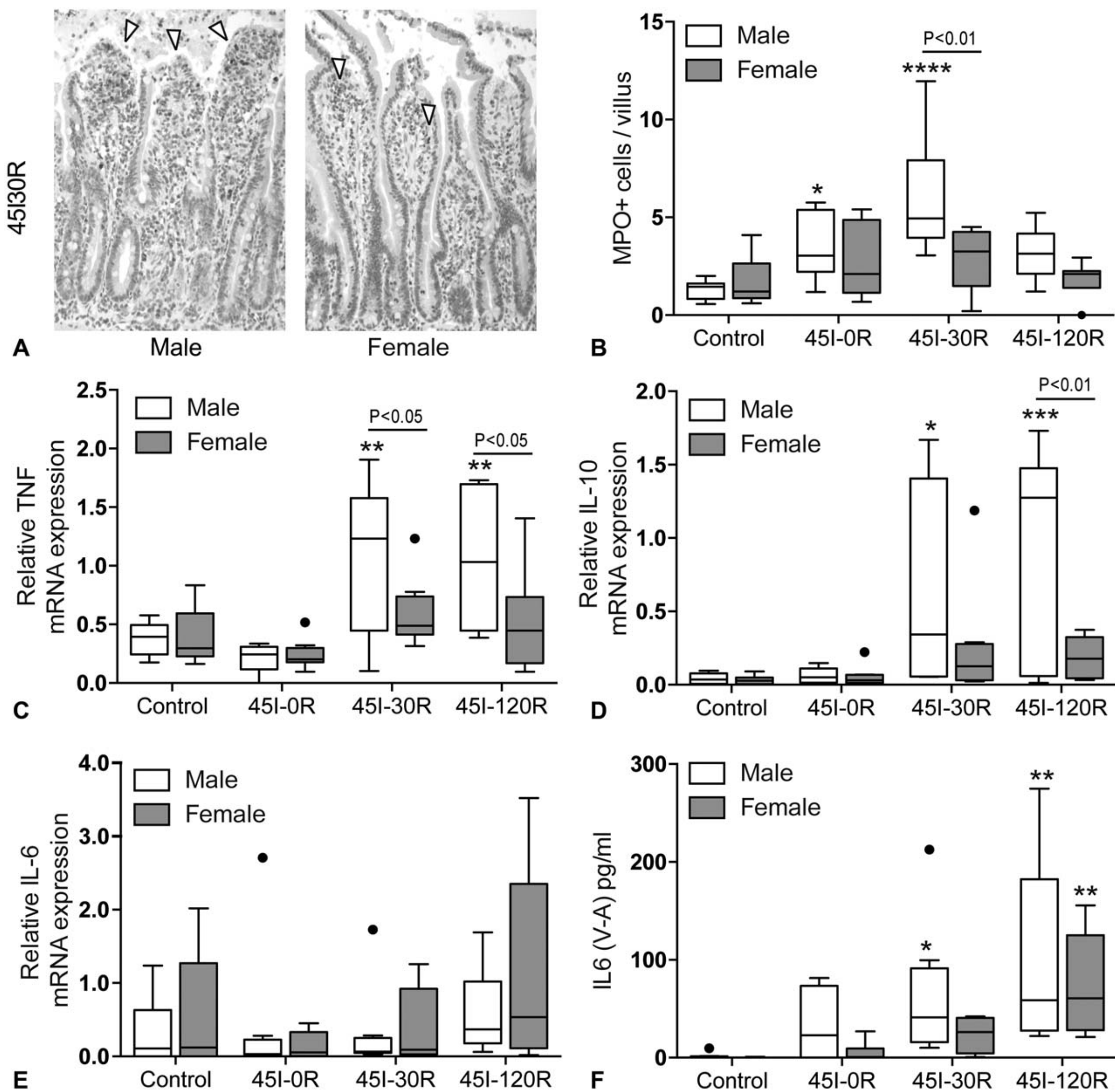

FIGURE 4. IR-induced inflammation is more pronounced in the male intestine than in the female intestine. (A) MPO staining showed abundant presence of circulating neutrophils in the lamina propria of male small intestine upon exposure to 45I-30R (left panel, arrows), while female small intestine exhibited only minor neutrophil influx (right panel, arrows). The histological features are representative for the tissue samples studied. (B) Quantification of MPO-positive cells showed a significant increase in neutrophil influx in male jejunum at 45I-0R and 45I-30R compared to control, but not in female jejunum. Male jejunal tissue displayed significantly more neutrophil influx at 45I-30R, compared to female jejunal tissue. (C) Relative TNF-α mRNA expression significantly increased in male small intestine after 45I-30R and 45I-120R when compared to control. The male TNF-α mRNA expression levels were significantly higher when compared to females at these time points. (D) Anti-inflammatory IL-10 mRNA expression was significantly increased in male small intestine at 45I-30R and 45I-120R. Males displayed significantly higher IL-10 mRNA expression when compared to females at 45I-120R. (E) Relative IL-6 mRNA expression did not significantly increase during IR in either male or female jejunum. (F) Arteriovenous concentration differences of IL-6 protein levels were significantly upregulated at 45I-30R and 45I-120R in males and at 45I-120R in females when compared to their respective controls. Protein levels approached significance between males and females during IR (overall sex effect $P = 0.05$; post hoc comparisons not significant). MPO. Control: males n = 8, females n = 8; 45I-0R: males n = 8, females n = 8; 45I-30R: males n = 8, females n = 8; 45I-120R: males n = 6, females n = 7. Cytokine expression. Males n = 7, females n = 8. 45I-0R: males n = 8, females n = 8. 45I-30R: males n = 8, females n = 8. 45I-120R: males n = 6, females n = 7. Magnification immunohistochemistry ×200. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control, $^{***}P < 0.001$ compared to control, $^{****}P < 0.0001$ compared to control. • indicates outlier; I, ischemia; R, reperfusion; IL-1, interleukin 1. IL. TNF-α indicates tumor necrosis factor alpha; IL-10, interleukin 10; IL-6, interleukin 6.

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damage than the male small intestine. Correspondingly, the female intestine displayed less ER stress and inflammatory responses. Our results should raise awareness among clinicians and draw attention to the pathophysiological differences of intestinal IR between male and female patients, for example after admission to the intensive care unit or after elective aortic or major abdominal surgery. Furthermore, our results indicate that future experimental studies on intestinal IR should consider sex differences and that the efficacy of therapeutic or preventive strategies should be tested in parallel in both sexes. Next to that, continued research into the cellular and molecular mechanisms responsible for these sex-based differences in response to intestinal IR is required. This research might ultimately provide targets for novel therapeutic interventions or preventive strategies, leading to improved outcomes for both male and female patients suffering intestinal ischemia and reperfusion in the near future.

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