# Ratio of Angiopoietin-2 to Angiopoietin-1 predicts mortality in acute lung injury induced by paraquat

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**Background:** To determine whether initial reactive oxygen species (ROS)-induced endothelial cell injury is involved in early death after paraquat intoxication and concentrations of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and von Willebrand factor (VWF) reflecting endothelial cell injury, we investigated the initial endothelial cell injury marker involved in the pathogenesis of death within 5 days after paraquat ingestion.

**Material/Methods:** Sixty patients with paraquat poisoning were prospectively enrolled. Plasma samples were collected at admission. Plasma concentrations of Ang-1, Ang-2, and VWF were measured by enzyme-linked immunosorbent assay. The patients were classified into 3 categories: survivors, early death (died within 5 days after ingestion), and late death (died more than 5 days after ingestion).

**Results:** The baseline concentration of Ang-2 and the Ang-2: Ang-1 ratio were significantly higher in patients who died (Ang-2 [pg/mL], 1012.75±468.02 *vs.* 1986.07±1675.37 [p=0.002]; Ang-2: Ang-1, 0.90±0.49 *vs.* 2.16±2.28 [p=0.002]). The Ang-2: Ang-1 ratio was significantly higher in the early death group (2.41±2.54) than in the survivors (0.90±0.49) and the late death group (1.33±0.64). The Ang-2: Ang-1 ratio was significantly associated with early death (OR, 2.602; 95% CI, 1.106–6.117; p=0.028) after adjusting for plasma levels of paraquat, age, $PCO_2$, and creatinine. VWF did not predict mortality.

**Conclusions:** Endothelial cell damage could be involved in the pathogenesis of early death following paraquat ingestion.

**key words:** paraquat • acute lung injury • angiopoietin • endothelial cells

## Background

Endothelial injury has been studied in septic and non-septic patients, both at risk for and with established acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]. Several clinical studies have demonstrated that circulating levels of angiopoietin-2 (Ang-2), which are associated with mortality, are elevated at the onset of critical illness or in those at risk for ALI/ARDS. This relates to vascular permeability and pulmonary dysfunction [2–12]. Experimental studies have demonstrated that angiopoietin-1 (Ang-1) and Ang-2 bind to endothelial tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 receptor (Tie2 receptor) [13]. Ang-1-induced activation of the Tie2 receptor results in vascular stabilization [14–16]. Ang-2 provokes pulmonary inflammation and vascular leakage, partly by preventing Ang-1 from binding the Tie2 receptor, thereby inhibiting the stabilizing effect of Ang-1 [16,17]. This suggests that Ang-2 is not only a marker but also a direct mediator in the pathogenesis of vascular permeability and pulmonary injury and dysfunction in septic and non-septic patients [18–20]. Furthermore, the ratio of Ang-2 concentration to Ang-1 concentration may be a prognostic biomarker of endothelial activation in ALI patients [10]. The antigen von Willebrand factor (VWF) is a macromolecular antigen that is produced predominantly by endothelial cells and in lesser amounts by platelets. VWF is a large multimeric glycoprotein present in blood plasma and produced constitutively in Weibel-Palade bodies, megakaryocytes ($\alpha$-granules of platelets), and subendothelial connective tissue [21]. VWF is not related to vascular permeability, but instead reflects endothelial damage. Some studies have shown that VWF is a marker of poor outcome in patients with ALI/ARDS [12,22,23].

Paraquat (1,1-dimethyl-4-4-bipyridium dichloride; PQ) is used not only as an herbicide but also to produce reactive oxygen species (ROS) in the oxidative stress model [24]. Paraquat mainly accumulates in the lungs, where it is retained even when its concentration in the blood starts to decrease. Subsequent redox cycling and generation of ROS trigger lung injury. ROS play a crucial role in the pathogenesis of various lung diseases such as asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome. Paraquat-induced lung injury might be a unique model of dominant ROS-induced lung injury [25,26]. ROS can lead to endothelial barrier dysfunction with subsequent increased permeability to fluid, macromolecules, and inflammatory cells. We hypothesized that endothelial cell injury could be involved in early death after paraquat intoxication and that concentrations of Ang-1, Ang-2, and VWF could reflect endothelial cell injury.

The aim of our study was to investigate whether Ang-2, VWF, and Ang-1 are related to early mortality. Therefore, we measured initial plasma levels of these markers in patients who intentionally ingested paraquat.

## Material and Methods

We prospectively collected samples from patients admitted to the Soonchunhyang University Cheonan Hospital for paraquat intoxication from March 2011 through August 2011.

Patients who had simultaneously ingested other pesticides were excluded. Standard medical emergency procedures were performed. In brief, gastric lavage was performed for all subjects who presented to the emergency room within 2 hours of paraquat ingestion. Patients who had ingested paraquat between 2 and 12 hours before presenting to the emergency room were administered 100 g of fuller's earth in 200 mL of 20% mannitol. Hemoperfusion was performed if the patient's urinary paraquat test result was positive on arrival at the emergency room. All procedures were performed after informed consent was obtained. The amount ingested was estimated based on the number of swallows (1 mouthful was considered 20 mL). High-resolution computed tomography (HRCT) was performed 7 days after ingestion, and again 1 week later if any abnormality was detected. On HRCT, mild lung lesion is <10% and severe lung lesion is ≥10%. Survivors were defined as those patients who survived more than 3 months after paraquat ingestion and had stable vital signs. This study was approved by the institutional review board at Soonchunhyang University Cheonan Hospital.

Blood samples were obtained at admission. The serial sample in 3 patients was obtained at admission; 6, 12, and 24 hours after admission; and 2, 3, 4, 5, 6, and 7 days after admission. The samples were stored at –70°C until analysis.

Paraquat level was measured by high-performance liquid chromatography with a Perkin Elmer Series 200 (Perkin Elmer, USA). In brief, PQ were separated on a reversed-phase column, Capcell Pak C18 UG120 (150×4.6 mm i.d.; particle size, 5 μm; Shiseido Co., Tokyo, Japan), by isocratic elution with a mixture of methanol and 200 mM phosphoric acid solution containing both 0.1 M diethyl amine and 12 mM sodium 1-heptane sulfonate (1:4, v/v) as the eluents [27].

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to measure Ang-1 (Quantikine Human Angiopoietin-1 ELISA kit, Cat DANG10; R&D systems, USA), Ang-2 (Quantikine Human Angiopoietin-2 ELISA kit, Cat DANG20; R&D systems, USA), and VWF (VWF Human ELISA kit, Cat #KA0512; Abnova, Taiwan).

### Statistical analysis

Data are presented as means ±SD for continuous variables and frequencies (percentages) for categorical variables. Differences between groups were evaluated with Student's $t$ test for continuous variables and $\chi^2$ test for categorical

**Table 1.** Clinical characteristics of 60 patients with paraquat poisoning.

| | |
|---|---|
| Age (years) | 51.75±17.89 |
| Men (cases)* | 36 (60%) |
| Estimated amount of paraquat dichloride (24.5% concentration) exposure (mL) | 83.89 [5, 500] |
| Time interval between exposure and hospital admission (hours) | 9.73±10.93 |
| Plasma paraquat level (µg/mL) | 36.67 [0.02, 618.66] |
| Mortality (cases) | 38 (63.3%) |
| WBC (/µl) | 16036.50±8435.83 |
| Hemoglobin (g/dl) | 13.61±2.02 |
| Platelets(/µl) | 231.43±94.95 |
| Albumin (g/dl) | 4.30±0.63 |
| Total bilirubin (mg/dl) | 0.82±0.82 |
| Aspartate aminotransferase (IU/L) | 43.18±50.01 |
| Alanine transaminase (IU/L) | 28.65±33.43 |
| Amylase (IU/L) | 388.00±704.73 |
| Lipase (IU/L) | 148.25±376.39 |
| Blood urea nitrogen (mg/dl) | 16.50±8.53 |
| Creatinine (mg/dl) | 1.49±1.26 |
| Sodium (mEq/L) | 139.70±8.65 |
| Chloride (mEq/L) | 101.52±7.29 |
| Potassium (mEq/L) | 3.56±0.87 |
| Uric acid (mg/dL) | 4.93±1.63 |
| pH | 7.39±0.07 |
| $pCO_2$ (mmHg) | 30.07±7.09 |
| $pO_2$ (mmHg) | 90.50±18.76 |
| $HCO_3^-$ (mEq/L) | 18.20±4.87 |

Mean ± Standard deviation [minimum, maximum], * number (percent).

variables. Multivariate logistic regression analysis was used to identify significant determinants of death after paraquat intoxication. The results of logistic regression analyses are reported as odds ratios (OR) with 95% confidence intervals (CI). Differences between groups were compared using Kruskal–Wallis test for 3 continuous variables. Values of p<0.05 were considered statistically significant. Statistical analyses were performed using SPSS for Windows (version 14.0; Chicago, IL, USA).

## Results

Characteristics of the 60 patients with acute paraquat poisoning at the time of their arrival are summarized in Table 1. Their mean age was 51.75±17.89 years, and 36 (60%) were men. The mean estimated amount of ingested paraquat dichloride was 83.89 mL (range, 5–500 mL). Overall mortality was 63.3%. Paraquat concentrations in blood ranged from 0.02 to 618.66 µg/mL. Mean Ang-1 level was 1.32±0.75 ng/mL. Mean Ang-2 level was 4.07±3.59 pg/mL. Mean VWF was 15.09±10.85 mU/mL. Mean Ang-2: Ang-1 ratio was 4.24±4.82.

Comparisons of Ang-1, Ang-2, Ang-2: Ang-1 ratio, and VWF between survivors and non-survivors are shown in Table 2. Although the 2 groups had no statistically significant difference in Ang-1, the Ang-2 and Ang-2: Ang-1 ratio were significantly higher in non-survivors than in survivors.

HRCT was done for 30 patients (22 survivors and 8 non-survivors). Mild lung lesion was found in 21 patients and severe lung lesion in 9 patients. According to HRCT, the 2 groups had no statistically significant difference in Ang-1, Ang-2, Ang-2: Ang-1 ratio, or VWF (not shown data).

Serial change of Ang-1, Ang-2, and Ang-2: Ang-1 ratio in 3 patients is shown in Figure 1. The Ang-2: Ang-1 ratio peaked within 1 day after paraquat ingestion, and then the level of the ratio decreased.

The patients were grouped into 3 categories: survivors, early death group (died within 5 days after ingestion), and late death group (died more than 5 days after ingestion). The Ang-2: Ang-1 ratio and plasma paraquat level were significantly higher in the early death group than in the others (Table 2). In addition, the $pCO_2$ and $HCO_3^-$ (mEq/L) were lower in this group.

Univariate analysis showed a statistically significant association between baseline plasma Ang-2 level and death (OR, 1.001; 95% CI, 1.000–1.002; p=0.017). Moreover, Ang-2: Ang-1 ratio was significantly associated with death (OR, 1.928; 95% CI, 1.116–3.331; p=0.019), as was plasma paraquat level (OR, 1.078; 95% CI, 1.052–1.137; p=0.006). Ang-1 (OR, 1; 95% CI, 0.999–1.001; p=0.326) and VWF (OR, 1.045; 95% CI, 0.993–1.100; p=0.091) were not associated with death.

In multivariate analysis, Ang-2: Ang-1 ratio was significantly associated with early death after adjustment for several combinations of potential confounders: plasma levels of paraquat (OR, 2.644; 95% CI, 1.206–5.795; p=0.015); plasma levels of paraquat, and age (OR, 2.906; 95% CI, 1.297–6.512; p=0.010); plasma levels of paraquat, age, and $PCO_2$ (OR, 2.644; 95% CI, 1.173–5.960; p=0.019); and plasma levels of paraquat, age, $PCO_2$, and creatinine (OR, 2.602; 95% CI, 1.106–6.117; p=0.028).

**Table 2.** Comparison of laboratory parameters between survivor and non-survivors.

| | Survivor (n=22) | Non-survivor (n=38) | p-value |
|---|---|---|---|
| Plasma paraquat level (µg/mL) | 0.45±0.52 | 56.69±116.94 | 0.005 |
| Ang-1 (ng/ml) | 1.27±0.51 | 1.33±0.86 | 0.748 |
| Ang-2 (ng/ml) | 2.53±1.17 | 4.97±4.18 | 0.002 |
| Ang-2/Ang-1 ratio | 2.25±1.23 | 5.40±5.70 | 0.002 |
| vWF (mU/ml) | 13.41±10.01 | 16.07±11.32 | 0.364 |
| WBC (/µl) | 11247.73±5210.61 | 18808.95±8748.55 | 0.000 |
| Hemoglobin (g/dl) | 13.68±1.44 | 13.57±2.30 | 0.850 |
| Platelets(/µl) | 207.00±70.99 | 245.58±104.66 | 0.130 |
| Albumin (g/dl) | 4.17±0.46 | 4.37±0.71 | 0.186 |
| Total bilirubin (mg/dl) | 0.85±0.83 | 0.80±0.82 | 0.822 |
| Aspartate aminotransferase (IU/L) | 41.00±58.51 | 44.45±45.17 | 0.799 |
| Alanine transaminase (IU/L) | 37.86±49.59 | 23.32±17.51 | 0.196 |
| Amylase (IU/L) | 174.77±141.86 | 511.45±858.97 | 0.023 |
| Lipase (IU/L) | 36.86±19.92 | 212.74±462.64 | 0.025 |
| Blood urea nitrogen (mg/dl) | 14.53±7.42 | 17.63±9.01 | 0.176 |
| Creatinine (mg/dl) | 1.20±1.57 | 1.65±1.02 | 0.177 |
| Sodium (mEq/L) | 139.77±5.72 | 139.66±10.04 | 0.961 |
| Chloride (mEq/L) | 103.18±5.50 | 100.53 ±8.06 | 0.180 |
| Potassium (mEq/L) | 3.90±0.43 | 3.36 ±1.00 | 0.006 |
| Uric acid (mg/dL) | 4.35±1.65 | 5.26±1.54 | 0.036 |
| pH | 7.40±0.04 | 7.38±0.08 | 0.092 |
| $pCO_2$ (mmHg) | 34.90±3.43 | 27.28 ±7.19 | 0.000 |
| $pO_2$ (mmHg) | 88.93±16.37 | 91.40±20.16 | 0.627 |
| $HCO_3^-$ (mEq/L) | 21.56±2.11 | 16.25±4.97 | 0.000 |

## Discussion

ALI induced by paraquat causes progressive pulmonary insufficiency. The toxicity of paraquat is due to its induction of redox cycling, which leads to oxidative stress-related cell death and inflammation. Because of selective accumulation in the lungs, especially in type II pneumocytes, paraquat causes severe lung injury manifested by edema, hemorrhage, interstitial inflammation, and progressive fibrosis [24,28].

The severity of paraquat ingestion poisoning can be classified into 3 categories [29]. First, patients who ingest less than 20 mg paraquat cation per kilogram of body weight may be asymptomatic or develop only gastrointestinal tract symptoms; they recover without sequelae. Second, patients who ingest between 20 and 40 mg paraquat cation per kilogram usually

die 5 days to several weeks after ingestion. The cause of death is progressive development of pulmonary fibrosis. Third, patients who ingest more than 40 mg paraquat cation per kilogram usually die within 1–5 days after ingestion, mainly because of multiorgan failure or shock.

We investigated whether initial endothelial cell injury could involve the ROS-induced injury model. We suspected that the cause of death in the early death group might be multiorgan failure due to endothelial cell injury.

Ang-2: Ang-1 ratio and Ang-2 were higher in the early death group than in the other groups. These findings suggest that endothelial cell damage may have been involved in the pathogenesis resulting from paraquat intoxication in the early death group.
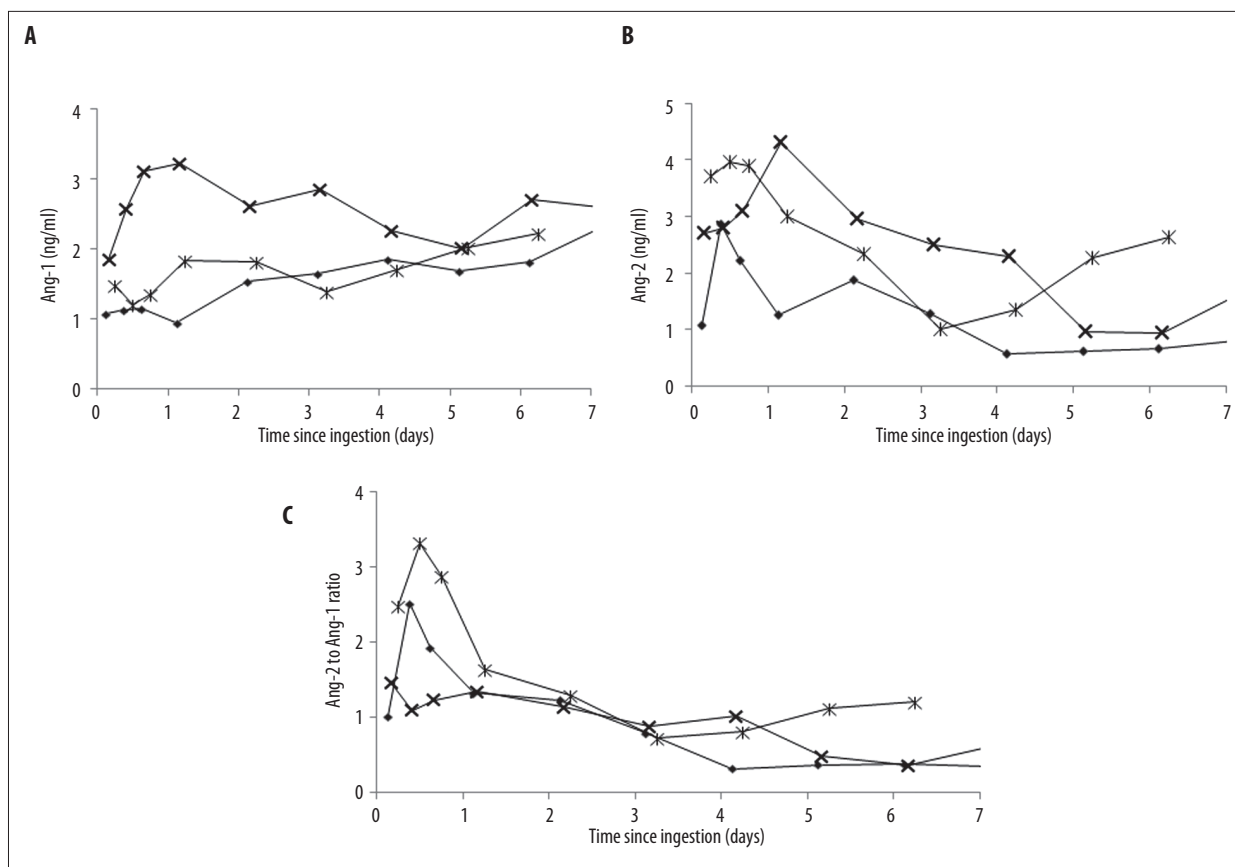
**Figure 1.** Serial change of Ang-1 (**A**), Ang-2 (**B**), and Ang-2: Ang-1 ratio (**C**) *vs.* time since ingestion (days) in 3 surviving patients. Each line represents the serum level of 1 patient.

Although it has previously been suggested that endothelial cell damage occurs after paraquat poisoning, the underlying mechanism has not been revealed. Some data showed that paraquat directly induced cytotoxicity in pulmonary microvascular endothelial cells [28]. Furthermore, lung inflammation indirectly induced by paraquat could evoke endothelial damage. Paraquat could cause an increase in vascular permeability that is attributed to endothelial cell injury. Our study showed that the Ang-2: Ang-1 ratio can predict mortality.

Some acute injury markers can predict mortality in cases of ALI/ARDS. Among these markers, Ang-2, Ang-2: Ang-1 ratio, and VWF can induce endothelial cell injury, which increases vascular permeability. Ang-2, Ang-2: Ang-1 ratio, and VWF may be good markers to predict mortality in sepsis models and septic patients [2,6,8–12]. Increased vascular permeability is the key mechanism in the pathogenesis of sepsis-induced lung injury. Therefore, initial endothelial cell injury markers could explain death in these models.

Paraquat-induced lung injury may be a good model for representing ROS-induced lung injury [25,26]. The main target cells are type II alveolar cells because of the accumulation of

paraquat. The paraquat-induced lung injury model might be different from the lipopolysaccharide model, because ROS-induced cell toxicity is a major mechanism in the paraquat model.

We showed that Ang-2: Ang-1 ratio can predict early mortality in multivariate analysis.

Ang-2 is proinflammatory and promotes vascular permeability. In contrast, Ang-1 stabilizes the endothelium. The Ang-2: Ang-1 ratio may reflect endothelial cell injury in the pulmonary microvasculature, which suggests that this mechanism could be involved in early damage to the pulmonary microvasculature and increased vascular permeability to paraquat.

VWF did not predict mortality in patients with paraquat poisoning. The use of VWF to predict ALD/ARDS in other situations has been controversial [12,23,30]. A study on poisoned patient sample time might be important because of the different dose and time interval after poisoning. Sabharwal et al showed that sequential plasma samples obtained from patients with ARDS had progressively increasing levels of VWF, up to days 4 and 8 [31]. Caflee et al showed that VWF did not have differential prognostic value for mortality depending on

the presence or absence of infection as a risk factor for ALI, but Ang-2 is a prognostic marker [19]. Indeed, further basic study is needed to understand when and how paraquat injures the pulmonary microvascular system.

The serial change of Ang-1, Ang-2, and Ang-2: Ang-1 ratio is shown in Figure 1. The Ang-2: Ang-1 ratio peaked within 1 day. We thought that paraquat-initiated endothelial injury might be associated with an early mechanism of pathogenesis and sequentially trigger inflammation and fibrosis.

According to HRCT, Ang-2: Ang-1 ratio does not reflect lung damage. Early death patients were excluded from this analysis because HRCT was performed 7 days after ingestion; this could be selection bias. ROS-directed lung injury may have been the principle pathogenic mechanism in the patients who underwent HRCT. Thus, according to HRCT, the endothelial damage marker was not different.

Our study had some limitations. First, we measured the sequential change of these markers in only 3 patients. Second, the origin of Ang-2 and Ang-1 was unknown. Regardless of origin, the Ang-2: Ang-1 ratio might reflect the endothelial damage that occurs after paraquat intoxication.

## Conclusions

In conclusion, endothelial cell damage could play a role in pathogenesis leading to early death after paraquat ingestion.

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