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Combined therapy with gas gangrene antitoxin and recombinant human soluble thrombomodulin for *Clostridium perfringens* sepsis in a rat model

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ABSTRACT

Cases of *Clostridium perfringens* septicemia, such as liver abscess, often develop a rapidly progressive intravascular hemolysis and coagulation; the mortality rate with current standard care including antibiotics and surgery is high. Herein, we firstly investigated the effects of gas gangrene antitoxin (GGA) (antitoxin against *C. perfringens*) and recombinant human soluble thrombomodulin (rTM) on the hemolysis, coagulation status, inflammatory process, and mortality in α-toxin-treated rats. Male 11-week-old Sprague Dawley rats were randomly divided into five groups: control group, α-toxin group, GGA group, rTM group, and combined GGA and rTM (combination group). After α-toxin injection, mortality and platelet counts, and hemolysis were observed for 6 h. The fibrin/fibrinogen degradation products (FDP), and plasma high-mobility group box 1 (HMGB1) were also measured at 6 h. The combination group demonstrated 100% survival compared with 50% survival in the α-toxin group and demonstrated significantly improved hemolysis, platelet counts, and lactate levels compared with those in the α-toxin group ($p < .01$). The FDP and HMGB1 levels in the combination therapy group were significantly lower than those in the α-toxin group ($p < .05$). Combination therapy with GGA and rTM administration is applicable as adjunct therapy for fatal *C. perfringens* sepsis.

1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic bacterium that exists in the human gastrointestinal tract and soil (Fraser and Collee, 1975). Cases with *C. perfringens* septicemia without gas gangrene (e.g., liver abscess, uterus abscess) often develop a rapidly progressive intravascular hemolysis and metabolic acidosis, with a high mortality rate ranging from 70% to 100% with standard intensive care (Ng et al., 2010; Rogstad et al., 1993; Simon et al., 2014). Median time between admission and death was reported to be only 8 h (van Bunderen et al., 2010). Several reports of fatal cases have been recently published because of its impact of hemolysis and high mortality rate (Hashiba et al., 2016; Meinders and Dijkstra, 2014; Sarvari et al., 2016; Yamaguchi et al., 2015; Yoshida et al., 2015). In such cases, α-toxin secreted by *C. perfringens* is the main toxin and is responsible for causing intravascular hemolysis followed by severe anemia, disseminated intravascular coagulopathy (DIC) (Daly et al., 2006; Ito et al., 2011), and multiple organ failure. High doses of penicillin, hyperbaric oxygen treatment, and surgery are the standard treatments of choice (van Bunderen et al., 2010). However, a better outcome has not been achieved with the current standard treatment.

Gas gangrene antitoxin, which is the antitoxin against gas gangrene toxins (*C. perfringens* Type A, *Clostridium septicum,* and

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Clostridium oedematiens), has been used as a treatment for gas gangrene for a long time. However, physicians of the current generation are not familiar with this therapeutic option because it has not been introduced as a treatment of choice in medical papers or congresses. This is mainly considered due to the limited demands for serum therapy, which have become rare especially in Japan for the sake of absolute vaccination policy and environmental hygiene (Nakai et al., 2003). Based on the pathophysiology, gas gangrene antitoxin can neutralize α-toxin secreted by *C. perfringens*. We have introduced the use of gas gangrene antitoxin in this type of *C. perfringens* septicemia as the optimal therapy based on the pathophysiological mechanism (Hifumi et al., 2013, 2010); however, no reliable scientific evidence to support its use is available thus far. In addition, after α-toxin induces inflammatory and coagulation responses (Oda et al., 2008; Ohsaka et al., 1978a, b), therapies directed against inflammatory mediators and anticoagulant therapies are also required to improve outcome.

Recombinant human soluble thrombomodulin (rTM), which is currently under phase III clinical trials for use in severe sepsis, has been shown to be effective for sepsis-induced DIC in a meta-analysis (Yamakawa et al., 2015). rTM could also be a candidate for the treatment of *C. perfringens*-induced DIC, because it serves as a negative feedback regulator of blood coagulation (Mohri et al., 1999). Moreover, there is a clinical report that rTM not only resolved the coagulation problem, but also prevented multiple organ failure associated with systemic inflammatory response in a patient with *C. perfringens* infection (Ito et al., 2011).

Based on this evidence, we hypothesized that combined therapy with gas gangrene antitoxin and rTM would elicit a beneficial effect on the symptoms and mortality in *C. perfringens* sepsis. To test this hypothesis, we examined the effects of gas gangrene antitoxin and rTM in α-toxin-treated rats.

2. Material and methods

2.1. Animal preparation

Male 11-week-old Sprague Dawley rats (CLEA JAPAN, Tokyo, Japan) were housed in separate cages in a temperature-controlled room under a 12 h:12 h light-dark cycle. They were fed a standard laboratory diet and water ad libitum. All surgical and experimental procedures were approved by the Animal Care and Use Committee and conformed to the Guidelines for Animal Experimentation.

Under general anesthesia using 4% of isoflurane induction, a polyethylene catheter (PE-60) was inserted into the abdominal aorta via the right femoral artery for blood pressure measurements. Another catheter (PE-50) was inserted into the inferior vena cava via the right femoral vein for administration of saline solution (1.5 ml/h) and drugs. Tracheostomy was also performed and a PE-240 catheter was inserted into trachea. Heart rate was triggered by the blood pressure pulse waveform. All catheters were filled with heparinized saline (100 U/ml).

Isoflurane was maintained at 1–2%, as it was necessary to maintain an adequate depth of anesthesia. After catheter placement and tracheostomy, the rats were stabilized for 1 h before the start of the experiment.

2.2. C. perfringens Type A test toxin (NIID Tokyo, Japan)

This test toxin is a crude α-toxin purified from the culture filtrate of *C. perfringens* Type A by 37% ammonium sulfate precipitations and QAE-Sepharose ion-exchange chromatography.

The toxin is a freeze-dried product, and one test dose of the toxin was defined and equal to five times the LD50 in mice. Alpha-toxin was resolved into phosphate buffered saline to administer it to the rats. The dose of α-toxin (1.5 test dose) was chosen based on preliminary experiment results: 4.5 test dose; 100% of mortality within 1 h, 0.5 test dose; 0% of mortality, and 1.5 test dose; 50% of mortality for 6 h post injection of α-toxin.

2.3. Gas gangrene antitoxin (NIID Tokyo, Japan)

Gas gangrene antitoxin is a freeze-dried product of equine immunoglobulin containing three types of antitoxins against the gas gangrene toxins (*C. perfringens* Type A, *C. septicum*, and *C. oedematiens*). This immunoglobulin is an F(ab′)2 fragment removed from the Fc fragments by pepsin digestion.

2.4. Recombinant human soluble thrombomodulin

rTM was generously provided by Asahi Kasei Pharma Co (Tokyo, Japan).

2.5. Experimental protocols

All rats received saline (1.5 ml/h for 6 h) as a fluid resuscitation. Rats were initially randomly divided into five groups to evaluate lethality as follows: (a) control group (n = 6); (b) 1.5 test dose of α-toxin (n = 6); (c) 20 U of gas gangrene antitoxin administered 1 h after α-toxin (1.5 test dose) administration (n = 6); (d) rTM (1 mg/kg) administered 30 min after α-toxin (1.5 test dose) administration (n = 6); and (e) rTM (1 mg/kg) followed by administration of 20 U of gas gangrene antitoxin (30 min and 1 h, respectively) after α-toxin (1.5 test dose) administration (n = 6).

Blood samples were collected at 0, 0.5, 1, 2, 3, 4.5, and 6 h after the start of the experiment (500 μl of blood was collected at each time point except for at 6 h [1 ml]). Blood samples were anticoagulated with sodium citrate and centrifuged immediately for 10 min at 3000 g, and the plasma supernatant was separated.

The arterial blood gases and lactate levels were analyzed by an ABL 700 (Radiometer, Copenhagen, Denmark). Platelet counts were measured by a hemocytometer and flow cytometry. The fibrin/fibrinogen degradation products (FDP) in serum were also measured using the latex turbidimetric immunoassay. Antithrombin III (AT-III) activities were measured with a chromogenic substrate using an automatic analyzer. AT-III levels of 100% was defined from samples of healthy dogs and cats. Commercial enzyme linked immunosorbent assay kits were used for measurement of plasma high-mobility group box 1 (HMGB1) (Shino-TEST, Kanazawa, Japan). FDP, AT-III, and HMGB1 were evaluated with blood samples collected at 6 h.

2.6. Principle of test in measurement of FDP

This product is a reagent for quantitative determination of FDPs utilizing immune aggregation of latex particles sensitized with mouse monoclonal antibody of 2 clones (5G5-E7 and 3G1D-1C9) to FDP IgG F(ab′)2 fragment when they react with FDP antigens in plasma and serum. Thus, latex particles aggregate in proportion to the concentration of FDPs in a specimen by an antigen-antibody reaction occurring when latex particles sensitized with mouse anti-human FDP monoclonal antibody react with FDPs in a specimen if FDP is present at a certain concentration or higher. The intensity of aggregation is measured between 500 and 800 nm and the FDP concentration in the specimen is determined from the calibration curve prepared with calibrator solutions by a similar reaction.