

Original Paper

# Urinary Trypsin Inhibitor Attenuates Acute Lung Injury by Improving Endothelial Progenitor Cells Functions

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## Key Words

Urinary Trypsin Inhibitor (UTI) • Endothelial progenitor cells (EPCs) • Acute lung injury (ALI) • Akt/eNOS

## Abstract

**Background:** Urinary Trypsin Inhibitor (UTI) is involved in various aspects of tissue repair, regeneration and development. However, the potential role of UTI in protection against acute lung injury (ALI) remains largely unknown. In the present study, we demonstrated that UTI treatment could ameliorate ALI induced by oleic acid (OA) treatment in rabbit model. **Methods:** Intravenous application of UTI (10000 U/kg/d) significantly improved the pathologies associated with OA-induced ALI. The lungs were stained with hematoxylin and eosin to score the lung injury. Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Plaque Plus. The proliferation and ability of tube structure formation of EPCs were observed and the level of phosphorylated Akt protein expression and eNOS protein expression were assayed. **Results:** Consistent with pathological scores, UTI treatment significantly reduced wet/dry ratio of OA injured lungs. A quantification of capillary density revealed that UTI treatment led to about 2 fold increase over uninjured control and about 1.5 fold increase over PBS treatment. The capacity for tube formation of EPCs on ECM gel was significantly reduced in the ALI group and recovered with UTI treatment. Quantification of western blot bands was summarized and showed that UTI treatment activates Akt/eNOS signaling. NO production could contribute to the improvement of EPCs function by UTI treatment. **Conclusions:** UTI-induced phosphorylation/activation of eNOS and Akt, increases the intracellular level of NO, thereby improving tube formation and proliferation function of EPCs. EPCs function is crucial for re-endothelialization after denuding injuries of arteries.

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## Introduction

Acute lung injury (ALI) is an acute progressive dyspnea and refractory hypoxemia caused by a variety of internal and external pulmonary factors. ALI and its more severe form, acute respiratory distress syndrome (ARDS), are the main causes of morbidity and mortality in various disease conditions, including heart/lung transplantations, acute pulmonary embolism, pulmonary thrombosis and ventilator induced lung injury [1-3]. The common pathological changes of ALI are acute inflammatory response and increase of pulmonary microvascular permeability, resulting in influx of protein-rich edema fluid and loss of epithelial barrier function [4]. Although a few approaches have been developed to address these issues, including anti-inflammation treatments, low tidal volume ventilation and fluid-conservative therapy, the mortality rate in patients with ALI/ ARDS remains 30 to 40% [5].

Urinary trypsin inhibitor (UTI), originally purified from the fresh urine of healthy men, could be used to inhibit the release of a variety of inflammatory factors from neutrophils and suppress the activity of neutrophil elastase (NE) [6]. Moreover, it also stabilizes lysosomal membrane and suppresses the release of destructive lysosomal enzymes, which preserves integrity of intracellular organelles [7]. In recent years, UTI was widely used in treating sepsis, the most life-threatening medical condition and main cause of ALI. Indeed, it has been reported that early application of UTI can attenuate the symptoms of ALI [8], however, the pharmacological actions and possible mechanisms underlying UTI mediated lung protection need to be further elucidated.

It has been demonstrated that up to 50% of lung capillaries are lost in animal models of ALI [9] and apoptosis and necrosis of lung endothelial cells is a hallmark of ALI/ARDS [10]. Thus, the therapies enhancing endothelial cell repair/regeneration and neovascularization could be an effective means to restore pulmonary function and reduce the severity of ALI/ARDS.

As one type of potential therapeutic agent in vascular regeneration, endothelial progenitor cells (EPCs) based therapy might be a promising treatment for ALI/ARDS. EPCs were initially described and defined as a special type of stem cells by Asahara et al. [11] and have been found in various organs, such as bone marrow, spleen, umbilical cord blood (CB) and peripheral blood (PB). They contribute to the formation of new blood vessels in postnatal life and are incorporated into injured vessels to become mature endothelial cells (ECs) in response to tissue injuries [12-14]. It has been shown that patients with pneumonia had high level of circulating EPCs in their blood within the first day of illness and the number of EPCs decreased 8 weeks after treatment and recovery [15], indicating the role of EPCs in repair of vasculature. Furthermore, the number of circulating EPCs has been positively linked with prognosis of patients with ALI [16]. In a left-sided rat lung transplant model, EPCs delivered through intravenous injection were able to integrate into the transplanted left lung suffering from ischemia-reperfusion injury during transplantation [17], indicating that EPCs play a role in protecting against ALI or pneumonia. In the present study, we hypothesized that the UTI can improve EPC-mediated vascular repair, which contributed to treatment of ALI. We utilized a rabbit model of ALI induced by oleic acid (OA) and explored potential beneficial effects of UTI treatments. The reason for using OA-induced ALI was OA has been demonstrated to cause injury to lung endothelial cells. If UTI can promote EPCs function, we might observe beneficial effects of UTI following OA injury. It was shown that treatment of UTI significantly improved pathologies of the lung associated with OA application. Specifically, UTI treatment improved vascularization of injured lungs. To test the molecular mechanism, we found that UTI could improve proliferation and functions of EPCs through upregulating Akt/eNOS signaling axis. Taken together, our studies revealed a novel function of UTI on regulation of EPCs for treatment of ALI.

## Materials and Methods

### *Animals*

Male, New Zealand White rabbits (2.1–2.5 kg) were purchased from the Animal Center of Zhongshan School of Medicine, Sun Yat-sen University (Guangzhou, China). All animals were caged at room temperature and allowed to eat and drink ad libitum. The current study was conducted according to the guidelines of the Animal Care Review Board of the Guangdong general hospital Committee on Animal Care.

### *Animal model*

The rabbits were randomly grouped into 3 groups (4 animals per group): one control (PBS treatment) group, one OA treated group, three OA+UTI treated groups. Rabbits were initially anesthetized with ketamine hydrochloride (25 mg/kg, i.v.; Rogar/STB, Montreal, PQ, Canada), and then maintained by continuous infusion of ketamine at a rate of 0.5 mg/kg/h during OA and/or UTI. The OA treatment group received 0.1 ml/kg/h, i.v. of OA (Sigma-Aldrich Corp) for 2 h [18]. The control group received 0.1 ml/kg/h, i.v. of PBS for 2 h. The OA +UTI treatment animals were treated with UTI (Techpool Biochemical Pharmaceutical Co., Ltd., 10000 U/kg/d, intravenous infusion) [19]. The first treatment of UTI was 30 minutes before OA infusion. The Second and third administrations of UTI were delivered 24 and 48 hours after OA infusion.

### *EPCs Isolation and Cultivation*

Peripheral blood was obtained from New Zealand White rabbits via the peripheral ear artery (10 ml/kg). Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Plaque Plus (Amersham Biosciences). Subsequently, mononuclear cells were washed and then incubated on culture dishes. Unselected mononuclear cells were plated on fibronectin-coated culture dishes (Biocoat; Becton Dickinson Labware, Franklin Lakes, NJ) at a density of  $10^6$  cells/mL in Medium 199 (Invitrogen, Carlsbad, CA), supplemented with 20% fetal bovine serum, 100 U/mL penicillin/streptomycin (Invitrogen), and 0.05 mg/mL bovine pituitary extract (Invitrogen). Under daily observation, after 4 days of culturing, media were changed and non-adherent cells were removed; attached early EPCs appeared. Thereafter, media were replaced after 2 days.

### *Tube-formation Assay*

An ECM gel (Sigma) placed on a 96-well culture plate at 37 °C for 1 h to allow solidification after thawed at 4 °C overnight. EPCs treated with or without PA were harvested and seeded (10,000 cells/well) on the top of the solidified ECM gel in EBM-2 medium supplemented with 0.5% BSA and VEGF (100 ng/mL). Cells were incubated at 37 °C for 12 h. Tube formation was defined as a structure exhibiting a length 4 fold more than its width. The networks of tubes were photographed from six randomly chosen fields with a microscope. The total length of the tube structures in each photograph was measured using Adobe Photoshop software (Adobe, San Jose, CA).

### *Proliferation Assay*

Proliferation of EPCs was determined by direct counting six random high-power microscopic fields ( $\times 100$ ) and b-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. EPCs were supplemented with MTT (0.5 mg/mL; Sigma) and incubated for 4 h for proliferation assay. The blue formazan was dissolved with dimethyl sulfoxide and measured at 550/650 nm.

### *Histopathological examination*

The lungs were embedded in paraffin and the sections were stained with hematoxylin and eosin (H&E). Two qualified pathologists, blinded with the treatments, scored the lung injury, according to combined assessments of alveolar congestion, hemorrhage, edema of Immunostaining assays.

### *Determination of NO Generation*

NO is unstable, but it produces the stable end products nitrite and nitrate. Hence, the best index of NO generation is the sum of nitrite and nitrate. Culture media were harvested and stored at -80°C until used for the assays. Levels of nitrite and nitrate were measured as described previously. Briefly, nitrate was converted to nitrite with nitrate reductase, and total nitrite was reacted to the Griess reagent. Absorbance of the color product was determined at 540 nm with a spectrophotometer.

## Western Blot Analysis

EPCs were harvested in Western blot lysis buffer and the lysates were cleared by centrifugation at 12,000 × g for 10 min at 4 °C. The proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes, then probed with one of the following primary antibodies against t-Akt, t-eNOS, p-Akt, p-eNOS. All these antibodies were polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies bound to the target proteins were then detected by horseradish peroxidase (HRP) -conjugated anti-rabbit IgG (Promega, Madison, WI) and visualized with enhanced chemiluminescent detection kit (Pierce Biotechnology, Rockford, IL). Intensities of all target bands were normalized with that of the protein loading control GAPDH band calculated by the FluorChem 8900 software system (Alpha Innotech, San Leandro, CA) [20].

## Statistical analysis

Data were generally expressed as mean±SD. SPSS software version 11.0 (SPSS, Inc., Chicago, IL) was used for statistical analyses. Statistical significance among mean values was evaluated by one-way ANOVA tests for measurement data, and LSD-t test for comparison between each other. Differences were considered significant when p value was P<0.05.

## Results

### *UTI reduces the pathologies associated with ALI*

In order to test the function of UTI in treating of ALI, we utilized our established rabbit model of ALI by oleic acid (OA) treatment [18]. In this study, the changes induced by OA resembles ALI in many morphological, histological and physiological aspects, included edema, hemorrhage and atelectasis in the ALI group (Fig. 1A, 1B), indicating our ALI model was successfully generated. We found that intravenous infusion of UTI (10000 U/kg/d) could significantly improve pathologies associated with ALI, as demonstrated by histological images (Fig. 1A) and pathologists' evaluations (Fig. 1B) included edema, hemorrhage, atelectasis, infiltration of inflammatory cells and total lung injury histology scores. The edema of the lungs following ALI was also quantified by measurement of wet/dry weight ratio between the ALI and UTI treatment groups (Fig. 1C). Consistent with pathological scores, UTI treatment significantly reduced wet/dry ratio of OA injured lungs. Taken together, our *in vivo* OA-induced ALI study suggested a protective function of UTI.

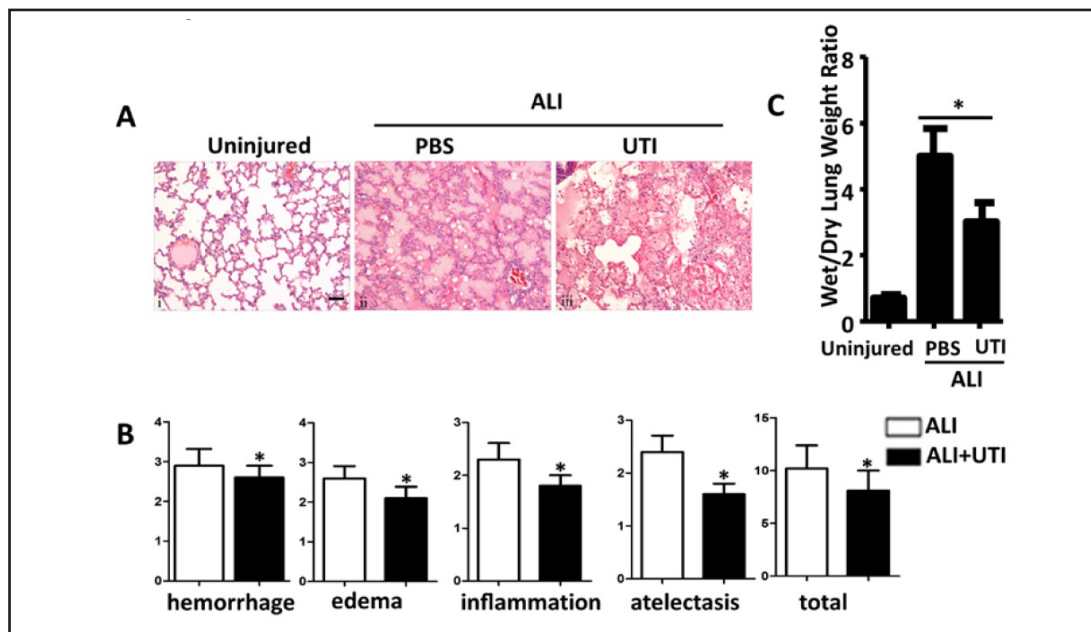
### *UTI treatment increases capillary density of OA injured lungs*

To further dissect the beneficial effects of UTI treatment, histologic evaluation of lung sections was performed to quantify capillary density, an index of neovascularization. As shown in Fig. 2, UTI treatment led to a significant enhancement of capillary density as compared to the uninjured group and PBS treatment group as controls. A quantification of capillary density revealed that UTI treatment led to about 2 fold increase over uninjured control and about 1.5 fold increase over PBS treatment, respectively (\*P<0.05).

### *UTI Increase Proliferation of EPCs*

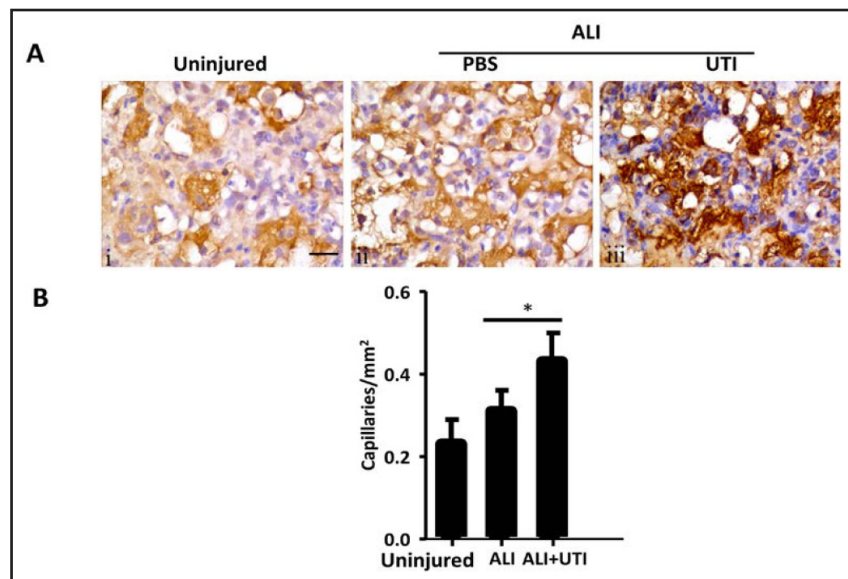
Since EPCs play a critical role on neovascularization after ALI [16], we hypothesized that UTI treatment might improve repair of ALI by enhancing function of EPCs. To answer this question, we first tested the effects of UTI on proliferation of EPCs. After isolating EPCs from animals with or without OA treatment, we measured proliferation of EPCs by MTT assay. As shown in Fig. 3A, the proliferation ability of the EPCs from uninjured animals was highest among all groups. Interestingly, after treated with UTI, the proliferation of EPCs from animals with ALI was significantly improved (P<0.05). In addition, the function of EPCs was also measured by tube formation assay, consistent with observations in proliferation assay, the capacity for tube formation of EPCs on ECM gel was significantly reduced in the ALI group and recovered with UTI treatment (Fig. 3B, 3C). Thus, our data suggested that ALI induced dysfunction of EPCs can be rescued by treatment of UTI.





**Fig. 1.** UTI infusion improves pathologies associated with OA induced ALI. (A) Histological changes in OA induced ALI with or without UTI treatment were shown in the representative images. Severe lung edema, hemorrhage, atelectasis and inflammatory cell infiltration were observed in OA treatment group, there was less damage in UTI treatment group (H&E, magnification  $\times 100$ ). (B) Lung injury scores in the indicated groups were evaluated by qualified pathologists. \*:  $p < 0.05$  in UTI-treatment group vs. ALI group. (C) Wet-to-dry weight ratios of the lungs from each experimental group were measured. UTI treatment reduced wet-to-dry ratio (\* $P < 0.05$ ). Scale bar: 50  $\mu\text{m}$ .

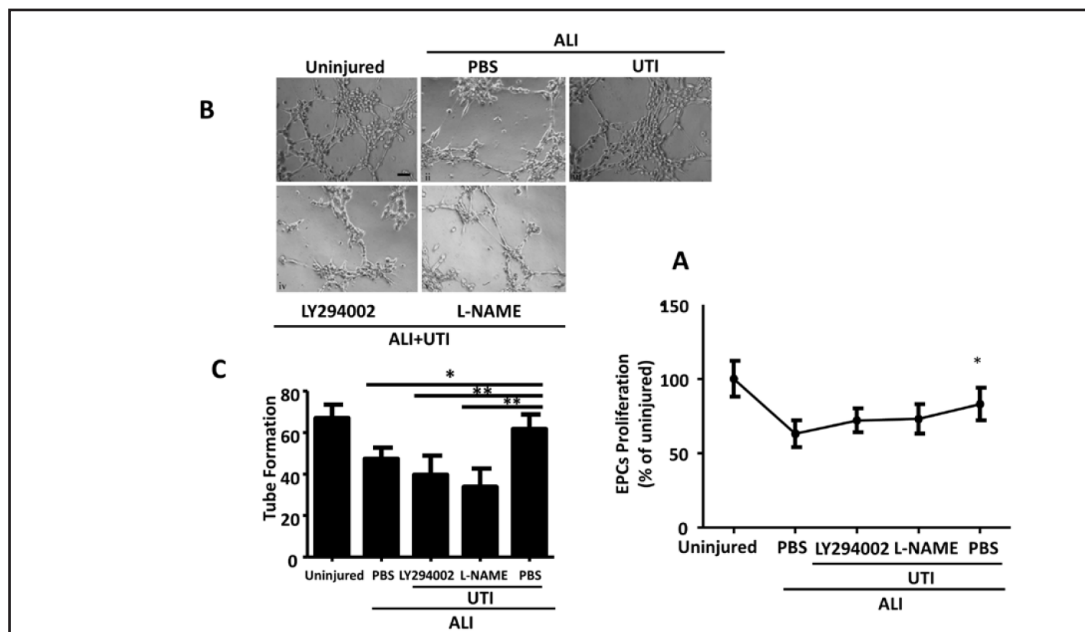
**Fig. 2.** UTI treatment increases capillary density of the injured lungs. (A) Histologic evaluation of capillaries in OA injured lung treated with or without UTI. (B) Capillary density was significantly increased in group receiving UTI versus the ALI control group at day 2 post OA treatment. (\* $P < 0.05$ ). (magnification  $\times 100$ ). Scale bar: 50  $\mu\text{m}$ .



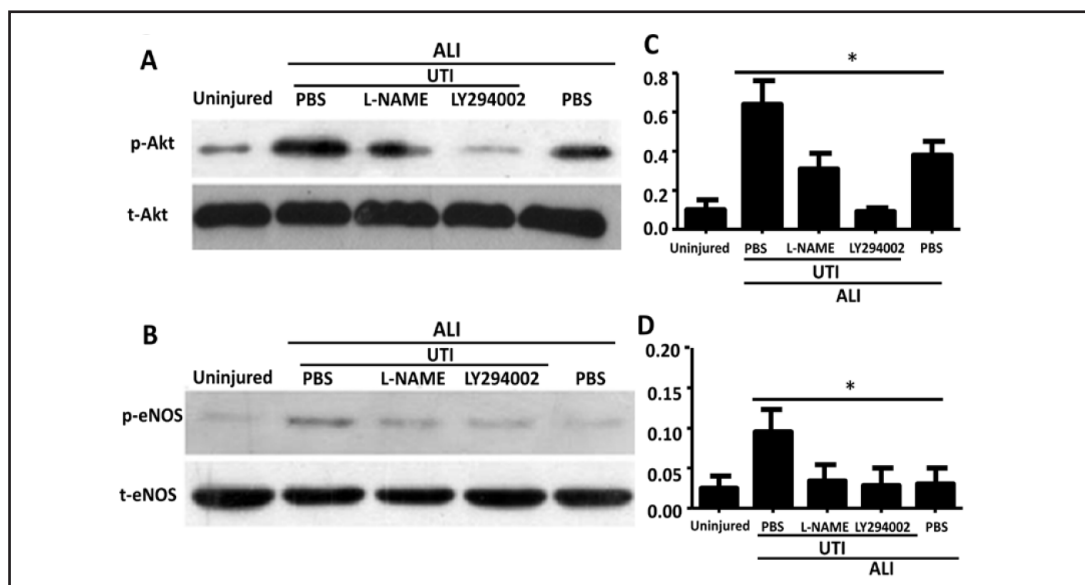
#### UTI improves functions of EPCs through Akt/eNOS signaling pathway

Akt/eNOS pathway plays a pivotal role in the regulation of EPCs functions. We therefore investigated the effects of PA on Akt/eNOS signal pathway in EPCs.

Immunoblotting showed that UTI treatment activated Akt signaling as evidenced by the enhancement of specific phosphorylated (activated) Akt at the serine 473 phosphorylation site (Fig. 4A). It has been shown that eNOS is regulated by Akt through posttranslational phosphorylation at its Ser1177 site in endothelial cells (ECs) [21]. To determine whether UTI had any effect on eNOS activity in EPCs, we examined the eNOS activation by measuring



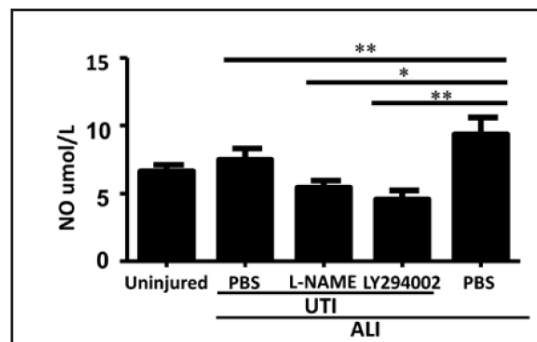
**Fig. 3.** UTI treatment enhances proliferation and tube formation of EPCs. (A) MTT assay was performed for EPCs proliferation activity. Proliferation ability of EPCs was significantly increased in group receiving UTI treatment versus the ALI group (\* $P < 0.05$ ). Akt and eNOS inhibitors, L-NAME and LY294002, inhibit the effect of UTI on EPCs proliferation capacity. (B) The tube formation ability of EPCs is also enhanced by UTI treatment. ALI impairs the tube formation ability of EPCs which can be rescued by UTI treatment. Similar to proliferation assay, pretreatment of EPCs with LY294002 or L-NAME attenuated the effect of UTI. (\* $P < 0.05$ ; \*\*  $P < 0.01$ ). (magnification  $\times 100$ ). Scale bar: 50  $\mu$ m.



**Fig. 4.** UTI treatment activates Akt/eNOS signaling and enhances NO production. ALI induces EPCs AKT (A) and eNOS (B) phosphorylation without influence t-AKT. There was more significant phosphorylation in ALI treatment with UTI group compared with ALI group. LY294002 or L-NAME inhibited EPCs AKT and eNOS phosphorylation. (C, D) Quantification of western blot bands was summarized. Data were represented by mean  $\pm$  SEM. (n=3 independent experiment, \*;  $p < 0.05$ ).

its phosphorylation at Ser1177. As shown in Fig. 4B, significant increase of eNOS phosphorylation was observed with UTI treatment.

**Fig. 5.** UTI treatment enhances NO production in EPCs. UTI treatment increases NO liberation from EPCs. Akt/eNOS inhibitors, LY294002 or L-NAME, attenuated the effect of UTI on NO production of EPCs. Data were represented by mean  $\pm$  SEM, (n=3 independent experiment, \*P<0.05, \*\* P<0.01).



Since Akt/eNOS signal pathway was enhanced by UTI, we further assessed the effect of UTI on NO production, which has been shown to be critical for functions of EPCs [22]. Indeed, we found that an increase of NO was associated with increase of phosphorylated eNOS (Fig. 4B and Fig. 5). Since both eNOS and inducible NOS (iNOS) can regulate NO production, we also checked the effects of PA on iNOS expression. We found that UTI treatment failed to alter iNOS expression, suggesting the selective inhibition of eNOS activity by PA (data not shown). Taken together, we demonstrated that the increase in Akt/eNOS phosphorylation and NO production could contribute to the improvement of EPCs function by UTI treatment.

#### *Pharmacological inhibitors of Akt/eNOS pathway blocks beneficial effects of UTI*

To further confirm the beneficial effects of UTI are through Akt/eNOS signaling pathway, two pharmacological inhibitors against the Akt pathway (LY294002) and eNOS pathway (L-NAME) were used to treat EPCs together with UTI. As shown in Fig. 3, both proliferation and tube formation of EPCs were significantly blocked by treatments of inhibitors. Consistently, NO production induced by UTI was also blocked by these inhibitors.

## Discussion

It has been reported that early application of UTI can reduce the symptoms of ALI through enhancing angiogenesis and other mechanisms. However, the potential beneficial effect of UTI for treatment of OA-induced ALI has not been fully investigated. In the present study, we found that the changes induced by OA resemble ALI in morphological, histological and physiological aspects, which makes OA-induced ALI an ideal animal model to study the efficacy of UTI treatment. With this established animal model, we found that infusion of UTI significantly reduced edema, inflammation, atelectasis and area of hemorrhage in the lung sections as compared to PBS treatment. Further studies revealed that UTI treatment increased tube formation and proliferation of EPCs through activating the Akt/eNOS pathways. Finally, our data suggested that an increase in NO generation was the key effector linking beneficial effect of UTI on the vasculogenesis of injured lungs and proliferation activities of EPCs. Our finding may partly explain the mechanisms underlying UTI mediated protection against ALI.

In this study we chose a model of OA-induced ALI in New Zealand White rabbit because the rabbit is an extensively used model that remains relevant in the study of lung injury mechanisms. A recent study showed that the lung is the organ that is most vulnerable to the effects of OA. Both endothelial and alveolar epithelial cells were markedly sensitive to OA injury [23]. Moreover, the changes induced by OA resemble ALI in many morphological, histological and physiological aspects. It has been demonstrated that levels of OA are significantly elevated in patients with ALI/ARDS. Similarly, at-risk patients who subsequently developed ALI/ARDS also exhibited higher serum OA levels [24]. Patients with sepsis, of whom approximately 50% develop ARDS [25, 26], also exhibited a dramatic six-fold increase in plasma OA levels in comparison to plasma from healthy volunteers [27-29]. Together, these observations have led investigators to propose OA as a prognostic factor for ARDS

[4]. Furthermore, OA was able to injure the alveolar-capillary membrane integrity, resulting in a significantly increased endothelial permeability, capillarity damage and hemorrhage. Therefore, we believe the OA induced ALI was an ideal animal model for ALI in this study.

UTI is a therapeutic drug that has been used in the treatment of ALI caused by lipopolysaccharide in animals [30] and disseminated intravascular coagulation, acute pancreatitis, and pulmonary dysfunction in humans [31, 32]. In this study, we treated endotoxin-induced ALI rabbits with UTI and found the ratio of lung wet-to-dry weight was significantly lower in animals receiving UTI treatment. Consistent with previous reports, our histologic examinations also revealed significantly reduced edema, infiltration of inflammatory cells, atelectasis and area of hemorrhage in the lung sections of UTI-treated animals. Meanwhile, histologic evaluation of lung tissue sections retrieved from the ALI animal showed that capillary density was markedly increased in UTI group, but the mechanisms is not clear.

EPCs function is crucial for angiogenesis and re-endothelialization after denuding injuries of arteries. Tube formation assays provide insight into the mechanism by which EPCs function *in vivo*. Since UTI treatment dramatically increased capillary density and EPCs contribute greatly to neovascularization, we investigated the beneficial effects of UTI on ALI as a result of improved EPCs function. Firstly, significant differences were noted in the tube formation in UTI treated group. We demonstrated significant improved effects of UTI on the ability of EPCs to incorporate into tubules. Secondly, EPCs are considered as highly potent regenerating cells with high proliferation ability. Our results show that UTI increased not only the function of tube formation but also EPCs proliferation, which appear to involve the NO-mediated pathways.

It has been illustrated that the function of EPCs may be dependent on aspects of the cellular environment such as cell-to-cell communication or growth factor support, etc. NO is a significant regulator of EPCs. To minimize the possibility that our observations were the result of nonspecific pharmacological effects, we used inhibitors capable of blocking separate enzymes in the various pathways. L-NAME, eNOS inhibitor. When NO production was inhibited by L-NAME, a known inhibitor of eNOS, both EPCs proliferation and tube formation were decreased significantly. These results suggest that the effect of UTI on EPCs tube formation and proliferation may be mediated in part by improvement of NO generation.

Recently, EPCs were shown to express lower levels of eNOS (endothelial nitric oxide synthase) as compared with mature vascular endothelial cells [33], and transplantation of autologous EPCs overexpressing eNOS in injured vessels enhanced the vasculoprotective properties of reconstituted endothelium. We have previously demonstrated that palmitic and linoleic acids may impair eNOS phosphorylation at the Ser1177 site resulting in reduced NO production, which contributes to the dysfunction of EPCs [34]. The present study demonstrated that UTI significantly increased eNOS phosphorylation and bioavailable NO without affecting total eNOS. Expression and phosphorylation of eNOS are known to be essential for the survival, migration, and angiogenesis of either EPCs or endothelial cells [20, 35]. UTI may increase proliferation and functional competences, which occurs in part by regulating EPCs eNOS activity in ALI. Taken together, one may speculate that the increase of eNOS phosphorylation and NO production could contribute to the improvement of EPCs of UTI.

Phosphorylated Akt is available to phosphorylate eNOS, leading to eNOS activation [36]. Therefore, the level of Akt phosphorylation partially reflects eNOS activity [20]. We further showed that UTI increased both eNOS and Akt phosphorylation. LY294002, a specific inhibitor of Akt, inhibited Akt/eNOS phosphorylation and the function of EPCs. UTI activates eNOS activity through the Akt/eNOS pathway, leading to a significant decrease in NO generation, which may increase EPCs proliferation and promote overall function. These data suggested UTI promoted EPCs function by the Akt/eNOS pathway.

Together these results, in association with the NO dependence of the signaling pathways mediating the EPCs function, indicate that UTI-induced phosphorylation/activation of eNOS and Akt, increases the intracellular level of NO, thereby improving tube formation



and proliferation function of EPCs. The therapeutic benefits of UTI could be attributable, at least partly, to angiogenesis of the damaged pulmonary vascular wall and maintenance of alveolar-capillary barrier integrity in rabbits with ALI. Since EPCs function is crucial for re-endothelialization after denuding injuries of arteries, we will do further research to study UTI on re-endothelialization after ALI.

## Disclosure Statement

The authors declare no conflict of interest of this work.

## Acknowledgement

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