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ORIGINAL ARTICLE
Myocardial Disease

Different Characteristics of Peripartum Cardiomyopathy Between Patients Complicated With and Without Hypertensive Disorders

– Results From the Japanese Nationwide Survey of Peripartum Cardiomyopathy –

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Background: There has been no nationwide survey concerning peripartum cardiomyopathy (PPCM) among the Asian population, and clinical profiles of PPCM complicated with hypertensive disorders complicating pregnancy (HD) as the major risk factor of PPCM have not been characterized.

Methods and Results: A retrospective, nationwide survey of PPCM in 2007 and 2008 all over Japan was performed and the clinical characteristics were compared between patients with and without HD. We obtained data for 102 patients. HD during pregnancy occurred in 42 patients (41%). Patients with HD were older than those without HD (33.8 vs. 31.9 years old, $P<0.05$) and babies were delivered more frequently by Caesarean section (81% vs. 52%, $P<0.01$). Although cardiac parameters at diagnosis were similar in patients with and without HD, patients with HD were hospitalized for a shorter period and had better cardiac function after 7 months. Multivariate regression analysis revealed that HD was independently associated with a shorter hospital stay and a higher left ventricular ejection fraction at last follow up.

Conclusions: PPCM complicated with HD had different clinical characteristics from those without HD. This condition might be a unique subset of PPCM that is characterized by relatively swift recovery except in the cases of death. In order to prevent severe heart failure and maternal death, peripartum women should be treated with HD cautiously and must immediately undergo a cardiac examination as needed. (*Circ J* 2011; **75:** 1975–1981)

Key Words: Cardiomyopathy; Heart failure; Hypertension; Pregnancy; Prognosis

Peripartum cardiomyopathy (PPCM) and pregnancy-associated cardiomyopathy are rare but life-threatening conditions that occur during the peripartum period in previously healthy women. Although its etiology remains unknown, potential risk factors include advanced maternal age, multiparity, multiple gestation, African descent, use of tocolytic agents, preeclampsia, and chronic hypertension.[1–3] Next to African descent, Asian populations showed the second highest incidence of PPCM in a study performed in Southern California,[4] but there was no nationwide survey about PPCM in Asian counties. Hypertensive disorders complicating pregnancy (HD) are observed in up to 60% of PPCM patients,[5] but few studies have analyzed the differences in clinical characteristics between PPCM patients with and without HD. Therefore, this study was performed: (1) to characterize PPCM in Japanese women; and (2) to evaluate whether complications of PPCM with hypertension affects the prognosis for this condition.

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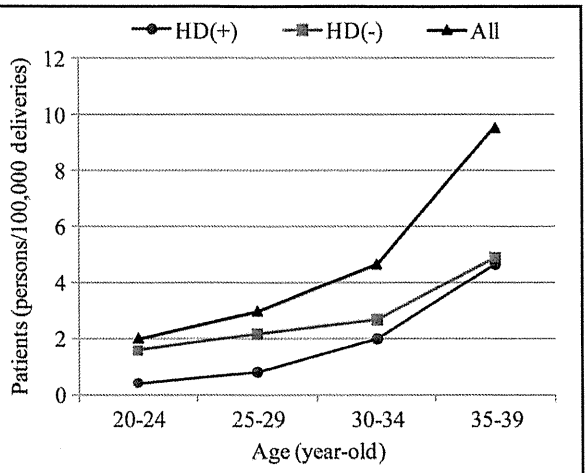

Figure 1. Incidence of PPCM per 100,000 deliveries in each age group. PPCM, peripartum cardiomyopathy; HD, hypertensive disorders complicating pregnancy.

Editorial p1827

Methods

A questionnaire survey of 1,444 professional medical organizations in Japan, including 1,030 departments of cardiology, 1,025 departments of obstetrics, and 431 emergency departments, was performed to identify patients with PPCM who were newly managed from January 2007 to December 2008. The diagnosis of PPCM was based on the following criteria: (1) development of heart failure during pregnancy or within the first 5 postpartum months; (2) no determinable etiology for cardiac failure; (3) no history of heart disease prior to pregnancy; (4) reduced left ventricular contraction based on a left ventricular ejection fraction (LVEF) <50% and/or a percent fractioning shortening (%FS) <30%. We modified the criteria established by Demakis and Rahimtoola[1] and those recommended by a workshop convened by the National Heart Lung and Blood Institute and the Office of Rare Diseases of the National Institute in Health.[6] Although classic diagnostic criteria of PPCM by Demakis and Rahimtoola limited the diagnosis to the last gestational month and first 5 months after delivery, Elkayam et al reported that clinical presentation and outcome of patients diagnosed early in pregnancy were similar to those of patients with traditional PPCM.[7] We included patients who developed heart failure during pregnancy and during the first 5 months after delivery in the present study, which was based on the report by Elkayam et al.

Age, parity, complications of pregnancy, time of diagnosis, symptoms, time and route of delivery, outcomes of mother and infant, length of hospital stay, and therapeutic information were collected as background data. Echocardiographic parameters and serum brain natriuretic peptide (BNP) levels at diagnosis, at hospital discharge, and at the last follow up were also obtained. If the patients were complicated with HD, the type and severity of hypertension, and the duration between the onset of HD and diagnosis of PPCM were also recorded.

HD were categorized according to the National High Blood Pressure Education Program Working Group Report on high blood pressure (BP) in pregnancy as: (1) gestational hypertension: systolic BP≥140 mmHg or diastolic BP≥90 mmHg for the first time during pregnancy, and no proteinuria (PU); (2) preeclampsia: systolic/diastolic BP≥140/90 mmHg after 20 weeks' gestation and PU≥300 mg/day or ≥1+dipstick; (3) eclampsia: seizures that cannot be attributed to other causes in a woman with preeclampsia; (4) preeclampsia superimposed on chronic hypertension: new-onset PU≥300 mg/day in hypertensive women without PU before 20 weeks' gestation or a sudden increase in PU or BP in women with hypertension and PU before 20 weeks' gestation; and (5) chronic hypertension: systolic/diastolic BP≥140/90 mmHg before pregnancy or diagnosed before 20 weeks' gestation.[8] The severity of preeclampsia was defined as mild for systolic/diastolic BP ≥140/90 mmHg and severe for systolic/diastolic BP≥160/110 mmHg. PU was defined as mild for >300 mg/day and severe for >2.0 g/day. The number of deliveries in Japan in

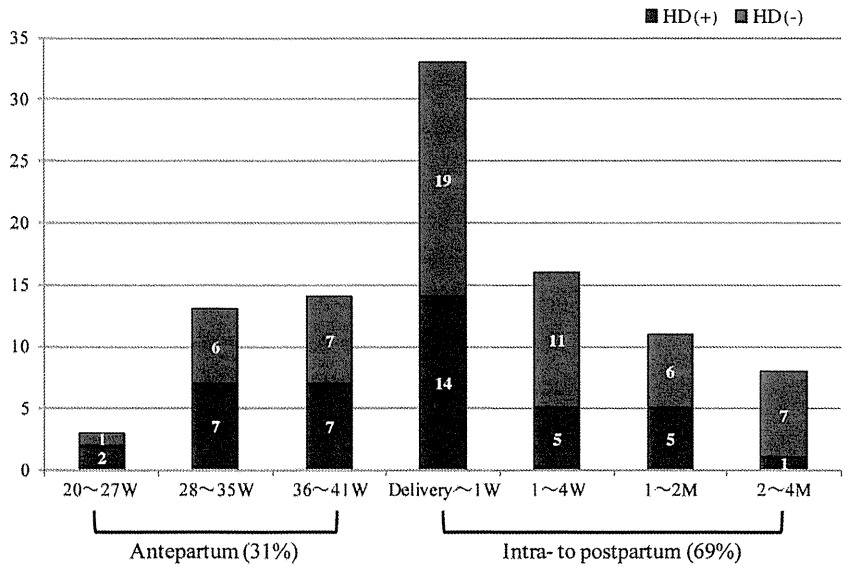

Figure 2. Time of diagnosis. HD, hypertensive disorders complicating pregnancy.

Table 1. Comparison of Patients' Background and Use of Medications at Discharge Between PPCM Patients Complicated With and Without HD

	HD (+) (n=42)	HD (–) (n=60)	P value*
Age (years)	33.8±4.2	31.9±4.1	<0.05
Parity	1.62±1.17	1.67±0.78	NS
Tocolytic therapy	6	8	NS
Twin pregnancy	7	8	NS
HD	42 (100%)	0	<0.0001
Gestational weeks of delivery (weeks)	36.4±3.7	37.5±2.4	NS
Route of delivery			
Vaginal delivery	8	27	<0.01
Cesarean section	34	29	
Medications at discharge			
ACE-I/ARB	26 (67%)	33 (63%)	NS
β-blocker	22 (56%)	30 (58%)	NS
Diuretics	26 (67%)	29 (56%)	NS
Anticoagulant	11 (28%)	11 (21%)	NS

PPCM, peripartum cardiomyopathy; HD, hypertensive disorders complicating pregnancy; NS, not significant; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.
*P value for comparison of the HD (+) and HD (–) groups.

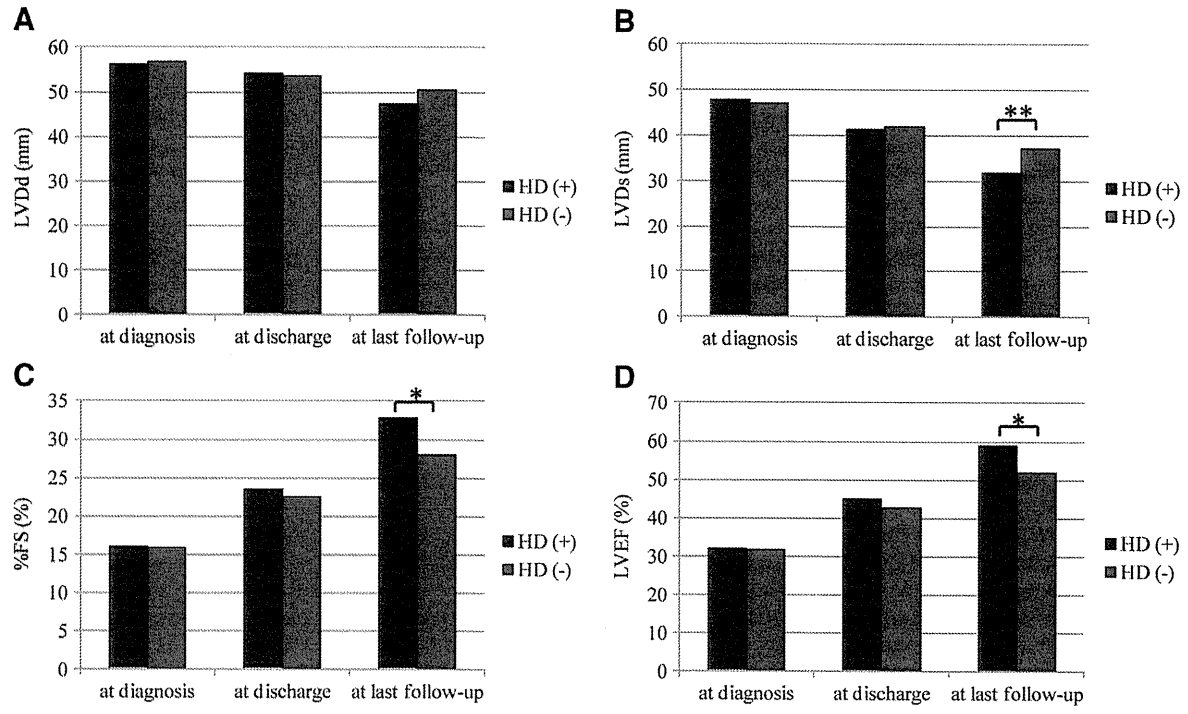

Figure 3. Changes of (**A**) LVDd, (**B**) LVDs, (**C**) %FS, and (**D**) LVEF in HD (+) and HD (–) groups. LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; %FS, % fractional shortening; LVEF, left ventricular ejection fraction; HD, hypertensive disorders complicating pregnancy. *P<0.05 for comparison of the HD (+) and HD (–) groups. **P<0.01 for comparison of the HD (+) and HD (–) groups.

each age group were taken from national statistics published by the Ministry of Health, Labour and Welfare.

Statistical significance was evaluated using paired and unpaired Student t-tests for comparisons between means. A chi-square test and a Fisher exact test were used for categorical data. Two-way ANOVA and correlation coefficient analysis were also used. Multivariate analysis was done to examine the correlations of length of hospital stay and LVEF at last follow up with variables such as age, parity, time of diagnosis, tocolytic therapy, twin pregnancy, HD and LVEF at diagnosis, which are considered as risk factors. All data were expressed as the mean±standard deviation. Statistical signifi-

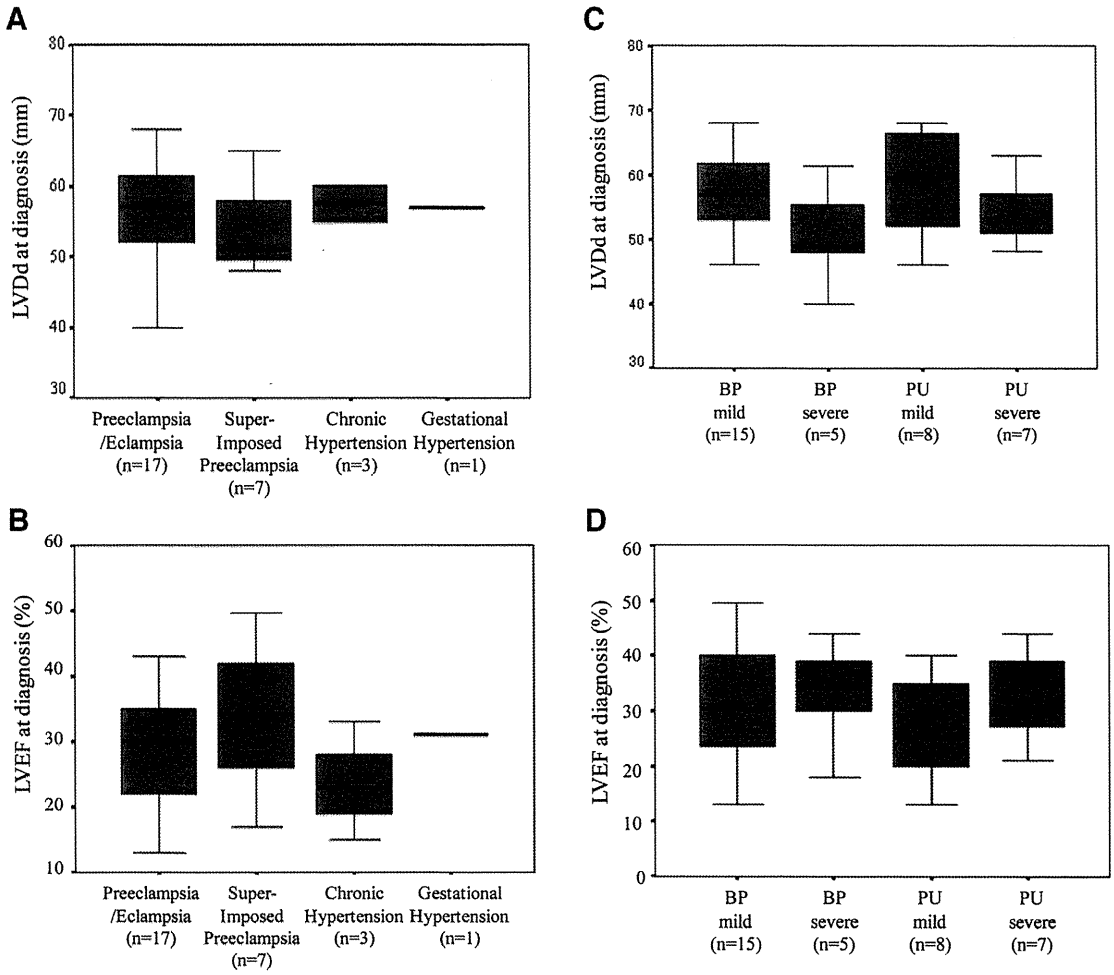

Figure 4. (**A**) LVDd and (**B**) LVEF at diagnosis in each type of HD, and (**C**) LVDd and (**D**) LVEF at diagnosis in preeclampsia patients with different severities of BP and PU. HD, hypertensive disorders complicating pregnancy; LVDd, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; BP, blood pressure; PU, proteinuria.

cance was defined as a P value <0.05. A software package (SPSS 11.0; SPSS, Chicago, IL, USA) was used for statistical analysis.

The Ethics Committee at the National Cerebral and Cardiovascular Center in Osaka, Japan approved the study in November 2008.

Results

Clinical Characteristics of All Patients

Out of 1,444 institutes, 1,049 (73%) responded. These responses included 102 cases fulfilling the inclusion criteria for PPCM. The estimated incidence of PPCM in Japan was 1/20,000 births. The mean age of the patients was 32.7 years old, with a range of 22–43 years old. Fifty-four percent of patients were primiparous women and the mean parity was 1.65±0.96. Tocolytic agents were used during pregnancy in 14%, twin pregnancy occurred in 15%, and HD was present in 42% of PPCM patients.

Diagnosis of PPCM was established antepartum in 31% and intra to postpartum in 69%. One-third of patients were diagnosed intrapartum to within 1 week after delivery. The major symptoms at onset were dyspnea in 80%, cough in 37%, and edema in 37%. With those complaints, 63% of patients were initially seen by an obstetrician and 12% of patients were seen by a general physician, and then referred to cardiologists. Only 9% were primarily seen by a cardiology specialist.

At diagnosis, an echocardiography showed the following mean values: left ventricular end-diastolic dimension (LVDd) 56.5±7.1 mm, left ventricular end-systolic dimension (LVDs) 47.8±8.1 mm, %FS 15.8±7.0%, and LVEF 31.6±12.0%. The mean serum BNP level was elevated to 1,258±1,028 pg/ml. There were only 4 patients whose serum BNP level was under 100 pg/ml.

The mortality rate was 4%. One patient who was at 34 weeks' gestation died from pulmonary edema on the day of admission, 1 patient died from acute heart failure 1 day after an emergency Caesarean section was performed because of obstructed labor at 37 weeks' gestation, 1 died from cardiac arrest 2 days after vaginal delivery despite implementation of percutaneous cardiopulmonary support, and another died

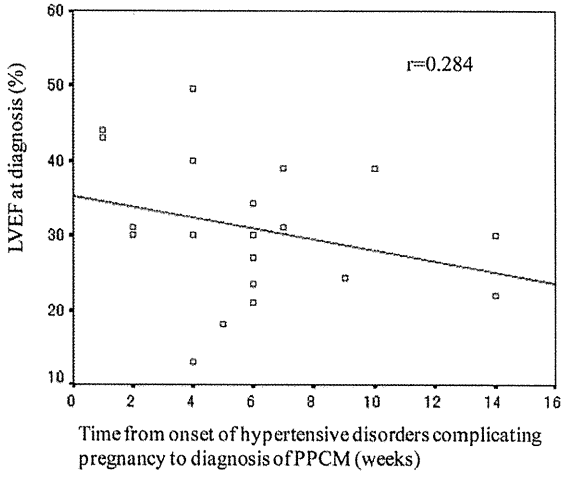

Figure 5. Relationship between LVEF at diagnosis and the time from onset of HD to diagnosis of PPCM. LVEF, left ventricular ejection fraction; HD, hypertensive disorders complicating pregnancy; PPCM, peripartum cardiomyopathy.

from worsening chronic heart failure more than 6 months after diagnosis.

Additionally, 2% of patients had severely deteriorated left ventricular function that required treatment with a left ventricular assist system (LVAS); 3% were transferred to other hospitals and no data were available for their prognosis; and further prognostic data for another 2% were not available. For the other 89% (91 patients), discharge from hospital occurred after a mean stay of 34.6 days. The clinical findings at discharge included mean values of LVDd 53.7±7.7 mm, LVDs 41.8±9.7 mm, %FS 22.8±8.9%, and LVEF 43.6±14.1%. The mean serum BNP at discharge was 211±277 pg/ml.

The mean follow-up period was 9.6±6.5 months for the 82 of 91 discharged patients. Echocardiography improved significantly, with values of LVDd 49.0±6.1 mm, LVDs 34.8±8.2 mm, %FS 29.6±8.3%, and LVEF 54.6±13.6%, and the mean serum BNP level had significantly decreased to 44±103 pg/ml. Sixty-three percent of patients recovered their LVEF over 50% after 6 months.

Comparison Between Patients With and Without HD

A total of 42 patients were complicated with HD in pregnancy [HD (+) group] and 60 patients did not have this complication [HD (–) group]. Hypertensive subcategories of PPCM patients are as follows: 18 patients with preeclampsia, 11 with preeclampsia superimposed on chronic hypertension, 3 with chronic hypertension, 1 with gestational hypertension, 1 with eclampsia, and 8 with an unknown subcategory. The incidence of PPCM per 100,000 deliveries (**Figure 1**) increased with maternal age, especially in the HD (+) group. This incidence was more than 10 times higher in 35- to 39-year-old women than in 20- to 24-year-old women in the HD (+) group (4.7 vs. 0.4 per 100,000 births, respectively), but only 3 times higher in the HD (–) group (4.91 vs. 1.59 per 100,000 births, respectively). The time of diagnosis of PPCM in the HD (+) and HD (–) groups showed a similar tendency (**Figure 2**). The clinical backgrounds of the HD (+) and HD (–) groups are compared in **Table 1**. Patients in the HD (+) group were significantly older and underwent a Caesarean section more frequently than those in the HD (–) group. At diagnosis, the

Table 2. Factors Correlated With the Length of Hospitalization

	Standardized coefficient	P value*
Age	0.074	0.509
Parity	–0.088	0.418
Antepartum onset	–0.002	0.988
Tocolytic therapy	0.134	0.219
Twin pregnancy	–0.199	0.072
HD	–0.248	0.027
LVEF at diagnosis	–0.420	<0.001

HD, hypertensive disorders complicating pregnancy; LVEF, left ventricular ejection fraction.
*P value for comparison of the HD (+) and HD (–) groups.

Table 3. Factors Correlated With LVEF at Last Follow up

	Standardized coefficient	P value*
Age	0.214	0.420
Parity	–0.069	0.116
Antepartum onset	–0.079	0.552
Tocolytic therapy	–0.101	0.476
Twin pregnancy	0.131	0.353
HD	0.277	0.042
LVEF at diagnosis	0.335	0.011
Follow-up period	0.054	0.686

Abbreviations as per Table 2.
*P value for comparison of the HD (+) and HD (–) groups.

2 groups had similar cardiac dimensions, systolic functions, and BNP levels; LVDd were 56.1±6.7 mm vs. 56.8±7.3 mm, LVDs were 47.1±7.3 mm vs. 48.3±8.6 mm, %FS were 16.0±6.7% vs. 15.8±7.2%, LVEF were 31.9±10.2% vs. 31.5±13.2%, and serum BNP were 1,114±884 pg/ml vs. 1,353±1,112 pg/ml in each HD (+) and HD (–) group, respectively.

Two deaths occurred in both the HD (+) and the HD (–) groups and 2 patients with LVAS in the HD (–) group also died. Among the discharged patients, the hospitalization period was shorter in the HD (+) group than in the HD (–) group (26.9 vs. 40.9 days). Use of medications at discharge was similar in the 2 groups (**Table 1**).

The mean observation periods were 7.9 months in the HD (+) group and 10.9 months in the HD (–) group. In a shorter period, cardiac parameters such as LVDs, %FS, and LVEF showed significantly greater improvement in the HD (+) group compared to the HD (–) group (**Figure 3**).

Both LVDd and LVEF at diagnosis, reflecting the degree of cardiac dysfunction, showed no significant relationship with the type of hypertension or severity of BP and PU (**Figure 4**). There was also no significant relationship of LVEF at diagnosis with the duration from onset of preeclampsia or superimposed preeclampsia to onset of heart failure, but there was a weak correlation of a longer duration of preeclampsia with a lower LVEF at diagnosis (r=0.284: **Figure 5**).

Factors Associated With the Length of Hospitalization and LVEF at Last Follow up

Table 2 shows the factors that correlate with the length of hospitalization among discharged patients. The better LVEF at diagnosis strongly predicts shorter hospitalization. HD is also associated with shorter hospital stay. Other risk factors such as age, parity, twin pregnancy, tocolytic therapy show no significant effect on the length of hospitalization. **Table 3**

shows the factors that correlate with LVEF at last follow up. Both LVEF at diagnosis and HD predict LVEF at last follow up.

Discussion

This nationwide study of PPCM in Japan is the first performed on an Asian population. The current study covered specialized obstetrics, cardiology and emergency departments from all over Japan, which suggests that our data are representative of the clinical features of PPCM. Interestingly, the background, risk factors, and prognosis of all cases were similar to a report from the USA in 2005.[7] This suggests that the etiology of PPCM might be similar in the USA and Japan beyond the difference of ethnicity, and we consider that this may be because both countries have similar medical standards and trend of pregnancy such as increased maternal age and a rate of artificial fertilization. However the incidence of PPCM in Japan is lower than that in the USA (1/20,000 births vs. 1/3,000–4,000 births).[7] Several reasons like ethnicity and lifestyle might attribute to this discrepancy, and there is a possibility that some patients are undiagnosed in Japan.

In our patient population, HD in pregnancy was the major complication of PPCM. Previous studies have found incidences of hypertensive states in PPCM ranging from 2 to 68%.[5,9,10] The incidence in this study was 41%, which is similar to the rates of 43% for HD found in the study by Elkayam et al,[7] 46% for hypertension in the study by Modi et al,[11] and 22% for preeclampsia in the study by Demakis et al,[12] respectively, and quite different from those found in Haiti (4%)[13] and South Africa (2%).[10] This might be explained by differences in race, lifestyle, and medical standards.

It remains controversial as to whether patients complicated with preeclampsia should be included in cases of PPCM. It is well known that preeclampsia affects organs including the brain, liver, kidney, and the hematopoietic system, and that these effects are usually reversible. However, it is generally thought that the heart is spared from deterioration in hypertension in pregnancy. In cases of preeclampsia, cardiac function is generally well maintained, based on previous studies using echocardiography (the findings include an increased afterload caused by hypertension and a diminished preload that is changeable depending on the degree of hydration).[14–16] A recent echocardiographic study by Rafik Hamad et al[17] showed that the E/E' ratio (where E is the early transmitral diastolic flow velocity, and E' is the early diastolic myocardial velocity) was elevated in preeclampsia patients compared with normal pregnant controls, indicating impaired diastolic left ventricular function. This impairment on echocardiography was accompanied by increased blood levels of amino-terminal pro-BNP, cystatin C, and several other cardiovascular biomarkers. It seems reasonable to hypothesize that impairment of diastolic function precedes impairment of systolic function, which is characteristic of PPCM, as in hypertensive cardiomyopathy aggravated to the end-stage dilated phase. However, our data showed no relationship between the severities of cardiac systolic dysfunction and hypertension, which appears contradictory. Because our data showed severe deterioration of left ventricular function in patients with HD as well as those without HD, it is reasonable to consider that these patients were suffering from cardiomyopathy. Also, a weak correlation of a longer duration of preeclampsia with a lower LVEF at diagnosis might suggest that hypertension might increase the severity of PPCM in the acute phase.[18]

Several theories have been proposed for the pathophysiological mechanism underlying the development of PPCM; this includes an autoimmune disorder,[19,20] viral myocarditis,[21] pregnancy-induced cardiac stress (hypervolemia, elevated heart rate, and thrombophilia[22]), and ethnic susceptibility.[2,6] In a recent study, van Spaendonck-Zwarts et al reported that a subset of PPCM is an initial manifestation of familial DCM.[23] Morales et al also reported that a proportion of PPCM and pregnancy-associated cardiomyopathy cases results from a genetic cause.[24] Heterogeneity is a common element in the pathogenesis of PPCM. In this study, the PPCM patients with HD had a shorter hospital stay than those without HD. The 2 groups of patients had the same left ventricular size and systolic dysfunction at diagnosis and at discharge. In contrast, parameters such as LVDs, %FS, and LVEF at the last follow up showed greater improvement in the hypertensive patients. Ntusi and Mayosi reviewed the etiology and risk factors of PPCM and mentioned that PPCM patients with HD showed good left ventricular recovery at 6 months.[25] But there has been no data to prove this concept except the current study. As supported by these data, PPCM with HD seems to be a characteristic subset of PPCM.

Recent data have shown that increased oxidative stress is proposed to aggravate proteolysis of full-length prolactin, and subsequently the 16kDa prolactin fragment, a cardiotoxin and endotheliotoxin, might contribute to the deterioration of PPCM.[26] Moreover, urinary prolactin and their isoforms of 14 and/or 16kDa prolactin are increased in preeclampsia patients.[27] Reuwer et al proposed a recent hypothesis for the increased co-existence of PPCM and preeclampsia based on the pathophysiology of the 2 conditions sharing the same molecular pathway.[28] The current study might suggest that hypertension in pregnancy is not causative in the development of PPCM, but that a hypertensive state and PPCM are associated with other common factors.

In our study, the rate of death was similar between PPCM patients with and without HD. Goland et al reported predictors of major adverse events (MAE; death, heart transplantation, temporary circulatory support, cardiopulmonary arrest, request for intensive care, thromboembolic complication, or implantation of pacemaker and implantable cardioverter) among PPCM patients, and only baseline LVEF and non-Caucasian background were significant predictors.[29] This result can apply to the current study. We cannot prevent PPCM in patients complicated with HD because of MAE at the acute phase because their cardiac functions were severely deteriorated; this was also the case for those patients without HD. Thus, identification of patients who might develop PPCM might allow early intervention or prevention of the condition.

It is often difficult to diagnose whether a pregnant woman complaining of dyspnea or edema has heart failure or not. From a practical clinical point of view, we might suggest the use of the serum BNP level to diagnose heart failure in PPCM patients, as well as a chest X-ray. Moreover, we should treat peripartum women, especially those who are older in age, with HD cautiously and they should immediately undergo a cardiac examination to rule out PPCM as needed.

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Disclosures

None.

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Maternal death analysis from the Japanese autopsy registry for recent 16 years: significance of amniotic fluid embolism

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Abstract

Aim: To clarify the cause of maternal deaths, an autopsy is essential. However, there has been no systemic analysis of maternal death in Japan based on autopsy cases.

Material & Methods: Maternal death reports were retrieved from a large amount of registered autopsy data on maternal death in the series of 'Annual of pathological autopsy cases in Japan'. These files contain 468 015 autopsy records from 1989 to 2004. We collected 193 cases of maternal death due to direct obstetric causes. We recorded all the data into Excel files. Then we analyzed the causes of death and classified them into 11 categories.

Results: The causes of maternal death were as follows: amniotic fluid embolism (AFE), 24.3%; disseminated intravascular coagulation (DIC) related to pregnancy-induced hypertension, 21.2%; pulmonary thromboembolism, 13.0%; injury to the birth canal, 11.4%; medical and surgical complications, 9.8%; and atonic bleeding or DIC of unknown cause, 8.3%. A discrepancy between the clinical diagnosis and pathological diagnosis was frequently observed in cases of AFE, septic DIC and injury to the birth canal. AFE diagnosed by autopsy was often clinically diagnosed as atonic bleeding or DIC of unknown cause before death. Half of the cases of AFE diagnosed by autopsy were associated with DIC.

Conclusion: We found that AFE, DIC related to pregnancy-induced hypertension, pulmonary thromboembolism and injury to the birth canal were the major causes of maternal death in Japan. AFE had various clinical features such as uterine atony and DIC in addition to pulmonary cardiac collapse.

Key words: amniotic fluid embolism, DIC, Japanese autopsy registry, maternal death, pulmonary thromboembolism, uterine atony.

Introduction

Maternal death is a shocking event in obstetrical practice. In Japan, the maternal mortality rate (number of maternal deaths per 100 000 live births) was 5.6 in 2002–2006.[1] The rate has been decreasing since the 1970s and is now stable at around 5. The statistics for maternal mortality in Japan have been mainly

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derived from clinical diagnoses of death. Information provided by medical autopsies has played an important role in increasing the accuracy of cause-of-death reports and improving clinical practice. Autopsies may also provide important data on the causes of maternal death, which is essential for reducing maternal mortality and directing public health efforts. Therefore an autopsy is performed in nearly every case of maternal death.

The problem with examining maternal deaths in Japan is that an autopsy is not performed for these cases. Also, there is no information on how many cases of maternal deaths involve autopsy. To clarify the real cause of maternal deaths in Japan, an autopsy is essential. However, there has been no systemic analysis of maternal death in Japan based on autopsy cases. The aim of this study is to elucidate the cause of maternal deaths in Japan based on anatomical analysis. We found a large amount of pathological data on maternal death in the 'Annual of the pathological autopsy cases in Japan'edited by the Japanese Society of Pathology. The aim of this study was to analyze the pathological data, and to know the real cause of maternal deaths based on autopsy. Moreover, the rate of home delivery in 1990 was 0.1%, and in 2000 was 0.2%. From this point of view this analysis could reflect the real cause of maternal death in Japan.

Methods

To collect autopsy cases of maternal death, we used files from the 'Annual of pathological autopsy cases of Japan'. Data are recorded for all cases of autopsy of hospital deaths in Japan. Also, this report was based on complete autopsies with histologic examinations by authorized pathologists by the Japanese Society of Pathology. We selected cases where the women died during pregnancy or within 42 days of the completion of a pregnancy. We excluded deaths from traffic accidents and suicide. We recorded the clinical diagnosis, anatomic diagnosis and findings into Excel files (Microsoft Corporation, Redmond, WA, USA). Then we investigated the data and classified the cases into 11 categories. Three specialists in obstetrics checked the contents and confirmed the diagnosis. Some records of anatomical findings and diagnoses were not completely adjusted to current international classification of diseases (ICD) cords. We excluded DIC secondary to consumptive coagulopathy such as a massive bleeding of laceration from unknown cause of DIC category. Also, we did not include the cases of uterine atony derived from latent uterine rupture.

Results

Among the 468 015 recorded cases of autopsy from 1989 to 2004, we found 193 maternal deaths due to direct obstetric causes. In this period, the range of the Japanese population was 122 460 000 (1989) to 12 617 600 (2004). The average of the population per year during the period was 124 632 186. Live birth was 1 246 802 (1989) to 1 110 721 (2004). The average of live birth per year was 1 190 170. Total fertility rate in this period was from 1.57 (1989) to 1.29 (2004). The average of fertility rate per year was 1.42. Figure 1 shows the causes of maternal deaths detected in the autopsy records. Amniotic fluid embolism (AFE) was the most common cause with 47 cases (24.3%). DIC related to pregnancy induced hypertension (PIH) including eclampsia, HELLP syndrome, and the abruption of the placenta ranked second with 41 cases (21.2%). Third was pulmonary thromboembolism with 25 cases (13.0%) and fourth was injury to the birth canal with 22 cases (11.4%). Among these 22 cases, there were 7 cases of uterine rupture, 5 cases of cervical laceration, 3 cases of vaginal laceration, 3 cases of retroperitoneal hemorrhage of unknown cause, 3 cases of post-hemorrhage of

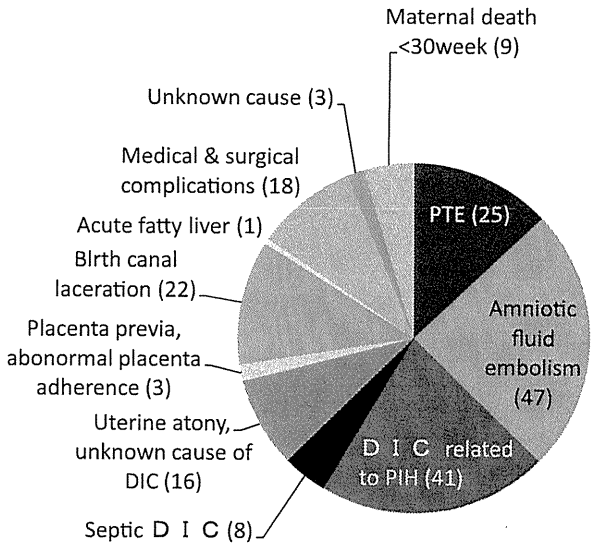

Figure 1 The cause of maternal death by autopsy in Japan from 1989 to2004. DIC, disseminated intravascular coagulation; PIH, pregnancy induced hypertension; PTE, pulmonary thromboembolism.

cesarean section, and 1 case of inversion of the uterus. The fifth leading cause of maternal death was medical and surgical complications with 18 cases (9.8%): 5 cases of rupture of an artery such as an aortic aneurysm, 4 cases of heart diseases (myopathy 3 cases, unknown cause of heat failure 1 case), 3 cases of hyperthyroidism, 2 cases of hepatitis, and 4 other cases. The sixth leading cause was uterine atony and uterine atony associated with DIC of unknown cause with 16 cases (8.3%), and the seventh cause was maternal death at less than 30 weeks of gestation with 9 cases (4.7%). Out of 9 cases of maternal death before 30 weeks of gestation, there were 4 cases of ectopic pregnancy, 3 cases of pulmonary thromboembolism, 1 case of abortion associated with DIC, and 1 case of invasive mole. The eight leading cause of maternal death was septic DIC with 8 cases (4.1%), ninth was placenta previa and placental abnormalities with 3 cases (1.6%), tenth was acute fatty liver with 1 case (0.5%), and the eleventh was unknown cause with 3 cases (1.1%).

The discrepancy between clinical and pathological diagnoses was analyzed. The rate of discrepancy was 49% for AFE, 5% for DIC related to PIH, 36% for pulmonary thromboembolism, 50% for birth canal laceration, 50% for septic DIC, 61% for medical and surgical complications and 17% for uterine atony and DIC of unknown cause. AFE, birth canal laceration, septic DIC and medical and surgical complications were frequently misdiagnosed (Fig. 2). AFE diagnosed by autopsy was often diagnosed clinically as atonic bleeding or DIC of unknown cause prior to death (Fig. 3).

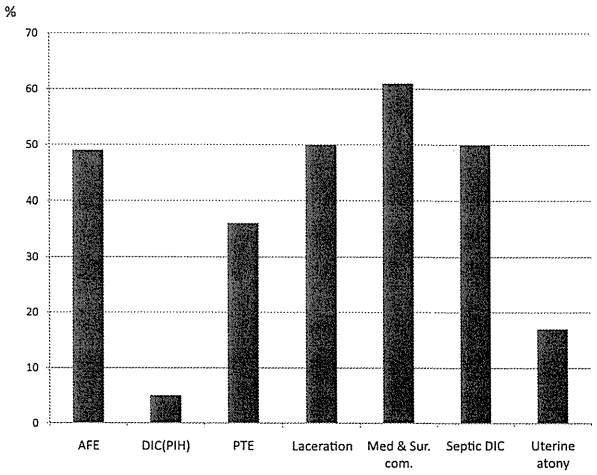

Figure 2 The discrepancy between clinical diagnosis and anatomical diagnosis. AFE, amniotic fluid embolism; DIC, disseminated intravascular coagulation; PIH, pregnancy induced hypertension; PTE, pulmonary thromboembolism; Med & Sur com, medical and surgical complication. Y-axis indicates coincidence percentage of clinical and anatomical diagnosis.

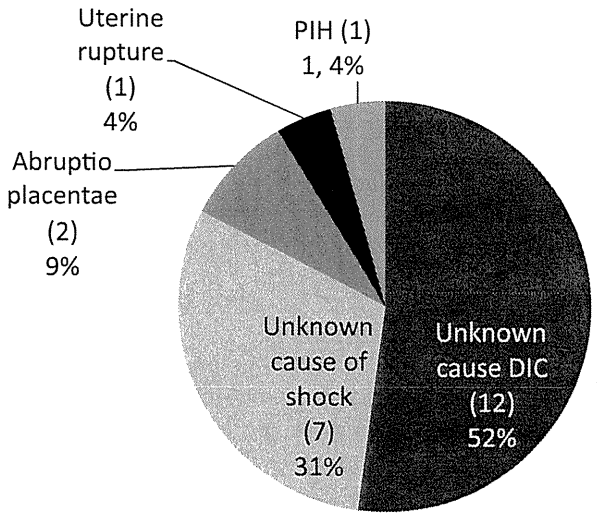

Figure 3 Distribution of clinical diagnosis of which the cases were anatomically diagnosed amniotic fluid embolism (AFE) and their clinical diagnosis were different from AFE. DIC, disseminated intravascular coagulation; PIH, pregnancy induced hypertension.

Discussion

We found that AFE, DIC related to PIH, pulmonary thromboembolism and injury to the birth canal were the major causes of maternal death in Japan. The results clearly showed that the critical diseases linked to maternal death are almost the same as in other developed nations.[2,3] To our knowledge, this is the first description of the causes of maternal death in Japan based on autopsy studies. In our study, anatomical diagnosis and clinical diagnosis were done by each hospital, and then their data was directly registered into the 'Annual of the pathological autopsy cases in Japan' report. For this reason the confidence level of diagnosis might differ in each hospital. However, specialists allowed by the Pathologic Association of Japan can perform and report the autopsy diagnosis into the report. Thus, the accuracy level of autopsy diagnosis is high.

We found that AFE was the most important cause of maternal deaths in Japan. Maternal death rate and its

causes are different from developing nations. Maternal death rate in developing nations is more than 10 times higher than that of developed nations.[4] Maternal hemorrhage, infection and abortion are the main causes of maternal death in developing nations.[5]

On the other hand, similar to our results, the leading causes of maternal death in developed nations were embolism and pregnancy induced hypertension.[6] Currently Steven L *et al.* reported that amniotic fluid embolism occupied 14% of 95 maternal deaths per 1 461 270 births in the US from 2001 to 2006.[7] This demonstrates that AFE is an important cause of maternal death in developed nations, which is also similar to our results. AFE in our results showed 24.8% of total maternal deaths, and is high compared to other reports. The definition of anatomical diagnosis of AFE is not established at present. In our study, the cases that amniotic fluid debris or fetal cells were present in several pulmonary arteries defined as anatomical AFE. From this definition we found that approximately 50% of anatomical diagnosis of AFE diagnosed clinically unknown cause of DIC or uterine atony (Figs 2,3). For this reason the incidence of AFE in our study may not compare to other reports.

Interestingly, the ranking of causes of maternal deaths based on official death certificates is different from our results. We compared our results with age matched maternal mortality data from the Japanese Mothers' and Childrens' Health & Welfare Association. The latter statistics were derived from death certificates, not from autopsies. According to the Annual report of Maternal and Child Statistics of Japan, in 1995, obstetric embolism including AFE and pulmonary thromboembolism ranked first (29.9%) among direct obstetrical causes, followed by PIH (28.4%), other direct obstetrical causes including medical and surgical complications (28.4%), post-partum hemorrhage, and placenta previa and abruptio placentae (4.5%).[1] In the year 2000, among direct obstetrical causes, obstetric embolism including amniotic embolism and pulmonary thromboembolism ranked first (22.6%), followed by placenta previa and abruption placentae (19.4%), other direct obstetrical causes including medical and surgical complications (19.4%), post-partum hemorrhage (16.4%), and PIH (12.9%). Obstetric embolism was the most frequent cause of death in both our study and the Mothers' and Childrens' Health & Welfare Association data. However, the rate of obstetric embolism including AFE and pulmonary thromboembolism from our data was 37.3%. The percentage of obstetric embolism among our autopsy data of maternal deaths was extremely high. The reason could be that half of AFE patients (23 cases) were not correctly diagnosed before death, because antemortem diagnosis of AFE is very difficult in the clinical environment. These results suggest that obstetricians should recognize that AFE has various signs and symptoms besides cardiopulmonary collapse. We would like to emphasize that DIC and uterine atony are also major symptoms of AFE. Recently, Gilbert described the complications of AFE, and reported that DIC and uterine atony are frequently associated with AFE.[8]

Our results and current findings suggest that some cases of AFE involved DIC or uterine atony rather than cardiopulmonary collapse. In fact, in spite of the presence of amniotic fluid debris in pulmonary arteries, some patients showed DIC or uterine atony mainly without cardiorespiratory symptoms. Awad *et al.* reported an AFE case in which the patient showed DIC of unknown cause and massive bleeding.[9]They found the cases of AFE presented with symptoms and signs other than the classical pattern of dyspnea, cyanosis and hypotension. They proposed that consumptive coagulopathy appears to be the 'forme frusta' of amniotic fluid embolism. Our findings support their hypothesis. We speculate that the unknown cause of uterine atony or unknown cause of post-partum DIC is attributed to the uterine type of AFE. Our hypothesis is shown in Figure 4.

Furthermore, several investigators also reported atypical AFE cases in which DIC appeared predominant.[10] Jang reported that amniotic debris in the vasculature of endocervix was found in some cases of AFE

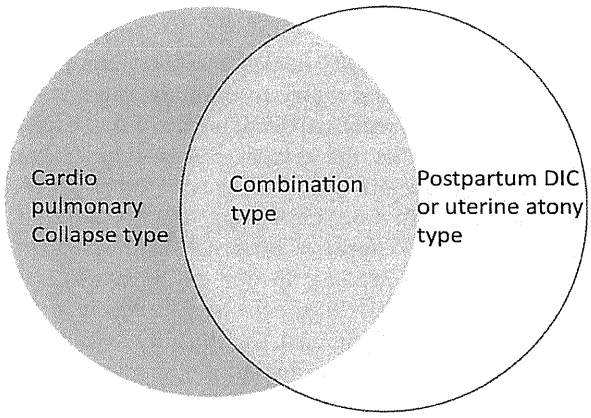

Figure 4 Hypothesis of three types of amniotic fluid embolism (AFE) based on autopsy. DIC, disseminated intravascular coagulation.

with DIC.[11] These findings may be compatible with our hypothesis. Such investigators suggest that AFE was similar to anaphylactoid shock. Serum complement levels are low in AFE.[12] Also, several reports founded that the severe vasoconstriction caused by anaphylactoid reaction was the main pathophysiology of AFE.[13,14] The aspect of anaphyractic reaction not only pulmonary arteries but also uterine vessels could be important to future investigations.

DIC related to PIH was a major cause according to our results, which was compatible with the statistics from death certificates. Improvements of management for PIH are still important in decreasing maternal mortality in Japan. Pulmonary thromboembolism is the third leading cause of maternal death. Currently, deep vein thrombosis is increasing in Japan as in other developed nations. Prophylactic guidelines were proposed by several medical societies in 2004. We hope that the incidence of deep vein thrombosis and pulmonary thromboembolism will be decreased by these guidelines.

Birth canal laceration was the fourth leading cause of maternal death. Uterine rupture, cervical laceration and vaginal laceration, in this order, were the major causes of laceration of the reproductive tract. Obstetricians must keep this in mind when they face postpartum massive bleeding.

We found that the rupture of vessels such as an aortic aneurysm, heart diseases (myopathy and heat failure), and hyperthyroidism are the major causes of medical and surgical complications. Arterial rupture induces the sudden onset of massive bleeding. It is well known to cause maternal deaths. Although it is difficult to detect such diseases during pregnancy in some cases, obstetricians should be careful regarding those diseases in antepartum care. We found four cases of heart diseases with cardiomyopathy the main cause among them. The cause of peripartum cardiomyopathy is still unknown and further investigations are needed. Hyperthyroidism is also an important disease for maternal deaths. We should recognize that hyperthyroidism is a disease linked to maternal death. The sixth cause of maternal deaths was uterine atony and uterine atony associated with DIC of unknown cause. This category of disease has not been well understood. A common pathological finding is that fibrin thrombosis is widely observed in the myometrium. We should continue to clarify the mechanism of this disease.

Maternal death at less at than 30 weeks of gestation ranked seventh. It seems to be ranked rather low; however, it might be due to a well-developed maternal check-up system especially from early stage of pregnancy in Japan. Ectopic pregnancy and thromboembolism are major causes among maternal death in early stage of pregnancy. As for the management early stage of pregnancy, obstetrician should pay attention not to misdiagnose these diseases.

In conclusion, we would like to propose several strategies to decrease the rate of maternal death in Japan. AFE has various clinical features such as uterine atony and DIC in addition to pulmonary cardiac collapse. Therefore, we should clarify the mechanism of AFE and improve the management of AFE. Notably, AFE is frequently associated with DIC. The early diagnosis and treatment of DIC will be important. Secondly, we should be aware of DIC related to PIH as a second cause of maternal death in Japan. PIH is the major cause of maternal death in all countries, but improvements in the management of DIC have been pointed for some time, and the incidence of PIH is decreasing year by year. However, our studies showed that PIH is still important in decreasing the rate of maternal deaths in Japan. When facing PIH cases, obstetricians should keep DIC in mind. Currently, prophylactic methods for pulmonary thromboembolism such as intermittent pressure pump and heparin administration are widely used. We should continue to try to reduce the incidence of thromboembolism. Birth canal laceration occurs at a constant rate in hospitals. Uterine rupture and cervical laceration should be recognized as critical diseases in obstetrical practice. Taken together, except amniotic fluid embolism, major causes of maternal deaths have been pointed out for a long time. Our results will contribute the diagnosis of obstetrical practice especially in severe maternal diseases. If we improve the management of such diseases, we believe that the maternal mortality rate in Japan will decrease in the near future.

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Sustained-release adrenomedullin ointment accelerates wound healing of pressure ulcers

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ABSTRACT

Pressure ulcers are one of the most common complications in elderly, incontinent or paralyzed patients. For the healing of pressure ulcers, the development of granulation tissue and reepithelialization is required. Adrenomedullin (AM), an endogenous vasodilator peptide, is reported to stimulate the proliferation and migration of various cells including endothelial cells, fibroblasts and keratinocytes. Therefore, we hypothesized that AM might accelerate the healing process of pressure ulcers in which these cells were involved. We developed a sustained-release ointment containing human recombinant AM, and applied it in a mouse model of pressure ulcer twice a day for 14 days. Human AM was efficiently absorbed in wound area, but its blood concentration was negligible. AM ointment significantly reduced the wound area on day 5 to 7 after injury. In addition, AM ointment accelerated the formation of granulation tissue and angiogenesis as well as lymphangiogenesis after 7 days of treatment. Immunological analysis revealed that Ki-67-positive proliferating cells in granulation tissue expressed AM receptors. In summary, sustained-release AM significantly improved wound healing of pressure ulcers through acceleration of granulation and induction of angiogenesis and lymphangiogenesis. Therefore, sustained-release AM ointment may be a novel therapeutic agent for pressure ulcers.

1. Introduction

Pressure ulcers are one of the most common complications in elderly, incontinent or paralyzed patients, resulting from neurological disease, cardiovascular disease and surgical procedures, and susceptibility to pressure ulcers occurs due to unrelieved pressure, shear force or friction [1]. Treatment of pressure ulcers includes pressure reduction, cleaning and surgical intervention; however, long-term therapy is necessary for most patients [1,2]. In the healing process of pressure ulcers, the development of granulation tissue and reepithelialization are critical.

Adrenomedullin (AM) is an endogenous vasodilator peptide [3] that has been shown to have proliferative, migrative and anti-apoptotic effects on various cells including vascular endothelial cells [4,5], smooth muscle cells [6], fibroblasts [7], and keratinocytes [8]. Furthermore, AM is produced in these cells including endothelial cells [9], fibroblasts [10], and keratinocytes [8,11] in response to proinflammatory cytokines. These cells are reported to possess its receptor complexes, calcitonin receptor-like receptor (CRLR)/receptor activity-modifying protein (RAMP)-1,-2, and -3 [12,13], indicating that AM stimulates the proliferation of these cells in an autocrine and/or paracrine manner [8,14]. Considering that the healing process of pressure ulcers involves granulation tissue, with invasion of the wound space in association with proliferation and migration of endothelial cells and fibroblasts, AM may contribute to the healing process of pressure ulcers.

Here, we showed the therapeutic potential of AM for the treatment of pressure ulcers. However, one of the greatest disadvantages associated with the use of recombinant AM is its rapid clearance after systemic administration [15]. Therefore, we developed a sustained-release AM ointment to overcome this problem related to the administration of AM in a pressure ulcer model. Our long-lasting drug delivery system allowed AM to be locally applied to a wound in the skin.

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Thus, the purposes of this study were 1) to investigate whether AM has therapeutic potential for the treatment of pressure ulcers, and 2) to investigate the underlying mechanisms of AM in the process of wound healing.

2. Materials and methods

2.1. Preparation of AM

Recombinant human AM was obtained from Shionogi & Co., Ltd. AM was dissolved in saline with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore). The chemical nature and content of AM in vials were verified by high-performance liquid chromatography [3] and radioimmunoassay [16]. All vials were stored frozen at −80 °C from the time of dispensing until the time of preparation for administration.

2.2. Analysis of AM release from ointment in vitro

Rehydrated recombinant human AM (500 μg/ml) was mixed in three kinds of ointment base (at 40 μg/g); white petrolatum (Cosmescience), polyethylene glycol (1:1 mixture of Macrogol 400 and 4000, Nikko Pharmaceutical), and anionic hydrogel (Hiviswako, Wako Pure Chemical Industries). Each ointment (1 g) was placed in a tube, 10 ml distilled water was added over the ointment, and 0.5 ml of supernatant was collected 3, 6, 12 and 24 h later. The concentration of AM in the supernatant was measured with an ELISA kit (Phoenix Pharmaceuticals) according to the manufacturer's instructions.

2.3. AM concentration in wound tissue and plasma

Human and mouse AM concentrations in plasma and wound tissue were measured with a radioimmunoassay kit (Shionogi and Phoenix Pharmaceuticals, respectively), as reported previously [17]. Human and mouse AM radioimmunoassay kits have no cross-reactivity. Briefly, each tissue was boiled in water to inactivate intrinsic proteases. After cooling, acetic acid was added and the mixture was homogenized. The supernatant of the extract, obtained after centrifugation, was lyophilized. For assay, the lyophilized material was dissolved in radioimmunoassay buffer, and the clear solution was subjected to radioimmunoassay. Plasma samples were analyzed without modification. The radioactivity was measured by a gamma counter (ARC-1000M, Aloka). All assay procedures were performed in duplicate.

2.4. Pressure ulcer model

We used 5-week-old male ICR mice (Japan SLC). A pressure ulcer model was produced by repeated induction of ischemia/reperfusion of the skin. Briefly, we anesthetized mice with isofluran (Escain, Mylan Inc), and removed the hair on the back using Epirat depilatory cream (Kanebo), and compressed the skin with a circular punch (Fujiwara Sangyo) for 4 h. After 20 h of reperfusion, we compressed the skin again for 4 h, followed by 20 h of reperfusion. The necrotic tissue was cut off with scissors the next day. All protocols were performed in accordance with the guidelines of the Animal Care and Ethics Committee of the Japanese National Cardiovascular Center Research Institute.

2.5. Study protocol

We randomly allocated ICR mice to three groups: mice with a pressure ulcer (without ointment group; n = 15), mice with a pressure ulcer to which hydrogel alone (50 mg, ointment only group; n = 15) was applied, and mice with a pressure ulcer treated with hydrogel containing AM (2 μg/50 mg, AM ointment group, n = 15). The wound area in each group was covered with a transparent dressing (Tegaderm, 3 M) immediately after application of the ointment. We applied the ointment twice a day and measured the wound area every day for 14 days [18].

2.6. Histological analysis

The wound tissue (n = 6 in each group) was excised on day 7, fixed in 4% formalin, embedded in paraffin, and processed for histological and immunohistological analysis. Tissue sections were stained with hematoxylin and eosin (H–E). To detect proliferative cells, vascular endothelial cells, and lymphatic endothelial cells, we performed immunohistochemical staining of Ki-67 (Dako Cytomation), von Willebrand factor (vWF, Chemicon), and LYVE-1 (R&D Systems).We also conducted CRLR (V-20, Santa Cruz Biotechnology) immunostaining of tissue sections. The images were obtained blindly using a computer-navigated microscope (BIOREVO, KEYENCE). The independent observer chose ten randomly selected areas within wound granulation tissue and the average number of cells positive for Ki-67, vWF, or LYVE-1 were calculated (magnifications: Ki-67: ×400, vWF and LYVE-1: ×200).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA were isolated from wound granulation tissue using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was reverse-transcribed into cDNA using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. PCR was carried out as follows: an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C. The specific primer pairs were: CRLR, 5′-TGTAATAA-CAGCACGCATGAG-3′ and 5′-GTTATTGGCCACTGCCGTGA-3′; RAMP-1, 5′-CACCATCTCTTCATGGTCACTG-3′ and 5′-CAATCGTGTGCGCCACGTGC-3′; RAMP-2, 5′-TGGATCTCGGCTTGGTGTGAC-3′ and 5′-GCAAGGTAG-GACATGTGTTTCG-3′; RAMP-3, 5′-TTGTGGTGAGTGTGCCCAGG-3′ and 5′-CCCATGATGTTGGTCTCCATC-3′ [19]. A set of GAPDH primers was used as an internal control.

2.8. Statistical analysis

Numerical values are expressed as mean ± S.E.M. Continuous variables were determined in four groups in this study. Therefore, for multiple comparisons of more than two groups, we performed one-way analysis of variance (ANOVA). If the result of ANOVA was significant, we used Newman–Keuls' procedure as a post hoc test. For repeated measurements such as chronological analysis, we performed two-way repeated ANOVA with Newman–Keuls' test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Controlled release of AM from ointment

We prepared three types of ointment base: anionic hydrogel, polyethylene glycol and white petrolatum, to determine the optimal base for the development of a sustained-release preparation of AM. AM in anionic hydrogel was gradually released over 24 h, whereas AM in polyethylene glycol was released rapidly, and AM in white petrolatum was hardly released (Fig. 1a). Thus, anionic hydrogel was considered a promising drug delivery base to examine the therapeutic effect of AM. To observe the absorption of AM in wound tissue and into the blood, we measured the concentration of human AM in wound tissue and plasma after local administration of human AM ointment on pressure ulcers in mice. The concentration of human AM in wound tissue was markedly elevated in AM ointment group (512.2 ± 66.7 fmol/g) (Fig. 1c). Although we could detect human AM

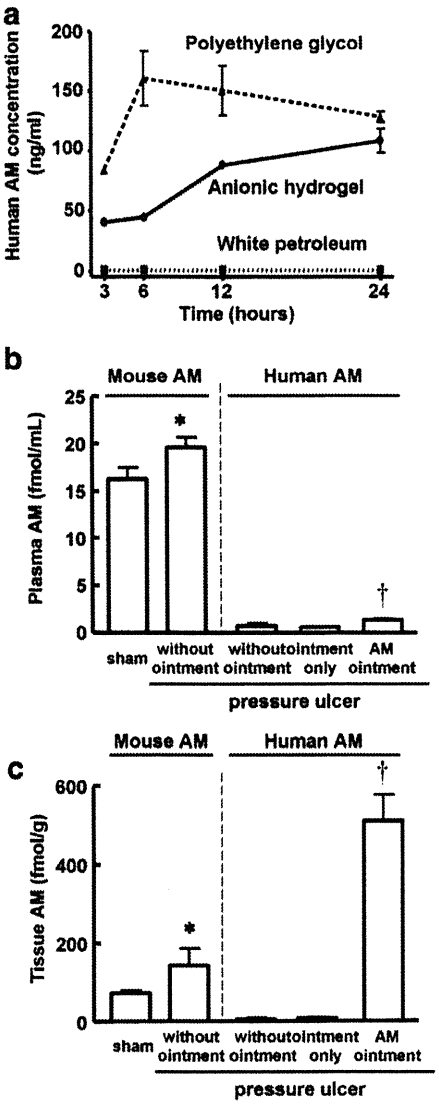

Fig. 1. AM release and absorption from ointment in vitro and in vivo. (a). Comparison of AM release from polyethylene glycol, white petroleum, and anionic hydrogel in vitro. AM in anionic hydrogel was gradually released over 24 h, while AM in polyethylene glycol was released rapidly, and AM in white petrolatum was hardly released. (b) Plasma concentration of mouse and human AM 1 h after treatment with AM ointment (anionic hydrogel). (c) The concentration of mouse and human AM in wound tissue. Mice received no surgical procedure were denoted as sham. The concentration of human AM in wound tissue was significantly elevated in the AM ointment group compared with without ointment and ointment only groups, but its concentration in plasma was negligible. N = 5 in each group.

immunoreactivity in plasma of AM ointment group, the concentration of human AM was significantly low (1.3 ± 0.12 fmol/ml of plasma) compared to that of mouse AM (19.6 ± 1.0 fmol/ml of plasma) (Fig. 1b). In addition, no significant change in blood pressure or heart rate was observed by human AM treatment (data not shown).

3.2. Effect of AM-containing ointment on pressure ulcer

To examine the therapeutic effect of AM ointment on the healing process of pressure ulcers, we applied AM ointment (2 μg AM in 50 mg hydrogel) twice a day, and measured the wound area for two weeks. AM significantly accelerated wound healing on days 5 to 7 after injury, compared to that in without ointment and ointment only groups (Fig. 2a,b). H–E staining of wound tissue on day 7 showed thicker granulation tissue in AM ointment group compared to that in without ointment and ointment only groups, and the newly formed granulation tissue in AM ointment group contained a number of blood vessels compared to those in without ointment and ointment only groups (Fig. 2c, 3c,d).

3.3. Effect of AM-containing ointment on cell proliferation, angiogenesis and lymphangiogenesis

Immunohistochemical analysis of the granulation tissue on day 7 demonstrated that AM ointment significantly increased the number of Ki-67-positive proliferating cells (AM ointment group: 541 ± 46.4/mm^2, $p<0.001$ vs. without ointment group: 257.8 ± 24.5/mm^2 and ointment only group: 262.4 ± 24.2/mm^2) (Fig. 3a, b). AM also increased the number of von Willebrand factor (vWF)-positive vessels (AM ointment: 197.4 ± 10.8/mm^2, $p<0.01$ vs without ointment: 150.2 ± 3.3/mm^2 and ointment only: 142.2 ± 10.4/mm^2) (Fig. 3c,d), and LYVE-1-positive lymphatic vessels (AM ointment: 38.4 ± 3.8/mm^2, $p<0.001$ vs without ointment: 9.6 ± 5.6/mm^2 and ointment only: 5.4 ± 3.0/mm^2) (Fig. 3e,f).

3.4. Expression of CRLR, RAMP-1, -2 and -3 in wound tissue

Immunofluorescent analysis of granulation tissue on day 7 demonstrated that Ki-67-positive cells also expressed CRLR (Fig. 4a). To examine the expression of functional AM receptors in granulation tissue, we performed RT-PCR for CRLR, RAMP-1, -2, and -3 in 3 samples from each group. RT-PCR revealed that these AM receptors were expressed in all granulation tissues (Fig. 4b). RAMP-1 and -3 mRNA expression in granulation tissue was increased by the treatment with AM ointment (Fig. 4b).

4. Discussion

In this study, we showed that 1) anionic hydrogel is a promising ointment base for sustained release of AM, 2) AM containing ointment accelerated wound healing in a pressure ulcer model, and 3) AM administration induced angiogenesis and lymphangiogenesis in wound tissue.

AM is an endogenous vasodilator peptide, and continuous infusion is required because it has an extremely short duration of action [20] but continuous administration of AM may cause hypotension. In the present study, anionic hydrogel could slowly release AM, and AM absorbed in the wound area did not cause any change in blood pressure or heart rate. The hydrogel used in this study is a high-molecular-weight carboxyvinylic derivative, which is extensively used in the manufacture of pharmaceutical gels, and is highly suited for use in controlled-release systems for not only chemicals but also peptides including insulin [21].

The wound healing process is a complex cascade that relies on several mechanisms including a hypoxic phase, inflammatory phase, tissue formation phase, and remodeling phase [22,23], each of which involve distinct cell types [24]. In the tissue formation phase, angiogenesis, granulation, and reepithelialization occur, and endothelial cells [25], fibroblasts [7,8], and keratinocytes [8] are mainly involved [22,23]. It has been demonstrated that AM stimulates proliferation of all these cells and enhances their DNA synthesis and proliferative activity via its receptors [13,26–28]. AM ointment increased granulation tissue formation and accelerated skin wound healing from day 5 to day 7 after injury, suggesting the acceleration of granulation and reepithelialization. In addition, angiogenesis, which is necessary to sustain the newly formed granulation tissue, was induced by AM on day 7 after injury. We also confirmed that Ki-67-positve proliferating cells in would tissue expressed high level of AM receptors. Therefore, sustained release of AM would enhance the proliferation of endothelial cells, fibroblasts, and keratinocytes in wound tissue, leading to acceleration of wound healing in the tissue formation phase [8,14].

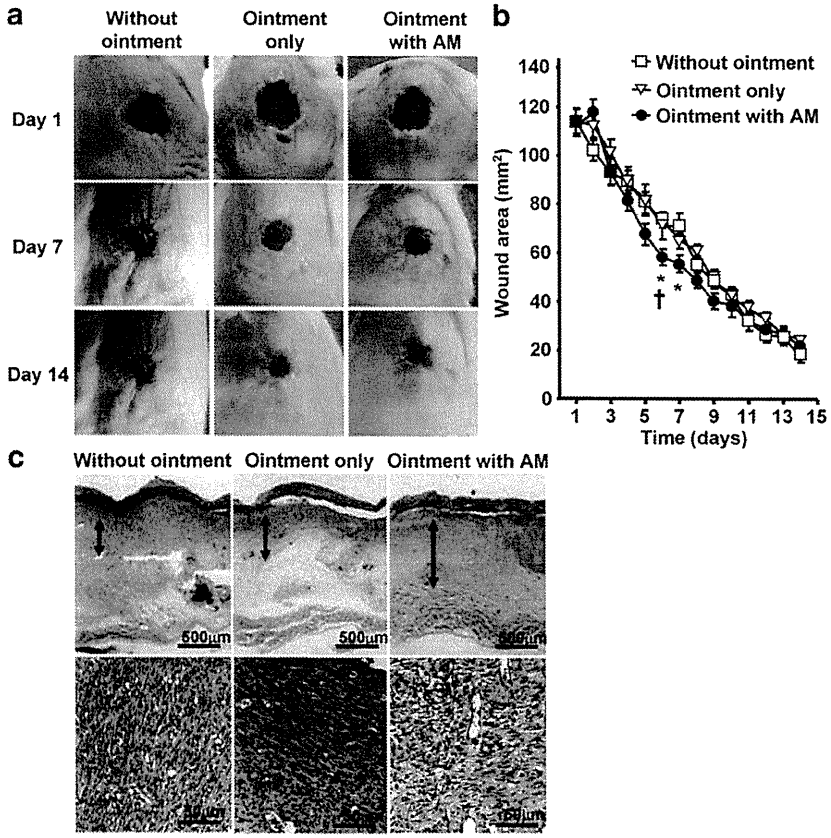

Fig. 2. Effect of AM ointment on pressure ulcer. (a) Gross appearance of wounds at indicated time points. (b) Time course of wound area. AM significantly accelerated wound healing in the early phase (days 5 to 7), compared to that in the without ointment and ointment only groups. N = 15 in each group. (c) Photomicrographs of granulation tissue of wound area stained with hematoxylin and eosin on day 7. Thicker granulation tissue was observed in the AM ointment group compared with that in the without ointment and ointment only groups, and the newly formed granulation tissue in the AM ointment group contained a number of large blood vessels. N = 6 in each group. Scale bars in upper panel of c, 500 μm; lower panel of c, 50 μm. *p<0.05 versus without ointment. †p<0.05 versus ointment only.

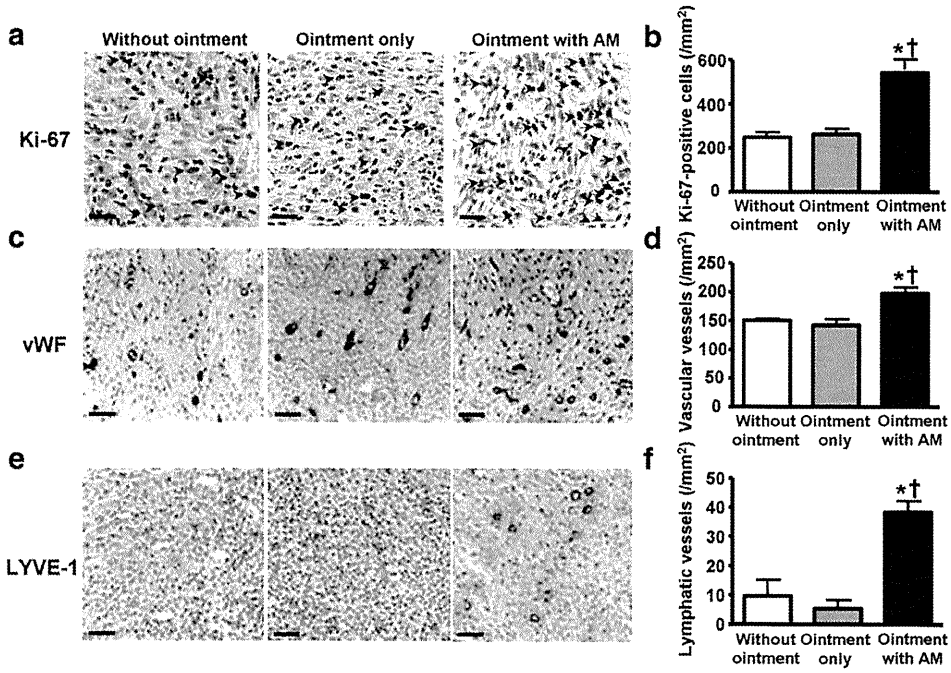

Fig. 3. Effects of AM ointment on cell proliferation, angiogenesis and lymphangiogenesis. (a,c,e) Representative microphotographs of wound tissue stained for Ki-67 (a), von Willebrand factor (vWF, c), and LYVE-1 (e) on day 7. (b,d,f) Semi-quantitative analysis of Ki-67 (b), vWF (d) and LYVE-1 (f)-positive cells. AM ointment significantly accelerated cell proliferation, angiogenesis, and lymphangiogenesis in wound tissue compared with that in the without ointment and ointment only groups. N = 6 in each group. Scale bars in a, 100 μm; in c and e, 50 μm. *p<0.05 versus without ointment. †p<0.05 versus ointment only.

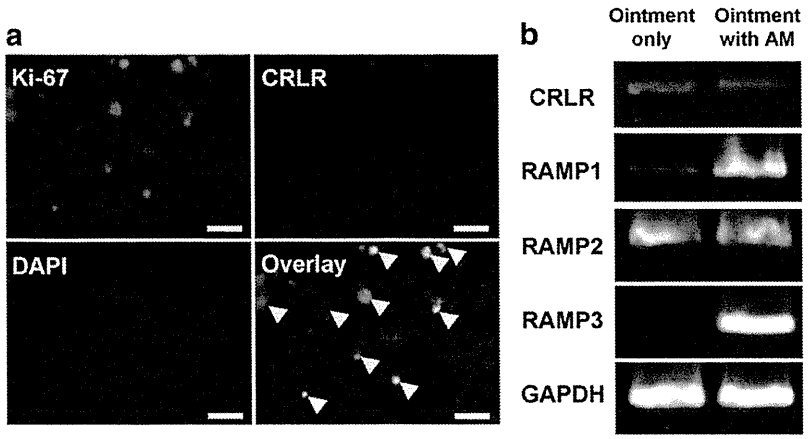

Fig. 4. Expression of CRLR and RAMPs in granulation tissue. (a) Immunostaining of CRLR and Ki-67 in sections was performed 7 days after induction of pressure ulcer. Arrow heads indicate double-stained cells. Proliferating Ki-67-positive cells in granulation tissue were also positive for CRLR. Ki-67: green, CRLR: red, DAPI: blue. Scale bar equals 20 μm. (b) RT-PCR revealed AM receptors including CRLR, RAMP-1, -2, and -3 were expressed in granulation tissue on day 7. The data shown are representative of three experiments. GAPDH was served as internal control.

Previous reports have shown that AM are essential for angiogenesis and vascular integrity [29,30]. Recently, we reported that AM is a major effector of lymphangiogenesis [31]. We demonstrated that AM accelerated proliferation, migration, and network formation of cultured lymphatic endothelial cells, and accelerated lymphangiogenesis in a mouse model of lymphedema [31]. Fritz-Six KL et al. also demonstrated that AM signaling is important for the development of lymphatic vasculature [32]. Because lymphangiogenesis as well as angiogenesis are crucial in the wound-healing process [33], we considered that administration of AM induced both angiogenesis and lymphangiogenesis, resulting in acceleration of wound healing in a mouse model of pressure ulcer. It is well known that blood and lymphatic vessels contribute to transportation of gases, liquids, nutrients, signaling molecules and circulating cells between tissues and organs [34]. Therefore, AM would contribute to acceleration of the healing process of pressure ulcers by not only inducing the formation of granulation but also improving local circulation through angiogenesis and lymphangiogenesis.

In summary, sustained-release AM accelerates wound healing of pressure ulcers through accelerating granulation and induction of angiogenesis and lymphangiogenesis. Therefore, sustained-release AM ointment may be a novel therapeutic agent for pressure ulcers.

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