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Effects of Thrombophilia and Antithrombotic Therapy on Embryonic Chromosomal Aberration Rates in Patients with Recurrent Pregnancy Loss

Nozomi Ouchi*, Toshiyuki Takeshita*, Sayuri Kasano, Ryoko Yokote, Mirei Yonezawa, Ryuhei Kurashina, Tomoko Ichikawa, Ikuno Kawabata and Yoshimitsu Kuwabara

Department of Obstetrics and Gynecology, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan

Background: Miscarriage occurs in 10-15% of pregnancies and recurrent pregnancy loss (RPL) occurs in 1% of couples hoping for a child. Various risk factors, such as thrombophilia, uterine malformation, and embryonic chromosomal aberration cause RPL. We hypothesized that antithrombotic therapy for RPL patients with thrombophilia would reduce miscarriage due to thrombophilia, which would reduce the total miscarriages and result in a relative increase in miscarriage due to embryonic chromosomal aberrations. In this study, we investigated the incidence of chromosomal aberrations in products of conception in RPL patients with and without antithrombotic therapy.

Methods: We performed a single-center, retrospective review of cases diagnosed as miscarriage with embryo chromosome analysis between July 1, 2000, and May 31, 2019. Rates of chromosomal aberration were compared between RPL patients with and without thrombophilia or antithrombotic therapy.

Results: One hundred and-ninety RPL cases were analyzed. The average age was 37.4 ± 4.3 years, and the average number of previous pregnancy losses was 2.2 ± 1.1. The overall chromosomal aberration rate was 67.4% (128/190). There was no difference in the chromosomal aberration rate between the factors for RPL, with or without thrombophilia, and antithrombotic therapy. Only advancing maternal age had significant correlation to increased embryo chromosomal aberration rates.

Conclusions: With or without antithrombotic therapy, miscarriage was caused by embryonic chromosome abnormalities at a certain rate. Antithrombotic therapy in RPL patients with thrombophilia may reduce abortions due to thrombophilia, which may also normalize the rate of embryonic chromosome aberrations in the subsequent miscarriages. (J Nippon Med Sch 2022; 89: 40–46)

Key words: abortion, chromosome aberrations, aspirin, heparin, antiphospholipid antibodies

Introduction

Miscarriage occurs in approximately 10 to 15% of pregnancies[1,2], and the majority of early pregnancy losses are caused by embryonic chromosomal abnormalities[3–6].

It is well known that fetal chromosomes are largely affected by maternal age. Grande et al. reported that the chromosomal aberration rate of products of conception was 30% in patients below 25 years of age, and 89% for those over 39 years old[7]. Recently, paternal age, as well as maternal age, has been reported to increase the odds of spontaneous abortion[8], although a relationship between paternal age and embryonic chromosome abnormalities has not yet been established.

Recurrent pregnancy loss (RPL) is defined as two or more failed pregnancies by the American Society of Reproductive Medicine[9] and the European Society of Human Reproduction and Embryology[10]. Recurrent miscarriage, historically defined as three or more consecutive

* These two authors contributed equally to this work.
Correspondence to Toshiyuki Takeshita, MD, PhD, Department of Obstetrics and Gynecology, Graduate School of Medicine, Nippon Medical School, 1–1–5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan
E-mail: toshimac@nms.ac.jp
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abortions, is said to occur in 1% of couples hoping for a child[11]. The risk of further miscarriage increases after each successive pregnancy loss[12], from approximately 5-9% with no losses, to 12% after one, to 20% after two, and increasing up to 40-45% after three or more successive pregnancy losses[11,13,14]. However, the prevalence of abnormal results in evidence-based and investigative diagnostic tests does not differ among women with different numbers of pregnancy losses[15]. Thus, it is reasonable to start evaluation and treatment for RPL after two consecutive miscarriages[16]. The risk factors for RPL vary, but in a study done in Japan among 16 facilities, the risks were as follows: positive antiphospholipid antibodies, 8.7%; malformation of the uterus, 7.9%; thyroid dysfunction, 9.5%; parental karyotype abnormality (i.e. balanced translocation, 3.7%); factor XII deficiency, 7.6%; protein S deficiency, 4.3%; and unknown risk factors, 65.1%[17]. Among these conditions, patients with antiphospholipid syndrome are treated with low-dose aspirin and heparin combination therapy (LDA+Hep) in the subsequent pregnancy. Moreover, patients with thrombus predisposition, such as slightly positive antiphospholipid antibody or factor XII deficiency, are often treated with low-dose aspirin monotherapy (LDA) in Japan.

We hypothesized that antithrombotic therapy for RPL patients with thrombophilia would reduce the number of miscarriages due to thrombophilia, which would reduce the total number of miscarriages and result in a relative increase in miscarriage due to embryonic chromosomal aberrations.

Therefore, in this study, we investigated the incidence of chromosome abnormalities in products of conception in RPL patients with and without antithrombotic therapy.

Material and Methods

Cases

Three hundred and ten cases diagnosed as missed abortion with embryo chromosome analysis performed between July 1, 2000, and May 31, 2019, at Nippon Medical School Hospital were included in this study. In 14 cases, no results were obtained due to poor culture. In 83 cases, the medical record was not complete. These cases were excluded. We also excluded cases of sporadic abortion, twin abortion, hydatidiform mole, and fetal structural anomalies (i.e., bodystalk anomaly). Thus, 190 enrolled cases were analyzed due to the risk factors for RPL and chromosomal aberration. The study protocol was approved by the Ethical Committee of Nippon Medical School (approval number 30-05). All chromosomal analyses were performed with written informed consent obtained from patients.

Antithrombotic Therapy

LDA

Eighty-seven cases were administered LDA. The indications for LDA were positive tests for mild antiphospholipid antibodies, factor XII deficiency, increased platelet aggregation, protein C deficiency, and Protein S deficiency. Patients received LDA (100 mg/day) after ovulation until the diagnosis of abortion.

LDA + Hep

Thirty-three cases were treated with LDA + Hep. The indications for LDA + Hep included antiphospholipid syndrome, severe thrombophilia, and a history of thrombosis. Patients received LDA (100 mg/day) after ovulation and unfractionated heparin (10,000 unit/day) after pregnancy.

Classical and Non-Classical Antiphospholipid Antibodies

Classically, antiphospholipid antibody is defined as lupus anticoagulant, anti-cardiolipin antibody, immunoglobulin G, immunoglobulin M, and anti-CL β2 glycoprotein I complex antibody[18]. We considered antiphosphatidylethanolamine antibody Immunoglobulin G, Immunoglobulin M, and anti-phosphatidylserine / prothrombin antibody as other risk factors for thrombophilia and these were defined as non-classical antiphospholipid antibodies that were treated with LDA+Hep.

Chromosome Analyses of Chorionic Villi

Chorionic villi were collected at the time of surgical management of miscarriages under the diagnosis of missed abortion. Surgical management of miscarriages was performed with manual vacuum aspiration or dilation and evacuation. The gestational period at the time of surgery ranged from 6 to 16 weeks.

All specimens were cut into 1 cm cubes and washed carefully with normal saline to remove maternal blood and decidua under a stereomicroscope. These cubes were then collected in a sterile container of 10 mL of AmnioMAX complete medium (Gibco®, ThermoFisher Scientific, Dreieich, Germany). Cytogenetic analysis was performed using the G-banding method. Dissected chorionic villi were enzymatically digested with Trypsin-EDTA (Gibco/Life Technologies, Waltham, MA, USA) and collagenase Type 2 (Worthington Biochemical, Lakewood, NJ, USA) then cultured in α-MEM complete medium (Gibco/Life Technologies, Waltham, MA, USA) at 37 °C in a 5% CO2 incubator. At least 20 metaphases were analyzed using the G-banding method for each case.

Statistical Analysis

Statistical tests were performed using IBM SPSS ver. 12 (International Business Machines Corp., Armonk, NY, USA) and JMP Pro 9 (SAS Institute Inc., Cary, NC, USA). Significant differences in the experiments using the present study were determined by one-way ANOVA, Welch's *t* test, Mann-Whitney's U test, and Chi-squared test. All values are expressed as mean ± standard deviation. P-values < than 0.05 were considered statistically significant.

Table 1 Case characteristics

total case	190
maternal age (years)	37.4±4.3
gestational age (weeks)	8.5±1.9
previous abortions (times)	2.2±1.1
previous delivery (times)	0.2±0.7
case of non-pharmacotherapy (%)	70 (36.8)
case of low dose aspirin monotherapy (%)	87 (45.8)
case of low dose aspirin + heparin combined therapy (%)	33 (17.4)

Results

As shown in **Table 1**, the average age of patients was 37.4 ± 4.3 years, and the average number of previous pregnancy losses was 2.2 ± 1.1. Seventy cases received no medications, 87 cases were administered LDA, and 33 cases were administered LDA+Hep. The indication for RPL treatment and the number of cases per indication are shown in **Supplementary Table 1** (https://doi.org/10.1272/jnms.JNMS.2022_89-103).

Among the products of conception from women with RPL, 67.4% (128/190) had abnormal and 32.6% (62/190) had normal chromosomal analyses.

As shown in **Table 2**, the cases were divided into two groups according to the presence of an embryonic chromosomal abnormality. While there was no significant difference in the number of previous miscarriages (2.14 ± 0.99 times vs. 2.37 ± 1.28 times, p = 0.42), the maternal age was significantly older in the embryonic chromosomal aberration group (38.15 ± 4.34 years vs. 35.95 ± 3.91 years, p <0.001).

Table 3 shows the comparison between chromosomal aberration rates in those with or without thrombophilia and antithrombotic therapy adjusted by age. There was

Table 2 Patients Characteristics with and without embryo chromosome aberrations

	Chromosomal Aberrations N=128	No Aberrations N=62	P value
Maternal Age (years)	38.15±4.34	35.95±3.91	0.0006*
No. of previous miscarriages and/or stillbirth (n)	2.14±0.99	2.37±1.28	0.4207**

*Welch's *t* test

**Mann-Whitney's U test

Table 3 Chromosomal aberration rates with or without thrombophilia or antithrombotic therapy

Variable	YES	NO	P value	Odds Ratio	95CI
with one or more risk factors for RPL [a]	44/65 (67.7)	84/125 (67.2)	0.9453	1.0227	0.5392 – 1.9396
antiphospholipid syndrome [b]	16/23 (69.6)	112/167 (67.1)	0.8106	1.1224	0.4363 – 2.8878
Positive tests of APL (classical) [c]	38/54 (70.4)	90/136 (66.2)	0.5781	1.2139	0.6127 – 2.4048
Positive tests of APL (non-classical) [d]	24/34 (70.6)	104/156 (66.7)	0.6586	1.2000	0.5342 – 2.6957
protein S deficiency	12/16 (75.0)	75/116 (64.7)	0.4132	1.6400	0.4970 – 5.4119
fXII deficiency	33/48 (68.8)	55/85 (64.7)	0.6359	1.2000	0.5639 – 2.5538
antithrombotic therapy [e]	80/120 (66.7)	48/70 (68.6)	0.7871	0.9167	0.4875 – 1.7236
heparin therapy	24/33 (72.7)	104/157 (66.2)	0.4701	1.3250	0.5949 – 2.9513

a. Risk factors includes antiphospholipid syndrome, congenital uterine anomaly and parental chromosomal structural rearrangement.

b. Antiphospholipid syndrome includes seronegative antiphospholipid syndrome.

c. Lupus anticoagulant, anticardiolipin IgG, IgM antibodies, anti-β2GPI antibodies.

d. Antiphosphatidylethanolamine IgG, IgM antibodies, antiphosphatidylserine/prothrombin antibodies

e. Antithrombotic therapy includes LDA and LDA plus heparin

Table 4 Embryonic karyotype of abortuses after antithrombotic therapy for the prevention of miscarriage in patients with antiphospholipid syndrome

maternal age	no. of previous miscarriages and/or stillbirths	parity	Antiphospholipid antibodies	Other thrombophilias	karyotype1	karyotype2	karyotype3
45	4	0	LA		47, XX, +22		
44	2	0	aCLIgG		47, XY, +8,i (14) (q10)		
43	3	0	LA		47, XY, +10		
41	2	0	aCLIgG		47, XY, +16		
39	3	0	aCLIgG		48, XX, +13, +20		
39	4	0	aCLIgG	fXII deficiency	47, XX, +22		
38	2	0	aCLIgG		46, XY, add (5) (p14)	46, XY, del (5) (p14)	46, XY
36	4	1	aCLIgG, aCLIgM	fXII deficiency	47, XY, +15		
36	1	0	aCLIgG		47, XY, +16		
36	3	1	aCLIgM		47, XX, +22	46, XX	
47	2	0	aPEIgG		47, XX, +22		
41	2	0	aPEIgM		47, XX, +22		
41	3	1	aPS/PT	protein S deficiency	48, XY, +15, +20		
35	2	0	aCLIgG, aPEIgM	fXII deficiency	45, XX, -22	46, XX	
43	4	0	aCLIgG		47, XX, +2	46, XX	
38	2	1	aPEIgM	fXII deficiency	47, XY, +13		
41	4	0	aCLIgM		46, XX		
40	1	0	aCLIgG	fXII deficiency	46, XX		
38	2	0	aCLIgG		46, XX		
32	3	1	aCLIgM		46, XX		
30	1	0	LA, aCLIgG, aCLβ2GPI		46, XY		
33	4	1	aPS/PT	protein C deficiency, protein S deficiency	46, XY		
28	3	0	aPEIgM	fXII deficiency	46, XX		

no difference in the chromosomal aberration rate with or without any one or more of the risk factors for RPL (antiphospholipid syndrome, congenital uterine anomaly, and parental structural chromosome rearrangement). We also found no difference in the chromosomal aberration rate correlating to those with or without thrombophilia, such as antiphospholipid antibody syndrome, factor XII deficiency, and protein S deficiency. In addition, the embryonic chromosomal aberration rate in the antithrombotic therapy (LDA+Hep) group was 66.7%, which was not different from 68.6% in the group without antithrombotic therapy. Furthermore, there was no difference in the chromosomal aberration rate between the group with and without heparin therapy (72.7% vs. %66.2, P=0.4701). There was also no significant difference in maternal age or gestational weeks of miscarriage among the non-pharmacotherapy, LDA, and LDA + Hep groups, though the number of previous miscarriages was significantly lower in the non-pharmacotherapy group ($p < 0.01$).

Table 4 shows 23 cases of antiphospholipid syndrome (including seronegative antiphospholipid syndrome) who miscarried despite LDA + Hep therapy. The average maternal age was 38.4 ± 4.7 years. The average number of previous pregnancy losses was 2.6 ± 1.0. Embryonic chromosomal aberrations were observed in 16 of 23 cases (69.6%). Maternal age was significantly higher in the chromosomal aberration group (p=0.0245) (**Fig. 1**).

As for the course after miscarriage, among 22 cases with no pharmacotherapy and normal embryonic chromosomes, 10 patients planned to receive additional treatment such as LDA therapy in the subsequent pregnancy. Live births were obtained in 5 of 12 cases (41.7%) who did not receive additional treatment and in 7 of 10 cases (70%) who received additional treatment (Supplementary Fig. 1; https://doi.org/10.1272/jnms.JNMS.2022_89-103). Among 42 cases with normal embryonic chromosomes using antithrombotic therapy (LDA or LDA + Hep), subsequent pregnancy was confirmed in 24 cases and 13 cases obtained live births without changing the treatment policy. Eight patients had live births after additional treatment, such as globulin, heparin, and prednisolone.

Discussion

In this study, there was no significant difference in the

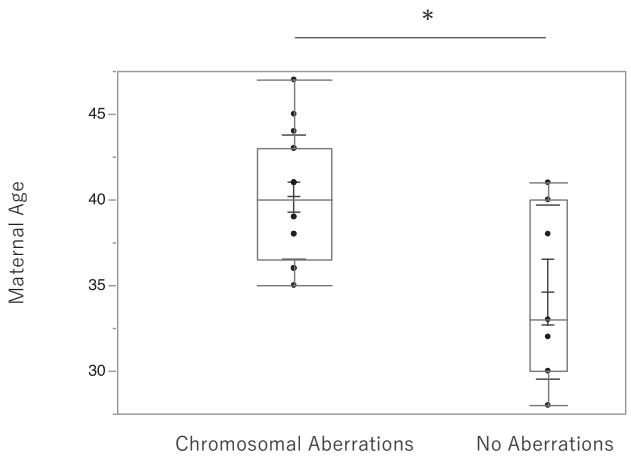

Fig. 1 Comparison of maternal age in patients with and without embryonal chromosomal aberrations after antithrombotic therapy for the prevention of miscarriage in patients with antiphospholipid syndrome
Maternal age was significantly higher in the group with chromosomal aberrations. Statistical analysis was performed using the Mann-Whitney U test. * P<0.05.

chromosomal aberration rate of products of conception with or without thrombophilia, even after antithrombotic therapy. We expected that most of the causes of miscarriage would be due to embryonic chromosomal abnormalities when thrombotic status was well controlled by appropriate antithrombotic therapy, however the fact was different.

Miscarriage rates generally increase with maternal age. The lowest risk of first trimester loss was in women in their 20s with an approximate risk of 8-10%, whereas in women aged 35-40 it varied from 17-25%, in women aged 40-45 it varied from 33-51%, and reached 57-75% in women aged above 45[19,20].

One of the causes of the increase in miscarriage rate with maternal age is the increase in the pregnancy rate of fetuses with chromosomal abnormalities. The risk for trisomy pregnancy increases with increasing maternal age[21]. Whereas sex chromosomal abnormalities, including Turner's syndrome or triploidy, are not influenced by maternal age as they are not secondary to maternal meiotic non-disjunction[21,22].

There are various reports on the association between the number of successive miscarriages and the rate of embryo chromosomal aberrations. In a report by Lan Yang et al., the rate of fetal chromosome abnormalities did not differ between their sporadic miscarriage group and recurrent spontaneous abortion group[23]. Stern et al. also reported that there was no difference in the frequency of abnormal karyotypes among abortuses from women with and without a history of recurrent spontaneous abortion (57%)[2]. On the other hand, according to a review by Zhang et al., the fetal chromosomal aberration rate in spontaneous abortion was 49.7%, but decreased in recurrent miscarriage to 40.4%[24]. In our previous report, the fetal chromosomal aberration rate decreased as the number of maternal past miscarriages increased as well, with the fetal chromosomal aberration rate being 72.4% for two previous miscarriages, 67.9% for three, and 58.5% for four or more[25]. Ogasawara et al. also reported that the fetal chromosomal aberration rate decreased as the number of miscarriages increased[4]. However, these studies have no comments regarding treatment and medication for recurrent miscarriage. It was suggested that as the number of miscarriages increases, miscarriages due to factors other than chromosomal abnormalities may increase.

According to Popescu et al., among patients with more than two pregnancy losses, 67% had abnormal microarray analysis and 74.6% of them had no RPL risk factors (such as antiphospholipid syndrome, congenital uterine anomaly, and parental chromosomal structural rearrangement). In contrast, 33% had normal microarray analysis and 84.8% of them had abnormal RPL workup[16]. In cases of recurrent miscarriage with normal fetal chromosomes, there is a high probability that RPL treatment is required, and scrutiny should be carried out.

In this study, embryonic chromosomal aberration rates were compared between patients with and without antithrombotic therapy and between those with and without antiphospholipid syndrome. No difference in chromosomal aberration rates was observed and the rates were between 64.7-75.0% in all groups. This abnormality rate was almost the same as or slightly higher than the chromosomal abnormality rate at the time of spontaneous abortion. Previous studies have also found differences in the fetal chromosomal aberration rate depending on the average maternal age of the population. Many studies on the population in the early 30s have a fetal chromosomal aberration rate in the 50% range[4]. However, Popescu et al. reported an aberration rate as high as 67% in a population averaged 35.7 years (ranged 26-45 years old)[16]. Lathi et al. reported rates of 63% in a population averaged 37.2 years (ranged 29-41 years old) [26]. Studies on populations in late 30s report aberration rates in the 60% range[5,6]. In addition, Du et al. reported that embryo chromosomal aberration rates increase with maternal age: 45.83% in age 20-24, 43.07% in age 25-29, 45.35% in age 30-34, 58.46% in age 35-39, and 75.51% in age 40 and

above [6]. The present study was conducted at a university hospital in Tokyo, Japan, due to which the age of the research population was high[27], averaging 37.4 years (range 26-47 years). In addition, there were 63 cases aged 40 years or older, accounting for one-third of the total. This is probably the reason why the embryo chromosome abnormality rate was high in our study.

In this study, the number of previous miscarriages was higher in the antithrombotic therapy group than in the non-pharmacotherapy group. Naturally, the rate of embryonic chromosomal aberrations should be lower in the antithrombotic group based on previous reports correlating the number of previous miscarriages and aberration rates. However, the embryo chromosomal aberration rate was similar between the antithrombotic and non-pharmacotherapy groups in this study. This may be because miscarriage factors other than the embryonic chromosomal abnormality, which may have increased as the number of miscarriages increased, were normalized with the use of LDA or LDA + Hep.

It may be considered that miscarriage with chromosomally normal cases found in both the antithrombotic therapy group and the non-pharmacotherapy group included miscarriage due to factors that had not been elucidated at the time of this study. The possibility of inflammation involving the uterine cavity, immune function, environmental poisons like tobacco, and vitamin deficiency may also be considered as causes of recurrent miscarriage in future[28,29].

As a limitation, approximately 30% of this study had normal villous chromosomes, though some of them may have been false negative villous chromosome results due to maternal tissue contamination. According to a report by Ruth et al., embryo chromosome tests normally result in 2.4 times more 46XX than 46XY. However, when compared with the DNA in the maternal blood, about 60% of the 46XX results did not contain fetal tissue. Excluding these, the sex of miscarried infants was 1:1[30]. In our study, results with 46XX were 3.2 times more than 46XY, so some of these results may not have accurately depicted embryonic chromosome.

In conclusion, regardless of miscarriage patients being with or without antithrombotic therapy, there was no significant difference in the percentage of embryonic chromosome abnormalities. In other words, with or without antithrombotic therapy, miscarriage was caused by embryonic chromosome aberration at a certain rate.

In addition, we understand that there are refractory RPL patients who have repeated miscarriage even after receiving RPL treatment. However, live births were obtained eventually in many cases of refractory RPL, so we must consider that not giving up is very important.

Conflict of Interest: None of the authors have anything to disclose. The authors report no conflicts of interest.

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