VII Summarized Guidelines

Table 20

Severity	Pathophysiology	Diagnosis / clinical course	Treatment	Daily life/exercise management*
I No dilatation	There is no evidence whether or not a history of Kawasaki disease is a factor associated with arteriosclerotic iesion.	Follow up patients for 5 years. Evaluate at 30 days, 60 days, 6 months, 1 year, and 5 year after onset with ECG, echocardiography, and, if necessary, chest X-ray. It is desirable that patients be evaluated with exercise ECG at the final examination.	Basically, no treatment is required during the remote phase. Patients with no coronary aneurysms after the acute phase may discontinue antiplatelet drugs such as aspirin.	No restriction is placed on daily life or exercise. Management Table: "No management needed" for children ≥5 years after onset. Consult with parents (or patients) to determine further man-agement. Lifetime prevention of lifestyle-related diseases is important. Junior and senior high school students should be educated on lifestyle-related diseases (blood lipid measurement, education on smoking cessation, and prevention of obesity).
II Transient dilatation during the acute phase	During the acute phase, histopathologically vasculitis develops in the outer layer of the tunica media and then expands to the intima in coronary arteries. Echocardiography reveals diffuse dilatation of coronary arteries, but these changes subside within 30 days after onset.			
III Regression	In many cases regression may occur 1 to 2 years after onset, particularly in small or medium aneurysms. In the segment with regression, decrease in coronary diastolic function, abnormal function of vascular endothelium, and substantial intimal hyperplasia have been reported.	Basically, follow patients annually with ECG, echocardiography, and chest X-ray up to entry into elementary school (age of 6, 7), and then with the same methods and exercise ECG in 4th grade (age 9, 10), at entry into junior high school (age 12, 13), and entry into senior high school (age 15, 16). Follow patients who had coronary aneurysms with a large internal diameter during the acute phase with an appropriate combination of imaging techniques**.		No restriction is placed on daily life or exercise. Follow the recommendations for Categories I and II.
IV Remaining coronary aneurysms	Aneurysms remaining during the convalescence phase or later are considered sequelae. Histopathologically, progression of inflammation leads to rupture of the internal elastic band, causing panangiitis. The internal and external elastic bands are broken into fragments and ruptured by arterial pressure to form aneurysms. Patients with giant aneurysms must be observed carefully for myocardial ischemia, since in such patients myocardial ischemia may develop even if no significant stenotic lesions are present.	Patients must be followed with exercise ECG and an appropriate combination of imaging techniques.** It is desirable that patients who had coronary aneurysms with a large internal diameter during the acute phase be evaluated with stress myocardial scintigraphy every 2 to 5 years to monitor for progression to stenotic lesions.	Continue treatment with antiplatelet agents such as aspirin. Anticoagulant therapy may be needed in patients with giant coronary aneurysms or thrombi in coronary aneurysms. CABG may be indicated for patients with giant coronary aneurysms not accompanied by signifcant stenotic lesions when myocardial ischemia has occurred.	No restriction is placed on daily life or exercise. Management Table: "E-allowed". Patients with giant aneurysms: Instruct as "D-prohibited" in the Management Table. In the second year after onset or later, "E-prohibited" is possible when no changes are noted.
V-a Coronary stenotic lesions (no findings of ischemia)	Thrombotic occlusion of medium or giant aneurysms may develop during the relatively early stage after onset. Sudden death may occur, though two-thirds patients with occlusion are asymptomatic. Patients often show improvement of myocardial ischemia due to the development of recanalized vessels and collateral flow after occlusion. Development/progression of regional stenosis during the remote phase is more prevalent in the left coronary artery than in the right coronary artery. The segments with greatest prevalence are the proximal segment or the main trunk of the left anterior descending artery. The risk of progression to stenosis/occlusion is higher in larger aneurysms. Stenosis may develop during long-term follow up.	Patients must be followed for life, and physicians must design the tailor-made management plan for individual patients. Follow-up examination must include exercise ECG and an appropriate combination of imaging techniques**. Although schedule may differ among individuals, patients are generally evaluated every 3 to 6 months.	Continue treatment with antiplatelet drugs such as aspirin. Use Ca-blockers, nitrates, β-blockers, ACE inhibitors, and angiotensin receptor II blockers to prevent ischemic attacks and heart failure.	No restriction is placed on daily life or exercise. Management Table: "E-allowed" for patients other than those with giant aneurysms. Explain the importance of drug treatment and ensure adherence, as well as symptoms which may occur and actions to be taken when ischemia develops. Patients must be followed at least annually until regression of aneurysms is documented.
V-b Coronary stenotic lesions (with findings of ischemia)			Follow the instructions for drug treatment in Category V-a. Consider CABG or appropriate PCI technique when exercise ECG or stress myocardial scintigraphy reveals ischemia.	Exercise shuld be restricted. Categorize in "D" or higher category based on patient condition. School sport club activities should be "prohibited". Select the most appropriate category from "A" to "D" on the basis of findings of exercise testing and evaluation of severity of myocardial ischemia. Educate patients well about the importance of drug treatment.

*See Table 19.
**Imaging techniques include echocardiography (including stress echocardiography), stress myocardial scintigraphy, selective CAG, IVUS, MRI, MRA, and MDCT.
CABG, coronary artery bypass grafting; ACE, angiotensin converting enzyme; PCI, percutaneous coronary intervention; CAG, coronary angiography; IVUS, intravascular ultrasound; MRI, magnetic resonance imaging; MRA, magnetic resonance angiography; MDCT, multi-row detector computed tomography.

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Appendix

Chair:
- Shunichi Ogawa, Department of Pediatrics, Nippon Medical School

Members:
- Teiji Akagi, Cardiac Intensive Care Unit, Okayama University Hospital
- Kiyoshi Baba, Department of Pediatrics, Osaka Developmental Rehabilitation Center
- Hisayoshi Fujiwara, Hyogo Prefectural Amagasaki Hospital
- Kenji Hamaoka, Department of Pediatric Cardiology and Nephrology, Kyoto Prefectural University of Medicine Graduate School of Medical Science
- Masahiro Ishii, Department of Pediatrics, Kitasato University
- Kensuke Karasawa, Department of Pediatrics, Nihon University School of Medicine
- Tsutomu Saji, First Department of Pediatrics, Toho University
- Tomoyoshi Sonobe, Department of Pediatrics, Japanese Red Cross Medical Center
- Atsuko Suzuki, Department of Pediatrics, Tokyo Teishin Hospital

Collaborators:
- Mamoru Ayusawa, Department of Pediatrics, Nihon University School of Medicine
- Ryuji Fukazawa, Department of Pediatrics, Nippon Medical School
- Kazuhiko Nishigaki, Second Department of Internal Medicine, Gifu University
- Hirotaro Ogino, Department of Pediatrics, Kansai Medical University
- Tomoo Okada, Department of Pediatrics, Nihon University School of Medicine

Independent Assessment Committee:
- Shigeyuki Echigo, Echigo Clinic
- Makoto Nakazawa, Pediatric and Lifelong Congenital Cardiology Institute, Southern Tohoku General Hospital
- Masami Ochi, Division of Cardiovascular Surgery, Nippon Medical School
- Tetsu Yamaguchi, Toranomon Hospital

(The affiliations of the members are as of March 2010)

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Fetal Reversed Constrictive Effect of Indomethacin and Postnatal Delayed Closure of the Ductus Arteriosus following Administration of Transplacental Magnesium Sulfate in Rats

Katsuaki Toyoshima[a, c] Kazuo Momma[a] Toshio Nakanishi[a, b]

[a]Section of Pediatric Cardiology, and [b]International Research and Educational Institute for Integrated Medical Sciences, Tokyo Women's Medical University, Tokyo, and [c]Section of Neonatology, Kanagawa Children's Medical Center, Yokohama, Japan

Key Words
Magnesium sulfate · Indomethacin · Ductus arteriosus · Preterm infant · Patent ductus arteriosus · Eclampsia · Tocolysis

Abstract
Background: Magnesium sulfate ($MgSO_4$) is used therapeutically for eclampsia and tocolysis. Some reports have suggested a relationship between therapeutic $MgSO_4$ and patent ductus arteriosus (DA) in preterm infants. ***Objectives:*** To clarify patent DA induction by $MgSO_4$ in preterm infants, we studied the increase in serum Mg concentrations and fetal dilatation and postnatal delayed closure of the ductus, using transplacental $MgSO_4$ in rats. ***Methods:*** Fetal and neonatal ductus diameters were measured with a microscope and a micrometer after rapid whole-body freezing. In the postnatal study, 21-day pregnant dams were administered a subcutaneous injection of $MgSO_4$ 1–3 h before delivery, and the ductus was studied 0, 15, 30, 60 and 120 min after birth. In the fetal study, $MgSO_4$ (1 g/kg) and indomethacin (10 mg/kg) were simultaneously administered to 21-day dams and the fetal ductus was studied 1, 2 and 4 h later. Serum Mg concentration was measured in the dams and newborns. ***Results:*** Neonatal Mg concentrations increased from 3.8 to 4.7 and 5.8 mg/dl at 1 and 3 h after maternal administration of $MgSO_4$. Following $MgSO_4$ administration 3 h before birth, closure of the neonatal DA was delayed. The ductus diameter was 0.88 mm (0.80 mm in control) at 0 min, and 0.26 mm (0.08 mm in the control) at 60 min after birth. In the fetal study, $MgSO_4$ initially reversed and later attenuated the ductus-constricting effect of indomethacin. ***Conclusions:*** Hypermagnesemia induced by transplacental $MgSO_4$ attenuates the fetal ductus-constricting effects of indomethacin, and delays postnatal ductal closure in rats.

Introduction

Magnesium sulfate ($MgSO_4$) is widely used as a first-line tocolytic agent [1–6]. It is also used as a prophylactic agent against seizures in pre-eclamptic women [7].

An elevated intracellular Ca^{2+} level is associated with the contraction of smooth muscles. $MgSO_4$ relaxes the uterine and vascular smooth muscles by increasing the serum and intracellular Mg^{2+} levels and acting as a Ca^{2+} antagonist, thus $MgSO_4$ prevents premature delivery and promotes vasodilatation [8, 9].

Recently, the incidence of patent ductus arteriosus (PDA) was compared between infants exposed to $MgSO_4$ and those who had not been exposed. The incidence of

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Katsuaki Toyoshima
Kanagawa Children's Medical Center
2-138-4, Mutsukawa, Minami-ku
Yokohama 232-8555 (Japan)
Tel. +81 45 711 2351, Fax +81 45 743 5797, E-Mail nqf37179@nifty.com

PDA was significantly higher in the group of infants exposed to $MgSO_4$ compared with the unexposed control group (67 vs. 60%, $p < 0.018$) [10].

We hypothesized that Mg^{2+} may affect the contraction mechanism of the ductus arteriosus (DA). However, a few reports have suggested a relationship between $MgSO_4$ and PDA [10–12].

The aim of this study was to assess whether hypermagnesemia, induced by transplacental $MgSO_4$, interferes with DA constriction.

Materials and Methods

Drugs

$MgSO_4$ (10% solution) was purchased from Towa Pharmaceutical Co. (Osaka, Japan). Indomethacin was purchased from Sigma Aldrich Co. (St Louis, Mo., USA). Indomethacin was diluted with lactose, and suspended in 1 ml of water for orogastric administration to dams.

Animals

Treatment conformed to the guiding principles of the American Physiological Society. The experiment was approved by the Ethical Committee of Animal Experiments of our Institute. Virgin Wistar rats (pregnancy period 21.5 days) were mated overnight from 17.00 to 9.00 h; the presence of sperm in vaginal smears fixed day 0 of pregnancy. The rats were housed in an environmentally controlled room, acclimatized to a 12-hour light/12-hour dark cycle, and maintained on commercial solid food and tap water ad libitum. Experiments were performed using the rat newborns delivered on the 21st gestational day. Treatment conformed to the guiding principles of the American Physiological Society. The experiment was approved by the Ethical Committee of Animal Experiments of our Institute.

Ductus Diameter Measurement

In the postnatal studies, following atlas dislocation and cesarean section of the near-term (21 days) dams, the newborn rats were incubated at room temperature (33°C). To study the in situ morphology of the postnatal DA, a rapid whole-body freezing method was used, as described in earlier studies [13, 14]. In brief, the rat newborns were frozen at 15, 30, 60 and 120 min after birth in acetone cooled to −80°C with dry ice. The frozen thorax was cut on a freezing microtome (Komatsu Solidate Co. Ltd., Tokyo, Japan) in the frontal plane, and the inner diameters of the ascending aorta, main pulmonary artery and DA were measured using a microscope (Nikon Binocular Stereoscopic Microscope, Nihon Kogaku Co., Tokyo, Japan) and a micrometer (Nikon Ocular Micrometer, Nihon Kogaku; fig. 1).

The DA of the neonatal rats was 1,200–800 μm in length, tubular along the middle three quarters of its length and horn shaped at the proximal and distal ends [15]. We measured the ductus every 100 μm in 8–12 planes. In ellipsoid images of the DA, the short axis was measured, assuming that the in situ DA was round in shape [15]. The narrowest DA diameter was used as the indicator of constriction.

In the fetal studies, the fetuses were delivered by caesarean section, with atlas dislocation of the dam. Immediately after birth, they were frozen with the umbilical cord and placenta intact in acetone cooled to −80°C with dry ice. The body weight of the frozen fetuses was measured, and 8–12 fetuses from each litter were studied. The ductus diameter of the fetuses was measured in the same manner as that used for the rat newborns. Constriction of the fetal DA may be not uniform, and it may be the most severe at the aortic end in some cases [16]. The narrowest DA diameter was used as the indicator of constriction.

Serum Mg Concentrations

We investigated serum Mg concentrations in the pregnant and newborn rats following the subcutaneous injection of $MgSO_4$ (1 g/kg) to 21-day pregnant rats (term: 21.5 days). Blood samples of pregnant rats were collected by cardiac puncture at 1 and 3 h after the subcutaneous injection of $MgSO_4$. A blood sample was collected from 10 rat newborns by making a deep cut to the neck within 5 min of birth. The controls were 10 rat newborns whose mothers had not been administered $MgSO_4$. These studies were repeated four times in order to obtain four samples each time. For measuring Mg concentration, serum was separated from the blood samples by centrifugation. Mg concentrations in serum were measured by atomic absorption spectrometry.

We examined serum Mg concentrations in the rat newborns of those dams that were subcutaneously injected $MgSO_4$ (1 g/kg) at 3 h before birth at 0 and 60 min after birth.

Postnatal Studies to Examine the Effect of $MgSO_4$ on the DA

The postnatal delaying effect of $MgSO_4$ on DA closure was studied using 5–12 rat newborns for each dose and time point that were considered for this study. The delaying effect of the $MgSO_4$ on postnatal ductus closure was studied by subcutaneous injection of $MgSO_4$ (1 g/kg) to dams on the 21st day of pregnancy. The newborns were delivered by cesarean section 1 or 3 h later. The ductus diameter was measured at 15, 30, 60 and 120 min after birth in both cases.

Fetal Studies to Examine the Effect of $MgSO_4$ on the DA

At near-term (21 days), the dilating effects of $MgSO_4$ on the fetal ductus that was constricted by indomethacin were studied by the simultaneous administration of $MgSO_4$ and indomethacin to the dam, and examination of the DA at 1, 2 and 4 h later. Indomethacin (10 mg/kg) was administered through an orogastric tube along with 1 ml water. $MgSO_4$ (1 g/kg) was injected subcutaneously. The fetuses of the dams that had been injected with a vehicle served as controls.

Postnatal Studies to Examine the Effect of $MgSO_4$ on Respiratory State

In near-term dams (21 days), the suppression effects of $MgSO_4$ on postnatal respiratory states were studied by administering a subcutaneous injection of $MgSO_4$ (1 g/kg) to the dams 3 h before delivery and by monitoring the arterial oxygen saturation (SaO_2) after birth by using a Nellcor N-550^R pulse oximeter and applying the sensor to the neck of the rat newborns.

Photographs

The frontal section of the DA was photographed for observing the constriction by using a binocular stereoscopic microscope

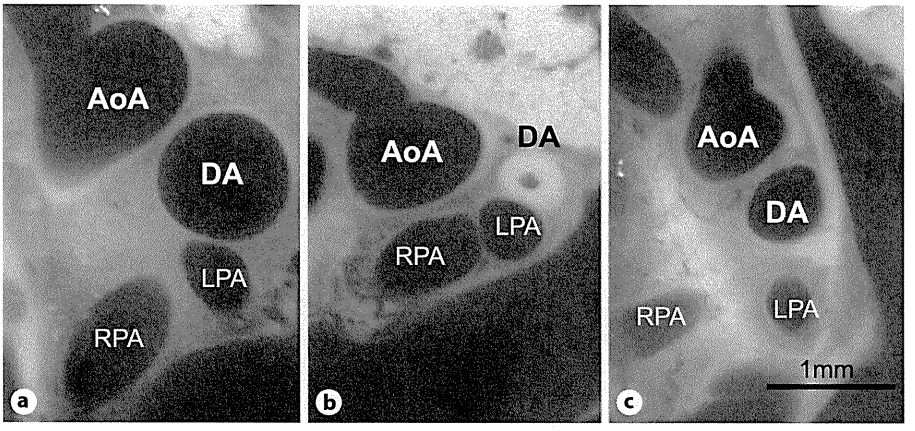

Fig. 1. Frontal cuts of the fetal and postnatal thorax at the level of the DA. **a** Dilated DA in a control fetus. **b** Constricted, thick-walled DA of a 30-min-old pup (control). **c** Half-constricted DA of a 30-min-old pup whose dam was injected with $MgSO_4$ (1 g/kg, s.c.) 3 h before delivery. AoA = Aortic arch; LPA = left pulmonary artery; RPA = right pulmonary artery.

(Wild M400 Photomacroscope, Wild Heerbrugg, Ltd., Heerbrugg, Switzerland) and color film (Reala, Fuji Film Co., Tokyo, Japan; fig. 1).

Statistics

The results are expressed as mean ± standard error of the mean (SEM). The statistical significance of the differences between the group means was determined using ANOVA and Bonferroni's method [17]. The difference was considered to be significant if the p value was less than 0.05.

Results

Serum Mg Concentrations

The serum Mg concentration before $MgSO_4$ injection was 2.7 ± 0.2 mg/dl (mean ± SEM) in the pregnant rats and 3.8 ± 0.1 mg/dl in the newborns. The serum Mg concentration peaked in the dams at 1 h after the subcutaneous injection of $MgSO_4$ (1 g/kg; fig. 2). The serum Mg concentrations in the rat newborns after the subcutaneous injection of $MgSO_4$ (1 g/kg) to the dams increased slowly, and were 4.7 and 5.8 mg/dl at 1 and 3 h later, respectively (fig. 2).

The serum Mg concentrations in the rat newborns after subcutaneous injection of $MgSO_4$ (1 g/kg) in the dams at 3 h before birth decreased slowly after birth (data not shown). The serum Mg levels in the rat newborns after injection of $MgSO_4$ in the dams at 3 h before birth were 5.8 and 5.5 mg/dl at 0 and 60 min after birth, respectively.

Postnatal Studies to Examine the Effect of $MgSO_4$ on DA

Postnatal death was not observed in this study. Figure 3 shows the time course of postnatal DA closure in

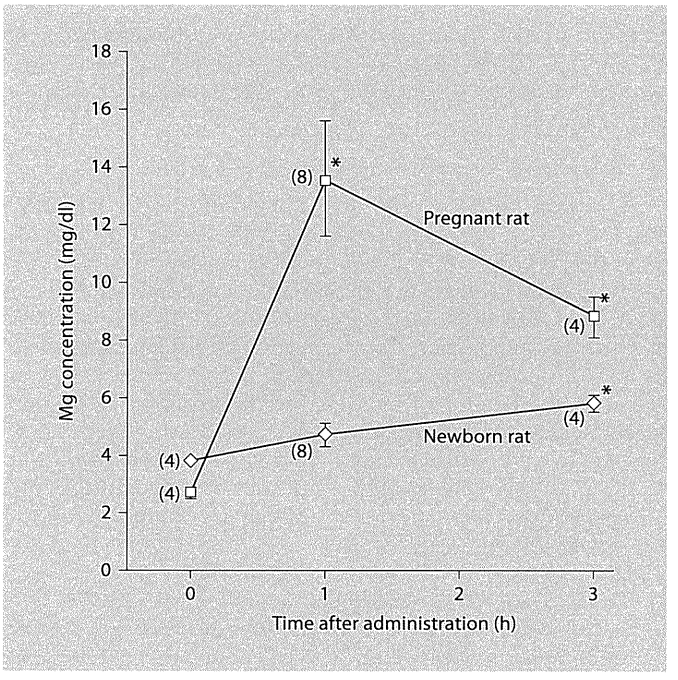

Fig. 2. Serum Mg concentrations in pregnant rats and rat newborns following injection of $MgSO_4$ (1 g/kg) to the pregnant dams. Figures in parentheses indicate number of samples. * $p < 0.05$ vs. control.

the controls and in rats with transplacental exposure to $MgSO_4$ (subcutaneous injection of $MgSO_4$, 1 g/kg, to the dams) at 1 h or 3 h before birth. In control newborns, the DA constricted rapidly after birth, and the inner diameter was 0.80 and 0.08 mm at 0 and 60 min after birth, respectively. Following the maternal administration of $MgSO_4$ 1 h before delivery, neonatal ductal closure was significantly delayed, and the DA diameter was 0.82 and

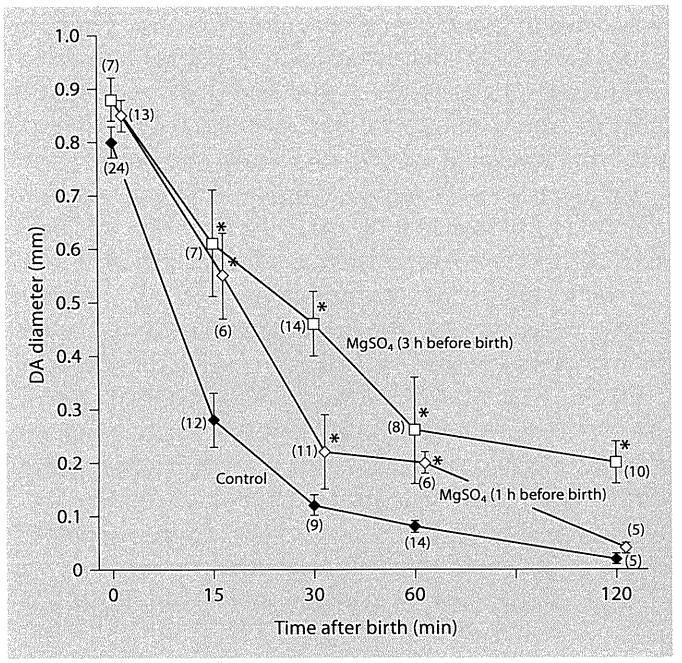

Fig. 3. Time course of postnatal ductal closure in the control rats and in rats with maternal $MgSO_4$ exposure (dams administered subcutaneous injection of 1 g/kg $MgSO_4$) at 1 and 3 h before birth. The course of the ductal closure was mildly delayed in rats with maternal $MgSO_4$ exposure 1 h before birth and more delayed in rats with maternal $MgSO_4$ exposure 3 h before birth. Figures in parentheses indicate number of animals. * $p < 0.05$ vs. control.

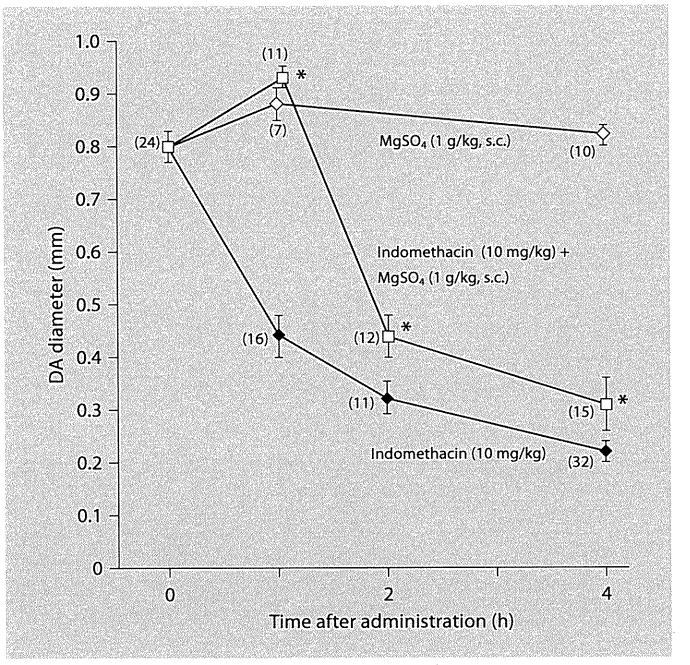

Fig. 4. Constriction of the fetal DA by indomethacin (10 mg/kg, orogastric) was counteracted by the simultaneous subcutaneous injection of $MgSO_4$ (1 g/kg) in near-term rats. At 1 h after administration, $MgSO_4$ (1 g/kg) completely reversed constriction of the fetal DA by indomethacin. $MgSO_4$ was partially effective in dilating the DA, at 2 and 4 h after administration. Figures in parentheses indicate number of animals. * $p < 0.05$ vs. indomethacin.

0.20 mm at 0 and 60 min after birth, respectively. Following the maternal administration of $MgSO_4$ 3 h before birth, neonatal ductal closure was delayed more than 1 h, and the DA diameter was 0.88 and 0.26 mm at 0 and 60 min after birth, respectively.

Fetal Studies to Examine the Effect of $MgSO_4$ on DA

Fetal death was not observed in this study. The orogastric administration of 10 mg/kg of indomethacin to near-term rats induced progressive severe fetal DA constriction. The inner diameter of the DA was 0.22 mm at 4 h after the administration of indomethacin (fig. 4). The subcutaneous administration of $MgSO_4$ (1 mg/kg) to the dams did not induce a significant ductus dilatation of the fetal DA, and the inner diameter of the DA was 0.88 at 1 h after the subcutaneous administration of $MgSO_4$ (1 g/kg).

The ductus-dilating effects of $MgSO_4$ were apparent following the simultaneous administration of $MgSO_4$ and indomethacin (fig. 4). One hour after the administration of $MgSO_4$ and indomethacin, the ductus-constricting effect of indomethacin was completely reversed, and the ductus showed dilatation. At 2 and 4 h after the administration of $MgSO_4$ and indomethacin, $MgSO_4$ counteracted the ductal constriction by indomethacin, and the ductus diameter was significantly larger than that when indomethacin alone was administered (fig. 4).

Postnatal Studies to Examine the Effect of $MgSO_4$ on Respiratory State

There was no difference in the SaO_2 level between the control newborns and the newborns with transplacental $MgSO_4$ exposure (dams administered 1 g/kg $MgSO_4$) 3 h before the birth (fig. 5).

Discussion

In this in vivo study, we have shown for the first time that postnatal DA closure is delayed following transplacental $MgSO_4$ exposure in rats. The respiratory suppression effect of $MgSO_4$ was not apparent in this study. The

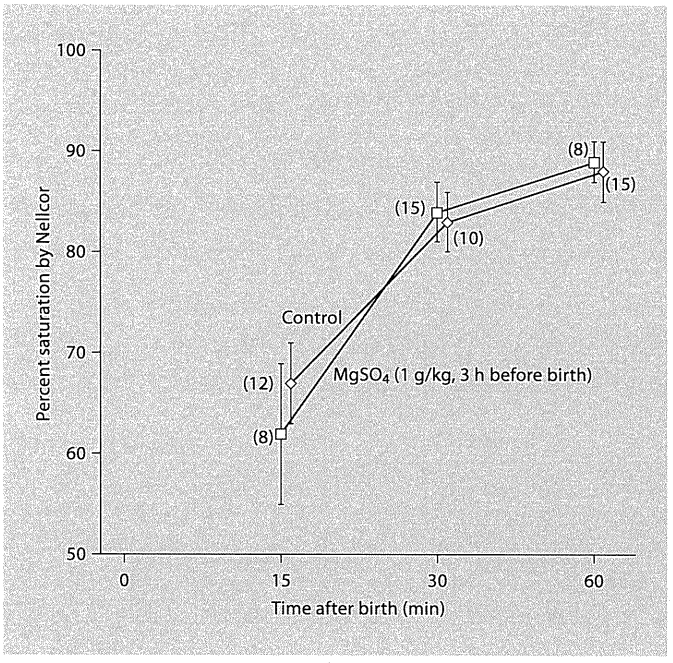

Fig. 5. SaO_2 of rat newborns following injection of $MgSO_4$ (1 g/kg) to the pregnant dams 3 h before delivery. There was no difference in SaO_2 between the control newborns and the newborns with transplacental $MgSO_4$ exposure. Figures in parentheses indicate number of animals.

course of the ductal constriction was delayed in the rat newborns that received $MgSO_4$ (1 g/kg) subcutaneously either 1 or 3 h before birth. The serum Mg concentration in the rat newborns, 1 and 3 h after the subcutaneous injection of $MgSO_4$ (1 g/kg) was 4.7 and 5.8 mg/dl, respectively. The serum Mg concentration in the rat newborns was similar to that observed in preterm infants with maternal administration of $MgSO_4$ for tocolysis [11, 18–20].

$MgSO_4$ has been widely used as a first-line agent for the prophylaxis of eclampsia and preterm labor [2–9, 11, 13, 18, 21]. $MgSO_4$ relaxes the uterine smooth muscles by increasing the serum and intracellular Mg levels and playing the role of a Ca^{2+} antagonist [8]. $MgSO_4$ is usually administered intravenously as an initial bolus of 4–6 g over 30 min, followed by a maintenance infusion of 1–3 g/h [18]. Serum Mg levels of 5–8 mg/dl are considered the therapeutic dose for inhibiting myometrial activity [18]. Maternal side effects secondary to $MgSO_4$ administration are typically dose related [18]. Mg readily crosses the placenta, achieving fetal steady-state levels within hours of the start of treatment [8, 11, 18, 21]. At cord Mg concentrations between 4 and 11 mg/dl, respiratory depression and motor depression have been observed. Clinically, serum Mg concentration in newborn infants usually exceeds maternal levels and leads to hypermagnesemia, with cord concentrations 70–100% higher than the maternal levels [19, 22–24]. Our study showed an initial rapid increase in the maternal magnesium concentration followed by a gradual increase in the fetal Mg concentration during the 3 h after $MgSO_4$ administration.

There have been a few reports suggesting a relationship between $MgSO_4$ and premature PDA [10–12]. Since the vessel wall of the DA is rich in smooth muscle, an elevated intracellular Ca^{2+} level is associated with DA constriction [25]. Studies in rats have shown that calcium channel blockers such as verapamil, when administered immediately after birth inhibit the spontaneous constriction of the DA [26]. High Mg concentrations competitively inhibit several calcium-dependent reactions, resulting in the dilatation of resistance vessels and the DA.

We studied the effect of $MgSO_4$ at a dose of 1 g/kg in this study. At this dose, $MgSO_4$ when administered alone has a weak ductus-dilating effect, and attenuates the ductus-constricting effect of indomethacin in rat fetuses. In our previous study, we have shown that the phosphodiesterase (PDE)-5 inhibitor (sildenafil), PDE-3 inhibitors (amrinone and milrinone) and the α-human atrial natriuretic peptide (hANP) dilate the fetal and postnatal constricted DA in a dose-dependent manner [27–29]. The ductus-dilating effects of 1 g/kg $MgSO_4$ are weaker than those of sildenafil, amrinone, milrinone and hANP. The ductus-dilating effects of $MgSO_4$ are not strong enough to be utilized for maintaining the patency of the DA in infants with DA-dependent congenital heart disease.

A few studies have reported the clinical time course of neonatal hypermagnesemia. The $MgSO_4$ accumulates in fetal tissues [8, 21]. McGuinness et al. [19] studied 23 term infants whose mothers had received intravenous $MgSO_4$ for pre-eclampsia and compared them with 14 control infants. The mean serum Mg concentration in the umbilical artery was 1.8 ± 0.1 mg/dl (mean ± SEM) in the control infants and 3.5 ± 0.2 mg/dl in the treated infants. Examination of the Mg levels in neonatal blood samples at 2, 12, and 24 h after delivery revealed that the Mg levels were higher in the treated infants than in the control infants [19]. Dangman and Rosen [30] have reported high serum Mg concentrations during the first 7 days of life in 16 preterm infants and 5 full-term infants with mothers who have been treated with $MgSO_4$. The mean serum Mg concentration in the umbilical artery increased from 3.7 to 4.2 mg/dl at 2 h after birth. The serum Mg half-life was

43 h and the Mg levels did not return to the baseline level until 1 week of age [30]. Rantonen et al. [11] reported that premature infants who received $MgSO_4$ transplacentally (administration period: 44 ± 46 h) developed hypermagnesemia for 3 days after birth. The mean serum Mg concentrations were 1.9 ± 0.2 mg/dl in the control infants and 4.4 ± 1.4 mg/dl in the treated infants on day 0, and increased to 2.2 ± 0.5 mg/dl in the control infants and 3.4 ± 1.0 mg/dl in the treated infants on day 3. In a study regarding the effects of maternal $MgSO_4$ treatment on the neonatal secretory response of the parathyroid hormone in preterm infants during the first 2 weeks of life, antenatal $MgSO_4$ exposure resulted in hypermagnesemia during the first 3–7 days of life [11]. Donovan et al. [31] reported the serum Mg levels in infants born after 32–44 weeks of gestation and whose mothers had received $MgSO_4$ for pre-eclampsia. The serum Mg concentrations were higher in preterm and asphyxiated infants [31]. After $MgSO_4$ therapy, the total Mg concentrations were significantly increased and the fetal levels paralleled maternal levels [32].

In human clinical practice, hypermagnesemia remains for a few days after birth. If alleviation of hypermagnesemia is observed after birth, the constrictive effect on the DA of indomethacin might be recovered. But the persistent hypermagnesemia in preterm infants may delay postnatal ductal constriction and attenuate the ductal constrictive effect of indomethacin and ibuprofen. Switching from therapeutic strategies to surgical ones in the early phase of the clinical course, and not adhering to intravenous indomethacin and ibuprofen therapy, may be beneficial in preventing cardiac failure or pulmonary hemorrhage associated with symptomatic PDA.

Our studies did not elaborate on the fact that these experimental data, unlike the clinical data, apply to the term neonate. However, PDE-3 and PDE-5 inhibitors dilated the fetal DA constricted by indomethacin more sensitively in preterm than in near-term rats [27, 28]. We speculate that the hypermagnesemia in preterm infants, more than in term infants, may delay postnatal ductal constriction and attenuate the ductal constrictive effect of indomethacin in clinical settings. We administered higher doses of $MgSO_4$ and indomethacin compared with clinical human dosages to clarify the effects of these drugs. Therefore, in clinical practice the effects of the drugs will be milder than the effect observed in our preclinical study using animals.

Clinical observations of the effect of hypermagnesemia on the DA should be made in the future.

Conclusions

$MgSO_4$ has an attenuating effect on fetal and neonatal ductal constriction in rat. This suggests the possibility that premature infants who receive transplacental $MgSO_4$ may be more likely to develop symptomatic PDA postnatally. In addition, the ductus-constricting effects of indomethacin on PDA may be attenuated in premature infants with antenatal $MgSO_4$ exposure.

Acknowledgements

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Increased P-Selectin Expression on Platelets and Decreased Plasma Thrombomodulin in Fontan Patients

Hidemi Kajimoto, MD; Makoto Nakazawa, MD*; Kagari Murasaki, MD**; Nobuhisa Hagiwara, MD**; Toshio Nakanishi, MD

Background: Thromboembolic events account for significant morbidity and mortality after the Fontan procedure, but the underlying mechanisms remain unclear. P-selectin on platelets indicates platelet activation. Thrombomodulin (TM), a receptor for thrombin and a major anticoagulant proteoglycan on the endothelial membrane, reflects the anticoagulant activity of the endothelium. The present study investigated the hypothesis that the balance between platelet activation and endothelial biological function is impaired in Fontan patients.
Methods and Results: Platelet P-selectin as a marker of platelet activation, plasma TM levels and protein C activity, as markers of anticoagulant activity of the endothelium, and thrombin-antithrombin complex III (TAT) were examined in 43 Fontan patients. P-selectin levels on platelets (4.5±1.4 vs 3.4±0.4 mean fluorescence intensity, P<0.001) and TAT levels (80.2±322.6 vs 1.9±0.9 ng/ml, P<0.05) were significantly higher in Fontan patients than in control subjects. On the other hand, plasma TM levels (1.5±0.8 vs 2.2±0.3 FU/ml, P<0.01) and protein C activity (71±35 vs 118±25%, P<0.001) were significantly lower in Fontan patients compared with controls. These abnormalities were not seen in patients after other surgical procedures for congenital heart disease.
Conclusions: Platelet activation is enhanced and endothelial function is impaired in patients after the Fontan procedure, which may partly explain the thromboembolic complications in Fontan patients. (*Circ J* 2009; **73:** 1705–1710)

Key Words: Congenital heart disease; Coagulation; Endothelium

A Fontan circulation is associated with an increased risk of thromboembolism[1–3] and the prevalence of thromboembolic events has been reported to range from 3% to 30% among Fontan patients.[4–8] Various abnormalities in the coagulation system have been postulated as the mechanism of the increased incidence of thromboembolism in Fontan patients, including decreased levels of protein C, protein S, and antithrombin III.[1,9,10] Although platelets play an important role in thrombus formation, and collagen-induced and adenosine-induced platelet aggregation are elevated in patients after the Fontan procedure,[11] whether platelets are indeed activated after the Fontan procedure remains unknown.

P-selectin is an adhesion molecule found in the secretory granules of platelets, and is mobilized to the membrane surface on activation.[12–18] Activated platelets expressing P-selectin on the surface release their granule contents, which facilitates the adhesion of platelets and neutrophils to the endothelium and causes platelet aggregation and thrombus enlargement by recruitment of leucocytes and platelets.[19] Thus P-selectin expressed on platelets is likely to play an important role in thrombus formation. Measurement of P-selectin expression on the platelet surface is a very sensitive method of determining platelet activation.[12,20–22] However, there have not been any studies evaluating platelet P-selectin in Fontan patients.

Because platelets are activated under conditions of endothelial dysfunction,[23,24] in the current study, we measured plasma thrombomodulin (TM) levels as a marker of endothelial function. TM is expressed mainly on the surface of vascular endothelial cells, it suppresses blood coagulation, and is a key component of the protein C anticoagulant pathway. TM converts thrombin from a procoagulant to an anticoagulant protease by increasing the rate of protein C activation.[25] We also measured protein C activity level as a marker of the anticoagulant pathway and the plasma thrombin–antithrombin complex III (TAT) level was measured to evaluate coagulability.

Methods

Patients

We studied 43 patients who had undergone the Fontan procedure (**Table 1**). There were 27 men and 16 women, with a mean age of 13±11 years, ranging from 2 to 37 years. The interval after the Fontan procedure was 1 month to 19 years. Of them, 18 patients underwent direct right atrium–pulmonary artery connection (APC) and 25 patients underwent total cavopulmonary connection (TCPC); 18 Fontan patients did not receive drug therapy, and 25 patients were

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Department of Pediatric Cardiology, Heart Institute of Japan, Tokyo Women's Medical University, Tokyo, *Pediatric and Lifelong Congenital Cardiology Institute, Southern Tohoku General Hospital, Koriyama and **Department of Cardiology, Heart Institute of Japan, Tokyo Women's Medical University, Tokyo, Japan
Mailing address: Toshio Nakanishi, MD, Department of Pediatric Cardiology, Heart Institute of Japan, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. E-mail: pnakanis@hij.twmu.ac.jp

Table 1. Demographic Data of the Patients Undergoing Fontan Procedure

Patient no.	Age (years)	Diagnosis	Type of Fontan	Medication	Interval after Fontan (months)	P-selectin (MFI)	TM (FU/ml)	PC activity (%)	TAT (ng/ml)	BNP (pg/ml)
Fontan patients without thrombus										
1	2	SLV	TCPC	Aspirin, warfarin	1	3.34	2.1	47	3.1	29.2
2	3	TGA	TCPC	Aspirin	1	3.72	1.5	69	6.1	46.4
3	2	DORV	TCPC	Aspirin, warfarin	1	3.82	1.7	22	30.2	ND
4	2	Hypo RV, VSD	TCPC	–	1	5.1	2	49	784	ND
5	4	AVSD, DORV	TCPC	Aspirin, warfarin	1	3.76	1.3	28	2.3	9
6	2	SRV	TCPC	Aspirin, warfarin, heparin	1	4.03	<1.0	<10	19	ND
7	15	AVSD, DORV, TAPVC	TCPC	Ticlopidine	1	3.67	ND	ND	ND	393
8	4	PA/IVS	TCPC	Aspirin, warfarin	1	5.55	2.1	ND	73	28
9	2	DORV	TCPC	–	1	3.44	<1.0	19	3.2	13
10	18	TA	TCPC	Aspirin	2	4.62	ND	ND	ND	74.2
11	5	TA	TCPC	Aspirin	4	4.93	1.5	87	1.3	17.6
12	3	SRV	TCPC	Aspirin	8	4.25	1.7	61	32.7	61.7
13	3	SLV	TCPC	–	9	3.5	1.6	ND	ND	ND
14	2	DORV	TCPC	Aspirin, ticlopidine	9	4.61	ND	ND	4.6	ND
15	3	SRV	TCPC	–	10	3.12	2.5	69	<1.0	ND
16	2	SLV	TCPC	Aspirin	10	3.38	3.5	65	>80	29.8
17	14	PA/IVS	TCPC	Aspirin, warfarin	12	3.28	1.6	84	1.5	ND
18	5	SRV	TCPC	Warfarin	12	4.81	2.5	39	1.3	11.2
19	9	DORV	TCPC	Aspirin, warfarin	15	3.68	1.8	84	4.3	27.2
20	3	PA/IVS	TCPC	–	16	4.47	1.5	72	8.4	75.3
21	4	SRV	APC	–	30	5.81	1.2	75	30.9	111
22	6	TA	APC	–	42	5.8	1.8	67	<1.0	ND
23	6	DORV	APC	Aspirin, warfarin	43	7.6	1.9	73	2	144
24	9	TA	APC	–	78	6.04	1.2	110	<1.0	ND
25	11	SRV	APC	–	79	4.75	1.3	76	1.5	93
26	29	AVSD, hypo RV	APC	Aspirin, heparin	82	3.91	ND	90	ND	116
27*	14	AVSD, DORV, TAPVC	TCPC	Ticlopidine	82	4.94	<1.0	42	1,890	426
28	20	DORV	APC	Aspirin, warfarin	84	3	1.1	56	36.1	ND
29*	13	AVSD, DORV, TAPVC	TCPC	–	90	4.72	2	42	1.3	15.4
30	14	AVSD, DORV	TCPC	–	96	3.12	2.1	38	10.1	38.8
31	29	AVSD, DORV	TCPC	Aspirin	108	3.13	1.2	90	3.2	143
32	14	SRV	TCPC	–	120	3.53	2.9	136	21.9	29.6
33	17	MA, DORV	APC	–	120	4.1	ND	98	1.4	ND
34	19	SRV	APC	–	156	ND	2.3	148	1.7	30.9
35	24	AVSD, DORV	APC	Aspirin	158	9.14	<1.0	155	1.3	103
36*	19	SRV	APC	–	158	8.56	<1.0	35	2.1	72
37*	37	SLV, MA	APC	–	188	3.75	1.1	89	6.9	201
38	34	SRV	APC	–	204	4.65	1.4	112	2.3	107
39*	34	SRV	APC	–	204	4.46	<1.0	105	28.9	ND
Mean					57	4.53	1.5	72	88.5	90.6
SD					65	1.42	0.9	35	340.0	104
Fontan patients with thrombus										
40	31	SRV	APC	Aspirin, warfarin	153	3.3	1.2	91	1.2	488
41	31	TA	APC	Warfarin	169	6.2	1.3	66	3.4	255
42	24	PA/IVS	APC	Aspirin, warfarin	204	3.15	1.1	32	23	ND
43	27	TGA	APC	Warfarin	229	2.44	1.3	67	1.3	51.1
Mean					189	3.77	1.2	64	7.2	264.7
SD					34	1.66	0.1	24	10.6	219

*Cyanotic patients.
MFI, mean fluorescence intensity; TM, thrombomodulin; TAT, thrombin-antithrombin complex III; BNP, brain natriuretic peptide; SLV, single left ventricle; TCPC, total cavopulmonary connection; TGA, transposition of great arteries; DORV, double-outlet right ventricle; AVSD, atrioventricular septal defect; SRV, single right ventricle; TAPVC, total anomalous pulmonary venous connection; TA, tricuspid atresia; APC, atrial-pulmonary artery connection; MA, mitral atresia.

on aspirin, ticlopidine, warfarin or a combination with heparin.

Four patients in the present study developed thrombus and all had been on warfarin (**Table 1**). Thrombus was observed in the right atrium in patients 40, 41 and 42, and in the left atrium in patient 43 at the time of the blood test.

As disease controls, we also studied 23 patients with congenital heart disease who had undergone biventricular repair using 2 ventricles (14 men, 9 women; mean age 21± 9 years, range 5–35). The diagnosis was congenital aortic valve stenosis in 1 patient, atrioventricular septal defect in 3 patients, coarctation of the aorta in 1 patient, congenitally corrected transposition of the great arteries in 2 patients, double-outlet right ventricle in 4 patients, coronary aneurysm in 1 patient, annuloaortic ectasia in Marfan syndrome in 2 patients, pulmonary atresia with intact ventricular septum in 1 patient, a single left ventricle in 1 patient, a common arterial trunk in 1 patient, tetralogy of Fallot in 4 patients, and ventricular septal defect in 2 patients.

Twelve healthy subjects without cardiopulmonary disease (5 men, 7 women; mean age 24±5 years, range 18–33 years) served as healthy controls. All subjects gave informed consent for the present study.

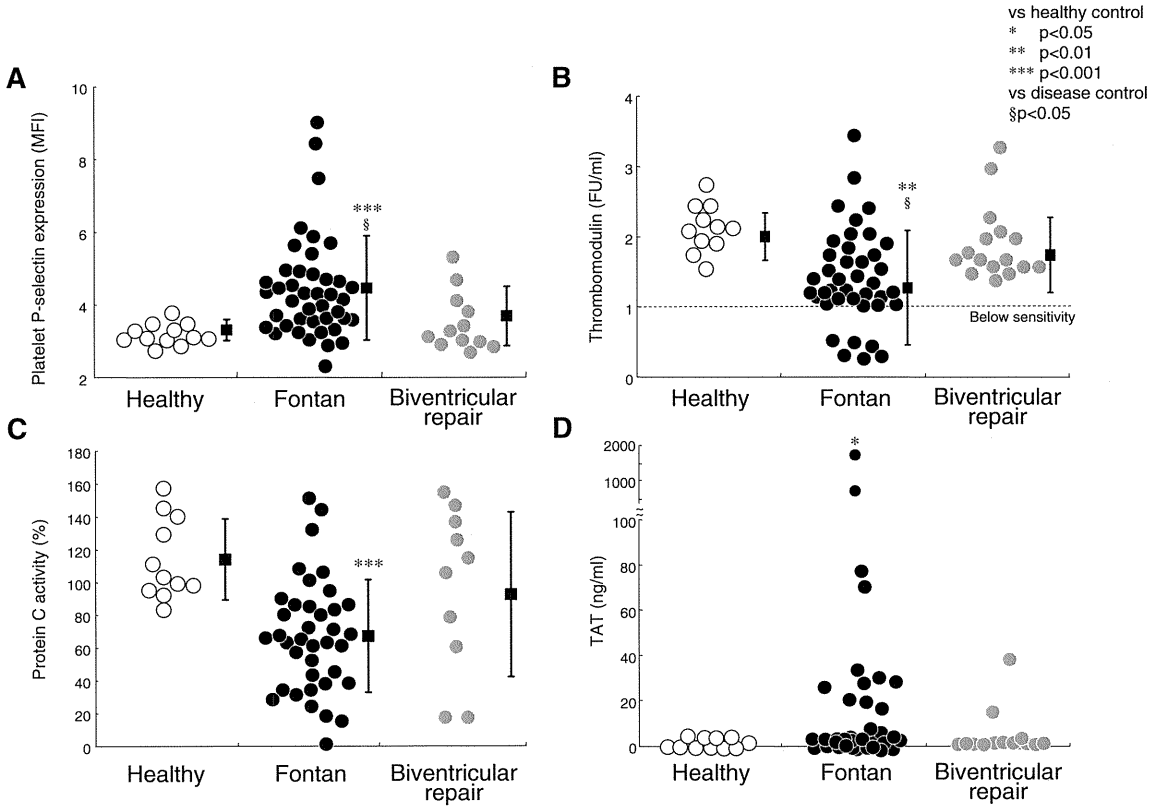

Figure 1. Expression of P-selectin on platelets (**A**), plasma thrombomodulin level (**B**), protein C activity (**C**), and thrombin–antithrombin complex III (TAT) level (**D**) in Fontan patients, patients who had undergone biventricular repair (disease controls), and healthy controls. Bar plots show the mean and standard deviation. (○) Control subjects, (●) Fontan patients, and (◍) disease controls. MFI, mean fluorescence intensity.

Methods

We tested blood samples taken from the patients while they were in a stable condition. None of the patients had undergone cardiac surgery or cardiac catheterization within 1 month of sampling. Whole blood was obtained from an antecubital vein without venous stasis using a 22-gauge needle. The first 3 drops of blood were discarded before the collection of blood used for analysis. The blood was mixed with 0.38% trisodium citrate. Immediately after the collection of the blood, we evaluated the expression of P-selectin on platelets as follows. Samples were analyzed on a FACS caliber flow cytometer with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A gate was set around the platelets, and 10,000 were counted from each sample. The platelets were incubated with fluorescein isothiocyanate-conjugated anti-P-selectin monoclonal antibody (AK4, mouse IgG1, Becton Dickinson) at room temperature for 30 min, and then fixed in 1% (vol/vol) paraformaldehyde and analyzed using flow cytometry. Platelet P-selectin was expressed as mean fluorescence intensity (MFI).

We measured plasma TM level, protein C activity, and TAT level using standard clinical laboratory methods. When the values of platelet P-selectin, plasma TM, protein C activity and/or TAT were markedly abnormal, we re-evaluated the data 1 week later to confirm that the data were indeed abnormal (data not shown).

Statistical Analysis

Data are expressed as the mean±standard deviation of the mean. Data of the Fontan patients, healthy controls, and disease controls were compared using the Mann-Whitney U test. Comparison of thromboembolic factors between the 2 types of Fontan procedure was by Student's t-test. Spearman's correlation coefficient by rank was used to evaluate the expression of P-selectin, TM and the interval after the Fontan operation. A level of $P<0.05$ was considered significant.

Results

The expression of P-selectin on platelets was significantly greater in the Fontan patients (4.5±1.4 MFI) than in the healthy controls (3.4±0.4; $P<0.001$) or the disease controls (3.7±0.8; $P<0.05$) (**Figure 1A**). Plasma TM levels were significantly lower in the Fontan patients (1.5±0.8) than in the healthy (2.2±0.3) and disease controls (1.9±0.5) (**Figure 1B**). Protein C activity was lower in the Fontan patients than in healthy controls (71±35 vs 118±25%; $P<0.001$) (**Figure 1C**). TAT levels were greater in the Fontan patients than in the healthy controls (80.2±322.6 vs 1.9±0.9 ng/ml; $P<0.05$) (**Figure 1D**).

Many Fontan patients showed elevated P-selectin expression on platelets, and only 1 of 4 patients who developed thrombus had elevated P-selectin expression on platelets and elevated TAT levels (**Table 1**). In all 4 patients who developed thrombus, however, plasma TM levels and protein C activity were low. Because the postoperative interval was longer in patients who developed thrombus, the relationships between P-selectin and plasma TM levels

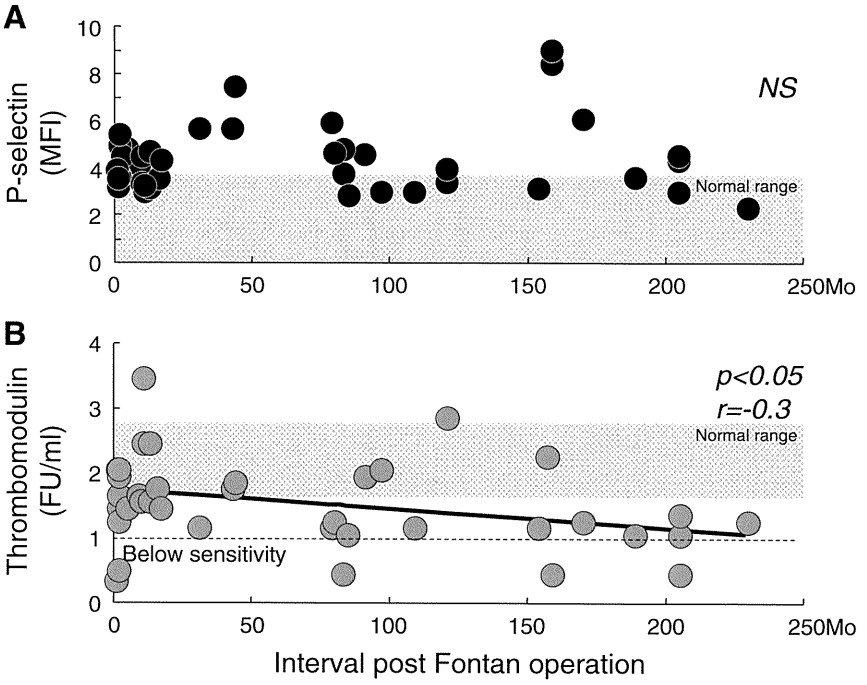

Figure 2. Relationship between platelet P-selectin (**A**) or thrombomodulin (**B**) level and the interval after the Fontan procedure. MFI, mean fluorescence intensity.

Table 2. Comparison of Thromboembolic Factors by Type of Fontan Procedure

	APC (n=18)	TCPC (n=25)	P value
Age at study (years)	21.2±10.6	7.5±7.0	<0.001
Interval after Fontan (months)	132±64	24±39	<0.001
Warfarin administration (%)	33	32	NS
P-selectin (MFI)	5.1±2.0	4.0±0.7	0.04
TM (FU/ml)	1.1±0.7	1.7±0.9	0.04
PC activity (%)	85.8±32.8	57.4±30.6	<0.01
TAT (ng/ml)	8.5±12.5	135.5±425.2	0.07

Abbreviations see in Table 1.

and the interval after the Fontan procedure were evaluated (**Figure 2**). Although there was no correlation between P-selectin expression and the interval after the Fontan procedure, there was a negative correlation between TM level and the postoperative interval ($P<0.05$, $r=-0.3$). In patients after APC, the platelet P-selectin was higher ($P<0.05$) and the plasma TM level ($P<0.05$) lower than in patients after TCPC. In patients after TCPC, protein C activity was lower than that in patients after APC ($P<0.01$) (**Table 2**). In addition, these thromboembolic factors were independent of the brain natriuretic peptide (BNP) values (**Table 1**).

Discussion

Platelet P-Selectin

The present study has demonstrated, for the first time, that P-selectin expression on platelets is elevated in patients after the Fontan procedure, indicating that platelet activation does occur in these patients. This characteristic of the Fontan patients was not observed in the disease controls who had undergone biventricular repair using 2 ventricles. We previously reported that increased expression of platelet P-selectin was a risk factor for thromboembolism in patients with cyanotic congenital heart disease.[26] After the Fontan procedure, patients do not have cyanosis but still have a high risk of thromboembolism. Elevated P-selectin level has also been reported in patients with congestive heart failure,[27–29] atrial fibrillation,[12] non-valvular atrial fibrillation after ischemic stroke,[30] and primary pulmonary hypertension.[18] Platelet activation under these conditions may be related to the increased shear stress and/or endothelial dysfunction.[23] Interestingly, many patients were receiving an antiplatelet drug (aspirin or ticlopidine) or a combination of an antiplatelet and an anticoagulant drug (heparin or warfarin), and their platelet P-selectin levels were still elevated. Chung et al demonstrated a significant reduction in the platelet P-selectin level following treatment of acute heart failure.[28] In the present study, there was no correlation between platelet P-selectin level and hemodynamic data (central venous pressure, diameter of the inferior vena cava, and BNP values) (**Table 1**; data for central venous pressure and diameter of the inferior vena cava are not shown). The effect of antiplatelet drugs on platelet P-selectin levels remains unclear. It is also unknown whether anticoagulation/antiplatelet therapy should be administered routinely to all Fontan patients.

Endothelial Dysfunction and Coagulability in Fontan Patients

TM facilitates the activation of protein C by thrombin.[25,31–35] Thrombin bound to TM cannot convert fibrinogen to fibrin or activate protein C. Activated protein C is known to

inhibit clotting factors V and VIII.[32–35] Therefore, TM acts as an intrinsic anticoagulant barrier between the blood and the endothelium,[25,36] playing a pivotal role in maintaining the coagulation balance. In our study, the findings of lower plasma TM levels and protein C activity suggest impaired endothelial function in Fontan patients (**Figures 1B, C**). Previous studies using near-infrared spectroscopy and flow-mediated vasodilatation have suggested that endothelial function in Fontan patients is impaired.[37,38] The endothelium in Fontan patients may be fatally damaged by the increased shear stress on the vessel wall caused by increased blood viscosity and/or by chronic hypoxemia before the Fontan procedure.[26,39]

Postoperative Interval After the Fontan Procedure

Although plasma TM levels and protein C activity were low in all patients who developed thrombus, only 1 of 4 patients demonstrated elevated P-selectin expression on platelets or elevated TAT level (**Figure 1**). The precise mechanisms by which thrombus is formed in patients after the Fontan procedure remain undetermined, but the process is most likely multifactorial. The elevated TAT levels detected in the present study suggest the presence of coagulation abnormalities after the Fontan procedure, which is in agreement with the findings of previous studies.[1,9,10] Thrombosis in the Fontan procedure is often silent and it is not clear exactly when the thrombus forms. Nevertheless, the probability of freedom from thromboembolism decreases with the interval after the Fontan procedure.[3,4] There was no correlation between P-selectin levels and the postoperative interval in the present study, but we speculate that platelets are activated irrespective of the time interval after the procedure. The negative correlation between TM level and the interval after the Fontan procedure suggests deterioration of endothelial function over time following this surgery (**Figure 2B**), which may partly explain the high prevalence of thromboembolic events long after the Fontan procedure.

Type of Fontan Procedure

Various types of Fontan procedure have evolved over the past 30 years, but the incidence of thromboembolic events in each type has not been defined. In this study, we showed that in patients after APC platelet P-selectin levels were higher and plasma TM levels lower than in patients after TCPC, although in patients after TCPC, protein C activity was lower than that in patients after APC. The condition of low TM level and activated platelet P-selectin is highly prevalent in patients after APC. TCPC, which is a newer technique, may have an advantage over APC in terms of thrombus formation. The long-term results of thromboembolism after TCPC remain to be studied.

Study Limitations

There are limitations in the present study. (1) The study population was heterogeneous with respect to age, anticoagulation therapy, and cardiac diagnosis. (2) A relatively small number of patients experienced episodes of thromboembolic events and patients with elevated P-selectin expression on the platelets did not necessarily develop episodes of thromboembolic events. (3) The true incidence of cardiac thrombus may have been underestimated, because transesophageal echocardiography was not performed on a routine basis in all of the patients. (4) We could not obtain set time interval data, because the patients' visits to the hospital were determined by their condition. (5) We could not determine which type of Fontan procedure was recommended, because modified TCPC was performed later in the study. Nevertheless, the present study results show that platelet activation and endothelial dysfunction occurs in Fontan patients. In future studies, we need to further examine the relationship between platelet activation and thrombus formation using more accurate modalities such as contrast computed tomography and/or transesophageal echocardiography on a routine basis.

In conclusion, platelet P-selectin and plasma TAT levels were elevated, whereas TM levels and protein C activation were decreased in Fontan patients. These abnormalities may be potential risk factors for thromboembolic events. Thrombogenicity still continues despite the finding that cyanosis is settled by the Fontan procedure.

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Delayed Neonatal Closure of the Ductus Arteriosus Following Early in utero Exposure to Indomethacin in the Rat

Kazuo Momma[a] Katsuaki Toyoshima[a, d] Kiyomi Ito[c] Kiyoshi Sugiyama[c]
Shinichiro Imamura[b, e] Fang Sun[b] Toshio Nakanishi[a, b]

[a]Section of Pediatric Cardiology and [b]International Research and Educational Institute for Integrated Medical Sciences, Women's Medical University, and [c]Department of Clinical Pharmacokinetics, Hoshi University, Tokyo, [d]Section of Perinatology, Kanagawa Children's Medical Center, Yokohama, and [e]Department of Medical Engineering, Faculty of Health Care Science, Himeji Dokkyo University, Himeji, Japan

Key Words
Ductus arteriosus · Patent ductus arteriosus · Prematurity · Indomethacin · Cyclooxygenase inhibitor · Prostaglandin · Prostanoid EP_4 receptor · L-NAME · Nitric oxide synthase inhibitor · Nitric oxide

Abstract
Background: Indomethacin is used to close the patent ductus arteriosus in premature infants and for tocolysis of preterm labor. Clinically and experimentally, early in utero exposure to indomethacin induces the paradoxical delay of postnatal closure of the ductus arteriosus. ***Objectives:*** To clarify the pharmacological nature of the delay of closure of the ductus arteriosus in the rat. ***Methods:*** We studied early in utero exposure to indomethacin (dose and timing) in addition to other drugs, inducing a delay in postnatal ductal closure. Pregnant rats at near term were studied by cesarean section on gestational day 21 (D21), incubated in room air at 33°C, followed by rapid whole-body freezing. ***Results:*** The delay in closure of the ductus arteriosus was dose dependent. A large dose of indomethacin (10 mg/kg) 1 or 2 days before birth induced a delay of 3–4 times. A timing study revealed maximum delay with administration of indomethacin 2 days before birth and minimum delay with administration 5 days before. Aspirin, ibuprofen, the selective COX1 inhibitor SC 560, the selective COX2 inhibitor rofecoxib and a prostaglandin EP_4 receptor blocker, ONO-208, all delayed neonatal ductal closure following maternal administration on D19 and D20. ***Conclusions:*** The delay by indomethacin was dose dependent. The maximum delay was induced by 2 doses of 10 mg/kg indomethacin on D19 and D20. The delay was induced by a decreased stimulus to the prostaglandin EP_4 receptor system in the last 2 days in utero. The delay was temporary with recovery 3 days or more after exposure.

Introduction

In neonatology, indomethacin and other cyclooxygenase (COX) inhibitors are used to close the patent ductus arteriosus (PDA) in premature babies [1, 2] by inhibiting COX and prostaglandin synthesis [3], with success rates

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Kazuo Momma, MD
Section of Pediatric Cardiology, Tokyo Women's Medical University
Kawadacho, Shinjuku-ku, Tokyo 162-8666 (Japan)
Tel. +81 3 3353 8111, Fax +81 3 3356 0441, E-Mail kmomma@iris.ocn.ne.jp