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Clopidogrel reduces the inflammatory response of lung in a rat model of decompression sickness

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ABSTRACT

Inflammation and platelet activation are critical phenomena in the setting of decompression sickness. Clopidogrel (Clo) inhibits platelet activation and may also reduce inflammation. The goal of this study was to investigate if Clo had a protective role in decompression sickness (DCS) through anti-inflammation way. Male Sprague–Dawley rats ($n = 111$) were assigned to three groups: control + vehicle group, DCS + vehicle, DCS + Clo group. The experimental group received 50 mg/kg of Clo or vehicle for 3 days, then compressed to 1,600 kPa (150 msw) in 28 s, maintained at 150 msw for 242 s and decompressed to surface at 3 m/s. In a control experiment, rats were also treated with vehicle for 3 days and maintained at atmospheric pressure for an equivalent period of time. Clinical assessment took place over a period of 30 min after surfacing. At the end, blood samples were collected for blood cells counts and cytokine detection. The pathology and the wet/dry ratio of lung tissues, immunohistochemical detection of lung tissue CD41 expression, the numbers of P-selectin positive platelets and platelet–leukocyte conjugates in blood were tested. We found that Clo significantly reduced the DCS mortality risk (mortality rate: 11/45 with Clo vs. 28/46 in the untreated group, $P < 0.01$). Clo reduced the lung injury, the wet/dry ratio of lung, the accumulation of platelet and leukocyte in lung, the fall in platelet count, the WBC count, the numbers of activated platelets and platelet–leukocyte complexes in peripheral blood. It was concluded that Clo can play a protective role in decompression sickness through reducing post-decompression platelet activation and inflammatory process.

1. Introduction

In recent years, fast buoyant ascent escape is applied to submarine accident rescue. However, if some mistakes happened, such as pressurized stay too long, too slow decompression and other factors may cause the occurrence of acute decompression sickness (DCS). Annual reports of diving fatality cases collected by the Divers Alert Network (DAN) indicated that the most common procedural incidents in recreational dive were buoyancy problems followed by rapid ascent (Pollock et al., 2008).

DCS is believed to be initiated by the formation of gas bubbles in tissue and blood. Vascular bubble formation contributes to endothelial injury leading to vascular obstruction, microcirculatory alterations, activation of coagulation cascades and inflammation (Ersson et al., 1998). It is now believed that severe DCS is not simply a localized phenomenon but a systemic (Gempp et al., 2008; Montcalm-Smith et al., 2008). Post-dive manifestation of DCS can be effectively treated by hyperbaric oxygenation; however, hyperbaric oxygen treatment is not always available immediately. So it is very important to find effective protective measures to reduce or prevent the development of DCS.

Platelet activation and inflammation are associated with DCS. Circulating bubbles induced by rapid decompression may impose a stress to tissues and affect both the blood vessel luminal surfactant layer and endothelial activation, resulting in subsequent (Levett and Millar, 2008) platelet and leukocyte aggregation, cytokine release, complement activation, and coagulation cascades (Bigley et al., 2008; Chen et al., 2011; Thom et al., 2011). Studies had found the relationship between the post-dive decrease in platelet count and the DCS severity (Pontier et al., 2012). Some researchers also found altered TNF-α and IL-6 levels in a rat model of DCS (Bigley et al., 2008) and a positive correlation between susceptibility to DCS and sensitivity to complement activation (Nyquist et al., 2007).

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Aggregated platelets can modulate the inflammatory response. Gawaz et al. (1998) have demonstrated that activated platelets stimulated the expression and release of inflammatory-related proteins in cultured endothelium. Moreover, in vivo studies have also suggested that platelets may participate in the inflammatory reaction through the activation of leukocytes (Neumann et al., 1999). Recently, Thom et al. (2012, 2013) found platelet–neutrophil interactions increased in divers after open-water SCUBA diving. Based on these researches, we hypothesized that the inflammatory response in DCS may be induced by aggregated platelets. In this study, we used clopidogrel, a P2Y12 adenosine diphosphate (ADP) receptor antagonist to do further research.

The P2Y12 receptor is essential for ADP-induced platelet aggregation (Daniel et al., 1998; Jin et al., 1998) and in thrombus growth and stability (van Gestel et al., 2003). Clopidogrel is converted to an active metabolite in the liver and the active metabolite irreversibly inactivates the P2Y12 receptor (Cattaneo, 2007). Antagonism of the P2Y12 receptor diminishes the extent of release from platelets of both the alpha and dense granules. These granules can stimulate peripheral blood leukocytes and inflammation responses directly or indirectly (Klinger and Jelkmann, 2002). Studies and clinical trials have showed that clopidogrel had anti-inflammatory effects in acute coronary syndrome (Molero et al., 2005) and acute lung injury (Zarbock et al., 2006).

In this study, we tested the anti-inflammatory effects of clopidogrel in a rat model of DCS, we also wanted to explore the relation between the activated platelets and inflammation response in DCS.

2. Materials and methods

2.1. Animal

Male Sprague–Dawley rats (Rattus Norvegicus) (230–240 g, 8 w, male) were purchased and bred in our Animal Resources Laboratory. The rats were maintained in our facility for 1 week before the experiments. All aspects of this study were reviewed and approved by the Institutional Animal Care and Use Committee.

2.2. Experimental groups

111 Male Sprague–Dawley rats were divided into three groups: the first group with administered treatment before the compression and decompression protocol (DCS + Clo group, $n = 45$), the second group with a placebo pretreatment before the same hyperbaric exposure and the same decompression protocol as the first group (DCS + vehicle group, $n = 46$), and the last group with a placebo pretreatment before an atmospheric exposure (no compression and decompression protocol, control + vehicle group, $n = 20$). Clo was dissolved in sterile water and administered by gavage using a rodent feeding needle. Clo (Sanofi Recherche, Toulouse, France) was delivered once a day (50 mg/kg) for 3 days before the hyperbaric exposure (Pontier et al., 2011). The DCS and control groups with placebo pretreatment received an oral gavage administration with sterile water (the same volume as Clo group).

2.3. Decompression protocol

In the DCS + vehicle and DCS + Clo groups, animals were placed in a cage containing 15 animals, and the whole group was compressed together in a hyperbaric chamber (Hongyuan, Yantai, China). Simulated fast buoyancy ascent escape was controlled by a self-edited computer compression-decompression process. Rats were compressed to1.6 MPa (150 m) with air in 28 s (the chamber pressure would increase doubly in every 7 s) and maintained at pressure for 242 s while breathing air. At the end of the exposure period, rats were decompressed to surface at a rate of 3 m/s. Carbon dioxide, temperature and humidity levels were measured with the sensors which were placed in chamber. CO_2 levels were kept below 300 ppm by continuous circulation of gases in the chamber through a soda-lime canister. Humidity was controlled with silica gel, and the ambient temperature was adjusted to 27 °C by central air condition.

After decompression, rats were observed during a 30-min period. In the previous study, we found that rats suffered with DCS can categorized into 3 scales. 1 for no signs, 2 for neurological DCS signs included walking difficulties and forelimb and/or hindlimb paralysis, 3 for pulmonary DCS signs when the animals exhibited pronounced respiratory maneuvers and died within 5 min after surfacing. This was consisted with Pontier et al. (2008). Following the 30-min post-decompression observation period, all the rats survived were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) for blood sample collection and killed by exsanguination.

2.4. Blood sampling techniques and white cell, platelet count

For white cell and platelet count analysis, 1 mL blood sample was taken from abdominal vein into vacutainer through infusion needle. The samples were fixed by EDTA (12 mM) to avoid coagulation and analyzed with a blood counter (Abbott, Illinois, USA).

2.5. Histological examination

Six rats from each group were anesthetized with pentobarbital (100 mg/kg i.p.) post exposure. The bodies were first flushed with phosphate-buffered saline (PBS) followed by periodate–lysine–paraformaldehyde (PLP). The lungs were removed and fixed for 2 h before dehydration and embedding in paraffin. 4 μm-thick sections were cut and placed on glass slides, then stained with hematoxylin and eosin (H&E) for routine histologic analysis. From each section, 10 random areas were examined at a magnification of ×400. Within each field, lung injury was scored according to: edema, infiltration of inflammatory cells, and hemorrhage. Each feature was graded as: 0, absent and appears normal; (1) light; (2) moderate; (3) strong; (4) intense. The three resulting scores were added and used as the lung injury score for that section (Chen et al., 2006).

2.6. Wet/dry lung weight ratio

The right upper lung lobe was excised at the end of the experiment and was weighed to determine the final wet lung weight and then dried in an oven at 80 °C for 48 h before being weighed again to give dry weight. The W/D weight ratio was then calculated ($n = 6$ per group).

2.7. Immunohistochemical analysis for CD41

Immunohistochemistry was performed for the detection of CD41 proteins in lung tissue. Lungs were perfused with buffered 1% paraformaldehyde, cut into 2- to 6-mm sections, placed in 10% buffered formalin, and embedded in paraffin. Embedded lung sections were further sliced into 5 μm sections and rehydrated in water. The sections were treated with 0.9% hydrogen peroxide in mqH_2O for 30 min to inactivate any endogenous peroxidases. After washing once with PBS (pH 7.4), sections were incubated with 10% normal goat serum in PBS for 30 min to block nonspecific binding