Forensic Science International 254 (2015) 126–132

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Inflammatory responses to neutral fat and fatty acids in multiple organs in a rat model of fat embolism syndrome

Meri Takada *, Shoetsu Chiba, Tomonori Nagai, Hiroshi Takeshita, Sanae Kanno, Toru Ikawa, Kana Sakamoto, Morihisa Sagi, Kazue Ichiba, Toshiji Mukai

Department of Legal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ward, Kawasaki, Kanagawa 216-8511, Japan

ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form 19 June 2015
Accepted 8 July 2015
Available online 18 July 2015

Keywords:
Fat embolism syndrome
Inflammatory cytokine
Neutral fat
Fatty acid
Rat

ABSTRACT

Fat embolism syndrome (FES) is a common complication of long bone fractures. FES is rare but with significant morbidity and occasional fatalities. Studies of animal models of FES are numerous; however, few studies compare inflammatory reactions in multiple organs. The present study investigated the effect of neutral fat and fatty acids, which cause changes in multiple organs and induce FES. Using rats we evaluated the ratio of lung-to-body weight and conducted histological analyses and quantitative analysis of inflammatory cytokine mRNAs in the lungs following intravenous administration of neutral fat or fatty acids. Neutral fat increased the ratio of lung-to-body weight, and neutral fat formed emboli in lung capillaries. The levels of interleukin-1 beta (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α) in the lungs increased after injection of neutral fat and oleic acid. Analysis of the histologic changes revealed that the highest numbers of fat droplets, occluding the capillaries of the lungs, kidney, heart, and brain formed 12 h after the injection of neutral fat and fat droplets gradually diminished 48 h later. Fat droplets were not detected in any organs after the injection of oleic acid. IL-1β and TNF-α levels in the lungs were elevated 9–24 h after the injection of neutral fat, although IL-6 levels peaked at 6 h. After injection of oleic acid, peak levels of IL-1β, IL-6, and TNF-α were detected at 6 h, and IL-6 again increased in all organs and plasma at 15 h. Neutral fat, but not fatty acids, formed emboli in the capillaries of multiple organs. These findings suggest that neutral fat increased inflammatory cytokine levels by forming emboli in organ capillaries, particularly in the lungs, while oleic acid augmented inflammatory cytokine levels by stimulating endothelial cells of multiple organs.

1. Introduction

Fat embolism (FE) is fat within the circulation, which can produce embolic phenomena, with or without clinical sequelae [1]. It is generally assumed that the fat is embolized into different organs, notably the lungs [2]. Pulmonary fat embolism (PFE) mainly occurs due to long-bone fractures, but can also occur due to fat crushing [2]. PFE causes acute respiratory distress, and the resulting hypoxia can lead to sudden death. Moreover fat embolism syndrome (FES) is a serious manifestation of FE involving multiple organs and is potentially life threatening [3]. FES is fat in the circulation associated with an identifiable clinical pattern of symptoms and signs [1]. Symptoms usually occur 24–72 h after trauma (particularly after fractures of the pelvis or long bones) and are predominantly pulmonary (tachypnea, dyspnea), neurological (confusion, drowsiness or coma), and dermatological (petechial rash) [4]. Clinically, only 1–10% of the patients develop FES, with a mortality rate of 5–10% [5].

There are two theories on the cause of FES. One theory maintains that intramedullary fat is forced into the circulation as emboli through a fracture or because of increased intramedullary pressure that is generated by certain orthopedic procedures [6]. These fat droplets are deposited in the pulmonary capillary beds and travel through such as arteriovenous shunts, anastomosis, and patent foramen ovale to the brain or multiple organs [7,8]. The second theory posits that free fatty acids (FFAs) are liberated when lipase produced by the lungs acts on embolic fats. FFAs are toxic to pulmonary endothelial cells and pneumocytes [6] and they cause interstitial and alveolar hemorrhage, edema, and chemical pneumonitis by producing endothelial damage, inactivating lung surfactant, and increasing permeability. These events often induce acute respiratory distress syndrome (ARDS) [9].

* Corresponding author. Tel.: +81 44 977 8111; fax: +81 44 977 3902.
E-mail address: m2takada@marianna-u.ac.jp (M. Takada).

http://dx.doi.org/10.1016/j.forsciint.2015.07.011

Zenker described the first autopsy case of FE with the presence of pulmonary capillary fat deposition in a patient who suffered from a crush injury in 1861 [10]. In 1873, the surgeon Bergmann described the first clinical case of FES in a patient with a fractured femur [11]. Since 1860, more than 2,000 papers have been published on the process of fat embolization and FES [12]; however the pathophysiologic mechanisms of FES have yet to be fully elucidated.

Most studies and case reports on FES are published in journals focused on the fields of orthopedics and emergency medicine because it generally occurs after a trauma such as pelvic or long-bone fractures [4], which are considered emergencies. However, we maintain and emphasize that FES is also significant for legal medicine because the cause of death is revealed only by postmortem examination that includes fat staining. Because some manifestations of FES are common to other critical illness, and there are no biochemical tests specific for FES, the clinical diagnosis can be very difficult.

Although various experiments using FES animal models employ injection of triolein or fatty acids [13–17], most studies focus on the relationship between FES and inflammatory reactions in the lungs because fat droplets in the blood stream mainly occlude lung capillaries and cause respiratory disorders. Long bones are very thin and it is hard to extract bone marrow fat from rats; therefore, we decided to use subcutaneous fat because its main components are very similar to those of bone marrow fat [18]. We also used fatty acids. In the present study, we induced FES in rats and compared the extent of inflammatory reactions in multiple organs and plasma analyzing inflammatory cytokine (Interleukin 1 beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α)) proteins, which reflect the activities of the cognate mRNA and have higher reliability.

2. Materials and methods

2.1. Materials

Test substances were neutral fat and fatty acids. Neutral fat was prepared by anesthetizing the rat, removing the subcutaneous fat, and grinding it in liquid nitrogen. The crushed fat (20 g) was mixed with 30 mL of distilled water and centrifuged for 10 min at 3000 rpm and the supernatant was used as neutral fat. Saturated fatty acids palmitic acid (C16:0) and stearic acid (C18:0) as well as the unsaturated fatty acids oleic acid (18:1 (cis-9)) and linoleic acid (18:2(n-6)) were purchased from Wako Pure Chemical Industries, Ltd, Japan. Neutral fat and fatty acids were emulsified with lecithin from soybeans (Wako) and glycerol (Wako) and then sonicated to produce fatty micelles as described by Eguchi et al. [19]. Pentobarbital sodium salt solution (Tokyo Chemical Industry, Japan) was used to anesthetize the rats. The organs were removed and soaked immediately in RNA later® (Life Technologies Corporation, USA) to stabilize and protect the integrity of RNA.

For histological analysis, 10% neutral-buffered formaldehyde (Wako) was used to fix the samples, Tissue Tech O.C.T. compound (Sakura Finetek Japan, Japan) was used to embed the samples, and a Leica CM 1850 Cryostat (Leica Biosystems Nussloch GmbH, Germany) was used to prepare thin sections that were stained for fat using Sudan III (Wako).

Total RNA was extracted from the lungs using the SV Total RNA Isolation System (Promega, USA). RNA was quantitated using the Go Taq 2-Step RT-qPCR System kit (Promega) two-step reverse transcription-quantitative PCR (RT-qPCR) protocol. The StepOne-Plus Real-Time PCR System (Life Technologies) was used for real-time PCR. Proteins were extracted from organs using the Detergent-Free Protein Extraction Kit for Animal Cultured Cells and Tissues (Invent Biotechnologies, USA). Quantikine® Enzyme-Linked Immunosorbent Assay (ELISA) Rat IL-1β (No. RLB00), Quantikine® ELISA IL-6 (No. R6000B), and Quantikine® ELISA TNF-α (No. RTA00) immunoassays (R&D Systems, USA) were used to determine the quantity of these inflammatory cytokines. A 2030 Arvo X Multilabel Reader (Perkin Elmer, Japan) was used to measure the absorbance of the samples.

2.2. Animals

We used 10-week-old male Wistar rats, weighing 300 ± 20 g. The animals were obtained from SLC, Japan and housed in humidity- and temperature-controlled ventilated cages on a 12-h light/dark cycle with free access to standard laboratory food and tap water. The rats were anesthetized with an intraperitoneal injection of 6.5% pentobarbital sodium salt solution (65 mg/kg body weight). The rats were randomly divided into groups of three animals each and injected with 600 μL/kg of neutral fat, oleic acid, or linoleic acid or 0.3 g/kg of palmitic acid or stearic acid through the right femoral veins. The emulsified fats were intravenously administered at the same molar concentration. The control groups were injected with the emulsifier (lecithin and glycerol) and physiological saline.

Six to 48 h after the injection of fats, three rats in each group were euthanized at 8 and 12 h for histological analysis and inflammatory cytokine mRNA analysis, and at 6, 9, 12, 15, 18, 24, and 48 h for inflammatory cytokine protein analysis by intraperitoneal administration of a lethal dose of 6.5% pentobarbital sodium salt solution. Blood was collected immediately after death followed by the removal of the lungs, heart, liver, kidneys, and brain. Blood was immediately centrifuged at 3000 rpm for 5 min, and the supernatant fluid (plasma) was used for analyses. The lungs of each rat were weighed to determine the lung-to-body weight ratio. Every group included three rats to comply with institutional requirements for the humane treatment of animals.

2.3. Histological analysis

We analyzed the histological characteristics of multiple organs harvested between 6 and 48 h after the injection of fats. Segments of each organ were immersed in 10% neutral-buffered formalin fixative for 3 days. On the fourth day, specimens were rinsed in tap water to remove the fixative and consecutively soaked in 10%, 20%, and 30% sucrose solutions for one day each. Organs were sectioned, embedded in O.C.T compound, and frozen in liquid nitrogen. Frozen sections were sliced into 5-μm-thick sections using a Leica CM 1850 Cryostat, and fats were stained with Sudan III.

2.4. Quantitative analysis of inflammatory cytokine mRNAs levels

Quantitative real-time RT-PCR analysis was used to determine the levels of IL-1β, IL-6, and TNF-α mRNAs in rat lungs 8 h after the injection of emulsified fats. First, total RNA was extracted from 60 mg of the right-upper lung of each rat as described above. The RT cycling conditions were 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. Ten micrograms of total RNA was reverse-transcribed as described above. The relative levels of inflammatory cytokine mRNA levels were determined as described above. The cycling conditions were as follows: 95 °C for 2 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. We confirmed that the melting curves of each product synthesized using the respective specific primers were identical.

2.5. Quantitative analysis of inflammatory cytokine levels

We determined the levels of inflammatory cytokine expression in multiple organs and plasma at 6, 9, 12, 15, 18, 24, and 48 h after