Early surgery increases mitochondrial DNA release and lung injury in a model of elderly hip fracture and chronic obstructive pulmonary disease

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Abstract. Hip fractures are one of the most common injuries in elderly individuals and are associated with a high incidence of complications and mortality. Clinical guidelines recommend early reparative surgery within 24-48 h from hospital admission; however, it is currently unknown whether this principle of early surgery is applicable for patients with hip fracture and chronic obstructive pulmonary disease (COPD). To investigate the systemic inflammatory response and lung injury as a result early surgery in elderly patients with hip fracture and COPD, a COPD model was created, by daily exposure to cigarette smoke, and evaluated. Rats (5 months of age) were exposed to cigarette smoking for 37 weeks to create a COPD group. Rats not exposed to cigarette smoke formed the control group. All rats experienced hip fracture, which was subsequently treated with surgery at 24 h (early fixation; EF) or 72 h (late fixation; LF) after fracture, respectively. Serum mitochondrial DNA (mtDNA), tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-10 were measured at 2 and 24 h after surgery. Cytokine and myeloperoxidase (MPO) activity in the lung tissue were measured and assessed via bronchoalveolar lavage. The serum mtDNA, IL-6 and IL-10 levels in the control group and in the COPD group increased rapidly at 2 h and peaked at 24 h, while TNF-α levels peaked at 2 h and subsequently decreased. Rats that received EF in the COPD group demonstrated a significant increase of TNF-α ($P<0.001$ at 2 h), IL-6 ($P<0.001$ at 2 and 24 h), IL-10 ($P=0.010$ at 2 h and $P=0.001$ at 24 h) and mtDNA ($P<0.001$ at 24 h) compared with the rats that received LF. LF in experimental rats also significantly reduced the severity of MPO activity ($P<0.001$ and $P=0.001$) and permeability ($P=0.009$ and $P=0.018$) in pulmonary samples at 2 or 24 h, respectively, compared with EF. However, LF in the control group did not demonstrate a significant advantage at reducing MPO and permeability in serum and pulmonary samples. The present study indicated that early surgery increased mtDNA and cytokine release in a model of elderly hip fracture with COPD, and LF may reduce the severity of the inflammatory response and degree of permeability in pulmonary tissues.

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Introduction

Hip fractures are one of the most common injuries in elderly individuals and are associated with a high incidence of complications and mortality (1-3). In the USA, ~300,000 hip fractures occur annually, and this number is expected to rise with the growth of elderly populations (4). Furthermore, mortality in the elderly may reach 10% at 1 month, 20% at 4 months and 30% at 1 year following hip fracture (5). The majority of reports have indicated that early surgery improves certain outcomes for the patient, including length of stay, the incidence of pressure sores, lung infection and return to independent living (6,7). Therefore, it would appear prudent to surgically treat elderly patients with hip fractures within the first 48 h of admission to hospital, and everything possible should be done to ensure the majority of patients are surgically treated within 1-2 days. However, the principle of early surgery may not be feasible for high-risk and physiologically unstable patients (8). It may be questioned whether this principle of early surgery is applicable to all patients with hip fracture. Limited studies have focused on the adverse effects of early surgery in elderly patients with hip fracture and impaired lung conditions.

It is understood that hip fracture and accompanying early surgery has an adverse effect on the lungs of elderly patients, and postoperative lung infections are associated with impaired lung conditions (9). Chronic lung disease is a compelling reason for delaying surgery and patients often require additional treatments and tests that are time-consuming. Chronic obstructive pulmonary disease (COPD), including chronic bronchitis and emphysema, is a type of obstructive lung disease characterized by chronically poor airflow (10). It is typically progressive in nature and not fully reversible. Individuals who smoke and have COPD will have a 60-70% higher risk of mortality following proximal femur fracture than those without (11). A growing body of evidence has suggested that hip fracture induces substantial mitochondrial (mt)DNA release, systemic inflammatory response and lung injury in the elderly (12). Immediate intramedullary nailing surgery could

aggravate the above pathological states (12). Similarly, it was previously demonstrated by the current group that hip fracture and surgery also elevate systemic proinflammatory mediators in elderly patients, and the inflammatory response may serve a key role in postoperative lung dysfunction (13).

Previous results have suggested that early surgery increases mtDNA and cytokine release, which may subsequently aggravate the systemic inflammatory response and lung injury induced by elderly hip fracture (14-16). Hip fracture and surgery in elderly patients may induce systemic inflammatory responses and lung injury, which increases the risk of pulmonary infection and mortality during the post-injury period (12,13). Therefore, it may be hypothesized that late surgery may be beneficial for survival by reducing the magnitude of the inflammatory response. However, the approach of reducing the inflammatory response is unknown as little research has focused on the impact of surgery on elderly patients with COPD.

We propose selective surgery as a solution for these high-risk patients with severe COPD, in whom early fixation may be associated with high inflammatory response and secondary injury to the lungs. The aim of the present study was to evaluate the adverse effect of early surgery in a model of elderly hip fracture with COPD.

Materials and methods

Animal care. A total of 40 adult male Wistar rats (5 months old; weight, 657±41 g) were obtained from the Animal Center of the Chinese Academy of Medical Sciences (Beijing, China). The rats were allowed to acclimatize to the laboratory conditions for 1 week under a 12-h light/dark cycle at a constant temperature (22±2˚C) and a relative humidity of 50-70% with free access to rodent chow and water. The model used in the present study mimics a person who started smoking at 19 years old, with an expected life expenditure of 80 years old (17). All rats were maintained according to international guidelines on the ethical use of animals in experiments and the present study was approved by the Institutional Review Board of the Beijing Army General Hospital (Beijing, China).

COPD model. A COPD model was created, as described in our previous study (18), and rats were exposed to cigarette smoke daily for 1-2 h for up to 37 weeks. Commercial filtered cigarettes (Zhong Nan Hai, Beijing Cigarette Factory, Beijing, China) containing 14 mg tar (equivalent to 1.5-fold of the tar quantity in the Kentucky Reference Cigarette 2R4F) and 1.4 mg nicotine (equivalent to 1.4-fold of the nicotine quantity in the Kentucky Reference Cigarette 2R4F) per cigarette were used in the present study (19).

The exposure system used was as previously described (18) and had a volume of 200l. Cigarette smoke was delivered into the exposure chamber through a tube connected to a fan. The cigarette smoke was passed undiluted through the tube into the chamber by a fan on top. A group of 10 rats were placed in the inhalation chamber. The COPD model was evaluated through respiratory function and histological structure of lungs, as previously described (18). The study progressed to the next step only when the establishment of COPD was successful.

Grouping and procedure of fracture and fixation. A control group (n=10) and a COPD group (n=10) were included in the present study. Control rats were not subjected to tobacco smoke exposure, whereas COPD rats experienced persistent tobacco exposure for 37 weeks. The control and COPD groups experienced hip fracture and either early or late fixation (EF and LF, respectively). According to our previous study (18), the peak of the inflammatory response in remote organs was at 24-48 h post-fracture, followed by a decrease of the inflammatory response. Surgery was implemented at 24 h after fracture (EF) or 72 h post-fracture (LF) in control rats and rats with COPD.

Hip fracture models were created as previously described (18), using a blunt guillotine device with a weight of 500 g, and temporarily fixed by plaster. The trochanteric fossa was prepared and the medullary canal opened via a 2-cm skin incision. Sequential 1x1 mm nailing was then inserted. All rats had to survive until the end of the study period (24 h after stabilization). Each group contained 6 rats. Alternative rats were added in time if mortality occurred due to various reasons (such as fighting).

Serum mtDNA isolation. Blood samples for mtDNA extraction were collected into EDTA-containing tubes. To obtain cell-free plasma, EDTA-blood samples were initially centrifuged at 900 x g for 10 min at room temperature (RT), and the plasma was transferred into a clear polypropylene tube. The plasma was centrifuged at 9,600 x g for 10 min at RT, and the upper portion of the plasma was transferred into another clear tube and stored at -80˚C. mtDNA was prepared from 200 μl plasma using a QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The same amount of DNA was used for each quantitative polymerase chain reaction (qPCR) using a SYBR Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) on a Mastercycler EP real lex (Eppendorf, Hamburg, Germany), as described below.

qPCR. According to a previous study (20), an mtDNA-230 primer set was used to amplify a 230 bp long DNA fragment of non-apoptotic origin (forward 5'-CAGCCGCTATTAAAG GTTCG-3' and reverse 5'-CCTGGATTACTCCGGTCTGA-3'). The mtDNA plasmid was constructed using a TA cloning kit (Takara Bio, Inc., Otsu, Japan) as follows: The purified PCR products were linked into a pMD18-T vector (Takara Bio, Inc.), and the connective product was transformed into DH5α competent *Escherichia coli* (D9057A, Takara Bio, Inc.) according to the manufacturer's protocol. The positive *E. coli* clones were screened and enriched, and then mtDNA was extracted using the Qiagen Plasmid Midi kit (Qiagen GmbH) from the plasmid and measured by NanoDrop™ One/OneC (Thermo Fisher Scientific, Inc.) when A260/280 ratio limited from 1.8-2.0. A standard curve was generated by six dilutions of DNA (range, 102-107 copies/μl) using an ABI 7500 ce detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR reactions were conducted in 96-well plates within a total volume of 20 μl/well containing the following reagents: 10 μl SYBR Premix Ex Taq II (2X), 0.8 μl forward primer, 0.8 μl reverse primer, 0.4 μl ROX reference Dye II (50X), 2 μl DNA and 6 μl double-distilled H_2O. Primer sequences were all synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The SYBR® Premix Ex Taq™ II PCR

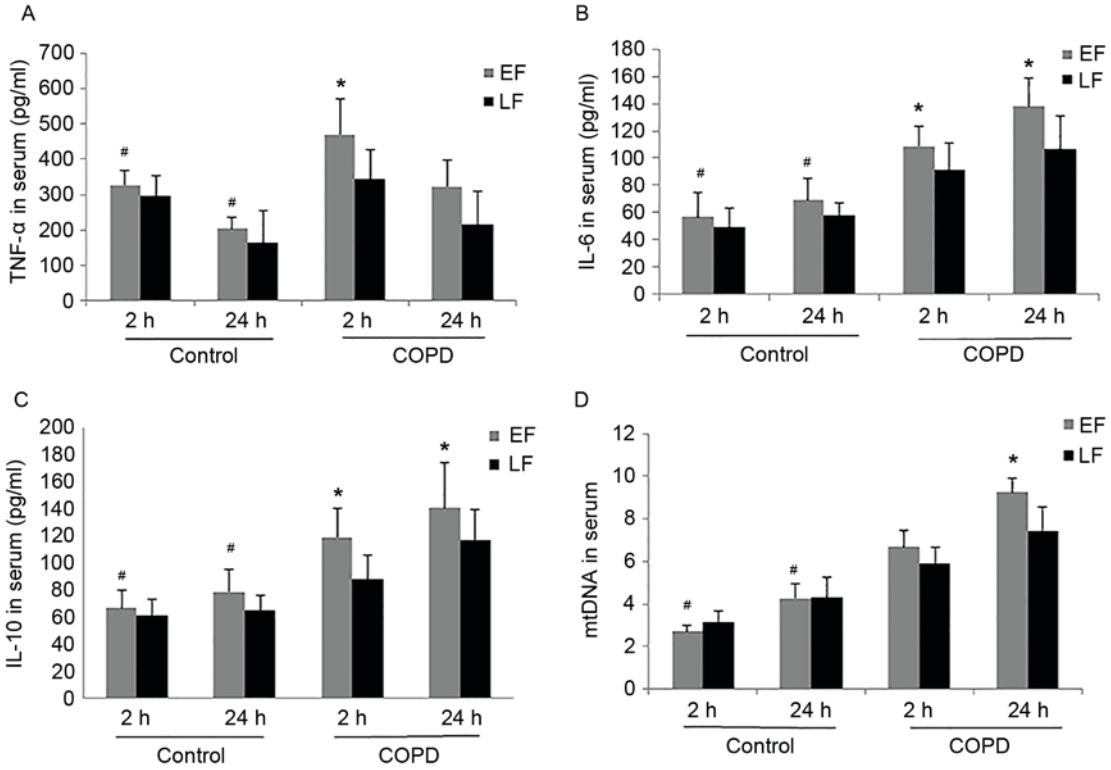

Figure 1. Levels of inflammatory cytokines and mtDNA in COPD and control groups following EF and LF. (A) TNF-α, (B) IL-6, (C) IL-10 and (D) mtDNA levels in serum of elderly rats in the control group and COPD group at 2 and 24 h after surgery. Data are presented as the mean + standard error of the mean (n=6/group). *P<0.05 vs. LF at the same time point; #P<0.05 vs. COPD group at the same time point. COPD, chronic obstructive pulmonary disease; EF, early fixation; LF, late fixation; TNF-α, tumor necrosis factor-α; IL, interleukin; mtDNA, mitochondrial DNA.

kit was purchased from Takara Bio, Inc. The PCR reaction was conducted using the following conditions: 95˚C for 30 sec, followed by 95˚C for 5 sec and 60˚C for 34 sec, repeated for 40 cycles. Each sample and DNA standard was analyzed in duplicate, and the mean value was used for quantification. A standard curve was created to quantify mtDNA concentration using purified mtDNA with CytB as the target, according to the protocol supplied with the SYBR® Premix Ex Taq™ II PCR kit. Only standard curves with a coefficient of correlation >0.96 were accepted.

Bronchoalveolar lavage fluid (BALF) and lung permeability analysis. Bronchoalveolar lavage (BAL) was performed to remove bronchoalveolar cells by instilling and withdrawing lavage solution (1.0 ml of 0.9% saline) three times via the tracheal tube before finally transferring it to a syringe. Recovery ranged from 70-90% of the instilled fluid. The recovered BALF was centrifuged at RT, 900 x g for 10 min, and the supernatant was extracted. Serum protein levels or BALF protein concentrations were quantified with the bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.), and the BAL-serum-protein ratio was accepted as a diagnostic procedure for pulmonary permeability changes and pulmonary damage/dysfunction.

Analysis of cytokines and myeloperoxidase (MPO) activity in lung tissue. In all rats, following the BAL procedure, pulmonary tissues (100 mg) from the middle lobe of the right lung (100 mg) were removed for homogenization, and were incubated at 4˚C for 1 h. The final homogenate was centrifuged at RT, 13,400 x g for 20 min. Tissue supernatants were used for cytokine assays for tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10, which were determined by avidin-biotin complex (ABC) ELISA (custom kit, R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol. Lowest limits of detection were 16 pg/mg for TNF-a, IL-6 and IL-10.

MPO activity is a sensitive index of tissue neutrophil infiltration, which was accepted as a marker of polymorphonuclear neutrophil (PMN) infiltration in pulmonary tissue. PMNs exert damaging effects through the release of proteolytic enzymes, reactive oxygen species and vasoactive substances when they have migrated into the lung tissue (21). According to the manufacturer's protocol, the level of MPO was measured using ABC ELISA (custom kit, R&D Systems, Inc. Minneapolis, MN, USA).

Statistical analysis. Data were presented as the mean ± standard error of the mean. Data were analyzed using SPSS v.16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Continuous variables were compared using the nonparametric Mann-Whitney test, while categorical variables were analyzed using Fisher's exact test. The changes of variables between groups were compared using analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes to serum TNF-α, IL-6, IL-10 and mtDNA levels. The present study examined the cytokines TNF-α, IL-6 and

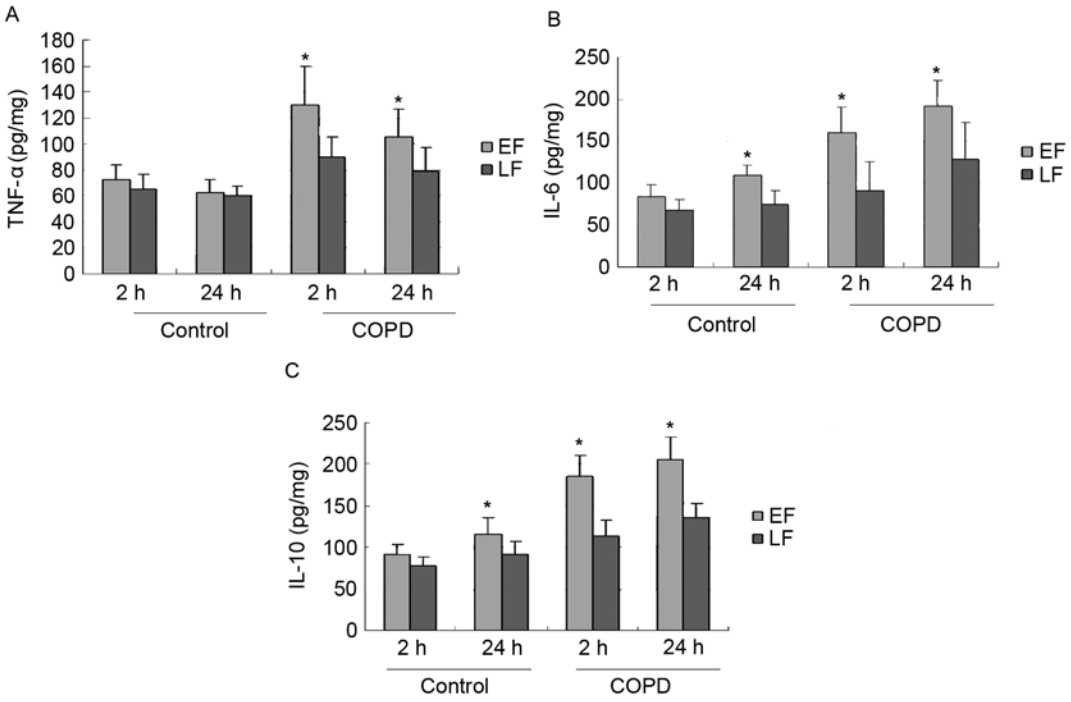

Figure 2. Levels of inflammatory cytokines in lung tissue extracts of rats from the COPD and control groups following EF and LF. (A) TNF-α, (B) IL-6 and (C) IL-10 levels in lung tissue extracts of elderly rats with or without COPD at 2 or 24 h after surgery. Data are presented as the mean + standard error of the mean (n=6/group). *P<0.05 vs. LF at the same time point. COPD, chronic obstructive pulmonary disease; EF, early fixation; LF, late fixation; TNF-α, tumor necrosis factor-α; IL, interleukin.

IL-10, and mtDNA levels in serum at 2 or 24 h after fixation surgery. ELISA analysis of cytokines and qPCR for mtDNA concentration from the experimental and control groups are demonstrated in Fig. 1. Serum IL-6 and IL-10, and mtDNA levels in the control group and in the COPD group increased rapidly at 2 h and peaked at 24 h; however, TNF-α peaked at 2 h and then decreased at 24 h. The serum concentrations of TNF-α, IL-6 and IL-10, and mtDNA in the COPD group increased significantly compared with those in the control group at 2 and 24 h after fixation (P<0.05; Fig. 1). There was no significant difference observed in the control group between EF and LF for TNF-α (P=0.203 and P=0.974), IL-6 (P=0.210 and P=0.103), IL-10 (P=0.554 and P=0.231) and mtDNA (P=0.247 and P=0.108) at 2 or 24 h, respectively. EF in rats with COPD induced a significant increase of TNF-α (P<0.001 at 2 h), IL-6 (P<0.001 at 2 and 24 h), IL-10 (P=0.010 at 2 h and P=0.001 at 24 h) and mtDNA (P<0.001 for 24 h) compared with the LF group (Fig. 1).

Inflammatory mediators in pulmonary extracts. The levels of TNF-α, IL-6 and IL-10 in lung tissue extracts of experimental and control aged rats are demonstrated in Fig. 2. LF in the COPD group induced a significant decrease of TNF-α (P=0.001 and P=0.006), IL-6 (P<0.001 and P=0.001) and IL-10 (P=0.035 and P<0.001) at 2 or 24 h, respectively, compared to the levels following EF. Although the levels of TNF-α (P=0.487), IL-6 (P=0.284) and IL-10 (P=0.220) following EF in the control group were higher at 2 h compared with those following LF, the differences were not statistically significant. At 24 h after fixation, IL-6 (P=0.043) and IL-10 (P=0.045) levels were significantly decreased following LF compared with EF in the control group.

Neutrophil infiltration and lung permeability. As demonstrated in Fig. 3A, MPO activity, a marker of PMN infiltration, following LF in the COPD group was significantly decreased at 2 (P<0.001) and 24 h (P=0.001) compared to EF in the COPD group. No significant differences were observed between EF and LF at 2 (P=0.504) or 24 h (P=0.668) for PMN infiltration in the control group.

The results demonstrated that the BAL-serum-protein ratio following EF in the COPD group was significantly elevated compared to LF at 2 (P=0.009) and 24 h (P=0.018; Fig. 3B). LF in the control group demonstrated a decrease in the BAL-serum-protein ratio at 2 h (P=0.572) and an increase at 24 h (P=0.866) compared to EF; however, these results were not statistically significant.

Discussion

It has been reported that COPD is the fourth most common cause of mortality in adults, responsible for ~2 million mortalities per year (22). Certain therapies used in COPD, such as oral and inhaled corticosteroids, have been demonstrated to increase the risk of osteoporosis, making elderly patients more prone to hip fracture (23). Two previous studies examined the contribution of a prior history of COPD on hip fracture prognosis. In a multicenter, retrospective study of 390 Medicare beneficiaries with hip fracture, a history of COPD was an independent predictor of 30-day mortality (24). In another retrospective study, individuals with COPD were found to have a 60-70% higher risk of mortality following hip fracture than those without COPD (25). COPD produced a marked increase of the inflammatory response (TNF-α, IL-6 and IL-10) following proximal femur fracture, which resulted in organ

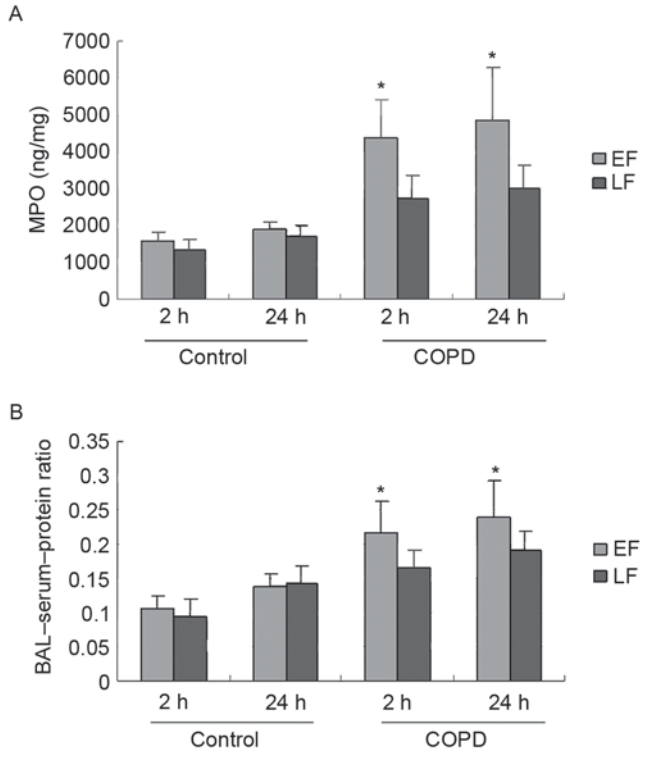

Figure 3. Neutrophil infiltration and lung permeability in rats from the COPD and control groups following EF and LF. (A) MPO activity and (B) BAL-serum-protein ratio in lung tissue extracts of elderly rats with or without COPD at 2 or 24 h after surgery. Data are presented as the mean + standard error of the mean (n=6/group). *P<0.05 vs. LF at the same time point. COPD, chronic obstructive pulmonary disease; EF, early fixation; LF, late fixation; MPO, myeloperoxidase; BAL, bronchoalveolar lavage.

injury (18). Hip fractures in elderly patients are known to have a reduced physiological reserve, have weaker connective tissue and often multiple additional pre-existing co-morbidities that make management more complicated (26). The dilemma of optimal timing of surgery for these high-risk patients in hip fracture is a challenge for all trauma surgeons.

Damage control orthopedics (DCO) is an approach that contains and stabilizes orthopedic injuries to enable improvement of the patient's overall physiology (21,27,28). Its purpose is to avoid worsening of the patient's condition by the 'second hit' of a major orthopedic procedure and to delay definitive fracture repair until a time when the overall condition of the patient is optimized (28). DCO has been suggested for the management of femoral shaft fractures in severely injured patients because patients were unable to tolerate the burden of early determinative surgery (21,29). A clinical study investigated the type and timing of surgery and demonstrated that improvements in the clinical status coincided with a less sustained inflammatory response if DCO was followed (30).

The results of the present study demonstrated that LF in the COPD group (surgery followed by peak of inflammatory response to trauma) resulted in significant decreases of cytokines (TNF-α, IL-6 and IL-10) and mtDNA in the circulation and pulmonary samples, and reduced the severity of injury to pulmonary tissues (MPO activity and permeability) compared to EF (surgery performed at the peak of the inflammatory response induced by trauma; early definitive fixation). The present results indicated that inflammatory infiltration may be reduced by modifying the timing of surgery, as demonstrated in an elderly rat model of COPD. It remains to be further determined whether this strategy may be introduced in clinical practice for elderly patients with hip fracture, in particular in those with COPD.

Notably, although LF in the control group was associated with reduced levels of cytokines (TNF-α, IL-6 and IL-10) in pulmonary samples at 24 h after surgery compared to EF, the difference of the level of MPO and permeability between these groups did not reach significance. This suggested that injury to pulmonary tissue induced by early surgery was similar to that of late surgery in normal elderly patients.

To the best of our knowledge, the present study was the first to evaluate the adverse effects of early surgery in a model of elderly hip fracture with COPD. Various studies have demonstrated that mtDNA is a critical activator of inflammation and the innate immune system (14,31). Some research reported that cellular injury released mtDNA into blood, which is a key link between trauma, inflammation, soluble immune response suppressor (SIRS) and acute lung injury in polytrauma patients and the elderly hip fracture model (12,13,16). mtDNA contains unmethylated CpG motifs that exhibit immune stimulatory capacities (31). Recently, our previous studies have demonstrated the mtDNA release induced by hip fracture induces the systemic inflammatory response and lung injury by activating the Toll-like receptor 9 (TLR9)/nuclear factor (NF)-κB pathway in rats (12,30), and may induce systemic inflammation through TLR9/NF-κB and p38/mitogen-activated protein kinase signaling, thereby initiating an immunological response characteristic of SIRS and acute respiratory distress syndrome (12,15,30). In the present study, the serum mtDNA concentration was increased rapidly and considerably when fixed at 24 h in EF and LF groups. Furthermore, the serum mtDNA concentration following EF was significantly higher at 2 and 24 h compared with those following LF in the COPD group.

Several limitations should be considered when interpreting the present results. Firstly, no continuous measurement in the same rat was obtained and the individual variability in cytokine release must therefore be acknowledged because a different set of rats was sacrificed at each time point. Secondly, the applicability of the present method of bone fracture and fixation to the clinical setting of hip fracture was unknown. The present method was used to induce injury due to its reproducibility and to maintain compliance with animal research guidelines.

In conclusion, the present study demonstrated that the inflammatory response was notably present in pulmonary samples and blood in the COPD model following surgery, and this was associated with an increase in MPO activity and permeability. LF in the COPD group significantly reduced severity of inflammatory infiltration, MPO activity and permeability in pulmonary samples compared to EF, suggesting the validity of DCO to reduce damage of organs in elderly patients with hip fracture and COPD.

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