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KLF2 and caveolin-1 as early indicators of acute lung injury induced by paraquat

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Abstract. – OBJECTIVE: The roles of Krüppel-like factors KLF2 and KLF4, and caveolin-1 (Cav-1) in acute lung injury (ALI) induced by paraquat (PQ) are insufficiently defined. Here we tested their expression in a rat model of ALI.

MATERIALS AND METHODS: 80 Wistar rats were divided into control group and groups exposed to PQ for 6, 12, and 24 hours (20 animals/group). ALI was assessed by behavioural and pathological scores. In addition, we quantified the concentration of KLF2, KLF4, and Cav-1 in serum by ELISA, and expression of these proteins at the mRNA and protein level in lung tissue (respectively, qPCR and Western blot).

RESULTS: Pathological scores were the highest at 12 hours after exposure to PQ. These changes correlated well with the kinetics of serum levels of KLF2, KLF4 and Cav-1. Thus, the lowest levels of the two former proteins were observed at 12 hours post-exposure, whereas Cav-1 levels peaked at this time point. In lung tissue, the kinetics of mRNA and protein expression of these proteins was different from the changes in the serum. Specifically, both KLF and Cav-1 mRNA expression changed significantly at 6 hours post-exposure to PQ, whereas KLF4 mRNA expression did not change significantly at any of the studied time points. Studies of at the protein level corroborated the observations of mRNA kinetics.

CONCLUSIONS: We demonstrate here that lung tissue expression of KLF2 and Cav-1 is modulated early in the pathogenesis of ALI. These proteins could, therefore, serve as molecular markers for early diagnosis of this clinical condition.

Key Words:
Paraquat, Acute lung injury, KLF2, KLF4, Cav-1.

Introduction

Paraquat (PQ) is a highly efficient heterocyclic herbicide widely employed around the world. It is a destructive organic quaternary ammonium. Even though it quickly inactivated after contact with soil, it still remains highly toxic to human and livestock. Mortality rate due to PQ ingestion can reach as high as 60-80% because of the lack of a specific antidote[1-3]. The absorptivity of PQ in the human body is 5-15%[4]. The main absorption paths are respiratory tract, skin and gastrointestinal tract, and most intoxications occur via ingestion. PQ is lethal when the concentration reaches 40 mg/kg weight. The mortality due to PQ poisoning is caused by acute pulmonary oedema, acute respiratory distress syndrome (ARDS) and acute liver, kidney, cardiac function failure. Even surviving through acute phase does not completely absolve an affected individual, as progressive pulmonary fibrosis injury may develop in the late stages, with late mortality being caused by pulmonary function failure.

Acute lung injury (ALI) is a medical condition which arises from direct or indirect inflammatory damage to alveolar capillary membrane. Clinical manifestations of ALI include refractory hypoxemia, aggravated severe dyspnoea, and non-cardiogenic pulmonary oedema. ALI transforms into ARDS as the condition progresses, and ARDS is associated with high morbidity and mortality. Therefore, proper prevention and treatment of ARDS are crucial.

KLF2 and KLF4 are the members in Krüppel-like transcription factors (KLFs), a class of zinc finger transcription factors that bind DNA. They are involved in multiple cell process including cell proliferation, apoptosis, and embryonic development[5,6]. In addition, KLF2 and KLF4 play important roles in inflammatory responses and oxidative stress, and can potentially be involved in the pathogenesis of ALI.

Caveolin-1 (Cav-1) is a major structural protein of caveolae and it is an important sig-

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nalling protein regulating vascular endothelial permeability, inflammation, and oxidative stress. Expression of Cav-1 in lung tissue may be helpful to prevent the occurrence of pulmonary oedema.

In our study, we tested expression of KLF2, KLF4 and Cav-1 in an experimental model of PQ-induced ALI.

Materials and Methods

Animals and Materials

All study protocol had been approved by local Animal Ethics Committee. Eighty healthy adult Wistar male rats (SPF grade) with a body weight of 250-290 g were used in this study. Paraquat was purchased from Syngenta Crop Protection (Nantong, Jiangsu, China). The KLF2, KLF4 and Cav-1 assay kits were purchased from Shanghai BlueGene Biotech (Shanghai, China). The Trizol RNA extraction reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). The qPCR kit was from TAKARA (Germany). The anti-KLF2 antibody was purchased from Abcam (Cambridge, UK), and anti-KLF4 and anti-Cav-1 antibodies were from Millipore (Billerica, MA, USA).

Animal Model

The rats were raised in Experimental Animal Centre for 1 week, and were fasted for 12 hours before the experiment, with free access to water. Rats were then randomly divided into 4 groups. Group A was comprised of control animals (control group), whereas the animals in Groups B, C and D were exposed to PQ for, respectively, 6, 12 and 24 hours. Each experimental group comprised of 20 animals.

PQ was administered by gavage, diluted from a 20% paraquat stock solution. PQ dose was 80 mg/kg weight, administered in 1 mL. Control animals received gavage (1 mL) with distilled water. After 6, 12, and 24 hours after gavage, activity of experimental animals was observed and recorded.

Gavage was administered as follows. A rat was fixed and its head was tilted upward to make the long axis of the body perpendicular to the ground. Gavage needle was inserted into one side of the mouth to reach the stomach. The depth of the needle was about 5 cm. PQ was slowly infused. When gavage was finished, the needle was maintained the gavage position for about 30 sec.

Specimen Collection and Downstream Assays

Rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g weight). Rats were immobilized in supine position and 5 mL of blood were collected by paracentesis of the inferior vena cava. After that, trachea was incised, and bronchoalveolar lavage (BAL) was done twice using 2 × 1 mL of normal saline. After BAL was collected, rats were sacrificed. Blood and BAL specimens were allowed to stand for 20 min and centrifuged to obtain supernatants. These were cryopreserved at -70° C.

Thoracotomy was then applied to collect lung tissue. Left lung tissue was used for histology. Right lung tissue was used to prepare a 10%-homogenate. After centrifugation, the supernatant was used to quantify expression of KLF2, KLF4, and Cav-1.

Lung Tissue Sectioning and Hematoxylin and Eosin Staining

Lung tissue was fixed in 10% formaldehyde and rinsed with normal saline. The liquid on the surface was carefully wiped with a filter paper. Then, the tissue was dehydrated overnight using 95%-100% alcohol in an automatic hydroextractor. Lung tissue was sliced into slices of 3 □m thickness. Slices were placed onto glass slides and stretched to remove folds. Slices were dewaxed twice using xylene (2 × 10 min). Afterwards, slides were rinsed again with normal saline and stained (5 min haematoxylin, rinsing, and 30 sec in 0.5% eosin). Slice were again dehydrated with alcohol, dealcoholized with xylene, and covered.

Lung Injury Score

Two pathology experts observed lung tissue slices under a ×400 magnification. Ten different visual fields were randomly selected in lung tissue slices for scoring. The average value was considered as the final result.

Detection of Wet/Dry Ratio (W/D) in Lung Tissue

Right anterior lung lobes were used in this analysis. Blood on the surface was wiped with a filter paper. Then, the weight was determined and considered as the wet weight of lung tissue (W). Afterwards, the lung tissue was baked at 80°C and weighed again. This was considered the dry weight (D). The wet/dry ratio was calculated as W/D.

KLF2, KLF4 and Cav-1 Protein and mRNA Expression

After thawing, serum specimens were analyzed for concentrations of KLF2, KLF4, and Cav-1 by ELISA according to manufacturer's instructions. mRNA expression of these signalling proteins was quantified after RNA extraction (Trizol protocol) and qPCR, and normalized to expression of glyceraldheyde 3-phosphate dehydrogenase (GADPH) mRNA as the internal reference gene.

In addition, we quantified expression of KLF2, KLF4 and Cav-1 by Western blot. Briefly, cells were lysed on ice for 30 min. Then, cells were subjected to ultrasonication. Lysates were centrifuged for 15 min at 4°C, 12,000 rpm. Supernatants were collected, protein concentration was quantified, and proteins were denatured by incubation with gel loading buffer and boiling for 5 mins. Twenty □g of protein were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred onto nitrocellulose membrane. After blocking with 5% skimmed milk powder in PBS-T (room temperature, 2-4 hours), membranes were probed with anti-KLF2 (1:1000 dilution), anti-KLF4 (1:500), anti-Cav-1 (1:500), or anti-GADPH (1:1000) antibodies overnight at 4°C. The horse radish peroxidase (HRP)-conjugated secondary antibody was used to detect primary antibody (2 hours at room temperature). Chemiluminence was visualized by ECL imaging system.

Statistical Analysis

The SPSS 18.0 (SPSS Inc., Chicago, IL, USA) statistical software was used for statistical analysis. Quantitative data were presented as mean ± SD. Data were tested for normal distribution and homogeneity of variance. The data that followed normal distribution and homogeneity of variance were compared using a one-factor Analysis of Variance (ANOVA). Paired comparisons were done using the LSD *t* test. The data that did not follow normal distribution and homogeneity of variance were analyzed using the Kruskal-Wallis H test. The p value of < 0.05 was considered as indicating significant differences.

Results

PQ Caused Significant Changes in Animal Activities and Lung Morphology

Rats in control group (Group A) had smooth breath, homogeneous and shiny hair, and moved as usual. In contrast, rats exposed to PQ (Groups B-D) exhibited specific symptoms including apathy, messy hair, reduced activity, increasing respiratory rate, cyanosis, no evasion when being grabbed, and more frequent defecating and urinating. Some rats exposed to PQ showed oral mucosal ulceration. The above symptoms were more pronounced with a longer exposure to PQ.

Lung colour in Group A was normal and showed smooth surface and no liquid exudation, edema, necrosis or haemorrhages. In contrast, hyperaemia, oedema, surface exudation, and scattered bleeding points could be observed in the lungs of animals exposed to PQ. Some rats showed hydrothorax. Again, the above symptoms were more pronounced in animals exposed for longer time to PQ.

Pathological Scores, Lung Tissue Water Content, and Respiratory Rates After Exposure to PQ

We then checked the condition of lung tissue according to the assessment criteria presented in Table I. We observed that animals in Group A (control animals) exhibited clear structure (thin and smooth) of alveolar walls and normal alveo-

Table I. ALI pathological scores.

Score	Observation parameters					
	Alveolar oedema	Pulmonary interstitial oedema	Red blood cells infiltration	Inflammatory cell infiltration	Hyaline membrane formation	Extent of atelectasis
0	No	No	No	No	No	No
1	< 25%	< 25%	< 25%	Small amount	Some	Some
2	25%-50%	25%-50%	25%-50%	Multiple	Small amount	Light to moderate
3	50%-75%	50%-75%	50%-75%	Clustered	Significant	Severe

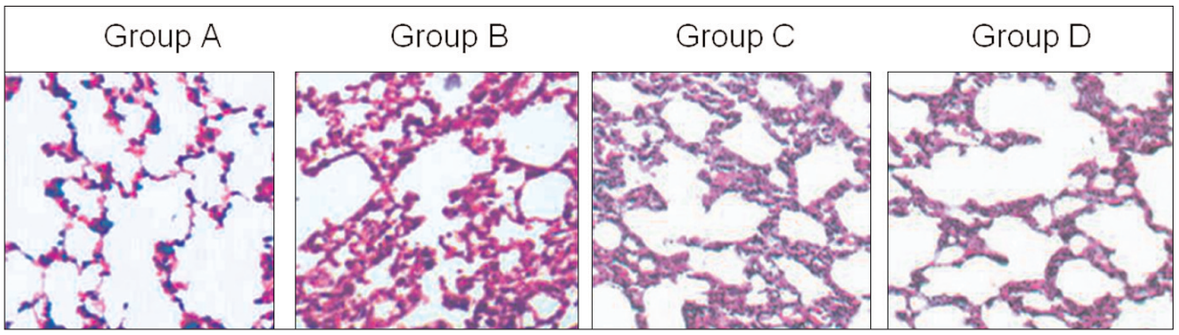

Figure 1. Hematoxylin & eosin staining of lung tissue. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ.

lar septa (Figure 1). However, experimental animals in Group B (6 hour post-exposure to PQ) showed a slightly narrower alveolar space, partial thickening of alveolar cavities, pulmonary hyperemia, mild oedema in pulmonary mesenchyme, and small focal inflammatory cell infiltration (Figure 1). The symptoms further aggravated with the increasing time post-exposure to PQ. Thus, in Group C (12 hours post-exposure), there was a narrowing or disappearing of alveolar space, significantly increased number of inflammatory cells in the alveolar cavity, pulmonary hyperemia, pulmonary mesenchyme oedema, and diffuse inflammatory cell infiltration (Figure 1). In Group D (24 hours post-exposure), there were alveolar space narrowing, inflammatory cells infiltration in the alveolar cavity, pulmonary hyperaemia, pulmonary mesenchymal oedema, and focal inflammatory cell infiltration (Figure 1). Not surprisingly, pathological scores in Groups B, C, and D were significantly higher than those in Group A (Figure 2, Table II; $p < 0.01$). The scores in Group C were significantly higher than those in Groups A and B (Figure 2, Table II; $p < 0.01$).

We next analyzed the wet/dry (W/D) ratio in experimental groups. The W/D ratio was significantly higher in experimental animals exposed to PQ (Groups B-C) compared with control group (Figure 3, Table II; $p < 0.01$). Moreover, the W / D ratio in Group C was significantly higher than those in Groups B and D (Figure 3, Table II; $p < 0.05$).

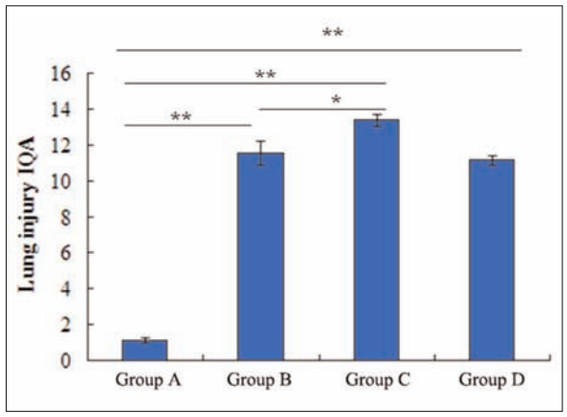

Figure 2. ALI pathological scores. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ. Pathological scores in Groups B, C, and D were significantly higher than that in Group A ($p < 0.01$). The score in Group C was significantly higher than that in Group B ($p < 0.05$).

Table II. ALI pathological scores, W/D ratio, and respiratory rate in experimental animals.

Groups	Pathological scores	W/D ratio	Respiratory rate (times/min)
Group A	1.12 ± 0.14	3.86 ± 0.16	60.24 ± 4.81
Group B	11.56 ± 0.68*	4.39 ± 0.23*	93.39 ± 6.43*
Group C	13.37 ± 0.36*,#	4.72 ± 0.28*,&	99.15 ± 5.85*,#
Group D	11.13 ± 0.27*	4.36 ± 0.25*	94.12 ±5.38*

Footnote: Data are expressed as mean ± SD of 20 animals. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ. W/D ratio: ratio of wet/dry lung tissue weight. *$p < 0.01$ *vs*. Group A, #$p < 0.01$ *vs*. Groups B and D, &$p < 0.05$ *vs*. Groups B and D.

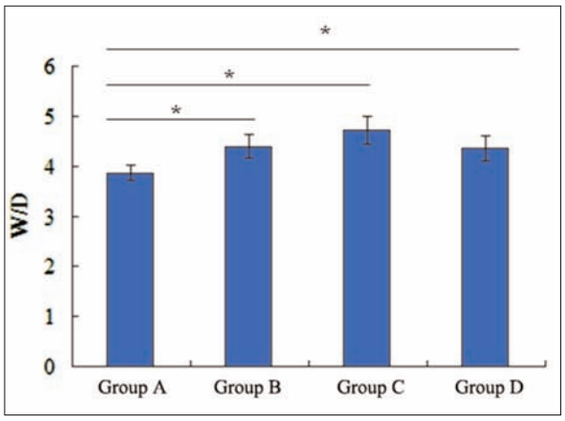

Figure 3. W/D ratio of lung tissue. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ. W/D: ratio of wet/dry lung tissue weight. W/D in Groups B, C, and D were significantly higher than that in Group A (*$p < 0.05$).

Afterwards, we quantified respiratory rates in control rats and rats exposed to PQ. In all animals, respiration was smooth before the experiment, with a frequency of approx. 60 times/min. Following the experiment, shortness of breath was observed in experimental animals exposed to PQ. Thus, two hours after gavage with PW, we observed respiratory distress, mild cyanosis, and increase in respiratory rates. After 4 hours post-exposure, cyanosis was more pronounced and respiratory rates reached about 100 times/min. After 24 hours, the cyanosis subsided, but respiratory rate still remained rapid.

Respiratory rates in animals exposed to PQ (Groups B, C, and D) were significantly higher than those in control animals (Table II; $p < 0.01$). In line with the preceding observations, we observed that respiratory rates in Group C were significantly higher than those in Groups B and D (Table II; $p < 0.01$).

Changes in KLF2, KLF4, and Cav-1 Expression Levels After Exposure to PQ

KLF2 and KLF4, the members in Krüppel-like transcription factors (KLFs), and Cav-1, a major structural protein of Caveolae proteins, may potentially be involved in the pathogenesis of PQ-induced ALI. We, therefore, tested systemic and local expression of these proteins after exposure to PQ.

In this regard, we first quantified serum levels of these proteins. KLF2 and KLF4 serum levels significantly decreased in the animals exposed to PQ (Figure 4, Table III; $p < 0.01$ *vs.* Group A). In line with the symptoms of ALI, the lowest levels of both studied proteins were observed at 12 hours post-exposure to PQ (Figure 4, Table III). Serum levels of Cav-1 increased in all experimental groups exposed to PQ (Figure 4, Table III; $p < 0.01$ *vs.* Group A), with the kinetics of Cav-1 serum levels reversely reflecting those of KLF2 and KLF4. Thus, the most pronounced changes of all three studied proteins were observed in the animals at 12 hours post-exposure to PQ (Figure 4, Table III).

We, then, obtained lung tissue samples and quantified expression of mRNAs of the afore-

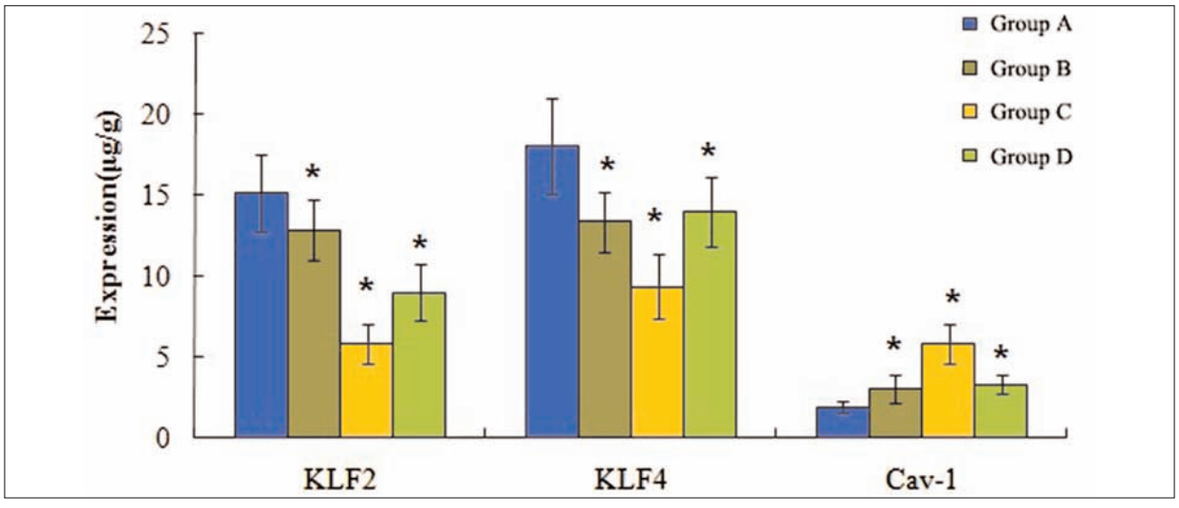

Figure 4. Serum concentrations of KLF2, KLF4 and Cav-1. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ.

Table III. KLF2, KLF4 and Cav-1 concentrations in serum.

Groups	Concentration in serum, μg/ml		
	KLF2	KLF4	Cav-1
Group A	15.03 ± 2.41	17.94 ± 2.96	1.84 ± 0.38
Group B	12.79 ± 1.84$^{\&,\wedge,\P}$	13.27 ± 3.88$^{\&}$	2.96 ± 0.87$^{\&}$
Group C	5.74 ± 1.18$^{\&}$	9.28 ± 1.97$^{\&,\S}$	5.76 ± 1.19$^{\#,\&}$
Group D	8.94 ± 1.75$^{\&}$	13.87 ± 2.18*	3.25 ± 0.56$^{\&}$

Footnote: Data are expressed as mean ± SD of 20 animals. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ. *$p < 0.05$ vs. Group A, $^{\#}p < 0.05$ vs. Group B, $^{\wedge}p < 0.05$ *vs.* Group C, $^{\P}p < 0.01$ *vs.* Group D, $^{\S}p < 0.01$ *vs.* Groups B and D.

mentioned proteins. Unlike serum proteins, there was no defined time kinetics of expression of these proteins in the lung. Thus, compared with control group, KLF2 mRNA expression in lung tissue of PQ-exposed animals was significantly lower (Figure 5, Table IV; $p < 0.05$) during initial hours post-exposure, but gradually reverted to normal levels 24 hours post-exposure (Figure 5, Table IV). In contrast, KLF4 mRNA expression did not change significantly during exposure to PQ. However, Cav-1 mRNA expression was significantly increased (Figure 5, Table IV; $p < 0.05$) compared with control group. Thereby, expression of KLF2 mRNA was negatively associated with progression of ALI, whereas Cav-1 mRNA expression showed a reverse trend.

We next wished to corroborate the findings at mRNA levels by studying protein expression of KLF2, KLF4, and Cav-1 in lung tissue. This expression was assessed by Western blot analysis. The results showed that compared with control group, expressions of KLF2 and KLF4 decreased in the animals exposed to PQ, whereas expression of Cav-1 increased (Figure 6). Thus, we could confirm our preceding observations on mRNA expression of these proteins.

Discussion

PQ is capable of aggregating in the patient's lung tissue, and this is largely a result of its reaction with alveolar cells. Due to a chemical structure of PQ similar to amines and existence of a transport system for these amines in type I and II alveolar epithelial cells, PQ can pass through cell membranes into the cells. Therefore, PQ aggregates in lung tissue of large amounts[7]. This is followed by the re-

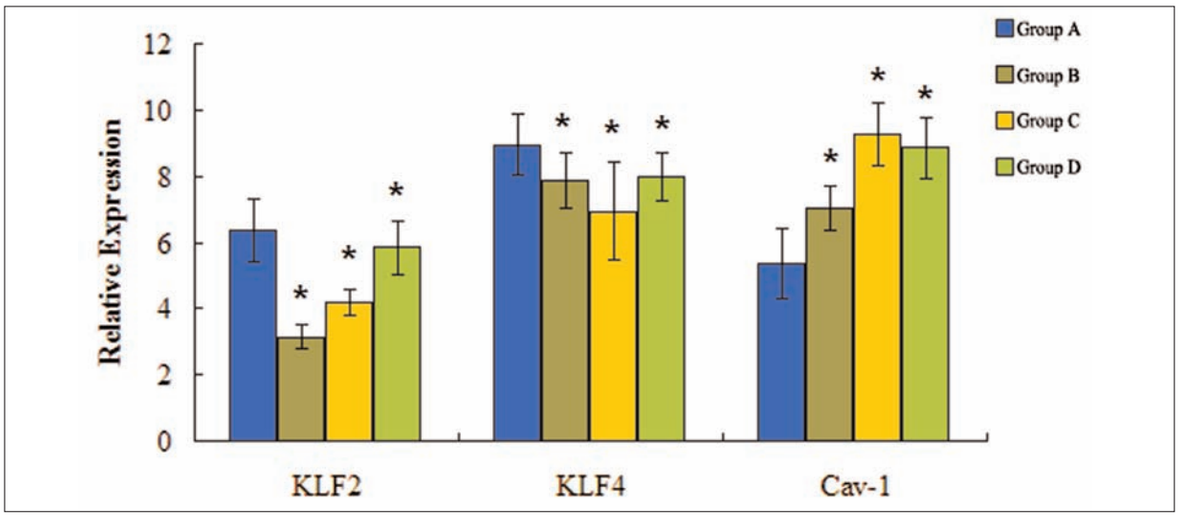

Figure 5. Expression of KLF2, KLF4 and Cav-1 mRNAs. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ.

Table IV. Expression of KLF2, KLF4 and Cav-1 mRNAs in lung tissue.

Groups	mRNA expression of lung tissue		
	KLF2	KLF4	Cav-1
Group A	6.38 ± 0.95	8.95 ± 0.93	5.37 ± 1.07
Group B	3.14 ± 0.37*	7.85 ± 0.83	7.03 ± 1.48$^{\&,\wedge}$
Group C	4.17 ± 0.38$^{*,\#}$	6.94 ± 1.48	9.26 ± 1.94$^{\&,\P}$
Group D	5.84 ± 0.79$^{*,\#}$	7.98 ± 0.74	8.86 ± 1.74$^{\&}$

Footnote: Data are expressed as mean ± SD. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ. $^{*}p < 0.05$ *vs*. Group A, $^{\#}p < 0.05$ *vs*. Group B, $^{\&}p < 0.01$ vs. Group A, $^{\wedge}p < 0.05$ *vs*. Group C, $^{\P}p < 0.01$ vs. Groups B and D.

lease of free radicals from alveolar compartments, which eventually leads to cell death.

In our study, we utilized a rat model of lung exposure to PQ. As expected, we observed extensive lung injury in animals exposed to PQ. Swelling and exudation was observed on the surface of lung tissue, including hemorrhagic spots or hemorrhagic petechiae, and hardened texture. The structure of lung tissue stained with hematoxylin-eosin showed significant morphological damage, with thickened and partially broken pulmonary septum. In addition, there was infiltration of red blood cells and inflammatory cells in alveolar space and pulmonary septum. Alveolar oedema was pronounced, and there was extensive formation of hyaline membrane. Atelectasis could also be observed.

We also studied expression of proteins involved in inflammatory responses in PQ-exposed animals. Inflammatory responses including production of inflammatory mediators lead to extensive lung injury in PQ intoxication. KLF2 and KLF4 are the members in Krüppel-like transcription factors. These proteins are involved in regulation of multiple vital processes including cell proliferation, apoptosis, and embryogenesis[8-11]. KLF2 was shown to inhibit pro-inflammatory activity of mononuclear cells, and overexpression of KLF2 suppressed the lipopolysaccharide-induced production of inflammatory cytokines[12]. In contrast, siRNA silencing of KLF2 increased expression of inflammatory genes. Similarly, KLF4 was shown to inhibit expression of the inflammatory cytokine interleukin-1□[13,14]. Our observations of diminished expression of KLF2 and, to a lesser extent, KLF4, support previous literature reports on the key role of these proteins in ALI.

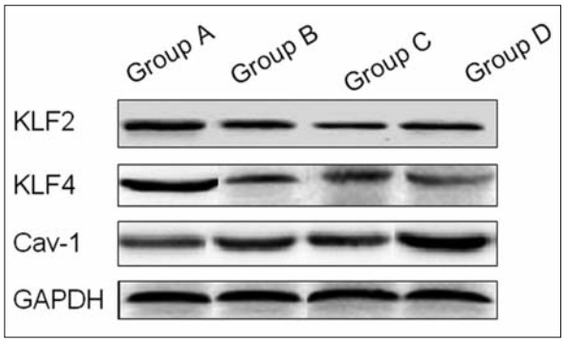

Figure 6. Western blot analysis of KLF2, KLF4 and Cav-1 protein expression. Group A: control animals. Groups B, C, and D: animals studied at, respectively, 6, 12 and 24 hours after exposure to PQ.

Cav-1 is another protein studied by us. This protein is thought to be involved in the pathogenesis of ALI by regulating vascular endothelial permeability, inflammatory reaction, and oxidative stress[15-17]. Still, the role of Cav-1 in ALI is presently unclear. In the present report, we demonstrate that Cav-1 expression in lung tissue (both mRNA and protein) was increased in the animals exposed to PQ. This could indicate protective role of this protein (i.e., to compensate for the injury subsequent to PQ), but could also indicate a harmful role (i.e., be involved in the development of ALI)[18]. We, however, tend to assume the former role of this protein. Also the literature seems to confirm protective role of Cav-1. Thus, mechanical ventilation was attenuated by CO inhalation only in mice expression Cav-1, but not in animals with knocked down protein[19].

Conclusions

We demonstrate here that lung tissue expression of KLF2 and Cav-1 is modulated early in the pathogenesis of ALI. These proteins could, therefore, serve as molecular markers for early diagnosis of this clinical condition.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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