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Original Article

Impact of different surgical traumas on postoperative ileus in rats and the mechanisms involved

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Abstract: The degree of postoperative ileus and the underlying pathophysiological mechanism among different types of surgical traumas have not been examined. The aim of this study was to investigate the inflammatory and oxidative stress changes in rat intestinal muscularis and gastrointestinal transit among three types of surgical traumas. Rats were randomized assigned to four groups: control group, intestinal manipulation (IM) group, intestinal ischemia/reperfusion injury (IR) group and peritoneal air exposure (AE) group. Gastrointestinal transit was measured 24 hours after surgery. Malondialdehyde (MDA), glutathione (GSH) and inflammatory mediators in intestinal muscularis were measured. Influx of neutrophil in intestinal muscularis was also determined. The degree of gastrointestinal motility impairment was equal between the IM and AE groups. However, the IR group was subject to a less impairment of gastrointestinal motility compared with the IM and AE groups. The IM group showed the most significant increase of inflammatory response, while the AE group showed the most significant increase of oxidative stress. The IR group showed a moderate increase of inflammatory response and oxidative stress. Rats subjected to IM, IR and AE could all develop into POI. We speculate that oxidative stress should be an equally important pathophysiological mechanism of POI as inflammation.

Keywords: Postoperative ileus, oxidative stress, inflammation, surgical trauma

Introduction

Postoperative ileus (POI), differs from mechanical bowel obstruction, is a transient episode of impaired of gastrointestinal motility after abdominal or other major surgery. It is characterized by nausea and vomiting, abdominal distension and bloating, delayed passage of flatus and defecation, and inability to progress to an oral diet [1]. POI is a major determinant of recovery after abdominal surgery and leads to increased morbidity and prolonged hospital stay, which is a great economic burden to health-care systems [2]. The annual costs related to POI have been estimated to be as much as US $ 1.47 billion in the USA alone [3]. Although a variety of strategies have been developed to prevent or minimize POI, including laparoscopic surgery and enhanced recovery programs (fast-track surgery), none of these methods have been completely successful in shortening the duration of POI [2].

Over the past decade, our insight into its pathophysiology has identified inflammation of the intestinal muscularis triggered by handling of the intestine as the main mechanism [4]. However, this evidence mostly based on animal model only involves manipulation of the intestine/colon but do not include other surgical traumas, such as ischemia/reperfusion injury and long term (hours) peritoneal air exposure during surgery. Therefore, from a clinical point of view, this animal model could not mimic all pathophysiological mechanism in variety of major abdominal surgery. In addition, a lot of trials showed that data obtained in this rodent model could not translate to the human situation [5-10].

POI could be induced by not only manipulation of the intestine, but also intestinal ischemia/reperfusion injury and peritoneal air exposure. The underlying pathophysiological mechanism, which is important for searching the therapeu-

tic targets, may be different between these different surgical traumas. Just as manipulation of the intestine, intestinal ischemia/reperfusion injury and long term (hours) peritoneal air exposure also happened commonly during major abdominal surgical procedures. However, the degree of POI and the underlying pathophysiological mechanism between manipulation of the intestine, intestinal ischemia/reperfusion injury and long term peritoneal air exposure have not been examined.

In view of the paucity of comparative information on POI induced by manipulation of the intestine, intestinal ischemia/reperfusion injury and peritoneal air exposure, in the present study, we examined inflammatory and oxidative stress changes in rat intestinal muscularis and gastrointestinal transit between these different surgical traumas.

Materials and methods

Animals

Healthy male Sprague-Dawley rats weighing 250-300 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. in China. The rats were housed in specific pathogen-free facilities and maintained under controlled temperatures (20-22°C), humidity (45-55%) and light (12 h light/12 h dark cycle) conditions with standard rat chow and water ad libitum. All experimental procedures were approved by the Institutional Animal Ethics Committee of Wenzhou Medical University. Studies were performed according to the guidelines of laboratory animal care (National Institutes of Health Publication No. 85-23, revised 1985).

Experimental groups and surgical procedures

Rats were randomly assigned to four different groups: control (CTL, sham operation) group, intestinal manipulation (IM) group, intestinal ischemia/reperfusion injury (IR) group and peritoneal air exposure (AE) group (n = 8 for each group). All the rats were fasted 16 h before operation, but had free access to water.

All surgical procedures were performed using standard sterile technique under general anesthesia with 2% pentobarbital sodium (3.5 mL/kg, subcutaneous injection). All rats underwent laparotomy via a midline abdominal incision about 3 cm. For the IM group, the small intestine was subjected to a standardized surgical manipulation as described previously [11]. For the IR group, the superior mesenteric artery was identified and occluded for 30 min [12]. The abdominal cavity was covered with moist gauze. For the AE group, the wound edge was retracted and the abdominal cavity was exposed to the air for 3 h. To avoided possible dehydration, physiological saline was sprayed to the peritoneal and viscera during exposure. For the control group, rats exclusively underwent laparotomy without any other surgical procedure. Six hours after surgery, all rats were given free access to standard rodent chow and water.

Measurement of gastrointestinal transit and collection of intestinal samples

Gastrointestinal transit was measured in all rats 24 hours postoperatively by evaluating the gastrointestinal distribution of liquid nonabsorbable fluorescein-labelled dextran (70,000 molecular weight, Sigma) as previously described (n = 8 for each group) [11]. Rats were administered fluorescein-labelled dextran (200 μl of 6.25 mg/ml solution in 0.9% saline) via oral gavage. Ninety minutes after administration, rats were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital sodium and the entire gastrointestinal tract was divided into 15 segments (one segment for the stomach, 10 segments for the small bowel, one segment for the caecum and three segments for the colon). Segments were opened and fluorescein-labelled dextran was washed out by saline (2 ml). Solutions were centrifuged at 12,000 rpm and clear supernatant was read at 494 nm/521 nm wavelength in a multiwall fluorescence reader. The distribution of the fluorescent dextran along the gastrointestinal tract was determined by calculating the geometric centre (GC): Σ (percent of total fluorescent signal per segment × segment number)/100.

Twenty-four hours after surgery, the rats (n = 8 for each group) were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital sodium, samples were collected from segment number seven of the small intestine. Muscularis from the small intestine was isolated and stored at -80°C. In addition, whole mount of intestine without isolating was fixed in paraformaldehyde (4%).

Table 1. Nucleotide sequences of oligonucleotide primers

Gene	Sense primer: 5' to 3'	Antisense primer: 5' to 3'
β-actin	TCACCAACTGGGACGATATG	GTTGGCCTTAGGGTTCAGAG
IL-1β	AAGAAGAAGATGGAAAAGCGGTT	TGCTTGAGAGGTGCTGATGTA
IL-6	AGTTGCCTTCTTGGGACTGATGT	GGTCTGTTGTGGGTGGTATCCTC
TNF-α	AGCAAACCACCAAGCGGAGG	CAGCCTTGTCCCTTGAAGAGAAC
IL-10	GGAGCAGGTGAAGAATGATTTTAAT	GTAGGCTTCTATGCAGTTGATG
iNOS	TCCTCAGGCTTGGGTCTTGTTAG	GGGTTTTCTCCACGTTGTTGTT
HO-1	ATGAGGAACTTTCAGAAGGGTC	GTGGGGCATAGACTGGGTT

IL, interleukin; TNF, tumour necrosis factor; iNOS, inducible nitric oxide synthase; HO-1, heme oxygenase isozyme 1.

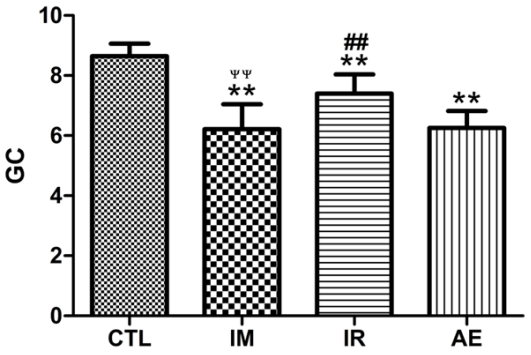

Figure 1. Results for gastrointestinal transit. GC, geometric centre; CTL, control; IM, intestinal manipulation; IR, intestinal ischemia/reperfusion injury; AE, peritoneal air exposure. Each bar represents mean ± S.D., $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the AE group, $^{\Psi\Psi}P < 0.01$ vs. the IR group.

Determination of protein concentration

Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Immunohistochemistry

Histochemical examination was performed on whole mounts of the intestine (n = 4 for each group). Transverse sections of the intestinal samples were fixed in paraformaldehyde (4%) and embedded in paraffin blocks. Sequential 4 μm sections were cut on charged slides. The sections were incubated with the primary antibodies: rabbit anti-myeloperoxidase (MPO) antibody (Abcam, UK, 1:100); rabbit anti- lymphocyte function antigen-1 (LFA-1) (CD11a/CD18) antibody (Abcam, UK, 1:100). After rinse in 0.01 M PBS, the sections were incubated with the secondary antibody for 20 min and then rinsed in PBS. Immunoreactivity was visualized using DAB (chromogen reagent) for 3 min at room temperature. Subsequently, the tissue specimen was counterstained with hematoxylin and mounted with cover slip. Finally the sections were inspected by light microscope at a magnification of × 200. MPO and LFA-1 positive cells were counted in 4 randomly chosen areas in each specimen.

Real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from the isolated intestinal muscularis (n = 8 for each group) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA purity and concentration were assessed by UV spectrophotometry (2.0 > A260:A280 > 1.8). cDNA was synthesized using RT kit (Toyobo, Osaka, Japan). The PCR reaction mixture was prepared using SYBR Green Real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan). Primer sequences were designed by the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The nucleotide sequences of oligonucleotide primers were listed in **Table 1**. Amplification of cDNA was performed with a 7500 real-time quantitative PCR instrument (Applied Biosystems, Carlsbad, CA, USA). The reaction parameters were incubation at 95°C for 1 min, then 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 1 min. All reactions were performed in triplicates and normalized using β-actin as an endogenous control gene. Relative data quantitation was performed by the ΔΔCT method.

Determination of intestinal muscularis malondialdehyde (MDA) and glutathione (GSH)

Samples (n = 8 for each group) were homogenized in ice-cold physiological saline and centrifuged at 500 × g for 15 min at 4°C. The level of MDA and GSH were measured by the methods of thiobabituric acid and 5, 5-dithio-bis-2-nitrobenzoic acid respectively [13], using commercial available kits purchased from Nanjing Jiancheng Bioengineering institute (Nanjing, China). The optical density (OD) was measured at a wavelength of 532 nm and 412 nm respec-

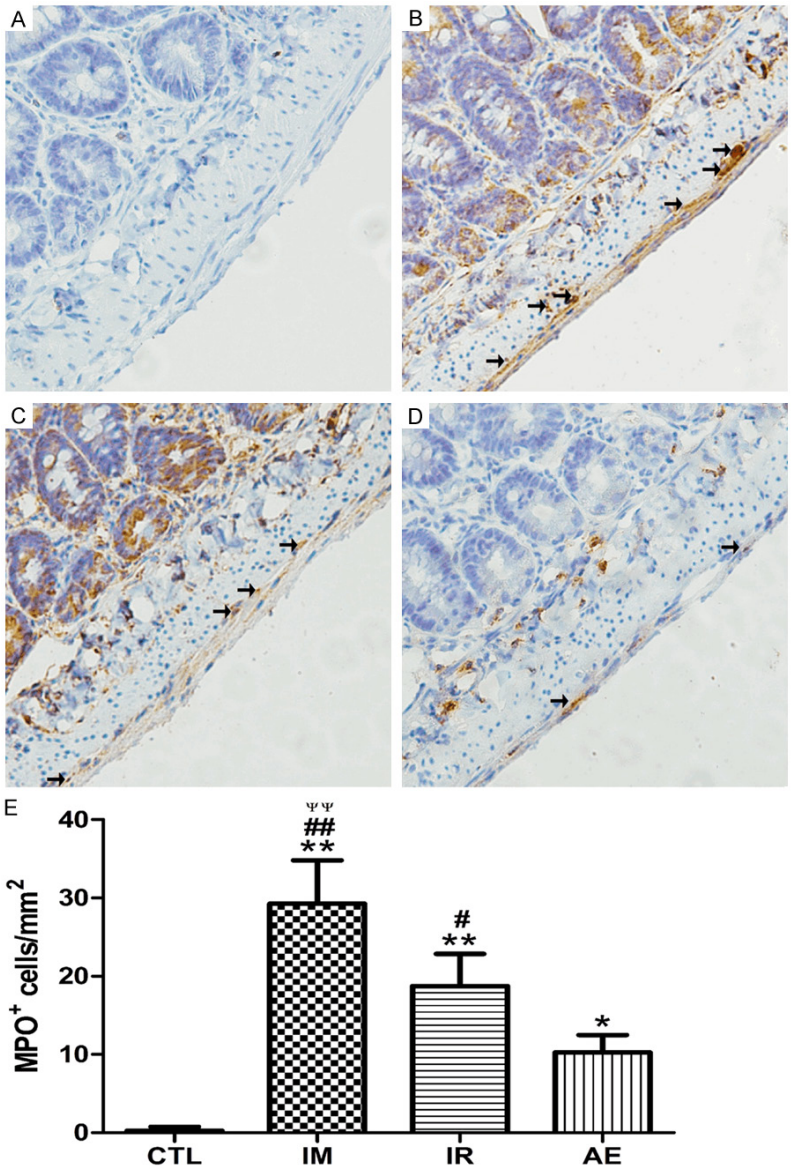

Figure 2. Immunohistochemistry results for MPO-positive cells in the intestinal muscularis (A, Control group; B, IM group; C, IR group; D, AE group). (E) Statistical analysis of the MPO-positive cells in the intestinal muscularis. MPO, myeloperoxidase; CTL, control; IM, intestinal manipulation; IR, intestinal ischemia/reperfusion injury; AE, peritoneal air exposure. Each bar represents mean ± S.D., $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the AE group, $^{\Psi\Psi}P < 0.01$ vs. the IR group.

tively, using a Varioskan Flash (Thermo Fisher Scientific Waltham, MA, USA).

Statistical analysis

All values were expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using one-way ANOVA, followed by post hoc tests (using Bonferroni's test) for multigroup comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Gastrointestinal transit

Figure 1 summarizes the calculated GC values for all groups. Higher values of GC indicate a more distal distribution of fluorescent signal and correspond to a more rapid gastrointestinal transit. Gastrointestinal transit significantly decreased in all experimental groups compared with control group ($P < 0.01$). No difference was observed in GC values between IM and AE groups ($P = 1$). However, the GC value of the IR group was higher than that of the IM and AE groups ($P < 0.01$).

Influx of neutrophil in the intestinal muscularis

MPO is a major protein constituent of the primary granules of vertebrate neutrophils. Influx of neutrophils was determined as number of MPO-positive cells in the intestinal muscularis 24 h after surgery. The number of MPO-positive cells in the IM, IR and AE groups significantly increased compared with that of the control group. Among these experimental groups, the increased number of MPO-positive cells was highest in the IM group (29.25 ± 5.56), moderate in IR group (18.75 ± 4.11) and lowest in the AE group (10.25 ± 2.22) (**Figure 2**).

LFA-1 is an adhesion molecule that is crucial in the process of transmigration and recruitment of leukocytes. In the intestinal muscularis, the number of LFA-1-positive cells significantly increased in the IM, IR and AE groups compared with that of the control group. The

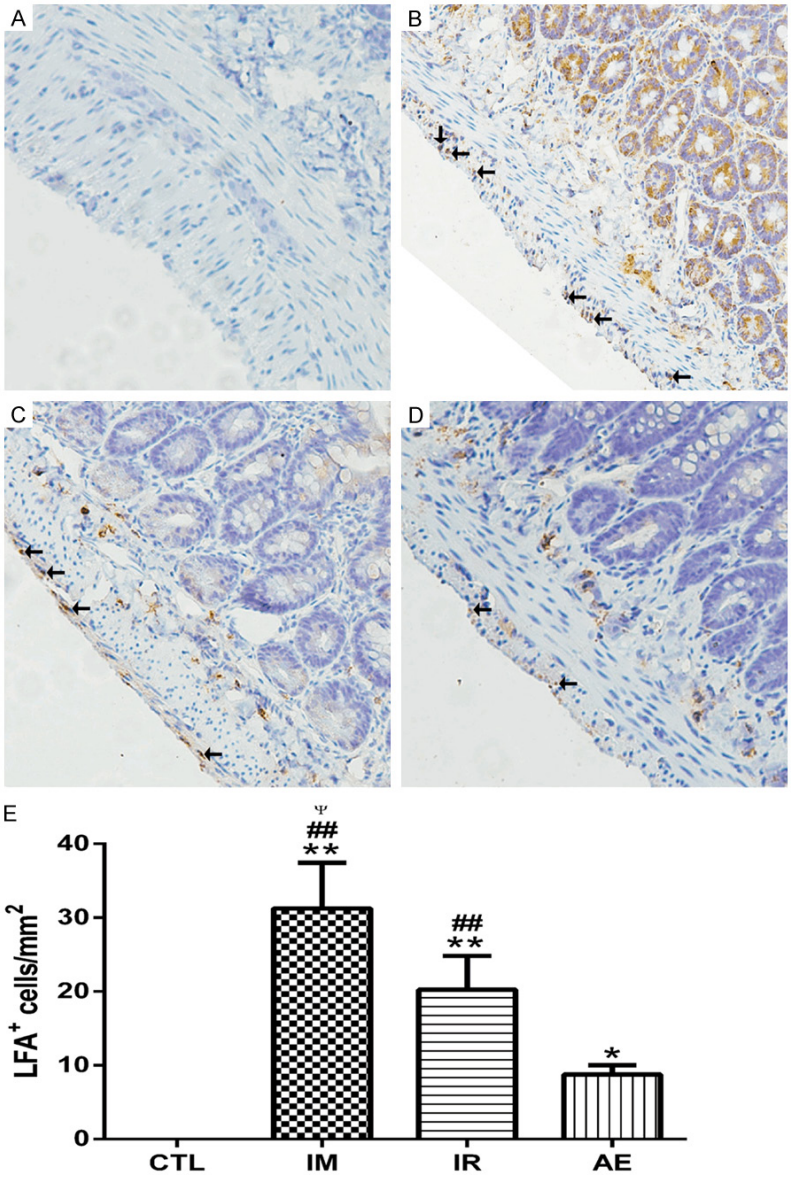

Figure 3. Immunohistochemistry results for LFA-1 positive cells in the intestinal muscularis (A, Control group; B, IM group; C, IR group; D, AE group) (200 ×). (E) Statistical analysis of the LFA-positive cells in the intestinal muscularis. LFA-1, lymphocyte function antigen-1; CTL, control; IM, intestinal manipulation; IR, intestinal ischemia/reperfusion injury; AE, peritoneal air exposure. Each bar represents mean ± S.D., $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the AE group, $^{\Psi}P < 0.05$ vs. the IR group.

increasing trends of LFA-1 among the three experimental groups were in consistent with that of the MPO-positive cells (IM group, 31.25 ± 6.18; IR group, 20.25 ± 4.57; AE group, 8.75 ± 1.26) (**Figure 3**).

Inflammatory mediators

The mRNA levels of inflammatory mediators (interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and IL-10) in the muscularis were measured by real-time quantitative PCR. The expressions of IL-1β, IL-6 and TNF-α mRNA significantly increased in the IM and IR groups compared with that of the AE and control groups, no significant difference was observed between the AE and control groups. In addition, the expressions of IL-1β, IL-6 and TNF-α mRNA in the IM group were significant higher than that of the IR group. The expression of IL-10 mRNA significantly increased in the IM, IR and AE groups compared with that of the control group. Among these experimental groups, the expression of IL-10 mRNA was highest in the IM group, moderate in IR group and lowest in the AE group (19.14, 13.16 and 6.36-fold increase over control group respectively) (**Figure 4A-D**).

Heme oxygenase isozyme 1 (HO-1) and inducible nitric oxide synthase (iNOS)

The expressions of HO-1 and iNOS mRNA significantly increased in the IM, IR and AE groups compared with that of the control group. However, no significant difference was observed among the experimental groups for the expression of HO-1 mRNA. In addition, the expression of iNOS mRNA in the IM and IR groups was significant higher than that of the AE group, but no significant difference was observed between the IM and IR groups (**Figure 4E**, **4F**).

The levels of MDA and GSH

The level of GSH significantly decreased in the IM, IR and AE groups compared with that of the

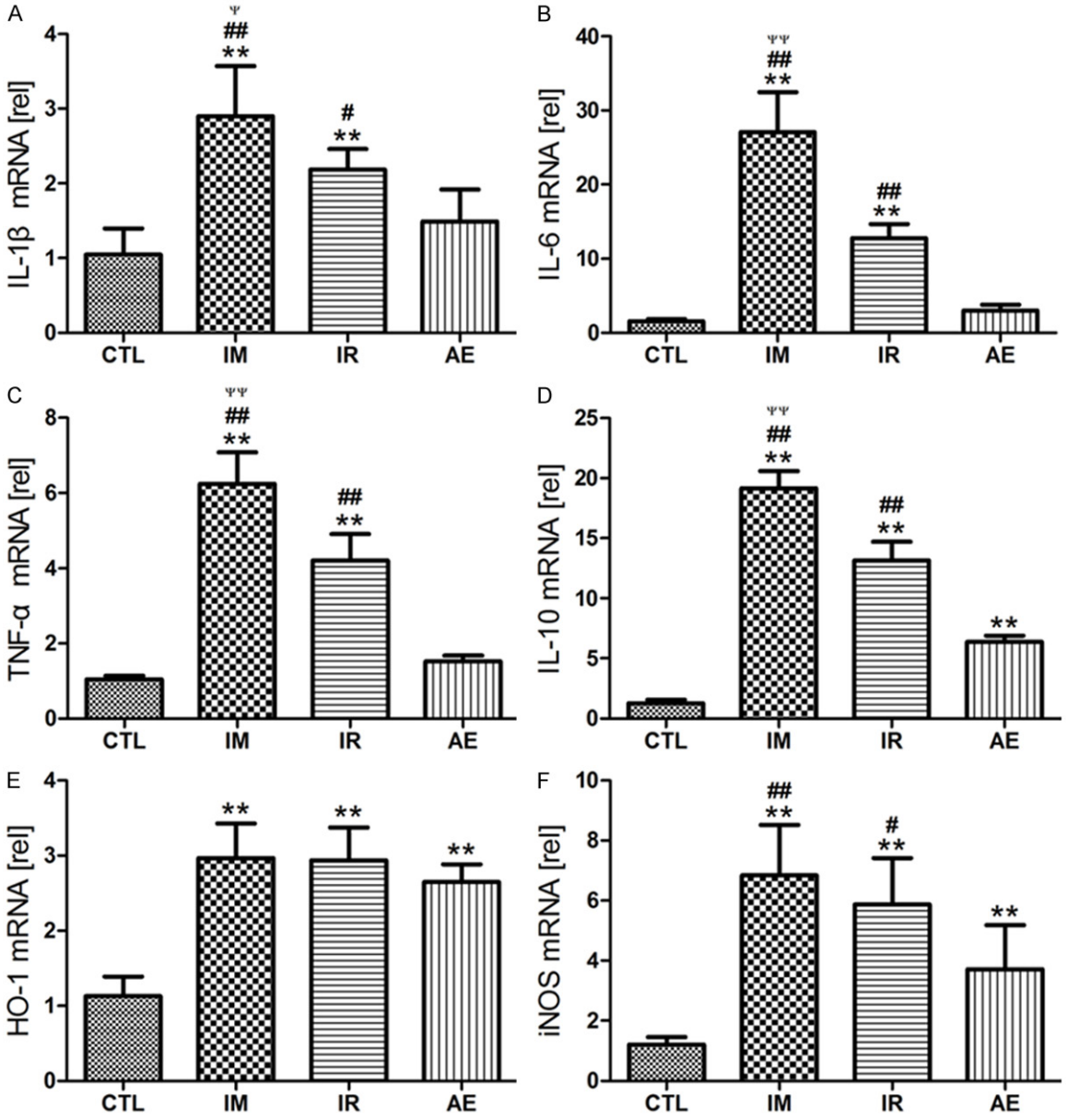

Figure 4. Real-time qPCR results for the expressions of IL-1β, IL-6, TNF-α, IL-10, HO-1 and iNOS mRNA in the intestinal muscularis. IL, interleukin; TNF, tumour necrosis factor; HO-1, heme oxygenase isozyme 1; iNOS, inducible nitric oxide synthase; CTL, control; IM, intestinal manipulation; IR, intestinal ischemia/reperfusion injury; AE, peritoneal air exposure. Each bar represents mean ± S.D., $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the AE group, $^{\Psi}P < 0.05$ and $^{\Psi\Psi}P < 0.01$ vs. the IR group.

control group. Among these experimental groups, the level of GSH was lowest in the AE group (6.84 ± 0.82 μmol/g protein), moderate in IM group (9.12 ± 1.38 μmol/g protein) and highest in the IR group (12.51 ± 1.56 μmol/g protein) (**Figure 5A**, **5B**). In contrast, the level of MDA significantly increased in the IM, IR and AE groups compared with that of the control group. Among these experimental groups, the level of MDA was highest in the AE group (0.33 ± 0.02 nmol/mg protein), moderate in IM group (0.28 ± 0.04 nmol/mg protein) and lowest in the IR group (0.23 ± 0.03 nmol/mg protein).

Discussion

In the present study, we demonstrated that rats subjected to intestinal manipulation, intestinal ischemia/reperfusion injury and peritoneal air exposure could all develop into POI. The degree

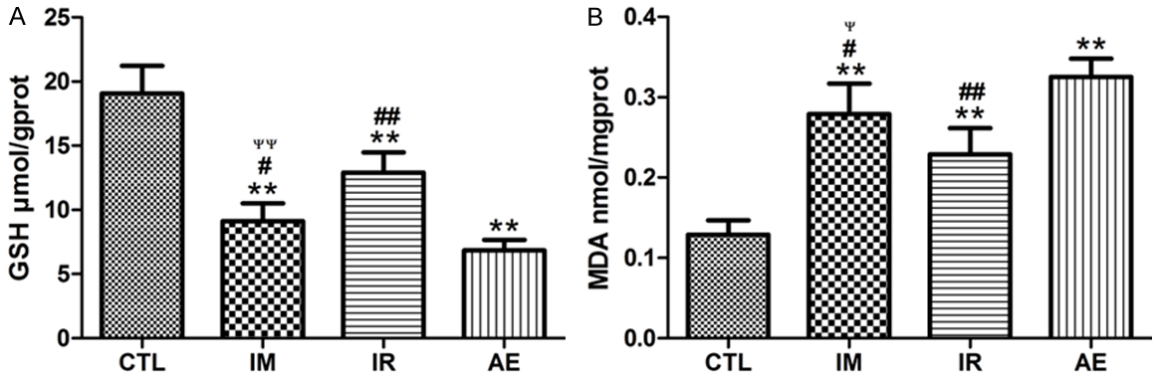

Figure 5. Results for the levels of GSH and MDA. CTL, control; IM intestinal manipulation, IR, intestinal ischemia/reperfusion injury; AE, peritoneal air exposure. Each bar represents mean ± S.D., $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the AE group, $^{\Psi}P < 0.05$ and $^{\Psi\Psi}P < 0.01$ vs. the IR group.

of gastrointestinal motility impairment was equal between the IM and AE groups. However, the IR group was subject to a less impairment of gastrointestinal motility compared with the IM and AE groups. For the underlying cellular mechanisms, the IM group showed a most significant increase of inflammatory response, while the AE group showed a most significant increase of oxidative stress. The IR group showed a moderate increase of the inflammatory response and oxidative stress.

It is well accepted that inflammation of the intestinal muscularis triggered by handling of the intestine during surgery is the main pathophysiological mechanism underlying POI [4]. The inflammation induced by intestinal manipulation activates resident macrophages present in the intestinal muscularis and causes circulating leukocytes (mainly neutrophils) to invade the muscularis. Invading leukocytes and activated resident macrophages produce prostaglandins and nitric oxide, compromising the intestinal contractile activity [2]. This mechanism is derived from data based on the animal models only involve manipulation of the intestine/colon [14]. Actually, POI is a common clinical condition arising after almost every abdominal surgery, including some surgical procedures that not involve manipulation of the intestine. Therefore, we speculate that the pathophysiological mechanism of POI may be different between patients undergoing different types of abdominal surgery. For example, patients with hypovolaemic shock before surgery, or patients undergoing major liver surgery that not involve intestinal manipulation but long time peritoneal air exposure. In the present study, we found that oxidative stress within intestinal muscularis, not inflammation, is the main mechanism of POI in the AE group. For the IR group, both oxidative stress and inflammation play an equally important role in the development of POI. Unsurprisingly, for the IM group, we demonstrated that intestinal inflammation is the main mechanism.

Oxidative stress represents an imbalance between the production of reactive oxygen species and the ability of the antioxidant defense system (i.e. antioxidant enzymes) to scavenge the reactive oxygen species (ROS) [15]. MDA is the most abundant lipid peroxidation product, which is a sensitive index to evaluate oxidative stress [16]. GSH is present in all mammalian tissues as the most abundant non protein thiol that defends against oxidative stress. The depletion of GSH was due to its reaction with ROS. Decreased levels of the antioxidants GSH has been documented in oxidative stress-related diseases [17].

LFA-1 is an adhesion molecule that is crucial in the process of transmigration and recruitment of leukocytes [18]. MPO is stored in large amounts inside azurophilic granules of neutrophils and has been detected in a wide range of inflammatory conditions [19]. In the present study, on one hand, the level of MDA significantly increased in the IM, IR and AE groups compared with that of the control group, and a highest level of MDA was observed in the AE group, which indicated a most significant increase of oxidative stress among these three experimental groups. On the other hand, the number of MPO and LFA-1 positive cells signifi-

cantly increased in the IM, IR and AE groups compared with that of the control group, but a lowest increased number of MPO and LFA-1 positive cells were observed in the AE group, which indicated a least significant increase of inflammation among these three experimental groups. This was further demonstrated by the expression of IL-1β, IL-6 and TNF-α mRNA, which was not significantly increased in the AE group compared with that of the control group. However, it is interesting that the degree of gastrointestinal motility impairment in the AE group was equal with that of the IM group, but worse than that of the IR group. All of these results indicate that, apart from the inflammatory response, oxidative stress could also cause a significant impairment of gastrointestinal motility.

In the animal models of intestinal manipulation induced POI, evidence has been accumulated that upregulation of iNOS in the resident macrophages greatly contributes to the impaired gastrointestinal motility and plays an essential role in the initiation of intestinal inflammation [14, 20]. In the present study, this evidence was confirmed in the IM group. However, the expression of iNOS mRNA in the AE group was significant lower than that of the IM and IR groups, but the degree of gastrointestinal motility impairment in the AE group was worse than that of the IR group, and equal with that of the IM group. Based on these findings, we speculated that oxidative stress within the intestinal muscularis may induce POI by mechanisms independent of iNOS upregulation, such as mitochondrial dysfunction of the intestinal muscle cells.

The heme oxygenase (HO) system is one the most critical cytoprotective mechanisms activated during cellular stress. It is now apparent that two isoenzymes of HO exist, heme oxygenase isozyme 1 (HO-1) and HO-2. The acute induction of HO-1 has been shown to have a beneficial effect protecting against oxidative stress and inflammation [21]. Results from our study showed that the expression of HO-1 mRNA significantly increased in the IM, IR and AE groups compared with that of the control group. No significant difference was observed among these three experimental groups. However, we speculate that the mechanism for induction of HO-1 may be different among these three groups, which was mainly induced by oxidative stress and inflammation for the AE and IM group, respectively. Both oxidative stress and inflammation play an important role for induction of HO-1 in the IR group.

In conclusion, our results showed that the impaired gastrointestinal motility could be induced by peritoneal air exposure, intestinal ischemia/reperfusion injury as well as intestinal manipulation in rats. The degree of gastrointestinal motility impairment was equal between the IM and AE groups, but the IR group was subject to a less impairment of gastrointestinal motility. Unlike the intestinal manipulation, oxidative stress, not inflammation, is the main pathophysiological mechanism for the peritoneal air exposure induced POI. In addition, both oxidative stress and inflammation play an important role in the intestinal ischemia/reperfusion injury induced POI. Therefore, we speculate that oxidative stress should be an equally important pathophysiological mechanism of POI as inflammation. Future studies should pay more attention to the oxidative stress induced POI and determine the exact underlying mechanism.

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Disclosure of conflict of interest

None.

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