was at least one fetus with oliguric oligohydramnios and at least one fetus with polyuric polyhydramnios. FLP was offered between 16 and 25 weeks. Preoperative ultrasound evaluation including fetal biometry and amniotic fluid volume estimation was performed, followed by a color and pulsed Doppler examination. In the Doppler studies, the occurrence of absent or reversed end-diastolic velocity in the umbilical artery, reverse flow in the ductus venosus, and pulsatile flow in the umbilical vein was recorded. The presence of fetal hydropic signs and placental location were also noted. This allowed to stage patients according to the Quintero staging system.[19]

Fetoscopic laser photocoagulation was performed on the basis of previously described methods for twin gestations.[10] Under regional or local anesthesia, a 3.8-mm trocar was percutaneously inserted into the recipient sac. A 3.5-mm diagnostic fetoscope and a 3-mm operating fetoscope (Richard Wolf, Knittlingen, Germany) or a 2-mm fetoscope with a 3-mm cannula (Karl Storz, Tuttlingen, Germany) were passed through a trocar. All vascular anastomoses between the fetuses were interrupted selectively using a Nd:YAG laser. In cases of MT triplets, identifying and coagulating all placental vascular anastomoses was attempted. Additional interventions were carried out at the discretion of the attending surgeon. When complete ablation of all placental vascular equators between the three triplets was technically difficult, only the vascular equator between the fetus identified as the donor and the one identified as the recipient was interrupted by laser. The amniotic fluid was subsequently drained through the cannula until the MVP reached <8 cm. All patients gave written informed consent to undergo FLP. Perioperative tocolysis as well as prophylactic antibiotics were provided. Postoperative care with weekly ultrasound evaluation, including pulsed Doppler assessment and delivery and neonatal management was provided by the referring perinatal centers. Delivery was determined according to obstetrical indications by the attending physician. Neonates were routinely examined by cerebral ultrasonography. Magnetic resonance imaging was performed when clinical examination or ultrasonography was suggestive of an abnormality. All infants were examined by neonatologists at 28 days of age. A color dye injection test of the placenta was prospectively planned to investigate the presence or absence of residual vascular anastomoses for the cases of surviving triplets that were involved in FFTS.

The outcome measurements were the rate of surviving triplets, the survival of at least one triplet, and the rate of major neurological complications in survivors at 28 days of age. Major neurological complications were defined as severe intraventricular hemorrhage (grade 3 or 4) and/or cystic periventricular leukomalacia.

RESULTS

Sixteen cases of triplet gestations, including nine DT and seven MT triplets, underwent FLP during the study period. Perioperative information is presented in Table 1. The placenta was anterior in six cases (three DT and three MT triplet gestations). Ten cases were classified as Quintero stage III or IV.[19]

For all 16 cases, FLP was completed without major maternal complications. The median gestational age at surgery was 21 weeks (range: 16–25 weeks) for DT cases and 20 weeks (range: 17–22 weeks) for MT cases. The median surgical time was 35 min (range: 25–75 min) for DT and 73 min (range: 58–125 min) for MT cases. For MT triplets, various measures were applied to complete the surgical procedure. Trocar assistance to displace the fetus was essential for the identification of vascular anastomoses obscured by the donor fetus in two cases of MT.[20] In another MT case, an intentional septostomy of the dividing membrane was performed to facilitate inspection of the entire vascular equator. Double uterine entry with trocars was used; one trocar was inserted into the sac of the recipient fetus, and the other was inserted into the sac of the nonaffected fetus with normal amniotic fluid volume. In this case, the two umbilical cords of the recipient fetus and the umbilical cord of the nonaffected fetus arose in close proximity to each other and were bound together within the dividing membrane. All vessels between the cords were shared ones, hence not truly defining a vascular equator. Therefore, only two vascular equators between the recipient fetus and the donor fetus, as well as between the nonaffected fetus and the donor fetus were ablated at surgery. In another case of MT triplets, comprising two donor fetuses and one recipient, the vascular equator between the donor fetus A and the donor fetus B was hidden beneath the donor fetus A; therefore, the vascular communications between the recipient fetus and the donor fetus A as well as the communications between the recipient fetus and the donor fetus B were interrupted.

Table 1 Perioperative data in cases of triplet gestations with feto-fetal transfusion syndrome that underwent fetoscopic laser photocoagulation

	DT (n=9)	MT (n=7)
Age, median [range]	32 [20–40]	30 [29–38]
Nulliparous, *n* (%)	7 (77.8)	4 (57.1)
ART, *n* (%)	3 (33.3)	0 (0.0)
Quintero stage, *n*		
I	2	0
II	1	3
III	6	3
IV	0	1
Anterior placenta, *n* (%)	3 (33.3)	3 (42.9)
Gestational age of FLP (weeks), median [range]	21 [16–25]	20 [17–22]
Operation time (minutes), median [range]	35 [25–75]	73 [58–125]

ART, assisted reproductive technology; FLP, fetoscopic laser photocoagulation.

Perinatal outcomes are presented in Table 2 on the basis of chorionicity. The detailed data of MT cases are presented in Table 3. There were no cases with persistence or recurrence of FFTS following FLP. However, in one DT case, twin anemia-polycythemia sequence developed after the procedure.[21] Chorioamniotic membrane separation after FLP was noted in one DT and one MT case. Among 27 fetuses involved in the DT, fetal demises occurred in six triplets (22%), who were all involved in FFTS. However, just one (5%)

Table 2 **Pregnancy and perinatal outcome at 28 days of age**

	DT (n=9)	MT (n=7)
GA of delivery (weeks), median [range]	32 [27–34]	29 [23–34]
Preterm birth		
<28 weeks, *n* (%)	1 (11.1)	1 (14.3)
<32 weeks, *n* (%)	4 (44.4)	5 (71.4)
Fetal demise, *n* (%)	6/27 (22.2)	1/21 (4.8)
One fetal demise, *n* (%)	2 (22.2)	1 (14.3)
Two fetal demise, *n* (%)	2 (22.2)	0 (0.0)
Three fetal demise, *n* (%)	0 (0.0)	0 (0.0)
ELBW infants, *n* (%)	2/21(9.5%)	8/20 (40.0%)
Neonatal death, *n*(%)	1/27 (22.2)	0/21 (0.0)
Grade III or IV IVH, *n*(%)	0 (0.0)	2/21 (9.5)
Cystic PVL, *n* (%)	0 (0.0)	0 (0.0)
Over all perinatal survival, *n* (%)	20/27 (74.1)	20/21 (95.2)
Three survival, *n* (%)	4 (44.4)	6 (85.7)
Two survival, *n* (%)	3 (33.3)	1 (14.3)
One survival, *n* (%)	2 (22.2)	0 (0.0)
Zero survival, *n* (%)	0 (0.0)	0 (0.0)
At least one survival, *n* (%)	9 (100.0)	7 (100.0)
Over all intact survival, *n* (%)	20/27 (74.1)	18/21 (85.7)

GA, gestational age; ELBW, extremely low birth weight less than 1000 g; IVH, intraventricular hemorrhage; PVL, periventricular leukomalasia.

of 21 MT triplets died *in utero* (case 2; Table 3). Double fetal demise occurred in two DT cases. The cause of death of one fetus appeared to be related to iatrogenic twin anemia-polycythemia sequence. However, the other demise could not be explained. The median gestational age at delivery was 32 weeks (range: 27–34 weeks) for DT cases and 29 weeks (range: 23–34 weeks) for MT cases. The incidence of preterm delivery <28 weeks was 11% (1/9) in DT and 14% (1/7) in MT; the incidence of preterm delivery <32 weeks was 44%(n=4) of nine in DT and 71% (5/7) in MT. The number of extremely low birth weight infants (<1000 g) was two (10%) of 21 live-born DT triplets and eight (40%) of 20 live-born MT triplets. All but one case of double fetal demise was delivered by cesarean section. One DT infant died in the neonatal period because of prematurity. Overall, perinatal survival at 28 days for DT cases was 74%, whereas it was 95% for MT triplets. At least one fetus survived in all 16 cases; however, all three survived till 28 days of age in 44% of DT cases and 86% of MT cases. Two MT neonates, born with extremely low birth weight, suffered severe intraventricular hemorrhages (cases 5 and 7; Table 3). The survival rates without neurological complications at 28 days of age were 74% for DT cases and 86% of MT triplet cases. One neonate required surgical treatment for a pseudo-amniotic band syndrome around an ankle due to the entrapment of the dividing membrane subsequent to intentional septostomy during FLP (case 1; Table 3).[22] A color dye injection test of the chorionic vessels was performed for four DT cases and six MT cases, in which all triplets involved

Table 3 **Detailed data on cases of monochorionic triamniotic triplets**

No	Quintero stage	Placental location	GA of FLP	Operation time (minutes)	Additional information on FLP	GA of delivery	Triplet A			Triplet B			Triplet C			Survival (n)
								BW	Outcome		BW	Outcome		BW	Outcome	
1	2	A	19	81	Intentional septostomy	29	R	1302	A and W	D	984	A and W	N	1270	A and W (PABS)	3
2	3	P	22	73	Trocar assistance	34	R	1614	A and W	D	-	FD	D	1762	A and W	2
3	4	P	18	84	Trocar assistance	29	R	916	A and W	D	1065	A and W	D	850	A and W	3
4	3	P	22	70		31	R	1264	A and W	D	1306	A and W	N	1328	A and W	3
5	2	P	20	125		28	R	963	A (IVH 3)	D	1052	A and W	N	957	A and W	3
6	2	A	20	58	VE between B and C could not be identified	32	R	1326	A and W	R	1372	A and W	D	1402	A and W	3
7	3	A	17	67	Two uterine entries. The UCs of A and C arose closely	23	R	670	A and W	D	590	A and W	N	568	A (IVH 4)	3

A, anterior; P, posterior; GA, gestational age (weeks); min, minutes; VE, vascular equator; UC, umbilical cord; R, recipient; D, donor; N, non affected triplet; BW, birth weight (gram); outcome, perinatal outcome at 28 days of age; A, alive; W, well; FD, fetal demise; IVH, intra-ventricular hemorrhage; PABS, pseudo amniotic band syndrome.

in FFTS resulted in live-birth. No patent placental vascular communication could be detected.

DISCUSSION

We describe our experience using FLP for FFTS in triplet gestations. The procedure could be completed in all cases without major maternal complications. As a consequence, all pregnancies ended with at least one survivor. In DT cases, the overall 28 days survival rate was 74%, and the intact survival rate was 74%. In MT triplets, the overall 28 days survival rate was 95%, and the intact survival rate was 86%.

Compared with trichorionic triamniotic triplet gestations, DT and MT triplet gestations have a higher perinatal mortality and morbidity.[1–3] Especially for MT, the perinatal mortality risk is 2.6 times higher than trichorionic triamniotic gestations.[3] In addition to a high incidence of premature birth, the specific cause of poor perinatal outcome of monochorionic triplets is thought to be chorionic vascular communications that can cause FFTS or other complications.[4–7] The actual incidence of FFTS in DT or MT triplet gestations is not well known; however, it is estimated to be up to 10%, which is similar to that in monochorionic twins.[3–5] FFTS in triplet gestations share adverse perinatal outcomes such as perinatal death and the neurological morbidity of typical twin–twin transfusion syndrome.[4–7] Survival rate with expectant management for FFTS in triplets was 36% according to review of the literature.[6] Because of the poor prognosis, selective feticide by umbilical cord coagulation has been applied to multiple pregnancies with FFTS.[6,7,13,14] In MT triplets, the third fetus can either act as the donor or recipient; it also can be apparently normal in terms of amniotic fluid volume. Currently, FLP can produce a favorable perinatal outcome, a high survival rate, and a lower neurological complication rate than what is reported for serial amnioreduction.[8–10] However, because reports of FLP in triplet gestations are limited, more data are welcomed.[6,13,15–18]

In six reports, the perinatal outcome in DT triplets that underwent FLP was similar to that of twins.[6,13,15–18] A meta-analysis[6] found that the survival rate among 237 DT triplets after FLP was 78% and that this was better than the rate reported from selective feticide or amniodrainage. According to the six studies, including ours, which reported to the prevalence of neurological complications among the surviving triplets up to at least 28 days of observation, the intact survival rate was 72.0% (range: 61.9–79.2%).[6,13,15–18]

However, the outcomes for MT triplets after FLP reported in earlier studies were less favorable. This may be due to technical limitations preventing achievement of complete obliteration of the vascular anastomoses.[6,13,15,16,18] Some authors raised questions about the feasibility and efficacy of FLP for MT triplets from their experiences and a literature review.[6,15,16] However, our results, in line with another study from the United States, demonstrated that FLP for MT triplets was feasible and as effective as it was for DT.[17] The survival rate at 28 days of 21 MT triplets in our study was 95%, higher than the 54% rate found with a literature review of 48 cases of MT triplets.[6] Because there can be vascular anastomoses between all fetuses in MT triplet gestations, the surgical technical issues can be more challenging. To complete coagulation of all communications among all triplets, we employed a variety of surgical techniques performed at the discretion of the surgeon; these included the use of the trocar to displace a donor fetus (cases 2 and 3; Table 3),[20] double uterine entry (case 7; Table 3), and intentional septostomy of the dividing membrane to enter the other sac (case 1; Table 3). We felt that these techniques facilitated identification of the vascular equator. Another reported technique that can ensure a complete procedure was to use amnioinfusion to create polyhydramnios in a sac for another uterine entry and amnioreduction to collapse a sac for a transmembranous identification of vascular communications from another sac.[17] However, these invasive maneuvers may increase the risk of premature delivery. In fact, the gestational age at delivery tended to be earlier for MT triplets compared with DT triplets.[17] A major limitation of our study may be the lack of the data on the long-term outcome of triplets. The long-term outcome of twins after laser surgery for twin–twin transfusion syndrome seems to be worse than that of those with short-term follow-up.[23] The long-term neurological status among twins after laser surgery could be affected by lower gestational age at birth and lower birth weight.[24]

CONCLUSION

In our hands, FLP was feasible and effective both in DT and MT triplets.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- The feasibility and efficacy of laser therapy for feto-fetal transfusion syndrome in dichorionic triplets has been recognized; however, because of technical difficulty, its qualities remain undetermined for monochorionic triplets.

WHAT DOES THIS STUDY ADD?

- Laser therapy for feto-fetal transfusion syndrome in both monochorionic and dichorionic triplets might be successful.
- We describe additional technical maneuvers that can fully evaluate the vascular equator between the triplets.

REFERENCES

1. Adegbite AL, Ward SB, Bajoria R. Perinatal outcome of spontaneously conceived triplet pregnancies in relation to chorionicity. Am J Obstet Gynecol 2005;193:1463–71.
2. Geipel A, Berg C, Katalinic A, *et al.* Prenatal diagnosis and obstetric outcomes in triplet pregnancies in relation to chorionicity. BJOG 2005;112:554–8.
3. Kawaguchi H, Ishii K, Yamamoto R, *et al.* Perinatal death of triplet pregnancies by chorionicity. Am J Obstet Gynecol 2013;209(1):36.e1–e7.
4. Lewi L, Jani J, Blickstein I, *et al.* The outcome of monochorionic diamniotic twin gestations in the era of invasive fetal therapy: a prospective cohort study. Am J Obstet Gynecol 2008;199:514 e1–8.

5. Nakayama S, Ishii K, Kawaguchi H, *et al.* Perinatal outcome of monochorionic diamniotic twin pregnancies managed from early gestation at a single center. J Obstet Gynaecol Res 2012;38:692–7.
6. Peeters SH, Middeldorp JM, Lopriore E, *et al.* Monochorionic triplets complicated by fetofetal transfusion syndrome: a case series and review of the literature. Fetal Diagn Ther 2012;32:239–45.
7. Chasen ST, Al-Kouatly HB, Ballabh P, *et al.* Outcomes of dichorionic triplet pregnancies. Am J Obstet Gynecol 2002;186:765–7.
8. Senat MV, Deprest J, Boulvain M, *et al.* Endoscopic laser surgery versus serial amnioreduction for severe twin-to-twin transfusion syndrome. N Engl J Med 2004;351:136–44.
9. Roberts D, Neilson JP, Kilby M, *et al.* Interventions for the treatment of twin–twin transfusion syndrome. Cochrane Database Syst Rev 2008;1:CD002073.
10. Sago H, Hayashi S, Saito M, *et al.* The outcome and prognostic factors of twin–twin transfusion syndrome following fetoscopic laser surgery. Prenat Diagn 2010;30:1185–91.
11. Hayashi A, Kikuchi A, Joshita N, *et al.* Monochorionic triplet pregnancy complicated by severe fetofetal transfusion. J Obstet Gynaecol Res 2005;31:414–20.
12. Ling PY, Leo MV, Rodis JF, *et al.* Amnioreduction in triplet fetofetal transfusion. Obstet Gynecol 2000;96:843.
13. Van Schoubroeck D, Lewi L, Ryan G, *et al.* Fetoscopic surgery in triplet pregnancies: a multicenter case series. Am J Obstet Gynecol 2004;191:1529–32.
14. Lewi L, Gratacos E, Ortibus E, *et al.* Pregnancy and infant outcome of 80 consecutive cord coagulations in complicated monochorionic multiple pregnancies. Am J Obstet Gynecol 2006;194:782–9.
15. Sepulveda W, Surerus E, Vandecruys H, *et al.* Fetofetal transfusion syndrome in triplet pregnancies: outcome after endoscopic laser surgery. Am J Obstet Gynecol 2005;192:161–4.
16. De Lia JE, Worthington D, Carr MH, *et al.* Placental laser surgery for severe previable feto-fetal transfusion syndrome in triplet gestation. Am J Perinatol 2009;26:559–64.
17. Chmait RH, Kontopoulos E, Bornick PW, *et al.* Triplets with feto-fetal transfusion syndrome treated with laser ablation: the USFetus experience. J Matern Fetal Neonatal Med 2010;23:361–5.
18. Diemert A, Diehl W, Huber A, *et al.* Laser therapy of twin-to-twin transfusion syndrome in triplet pregnancies. Ultrasound Obstet Gynecol 2010;35:71–4.
19. Quintero RA, Morales WJ, Allen MH, *et al.* Staging of twin–twin transfusion syndrome. J Perinatol 1999;19:550–5.
20. Quintero RA, Chmait RH, Bornick PW, *et al.* Trocar-assisted selective laser photocoagulation of communicating vessels: a technique for the laser treatment of patients with twin–twin transfusion syndrome with inaccessible anterior placentas. J Matern Fetal Neonatal Med 2010;23:330–4.
21. Slaghekke F, Kist WJ, Oepkes D, *et al.* Twin anemia-polycythemia sequence: diagnostic criteria, classification, perinatal management and outcome. Fetal Diagn Ther 2010;27:181–90.
22. Shamshirsaz AA, Shamshirsaz AA, Nayeri UA, *et al.* Pseudoamniotic band syndrome: a rare complication of monochorionic triplets with twin-to-twin transfusion syndrome. Prenat Diagn 2012;32:97–8.
23. Maschke C, Diemert A, Hecher K, *et al.* Long-term outcome after intrauterine laser treatment for twin–twin transfusion syndrome. Prenat Diagn 2011;31:647–53.
24. Lopriore E, Ortibus E, Acosta-Rojas R, *et al.* Risk factors for neurodevelopment impairment in twin–twin transfusion syndrome treated with fetoscopic laser surgery. Obstet Gynecol 2009;113:361–6.

Journal of Clinical Apheresis 00:00–00 (2014)

Brief Report

Successful Treatment of Severe Rhesus D-Incompatible Pregnancy With Repeated Double-Filtration Plasmapheresis

Koichi Kamei,[1]* Koushi Yamaguchi,[2] Mai Sato,[1] Masao Ogura,[1] Shuichi Ito,[1] Tomomi Okada,[2] Seiji Wada,[2] and Haruhiko Sago[2]

[1]Department of Nephrology and Rheumatology, National Center for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan

[2]Center of Maternal-Fetal, Neonatal, and Reproductive Medicine, National Center for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan

Fetal anemia is caused by Rhesus (RhD) sensitization as a result of RhD incompatibility during pregnancy. The severe form of this disease can cause hydrops fetalis leading to intrauterine death. We experienced a highly sensitized 39-year-old woman with B Rh-negative blood. She had a history of three induced abortions and experienced perinatal death associated with hydrops fetalis. During the pregnancy prior to her most recent one, she was treated with double-filtration plasmapheresis (DFPP), high dose γ-globulin and intrauterine fetal blood transfusion (IUT). For her most recent pregnancy, we performed only weekly or fortnightly DFPP from 13 weeks until delivery. Anti-D antibody titer was maintained between 32 and 256 without any signs of fetal anemia. IUT was not required at any stage of the pregnancy. No adverse events were observed. She successfully delivered a healthy male infant weighing 2,289 g by Cesarean section at 35 weeks. Repeated DFPP may be an effective and safe strategy to reduce antibody titers in highly sensitized women with RhD-incompatible pregnancy, avoiding the need for IUT. J. Clin. Apheresis 00:000–000, 2014.

Key words: rhesus D-incompatible pregnancy; double-filtration plasmapheresis; anti-D antibody; fetal anemia; intrauterine fetal blood transfusion

INTRODUCTION

Fetal anemia is a complication of maternal-fetal blood group incompatibility. The most common cause of such incompatibility is Rhesus D (RhD) sensitization. The severe form of this disease may result in hydrops fetalis and intrauterine death [1, 2]. RhD immunoglobulin prophylaxis can dramatically decrease the incidence of hemolytic disease of the fetus. However, this condition has been known to persist despite treatment [1, 2].

We previously reported a case of a highly sensitized female with RhD-incompatible pregnancy who received double-filtration plasmapheresis (DFPP), high-dose γ-globulin and intrauterine fetal blood transfusion (IUT). She delivered an infant at 34 weeks by Caesarean section [3]. Here, we report on this patient again. For her most recent pregnancy, she did not require IUT at any stage due to the use of repeated DFPP.

CASE REPORT

The patient was a 39-year-old female with B Rh-negative blood. She had induced abortion at 19, 21, and 26 years of age, and a Caesarian section at 23 years old for intrauterine fetal death associated with hydrops fetalis (1,090 g) at 28 weeks' gestation. No RhD immunoglobulin prophylaxis was administered during these episodes. At the pregnancy prior to her most recent one, as her anti-D antibody titer was 128 at 6 weeks of gestation, we performed double-filtration plasmapheresis (DFPP) and administered high dose γ-globulin at 15 weeks of gestation. However, anti-D antibody titer started to increase at 25 weeks and reached 1,024 at 27 weeks. Despite additional DFPP being performed five times from 27 weeks, fetal anemia progressed and intrauterine fetal blood transfusion (IUT) was eventually required. At delivery (34 weeks), anti-D antibody titer rose to 4,096.

*Correspondence to: Koichi Kamei; Department of Nephrology and Rheumatology, National Center for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan.
E-mail: kamei-k@ncchd.go.jp.

Received 22 June 2014; Accepted 31 October 2014
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/jca.21372

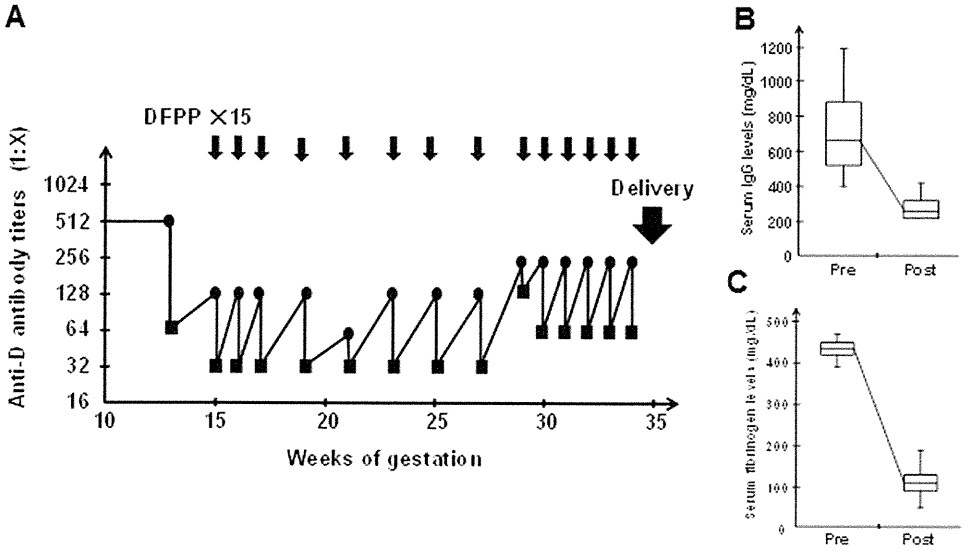

Fig. 1. Clinical course (**A**) Changes of anti-D antibody titers during treatment with DFPP. (**B**) Changes of serum immunoglobulin G (IgG) levels before and after DFPP. (**C**) Changes of serum fibrinogen levels before and after DFPP. (B) and (C) Boxes represent median and interquartile values and whiskers represent maximum and minimum values.

The patient visited our center during her last pregnancy at 10 weeks of gestation at the age of 39, 6 years after the prior pregnancy. At that stage, her anti-D antibody titer was 512. As high dose γ-globulin could not be administered due to its high cost and off-label use, DFPP was initiated from 13 weeks. We used the KM-9000 DFPP system (Kawasumi, Tokyo, Japan), Plasmaflo OP-05W (Asahi Kasei Medical, Tokyo, Japan) plasma-separating filter, and Cascadeflo EC-20W (Asahi Kasei Medical, Tokyo, Japan) as a second filter. Nafamostat was used for anticoagulant. The treated plasma volume was set to 4.0 L (1.3 plasma volumes) and the substitution fluid was 800 mL of 7.5% albumin. Blood was drawn from the left antecubital vein and infused into the right antecubital vein. Up until 29 weeks of gestation, DFPP was performed fortnightly with the titer maintained between 32 and 128 (Fig. 1a). At 29 weeks, the schedule was changed to once a week because the titer rose to 256. We evaluated fetal anemia using middle cerebral artery peak systolic velocity by Doppler ultrasonography. Fetal anemia was not detected during the pregnancy. No adverse events were observed and serum immunoglobulin G and fibrinogen were acceptable throughout the treatment period (Figs. 1b and 1c).

The patient delivered a healthy male infant (weight 2,289 g, Apgar score 8/9) by Cesarean section at 35 weeks. The infant showed 11.2 g/dL of hemoglobin, 36.0% of hematocrit, 6.4 mg/dL of total bilirubin, and RhD-positive blood type O. Direct Coombs test was positive. After a single course of blood exchange and phototherapy, the newborn was discharged at 17 days without any neurological problems.

DISCUSSION

We presented a case of a highly sensitized woman with RhD-incompatible pregnancy who achieved a successful delivery with no fetal anemia due to a treatment with repeated DFPP. Compared with simple plasma exchange, DFPP has a merit of less albumin substitution [4, 5]. As the half-life of nafamostat is 5–8 min, which is much shorter than heparin (1–1.5 h), it has a lower risk of bleeding. In view of that, we think that nafamostat is suitable for use as an anticoagulant in apheresis for pregnant women. During the pregnancy prior to her most recent one, after we performed DFPP and administered high dose γ-globulin at 15 weeks of gestation, we did not perform DFPP repeatedly until 27 weeks, resulting in the drastic rise of the antibody titer. We believe that we could control the anti-D antibody titer at acceptable levels throughout the course of the pregnancy by performing DFPP repeatedly from 13 weeks at the most recent pregnancy, although the presence of new maternal anti-anti leukocyte antigen antibodies from the same partner might have a blocking effect against severe hemolysis in the fetus [6]. No adverse events such as infection or bleeding tendency related to DFPP were observed.

The American Society of Apheresis proposed in 2013 that therapeutic plasma exchange should be considered for fetal hemolytic anemia until approximately 20 weeks of gestation, and subsequent to that, IUT should be the first-line treatment [7]. Although IUT is now deemed as a relatively safe option, adverse events such as fetal bradycardia, rapture of membranes, hemorrhage, and intrauterine infection can occur, with 2% fetal loss being reported [1, 8]. Repeated IUT may also

induce alloimmunization [2]. Furthermore, technical competence in IUT may vary among institutions. Considering all these factors, we propose the use of repeated DFPP to minimize the need for IUT.

In conclusion, DFPP may be an effective and sound strategy to lower antibody titers in highly sensitized women with RhD-incompatible pregnancy. By performing DFPP repeatedly, we could potentially avoid IUT. To further evaluate our findings, more cases are needed.

ACKNOWLEDGMENT

The authors would like to thank Dr. Julian Tang from the Department of Education for Clinical Research, NCCHD, for editing this manuscript.

REFERENCES

1. Moise KJ Jr. Management of rhesus alloimmunization in pregnancy. Obstet Gynecol 2008;112:164–176.
2. Papantoniou N, Sifakis S, Antsaklis A. Therapeutic management of fetal anemia: review of standard practice and alternative treatment options. J Perinat Med 2013;41:71–82.
3. Isojima S, Hisano M, Suzuki T, Sago H, Murashima A, Yamaguchi K. Early plasmapheresis followed by high-dose γ-globulin treatment saved a severely Rho-incompatible pregnancy. J Clin Apher 2011;26:216–218.
4. Hanafusa N, Noiri E, Yamashita T, Kondo Y, Suzuki M, Watanabe Y, Kanai T, Miyashita E, Tsuno NH, Fujii T, Kozuma S, Takahashi K, Taketani Y, Nakao A, Fujita T. Successful treatment by double filtrate plasmapheresis in a pregnant woman with the rare P blood group and a history of multiple early miscarriages. Ther Apher Dial 2006;10:498–503.
5. Taniguchi F, Horie S, Tsukihara S, Nagata N, Nishikawa K, Terakawa N. Successful management of a P-incompatible pregnancy using double filtration plasmapheresis. Gynecol Obstet Invest 2003;56:117–120.
6. Dooren MC, Kuijpers RW, Joekes EC, Huiskes E, Goldschmeding R, Overbeeke MA, von dem Borne AE, Engelfriet CP, Ouwehand WH. Protection against immune haemolytic disease of newborn infants by maternal monocyte-reactive IgG alloantibodies (anti-HLA-DR). Lancet 1992;339:1067–1070.
7. Schwartz J, Winters JL, Padmanabhan A, Balogun RA, Delaney M, Linenberger ML, Szczepiorkowski ZM, Williams ME, Wu Y, Shaz BH. Guidelines on the use of therapeutic apheresis in clinical practice-evidence-based approach from the Writing Committee of the American Society for Apheresis: the sixth special issue. J Clin Apher 2013;28:145–284.
8. Van Kamp IL, Klumper FJ, Oepkes D, Meerman RH, Scherjon SA, Vandenbussche FP, Kanhai HH. Complications of intrauterine intravascular transfusion for fetal anemia due to maternal red-cell alloimmunization. Am J Obstet Gynecol 2005;192:171–177.

Novel Insights from Clinical Practice

Fetal Diagnosis and Therapy

Fetal Diagn Ther
DOI: 10.1159/000365812

Received: April 16, 2014
Accepted after revision: July 7, 2014
Published online: January 21, 2015

Twin Anemia-Polycythemia Sequence after Laser Surgery for Twin-Twin Transfusion Syndrome and Maternal Morbidity

Kosuke Taniguchi[a] Masahiro Sumie[a] Rika Sugibayashi[a] Seiji Wada[a]
Kentaro Matsuoka[b] Haruhiko Sago[a]

[a]Center of Maternal-Fetal, Neonatal and Reproductive Medicine and [b]Departments of Pathology, National Center for Child Health and Development, 2–10–1 Okura, Setagaya-ku, Tokyo, Japan

Established Facts

- Twin anemia-polycythemia sequence (TAPS) is characterized by large inter-twin hemoglobin value differences without inter-twin amniotic fluid discordance.
- There is limited data on the maternal and fetal prognosis of TAPS.

Novel Insights

- Post-laser TAPS in a higher stage can cause severe maternal complications.

Key Words
Twin anemia-polycythemia sequence · Twin-twin transfusion syndrome · Fetoscopic laser photocoagulation

Abstract
Twin anemia-polycythemia sequence (TAPS) is characterized by large inter-twin hemoglobin value differences without inter-twin amniotic fluid discordance. The management of post-laser TAPS remains controversial. Hence, more studies on TAPS, together with the associated maternal complications and outcome of the fetuses and infants are needed. Between 2003 and 2012, we performed 287 cases of fetoscopic laser photocoagulation for twin-twin transfusion syndrome. Among the 114 who were placed under our care until delivery, three cases of TAPS occurred. In one case, we conducted intrauterine intravenous transfusion, while in the other two cases, we adopted expectant management. We performed an emergency caesarean section at 27–30 weeks of gestation in all cases due to a severe condition of anemia in the TAPS donor. Two cases with antenatal TAPS stage 4 had severe maternal complications; one had minute pulmo-

1015–3837/15/0000–0000$39.50/0

E-Mail karger@karger.com
www.karger.com/fdt

Haruhiko Sago, MD, PhD
Center of Maternal-Fetal, Neonatal and Reproductive Medicine
National Center for Child Health and Development
2–10–1 Okura, Setagaya-ku, Tokyo 157-8535 (Japan)
E-Mail sagou-h@ncchd.go.jp

nary embolism, while the other had Mirror syndrome. All three pairs of infants survived. One TAPS donor and one TAPS recipient had neurodevelopmental impairment; bilateral deafness at 9.5 years old and spastic paralysis at 2 years old, respectively. In conclusion, post-laser TAPS in a higher stage can cause severe maternal complications. Close observations for both fetuses and mothers are required for such cases.

Introduction

Twin anemia-polycythemia sequence (TAPS) is a novel form of feto-fetal transfusion in monochorionic twins first reported by Lopriore et al. [1]. It was characterized by large inter-twin hemoglobin value differences without inter-twin amniotic fluid discordance. The etiology of TAPS is postulated to be slow feto-fetal transfusion through minuscule anastomoses [2]. TAPS can occur not only spontaneously but also iatrogenically after fetoscopic laser photocoagulation (FLP) for twin-twin transfusion syndrome (TTTS). TAPS is estimated antenatally by fetal middle cerebral artery peak systolic velocity (MCA-PSV) measurements by Doppler ultrasonography [2]. As the management of antenatal TAPS remains undetermined, more studies of TAPS and the associated maternal complications, as well as outcome of the fetuses and infants, are needed.

Between 2003 and 2012, we performed 287 cases of FLP for TTTS of which 114 cases were placed under our care until delivery. Prenatal care with weekly ultrasound examination, including pulsed Doppler assessment, was provided for all the mothers. Subsequently, three cases of TAPS were discovered. The diagnosis of TAPS was based on MCA-PSV >1.5 Multiples of the Median (MoM) in the donor fetus that coincided with a decreased MCA-PSV <1.0 MoM in the recipient, in the absence of twin oligo-polyhydramnios sequence. We adopted antenatal and postnatal diagnostic criteria and stage classification of TAPS as per the report by Slaghekke et al. [2]. In this study, we described the three cases of TAPS with the associated maternal and fetal complications.

Case Presentation

Case 1

A 28-year-old Gravida 2, Para 1 Japanese woman with Quintero Stage II TTTS [3] underwent FLP with selective coagulation [4] at 18 + 4 weeks of gestation. On the ninth day after FLP, MCA-PSV of the TTTS donor increased gradually. On the 18th day after FLP (21 + 2 weeks of gestation), the TTTS donor had a high MCA-PSV (1.81 MoM), while the TTTS recipient had a low MCA-PSV (0.65 MoM). TAPS was suspected as the TTTS donor became TAPS donor and the TTTS recipient became TAPS recipient. The anemic state of the TTTS donor gradually worsened and eventually developed into hydrops fetalis with ascites and skin edema (antenatal TAPS stage 4). An emergency cesarean section was performed at 30 + 2 weeks (80 days after FLP).

Just after delivering the firstborn through a caesarean section, the patient complained of strong respiratory discomfort. In spite of oxygen supply, her PaO_2 level decreased. Auscultation of gallop rhythm was carried out. Computed tomography showed massive thrombosis (diameter, 1 cm; length, 10 cm) located on the left ovarian venous and inferior vena cava with no visible pulmonary embolism. Anticoagulant therapy was initiated (started with heparin, which was later replaced by warfarin) and a temporary inferior vena cava filter catheter was inserted because minute pulmonary embolism was suspected. She was discharged 24 days after the caesarean section.

A placental study performed with colored dye showed one minuscule residual arterio-venous anastomosis from the TTTS donor to the TTTS recipient (less than 1 mm and located center of the placenta) (fig. 1a, b). Tables 1 and 2 show the clinical course as well as neonatal and long-term outcomes. Postnatal TAPS stage had the value 5. Both twins required intensive care at the neonatal intensive care unit (NICU). At nine months old, magnetic resonance imaging (MRI) revealed slight ventriculomegaly with brain atrophy and a reduction in volume of white matter (fig. 2a). No auditory brainstem response was exhibited in the TAPS donor (TTTS donor). Home oxygen therapy for chronic lung disease was needed until five years old. At three years of age, Tsumori's Mental Development Test [5] showed that the TAPS donor experienced retardation in motor and cognitive adaptation, as well as social behavior section. At seven years of age WISC-IV revealed comprehensive retardation (FSIQ point was 56). The TAPS donor still requires hearing aids for both ears. On the other hand, at three years of age, the TAPS recipient showed no neurodevelopmental impairment when assessed by Tsumori's Mental Development Test.

Case 2

A 31-year-old Gravida 2, Para 1 Japanese woman was referred to our institution at 17 weeks of gestation due to TTTS, and subsequently diagnosed with Quintero stage III TTTS. FLP was performed at 17 + 4 weeks of gestation. The TTTS donor had a high MCA-PSV (2.07 MoM), while the TTTS recipient had a low MCA-PSV (0.87 MoM) on the 7th day after FLP (18 + 4 weeks of gestation). The TTTS donor became a TAPS donor and the TTTS recipient became a TAPS recipient. The TAPS donor (TTTS donor) had hydropic symptom on the 62th day after FLP (26 + 2 weeks of gestation). Mirror syndrome was suspected due to increasing placental thickness, excess maternal body weight gain and significant edema. The mother exhibited anemia, as well as evaluated uric acid and severe hypoproteinemia without proteinuria. Fetal heart rate pattern of the TAPS donor (TTTS donor) indicated a sinusoidal pattern in cardiotocogram. Intrauterine intravenous transfusion (IUT) was eventually performed on the 66th day after FLP (26 + 6 weeks of gestation) to improve anemia in the TAPS donor. However, the MCA-PSV in the TAPS donor (TTTS donor) did not declined adequately and the hydropic conditions worsened (antena-

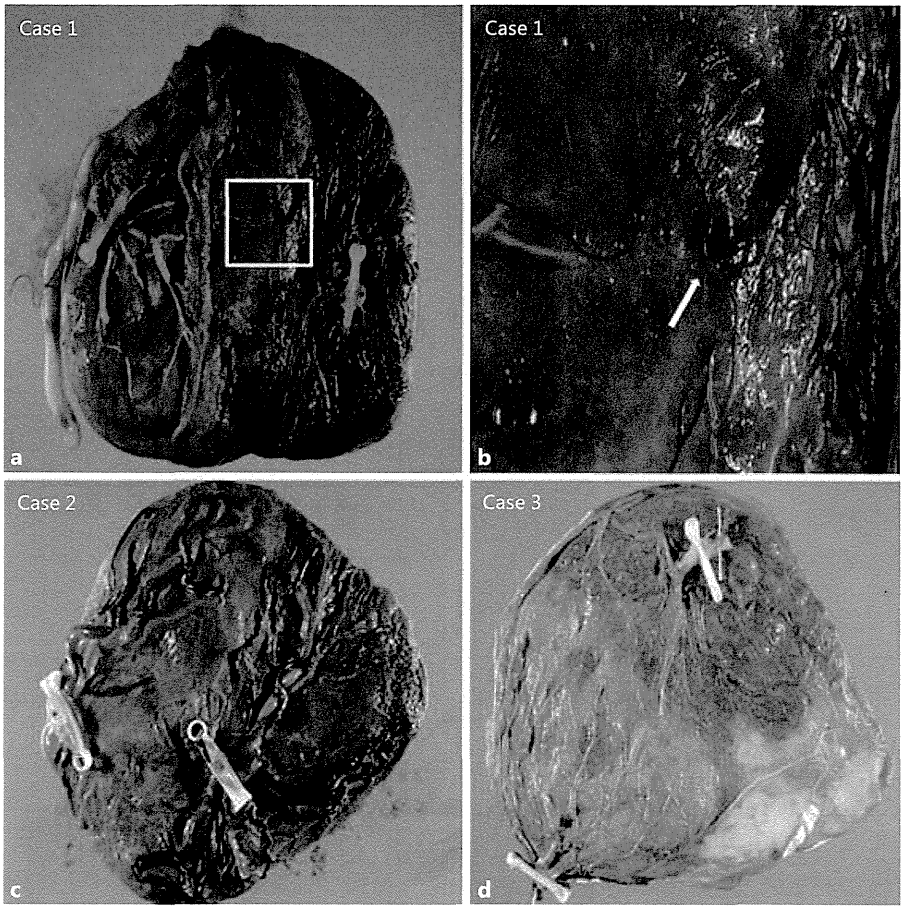

Fig. 1. Placenta pathological studies. **a** Case 1; Colored dye showed one minuscule residual arterio-venous anastomosis from the TTTS donor to the TTTS recipient (less than 1 mm and located center of the placenta) (white square). **b** Case 1; Close up of minuscule residual arterio-venous anastomosis. (white arrow). **c** Case 2; Resin injection showed no residual anastomoses. **d** Case 3; Colored dye showed no residual anastomoses. We routinely use darker colors (blue or green) for arteries and lighter colors (red or yellow) for veins.

Table 1. Baseline characteristics and perinatal managements and outcomes

	Case 1	Case 2	Case 3
Maternal age (G and P)	28 (G2P1)	31 (G2P1)	40 (G10P4)
Quintero stage of TTTS	II	III	II
GA at FLP	18 weeks 4 day	17 weeks 4 day	17 weeks 4 day
GA and POD to FLP at diagnosing TAPS	21 weeks (18th day after FLP)	18 weeks (7th day after FLP)	22 weeks (31th day after FLP)
Antenatal treatment	no (expectant management)	IUT	no (expectant management)
GA and POD to FLP at CS	30 weeks 2 day (80th day after FLP)	27 weeks 2 day (69th day after FLP)	28 weeks 1 day (55th day after FLP)
Fetal condition	hydrops fetails of TAPS donor	hydrops fetails of TAPS donor	no hydrops fetail
Maternal complication	deep vein thrombosis minute pulmonary embolism	Mirror syndrome pre-renal acute kidney disorder	no complication
Antenatal TAPS classification	stage 4	stage 4	stage 2
Residual placental anastomoses	one arterio-venous anastomoses	not detected	not detected

G = Gravida; P = parity; TTTS = twin-twin transfusion syndrome; GA = gestational age; FLP = fetoscopic laser photocoagulation; TAPS = twin anemia polythcemia sequence; POD = post-operative day; IUT = intrauterine transfusion; CS = caesarean section.

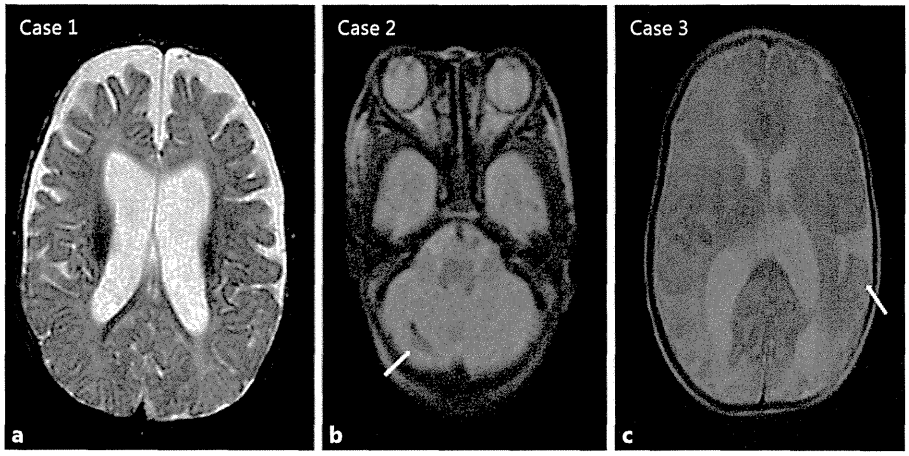

Fig. 2. MRI imaging. **a** Case 1; TAPS donor (TTTS donor) at nine months old. A slight ventriculomegaly and brain atropy, together with a volume reduction of white matter were observed. **b** Case 2; TAPS donor (TTTS donor) at four months old. A small right cerebellum hemorrhage trace (white arrow) were observed. **c** Case 3; TAPS recipient (TTTS donor) at three months old. Cortical dysplasia (deformity of the left perisylvian: white arrow), volume reduction of white matter and slight ventriculomegaly were observed.

Table 2. Neonatal and long-term outcomes

	Case 1		Case 2		Case 3	
TTTS TAPS	donor donor	recipient recipient	donor donor	recipient recipient	donor recipient	recipient donor
GA at birth	30 weeks 2 day		27 weeks 2 day		28 weeks 1 day	
Birth weight, g	990	1,330	934	958	928	808
Apgar score (1 min/5 min)	2/2	6/7	1/1	5/7	5/8	5/8
Neonatal Hb, g/dl	3.2	23.4	2.8	15.4	20.4	3.4
Absolute reticulocyte count (10 exp9/l)	14.4	3.8	15	7	5.3	35.5
Postnatal TAPS classification	stage 5	stage 5	stage 2	stage 2	stage 3	stage 3
Neonatal hematological treatments	blood transfusion	no	blood transfusion	no	no	blood transfusion
Day at discharge	11th month	80th day	104th day	90th day	88th day	88th day
MRI findings	brain atrophy, volume reduction of white matter ventriculomegaly	normal	cerebellum hemorrhage trace, subependymal hemorrhage	normal	cortical dysplasia, volume reduction of white matter, ventriculomegaly	normal
Neurodevelopmental impairment	bilateral deaf (9.5 years old)	normal (9.5 years old)	normal (2.8 years old)	normal (2.8 years old)	spastic paralysis (2 years old)	normal (2 years old)
Cognitive assessment	delay†	normal†	normal‡	normal‡	delay†	normal†

TTTS = Twin-twin transfusion syndrome; TAPS = twin anemia polycythemia sequence; GA = gestational age; MRI = magnetic resonance imaging; † Assessed by Tsumori's Mental Development Test. ‡ assessed by Kyoto Scale of Psychological Development.

tal TAPS stage 4). An emergency cesarean section was performed at 27 + 2 weeks of gestation. The postoperative course was eventful with massive hematoma at the incision of the uterus due to coagulopathy, together with pre-renal acute kidney disorder arising from intravascular dehydration. The mother was hospitalized for 26 days after the caesarean section.

A placental study performed with resin injection showed no residual anastomoses (fig. 1c). The postnatal TAPS stage had the value 2. The twins were placed in the NICU. While MRI (fig. 2b) on the 81st day revealed a small right cerebellum hemorrhage trace and old bilateral subependymal hemorrhage in the TAPS donor (TTTS donor), no neurodevelopmental impairment was found at 2.8 years old. At the same age, the TAPS recipient (TTTS recipient) had no neurodevelopmental impairment. Both twins were assessed by the Kyoto Scale of Psychological Development 2001 [6] and DENVER II [7].

Case 3

A 40-year-old Gravida 10, Para 4 Japanese woman diagnosed with Quintero stage II TTTS underwent FLP at 17 + 4 weeks of gestation. MCA-PSV of the TTTS recipient increased transiently after surgery, but was normalized seven days after FLP. Although the postoperative course was uneventful, the TTTS recipient experienced a sudden increase in MCA-PSV (1.55 MoM), while the TTTS donor had low MCA-PSV (0.6 MoM). Subsequently, TAPS was suspected on the 31st day after FLP (22 + 0 weeks of gestation). The TTTS donor became a TAPS recipient and TTTS recipient became a TAPS donor. Pericardial effusion emerged in the TAPS donor (TTTS recipient) on the 48th day after FLP (27 + 1 weeks of gestation) and the value of MCA-PSV gradually increased, reaching 2.3 MoM eventually. Fetal cardiac failure due to severe anemia (antenatal TAPS stage 2) was suspected. As the gestation period was over 28 weeks, a decision for cesarean section was made. It was performed on the 55th day after FLP (28 + 1 weeks).

A placental study performed with a colored dye showed no residual anastomoses (fig. 1d). Postnatal TAPS stage had the value 3. The twins were placed in the NICU. At three months old, MRI (fig. 2c) revealed that the TAPS recipient (TTTS donor) had cortical dysplasia (deformity of light perisylvian and hippocampus laterality) and a reduction in volume of white matter with slight ventriculomegaly, which led to spastic paralysis of right upper and lower limb at two years of age. The TAPS recipient (TTTS donor) had moderately delayed cognitive adaptation and sociolinguistics, while the TAPS donor (TTTS recipient) did not display any developmental delay when assessed by Tsumori's Mental Development Test.

Discussion

We experienced three TAPS cases (2.6%) in 114 cases of FLP for TTTS that were under our care until delivery. All three cases resulted in preterm delivery, with fetal treatment of IUT in one case. All six infants survived, but two infants had neurological problems. In two cases, maternal complications (minute pulmonary embolism and Mirror syndrome) occurred.

This study showed that severe post-laser TAPS can be associated with critical maternal morbidity. To date, there are no such reports. Multiple pregnancy and long-term bed rest for potential premature labor tend to lead to the development of maternal deep vein thrombosis (DVT) [8, 9], which in turn results in pulmonary embolism, as demonstrated in Case 1. Mirror syndrome has been reported in severe TTTS after FLP [10]; as was the clinical course displayed in Case 2. Both Cases 1 and 2 were antenatal TAPS stage 4, and experienced a long period between the occurrence of TAPS and delivery. A higher stage of TAPS and a longer period after the occurrence of TAPS may influence severe maternal complications. As such, close monitoring for both fetuses and mothers is required in TAPS cases.

The treatment options for antenatal TAPS include IUT in the donor, with or without partial exchange transfusion to the recipient, and FLP [11, 12]. Since Case 1 was a relatively older case, antenatal treatment was not an option at that time. We could not perform FLP for Cases 2 or 3 mainly due to an absence of amniotic fluid discordance, which was likely to cause technical difficulties. IUT was performed in Case 2 because of severe prematurity for delivery at 26 weeks of gestation. The MCA-PSV in the anemic donor did not decline adequately and the hydropic state remained after IUT. However, the outcome of both infants was favorable. A poor prognosis for post-laser TAPS donor despite IUT was recently reported [13]. We did not perform IUT in Case 3 since the gestational age was already over 28 weeks. While FLP is the causative treatment and can be effective for TAPS [11], it can be technically challenging to perform. As such, despite its effectiveness not being fully determined yet, we decided to perform IUT instead.

Several reports have suggested that there were more minuscule anastomoses than normal monochorionic diamniotic twin in TAPS cases [14, 15]. Although there was one minuscule arterio-venous residual anastomosis from donor to recipient in Case 1, we could not detect residual anastomoses by injection studies in the other two cases. In Case 2, as we could not observe any residual anastomosis on the placental surface, we performed resin injection in an attempt to detect deeper arterio-venous anastomosis [16]. However, we did not detect any anastomosis. In Case 3, we were unable to carry out a detailed search as the succenturiate placenta hampered the injection of a color dye into peripheral small anastomoses. In view of these situations, we are aware that there may be some technical flaws on our part in Cases 2 and 3.

A recent cohort study showed neurodevelopmental problems in post-laser TAPS [13]. The authors reported a survival rate of 80% but neurodevelopmental impairment and cognitive delay was found in nearly one in five post-laser TAPS infants who survived. In our case, all infants survived, but one (a TAPS donor, Case 1) was diagnosed with hypoacusis at nine months of age, while another (a TAPS recipient, Case 3) developed spastic paralysis. These children also experienced cognitive delay; particularly, the delay was severe in Case 1. On the other hand, antenatal TAPS staging was high in Case 2, but resulted in no neurodevelopmental impairment with abnormal findings on MRI in TAPS donor. While it is difficult to attribute the positive outcome of Case 2 to IUT, neurological impairments are known to be significantly present in post-laser TAPS with expectant managements.

In conclusion, post-laser TAPS in a higher stage can cause severe maternal complications. The neurological prognosis of post-laser TAPS may be poor in expectant managements. Close observations for both fetuses and mothers are required when in cases of TAPS.

Acknowledgment

We would like to thank Julian Tang of the Department of Education for Clinical Research, National Center for Child Health and Development, for proofreading, editing and rewriting parts of this manuscript.

References

1 Lopriore E, Middeldorp JM, Oepkes D, Kanhai HH, Walther FJ, Vandenbussche FP: Twin anemia-polycythemia sequence in two monochorionic twin pairs without oligo-polyhydramnios sequence. Placenta 2007;28:47–51.

2 Slaghekke F, Kist WJ, Oepkes D, Pasman SA, Middeldorp JM, Klumper FJ, Walther FJ, Vandenbussche FP, Lopriore E: Twin anemia-polycythemia sequence: diagnostic criteria, classification, perinatal management and outcome. Fetal Diagn Ther 2010;27:181–190.

3 Quintero RA, Morales WJ, Allen MH, Bornick PW, Johnson PK, Kruger M: Staging of twin-twin transfusion syndrome. J Perinatol 1999;19:550–555.

4 Sago H, Hayashi S, Saito M, Hasegawa H, Kawamoto H, Kato N, Nanba Y, Ito Y, Takahashi Y, Murotsuki J, Nakata M, Ishii K, Murakoshi T: The outcome and prognostic factors of twin-twin transfusion syndrome following fetoscopic laser surgery. Prenat Diagn 2010;30:1185–1191.

5 Matsuzaki T, Matsui M, Nakazawa J, Ichida F, Yagihara T: [Application of the Bayley scales of infant development as a developmental test for Japanese infants with congenital heart disease]. No To Hattatsu 2008;40:308–312.

6 Koyama T, Osada H, Tsujii H, Kurita H: Utility of the Kyoto scale of psychological development in cognitive assessment of children with pervasive developmental disorders. Psychiatry Clin Neurosci 2009;63:241–243.

7 Frankenburg WK, Dodds J, Archer P, Shapiro H, Bresnick B: The Denver II: a major revision and restandardization of the Denver developmental screening test. Pediatrics 1992;89:91–97.

8 Walker MC, Murphy KE, Pan S, Yang Q, Wen SW: Adverse maternal outcomes in multifetal pregnancies. BJOG 2004;111:1294–1296.

9 Kovacevich GJ, Gaich SA, Lavin JP, Hopkins MP, Crane SS, Stewart J, Nelson D, Lavin LM: The prevalence of thromboembolic events among women with extended bed rest prescribed as part of the treatment for premature labor or preterm premature rupture of membranes. Am J Obstet Gynecol 2000;182:1089–1092.

10 Hayashi S, Sago H, Hayashi R, Nakagawa S, Kitagawa M, Miyasaka K, Chiba T, Natori M: Manifestation of mirror syndrome after fetoscopic laser photocoagulation in severe twin-twin transfusion syndrome. Fetal Diagn Ther 2006;21:51–54.

11 Slaghekke F, Favre R, Peeters SH, Middeldorp JM, Weingertner AS, van Zwet EW, Klumper FJ, Oepkes D, Lopriore E: Laser surgery as a management option for twin anemia-polycythemia sequence. Ultrasound Obstet Gynecol 2014;44:304–310.

12 Genova L, Slaghekke F, Klumper FJ, Middeldorp JM, Steggerda SJ, Oepkes D, Lopriore E: Management of twin anemia-polycythemia sequence using intrauterine blood transfusion for the donor and partial exchange transfusion for the recipient. Fetal Diagn Ther 2013;34:121–126.

13 Slaghekke F, van Klink JM, Koopman HM, Middeldorp JM, Oepkes D, Lopriore E: Neurodevelopmental outcome in twin anemia-polycythemia sequence after laser surgery for twin-twin transfusion syndrome. Ultrasound Obstet Gynecol 2014;44:316–321.

14 Lopriore E, Slaghekke F, Middeldorp JM, Klumper FJ, Oepkes D, Vandenbussche FP: Residual anastomoses in twin-to-twin transfusion syndrome treated with selective fetoscopic laser surgery: localization, size, and consequences. Am J Obstet Gynecol 2009;201:66.e61–e64.

15 de Villiers SF, Slaghekke F, Middeldorp JM, Walther FJ, Oepkes D, Lopriore E: Placental characteristics in monochorionic twins with spontaneous versus post-laser twin anemia-polycythemia sequence. Placenta 2013;34:456–459.

16 Benirschke K: Pathology of the human placenta. New York, Springer, 2012.

Comparison of Angiogenic, Cytoprotective, and Immunosuppressive Properties of Human Amnion- and Chorion-Derived Mesenchymal Stem Cells

Kenichi Yamahara[1]*, Kazuhiko Harada[2], Makiko Ohshima[1], Shin Ishikane[2], Shunsuke Ohnishi[3], Hidetoshi Tsuda[1], Kentaro Otani[1], Akihiko Taguchi[4], Toshihiro Soma[5], Hiroyasu Ogawa[5], Shinji Katsuragi[6], Jun Yoshimatsu[6], Mariko Harada-Shiba[1,7], Kenji Kangawa[2], Tomoaki Ikeda[1,6,8]

1 Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan, **2** Department of Biochemistry, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan, **3** Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan, **4** Department of Regenerative Medicine Research, Institute of Biomedical Research and Innovation, Kobe, Hyogo, Japan, **5** Division of Hematology, Department of Internal Medicine, Hyogo Medical College, Nishinomiya, Hyogo, Japan, **6** Department of Perinatology, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan, **7** Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan, **8** Department of Obstetrics and Gynecology, Mie University School of Medicine, Tsu, Mie, Japan

Abstract

Although mesenchymal stem cells (MSCs) can be obtained from the fetal membrane (FM), little information is available regarding biological differences in MSCs derived from different layers of the FM or their therapeutic potential. Isolated MSCs from both amnion and chorion layers of FM showed similar morphological appearance, multipotency, and cell-surface antigen expression. Conditioned media obtained from amnion- and chorion-derived MSCs inhibited cell death caused by serum starvation or hypoxia in endothelial cells and cardiomyocytes. Amnion and chorion MSCs secreted significant amounts of angiogenic factors including HGF, IGF-1, VEGF, and bFGF, although differences in the cellular expression profile of these soluble factors were observed. Transplantation of human amnion or chorion MSCs significantly increased blood flow and capillary density in a murine hindlimb ischemia model. In addition, compared to human chorion MSCs, human amnion MSCs markedly reduced T-lymphocyte proliferation with the enhanced secretion of PGE2, and improved the pathological situation of a mouse model of acute graft-versus-host disease. Our results highlight that human amnion- and chorion-derived MSCs, which showed differences in their soluble factor secretion and angiogenic/immuno-suppressive function, could be ideal cell sources for regenerative medicine.

Citation: Yamahara K, Harada K, Ohshima M, Ishikane S, Ohnishi S, et al. (2014) Comparison of Angiogenic, Cytoprotective, and Immunosuppressive Properties of Human Amnion- and Chorion-Derived Mesenchymal Stem Cells. PLoS ONE 9(2): e88319. doi:10.1371/journal.pone.0088319

Editor: Atsushi Asakura, University of Minnesota Medical School, United States of America

Received September 1, 2013; **Accepted** January 6, 2014; **Published** February 14, 2014

Funding: This study was supported by a Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science, and Health Labour Sciences Research Grant/Research grants for Cardiovascular Disease, The Ministry of Health Labour and Welfare. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yamahara@ncvc.go.jp

Introduction

Mesenchymal stem cells (MSCs) residing within various tissues, including bone marrow [1] and adipose tissue [2], are reported to differentiate into various types of cells including osteoblasts, chondrocytes, and adipocytes. This multipotency renders MSCs an attractive therapeutic source for regenerative medicine. However, because an invasive procedure is required to obtain autologous bone marrow or adipose tissue-derived MSCs, an alternative source of MSCs that can be obtained non-invasively is desirable.

Appendages of the fetus, which consist of the placenta, umbilical cord, and fetal membrane (FM), are normally discarded after delivery as medical waste. A large quantity of MSCs could be obtained without harm from the human FM because of its size (> 40×40 cm), which represents an advantageous characteristic as a source of cell therapy. We have previously reported the therapeutic potential of rat FM-derived MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Although the FM is composed of the amnion and chorion, and both layers contain MSCs [9], it is technically difficult to separate these membranes as well as their MSCs in rat.

Thus, the purposes of this study were: 1) to isolate and characterize MSCs from human amnion and chorion; 2) to examine their differences in the expression profile of growth factors and cytokines; and 3) to investigate the therapeutic potential and difference of these MSCs using murine hindlimb ischemia and acute graft-versus-host disease (GVHD) models.

Materials and Methods

Ethics Statement

The study protocol and informed consent procedure were approved by the ethics committee of the National Cerebral and

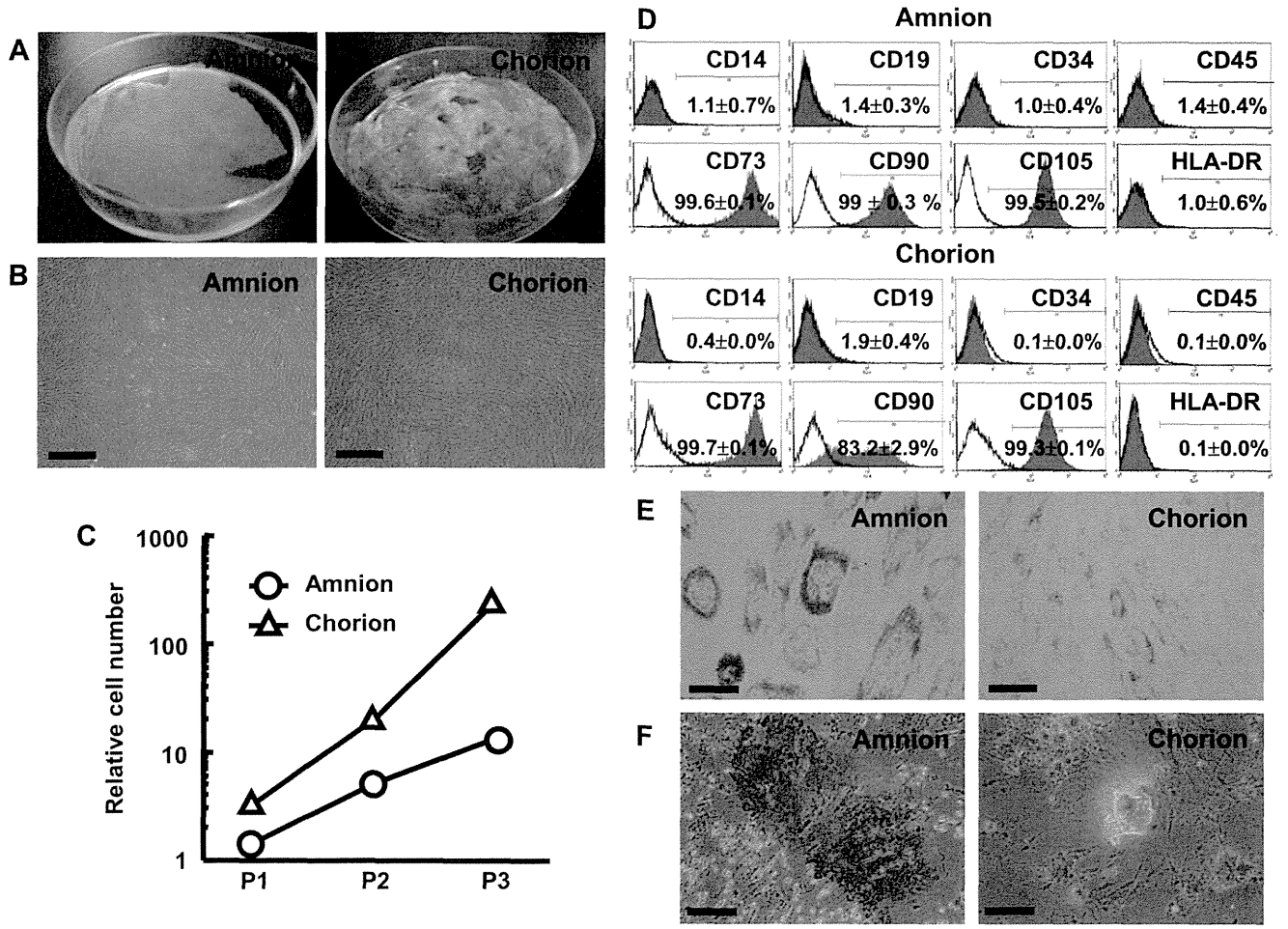

Figure 1. Characterization of human amnion- and chorion-derived MSCs. (A) Representative photographs of human amnion and chorion. (B) Photographs of cultured MSCs obtained from human amnion and chorion at passage 3. Scale bars = 500 μm. (C) Relative cell number of amnion- and chorion-derived MSCs at each passage. (D) FACS analysis of amnion and chorion MSCs. (E, F) Differentiation of amnion and chorion MSCs into adipocytes (E) and osteocytes (F). Scale bars = 100 (E) and 50 (F) μm.
doi:10.1371/journal.pone.0088319.g001

Cardiovascular Center (Permit Number: M18-042-4). Animal protocols were approved by the Animal Care Committee of the National Cerebral and Cardiovascular Center Research Institute (Permit Number: 13052). Animal studies were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

Isolation and Expansion of Amnion- and Chorion-derived MSCs from Human FMs

After obtaining written informed consent, FMs were obtained following cesarean section of healthy donor mothers. Amnion and chorion were separated by mechanical peeling of the FM, and digested with type-II collagenase solution (5 ml/g tissue and 300 U collagenase/mL, Worthington Biochemicals, Lakewood, NJ) for 1 h at 37°C in a waterbath shaker. After filtration with a mesh filter, cells were suspended in α-minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen), and incubated at 37°C with 5% CO_2 after plating on a dish. The adherent, spindle-shaped MSCs developed visible symmetric colonies by days 1 to 2.

Characterization of Human Amnion and Chorion MSCs

For defining FM-MSCs, we referred to the criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [10].

Cultured MSCs were analyzed by FACSCalibur (BD Biosciences). Cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal against human CD14 (clone M5E2), CD19 (clone HIB19), CD34 (clone 581), CD45 (clone HI30), CD73 (clone AD2), CD90 (clone 5E10), CD105 (clone 266), or HLA-DR (clone G46-6 (L243)), all purchased from BD Biosciences. Isotype identical antibodies served as controls.

To induce differentiation into osteocytes, MSCs were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions. After 14–17 days of differentiation, cells were fixed and stained with Alizarin Red S (Sigma-Aldrich, St. Louis, MO).

To induce adipocyte differentiation, MSCs were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methyl-

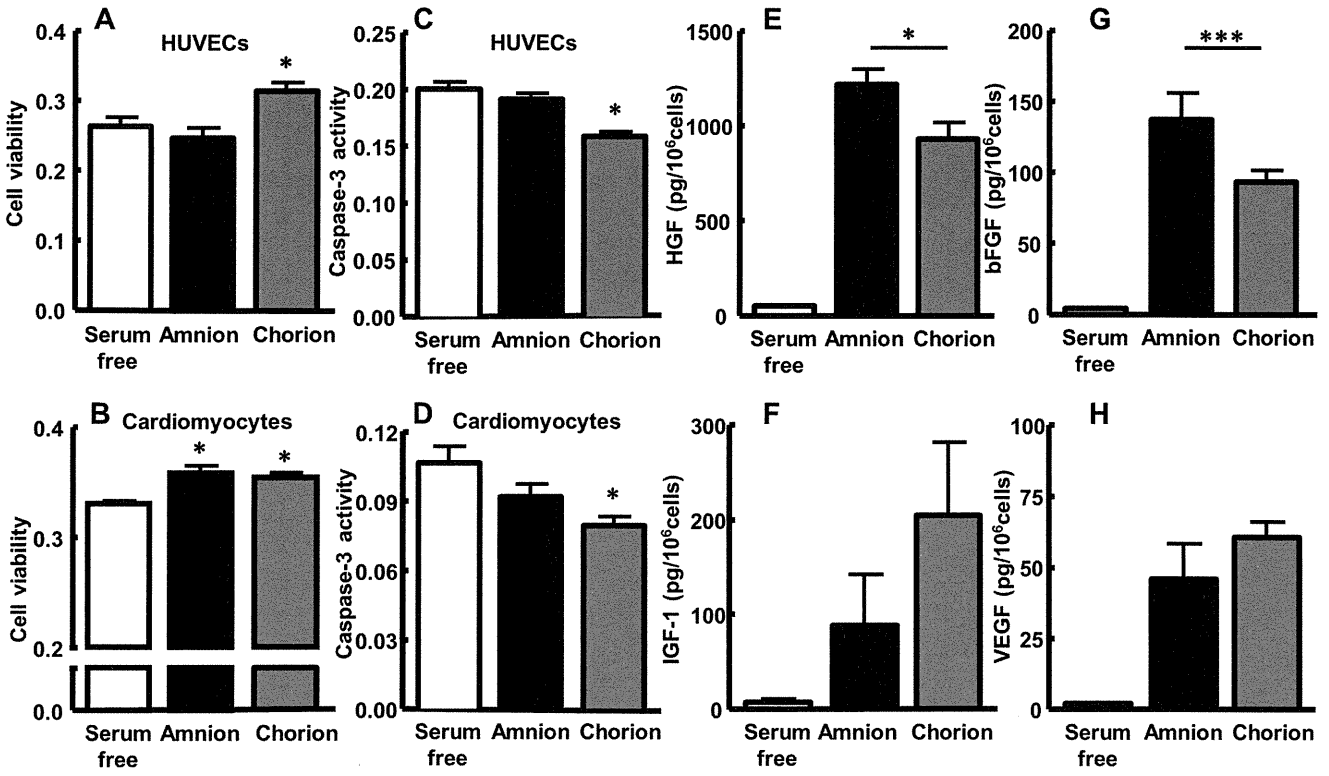

Figure 2. Growth factor secretion and the cytoprotective effect of amnion and chorion MSCs. (A–D) Cytoprotective effect of FM MSC-derived conditioned medium was analyzed by the MTS assay (A, B) and caspase-3 activity (C, D) in HUVECs (A, C) and cardiomyocytes (B, D). Values are mean ± SEM. *p<0.05 vs. serum-free. (E–H) Conditioned medium obtained from FM-derived MSCs was collected after incubation for 24 h. The concentration of HGF (E), IGF-1 (F), bFGF (G), and VEGF (H) in serum free conditioned medium was measured by ELISA. *p<0.05 and ***p<0.001.
doi:10.1371/journal.pone.0088319.g002

xanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako), 50 μM indomethacin (Wako), and 10 μg/mL insulin (Sigma-Aldrich) in α-MEM supplemented with 10% FCS. After 21 days of differentiation, adipocytes were stained with Oil Red O (Sigma-Aldrich).

Conditioned Medium Analysis of FM-MSC-associated Cytoprotective Function

Human umbilical vascular endothelial cells (HUVECs; Lonza, Basel, Switzerland) were seeded onto a collagen-coated plate and incubated in medium 199 (Invitrogen) supplemented with 20% FCS for 24 h. Neonatal rat cardiomyocytes were isolated from Lewis rats on postnatal day 1, as described previously [11], and seeded onto a laminin-coated plate followed by incubation in α-MEM supplemented with 10% FCS for 24 h. Cells were then subjected to serum deprivation with/without hypoxia (1% O_2) by culturing with serum-free medium or serum-free conditioned medium obtained from FM-MSCs cultured for 24 h. The cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium (MTS), indicative of cell viability, as well as caspase-3 activity, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI) and a CaspACE^{TM} Assay System Kit (Promega), according to the manufacturer's instructions.

Analysis of FM-MSC Production of Growth Factors and Prostaglandin E2

Conditioned media were collected from MSCs cultured in α-MEM with/without 10% FCS for 24 h (n = 4–6). The concentrations of the following growth factors were measured using ELISA kits: hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE2), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

FM-MSC Transplantation in the Hindlimb Ischemia Model

Six-week-old male KSN nude mice were anesthetized with pentobarbital, and the right common iliac artery was resected. After surgery, amnion MSCs (1×10^6 cells/50 μL PBS), chorion MSCs (1×10^6 cells/50 μL PBS), or PBS (50 μL PBS) was injected into the ischemic muscle with a 30-gauge needle at five different sites (n = 15 in each group). A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) was used to measure serial hindlimb blood flow for 7 days, as previously described [12].

Five and seven days after MSC transplantation, ischemic hindlimb tissues were obtained and snap-frozen. Frozen tissue sections were stained with anti-mouse CD31 antibody (BD Biosciences) to detect capillary endothelial cells. Ten fields were randomly selected to count the number of capillaries. The adjusted capillary number per muscle fiber was used to compare the differences in capillary density between the three groups.

In vitro CD4+ T cell Proliferation Assay

Peripheral blood mononuclear cells were prepared from buffy coats obtained from healthy donors by centrifugation through Ficoll-Paque (GE healthcare, Uppsala, Sweden). CD4+ T cells were isolated by magnetic bead depletion of CD8, CD14+,

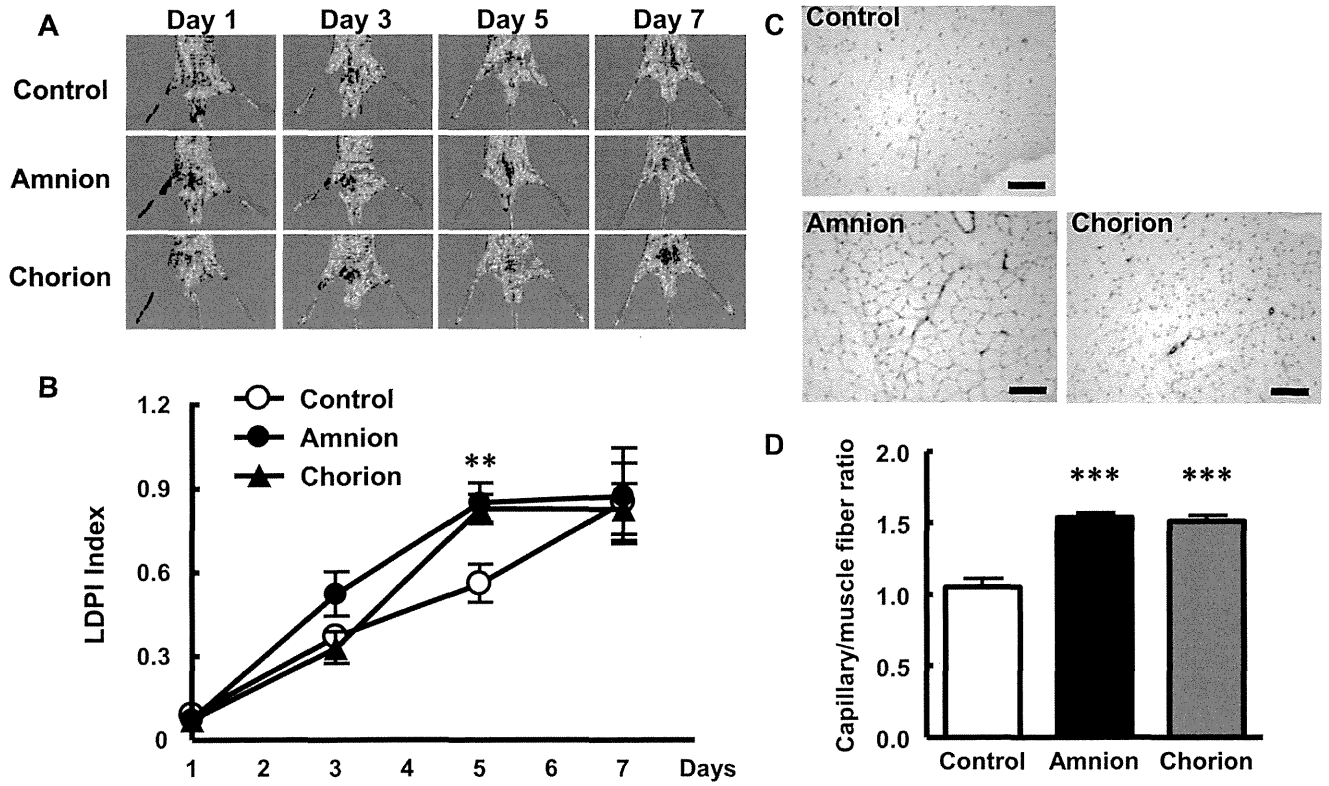

Figure 3. Angiogenic potential of amnion and chorion MSCs against hindlimb ischemia. (A) Representative images of serial hindlimb blood perfusion. Blood perfusion of ischemic hindlimb increased in the amnion and chorion MSC groups at day 5. (B) Quantitative analysis of hindlimb blood perfusion with the LDPI index, the ratio of ischemic to non-ischemic hindlimb blood perfusion. (C) Representative photographs of immunohistochemistry with anti-CD31 antibody. Scale bars = 100 μm. (D) Quantitative analysis of capillary density in ischemic hindlimb muscle at day 5 among the control, amnion, and chorion MSC groups. Capillary density is shown as the capillary-to-muscle-fiber ratio. Data are mean ± SEM. **p< 0.01 and ***p<0.001 vs. control.
doi:10.1371/journal.pone.0088319.g003

CD15+, CD16+, CD19+, CD36+, CD56+, CD123+, T cell receptor-gamma/delta, and glycophorin A–positive cells (CD4+ T Cell Isolation Kit) on an AutoMACS instrument (Miltenyi Biotec). CD4+ T cells (5×10^5 cells/well) were cultured with X-VIVO medium (Lonza, Walkersville, MD) containing 2% FBS and 5 μg/ml anti-CD28 antibody (clone CD28.2, BioLegend, San Diego, CA) in anti-CD3-precoated 24-well culture plates (clone OKT3, BioLegend). During in vitro proliferation of CD4+ T cells, human amnion-, chorion-, or bone marrow-derived (Lonza) MSCs were co-cultured at 5×10^4 cells/well. After 5 days of co-culturing, T cells were separated from the monolayer MSCs and counted with an automated cell counter (Countess, Invitrogen).

FM-MSC Transplantation into the Acute GVHD Model

Seven- to eight-week-old female B6C3F1 (recipient; C57BL/6×C3H/He; H-$2^{b/k}$) and BDF1 (donor; C57BL/6×DBA/2; H-$2^{b/d}$) mice were purchased from Japan SLC (Shizuoka, Japan). Recipient mice were lethally irradiated with 15 Gy total body irradiation (TBI; X-ray) split into two doses separated by 2 h. On the following day, donor-derived cells (1×10^7 bone marrow cells and 3×10^7 spleen cells) were suspended in 0.2 mL RPMI-1640 medium (Invitrogen) and transplanted via the tail vein into the post-irradiation recipient mice. On days 14, 17, 21, and 25 after hematopoietic stem cell transplantation, 1×10^5 amnion or chorion MSCs in 0.1 mL RPMI medium were transplanted via the tail vein. In the control group, the same amount of RPMI was infused via the tail vein. The severity of GVHD was evaluated by measuring the body weight of mice.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (S.E.M). Comparisons of parameters for more than three groups were made by one-way analysis of variance (ANOVA) followed by the Newman-Keuls' test. Comparisons of the time-course of the LDPI index were made by two-way ANOVA for repeated measures, followed by Bonferroni tests. A p value <0.05 was considered statistically significant.

Results

Characterization of Amnion and Chorion MSCs

From each human FM, 23.5±3.7 g amnion and 37.6±2.5 g chorion could be separated (n = 5 and n = 3, respectively) (Figure 1A). By enzymatic digestion, over one million cells per gram of the amnion ($1.9\pm0.2\times10^6$/g, n = 5) or chorion ($1.3\pm0.3\times10^7$/g, n = 3) were obtained. At passage 3, cultured cells from both layers were fibroblast-like, spindle-shaped cells, and there was no difference in morphology according to the origin of layers (Figure 1B). Cell-doubling time of amnion MSCs (32.2±1.13 h) was equal to that of chorion MSCs (34.1±1.94 h) (Figure 1C).

Both amnion- and chorion-derived MSCs expressed CD73, CD90, and CD105, but not CD14, CD19, CD34, CD45, or HLA-

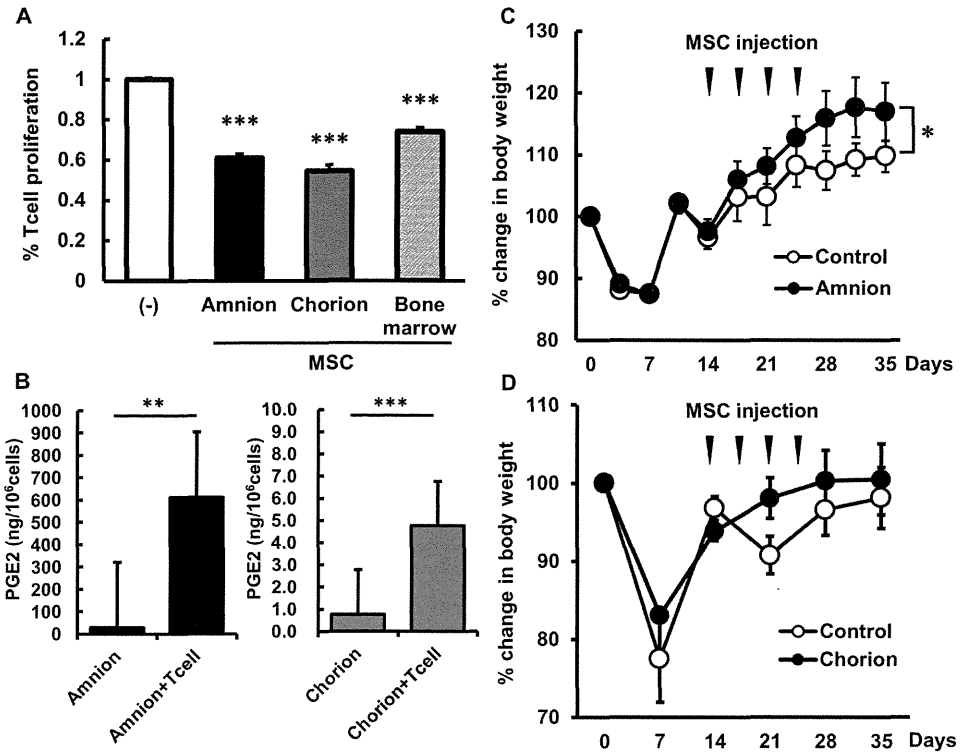

Figure 4. Immunosuppressive property of amnion and chorion MSCs. (A) Inhibition of human CD4+ T cell proliferation upon co-culture with human amnion, chorion, and bone marrow MSCs. (B) The concentration of PGE2 in FM-MSC-conditioned medium was measured by ELISA. Amnion MSCs secreted a significant amount of PGE2 compared with chorion MSCs. (C, D) Effect of human amnion (C) or chorion (D) MSC transplantation in a murine GVHD model. Treatment with amnion MSCs significantly reduced recipient weight loss in a mouse model of GVHD. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.
doi:10.1371/journal.pone.0088319.g004

DR (Figure 1D), which satisfied the criteria for identifying MSCs [10]. In addition, amnion and chorion MSCs could differentiate into adipocytes and osteocytes, as demonstrated by positive Oil Red O and Alizarin Red S staining, respectively (Figure 1E and 1F).

Cytoprotective Effects of Amnion and Chorion MSCs on Endothelial Cells and Cardiomyocytes

To evaluate the cytoprotective effect of amnion and chorion MSCs, we examined cell viability and apoptosis of HUVECs and neonatal rat cardiomyocytes cultured under serum deprivation. In the MTS assay, cell viability of cardiomyocytes was significantly increased when cultured with conditioned medium obtained from amnion and chorion MSCs (absorbance value: serum-free control 0.331 ± 0.002, amnion MSCs 0.359 ± 0.006; $p<0.001$, and chorion MSCs 0.355 ± 0.004; $p<0.01$ vs. control) (Figure 2B). Cell viability of HUVECs also increased when cultured with chorion MSC-derived conditioned medium (serum-free control 0.263 ± 0.013, amnion MSCs 0.247 ± 0.014, and chorion MSCs 0.313 ± 0.012; $p<0.05$ vs. control) (Figure 2A). Similarly, conditioned medium obtained from chorion MSCs significantly decreased the caspase-3 activity of HUVECs (absorbance value: serum-free control 0.201 ± 0.006 vs. chorion MSCs 0.159 ± 0.004; $p<0.001$) and cardiomyocytes (control 0.106 ± 0.007 vs. chorion MSCs 0.079 ± 0.004; $p<0.05$) (Figure 2C, D). Amnion MSC-derived conditioned medium also showed a tendency to decrease the caspase-3 activity of these cells, but without statistical significance.

Secretion of Growth Factors from Cultured Amnion- and Chorion-derived MSCs

To investigate the secretion of major growth factors from MSCs, we performed ELISA of HGF, IGF-1, bFGF, and VEGF. The differences in the cellular expression profile of the growth factors were observed in these FM-derived MSCs (Figure 2E–H). Among these growth factors, amnion MSCs secreted significant amounts of HGF (1217.2 ± 80.2 pg/10^6 cells; $p<0.001$ vs. chorion-MSC) and bFGF (137.2 ± 18.5 pg/10^6 cells; $p<0.05$ vs. chorion-MSC) compared with chorion MSCs (HGF: 932.5 ± 85.3 pg/10^6 cells, bFGF: 93.6 ± 8.1 pg/10^6 cells) (Figure 2E, G). There was no significant difference between amnion and chorion MSCs in the level of secreted IGF-1 (88.8 ± 53.4 pg/10^6 cells and 205 ± 77.0 pg/10^6 cells, respectively) and VEGF (46.1 ± 12.3 pg/10^6 cells and 60.7 ± 5.3 pg/10^6 cells, respectively) (Figure 2F, H).

Augmentation of Angiogenesis in the Ischemic Hindlimb after Human FM-MSC Transplantation

Analysis of LDPI revealed that accelerated limb perfusion was observed in the amnion and chorion MSC-transplanted groups (Figure 3A). The LDPI index was significantly higher in the amnion and chorion MSC groups (amnion MSCs: 0.85 ± 0.07; $p<0.01$, chorion MSCs: 0.83 ± 0.05; $p<0.01$) than in the control group (0.56 ± 0.07) 5 days after transplantation (Figure 3B). At 7 days after transplantation, there was no difference between the treated and control groups.

Immunostaining with the endothelial marker CD31 showed significant augmentation of capillaries in the amnion and chorion

MSC-treated groups compared with the control group (Figure 3E). The capillaries-to-muscle-fiber ratio of ischemic muscle at day 5 after transplantation was significantly increased in the amnion and chorion MSC groups (amnion MSCs: 1.53±0.03/muscle fiber; $p<0.001$, chorion MSCs: 1.51±0.04/muscle fiber; $p<0.001$) compared with the control group (1.05±0.06/muscle fiber; Figure 3F). At day7, the capillaries-to-muscle-fiber ratio of ischemic muscle was also increased in the amnion or chorion MSC-transplanted mice (amnion MSCs: 1.67±0.17/muscle fiber, chorion MSCs: 1.43±0.09/muscle fiber) compared to the control mice (1.36±0.11/muscle fiber).

Immunosuppressive Property of Human FM-MSCs

Although the number of T cells was markedly increased under proliferating conditions of human CD4+ T cells stimulated with anti-CD3 and -CD28 antibodies, the increase was significantly suppressed when co-cultured with amnion-, chorion-, or bone marrow-derived MSCs (61.1±1.8%, 54.6±3.0%, 74.0±2.1%, respectively. $p<0.001$ vs. control) (Figure 4A).

PGE2 is a well-known immune modulator in bone marrow MSCs [13] and we confirmed that amnion MSCs in culture secreted a significant amount of PGE2 (29.7±7.8 ng/10^6 cells), particularly when co-cultured with human CD4+ T cells (613.1±139.9 ng/10^6 cells; $p<0.01$ vs. amnion MSCs) (Figure 4B). In chorion MSCs, however, the concentration of PGE2 was relatively low (0.77±0.13 ng/10^6 cells) but significantly increased in co-culture with CD4+ T cells (4.76±0.47 ng/10^6 cells; $p<0.001$ vs. chorion MSCs). The experiments were repeated with two or three independent MSC/CD4+ T cell donor pairs and the data are presented as the measured mean levels.

In addition, to evaluate the potential of FM-MSCs to suppress acute GVHD, mice underwent allogeneic hematopoietic stem cell transplantation and treatment with human FM-MSCs. As shown in Figure 4C, the loss in body weight of recipient mice after allogeneic hematopoietic stem cell transplantation was significantly reduced with concomitant transplantation of human amnion-derived MSCs. In human chorion MSC-transplanted group, however, no significant changes in body weight was observed during the observation period (Figure 4D).

Discussion

Human MSCs derived from bone marrow or adipose tissue exert a regenerative effect in animal models and human patients [14]. In addition, several reports have described the therapeutic potential of transplanted cells derived from the appendages of the fetus, including amniotic epithelium cells [15], and amniotic fluid- [16], amnion-, and chorion-derived MSCs [17,18]. We have previously demonstrated the therapeutic potential of rat FM-MSCs using various rat models including hindlimb ischemia, autoimmune myocarditis, glomerulonephritis, renal ischemia-reperfusion injury, and myocardial infarction [3–8]. Recent studies including ours also revealed the angiogenic and immunosuppressive property of human fetal appendage-derived MSCs [14,18–20], but comparative studies of the therapeutic effects among these MSCs are lacking. Therefore, in this study, we examined the differences in the cellular function and therapeutic properties between human FM-derived amnion and chorion MSCs.

It is known that MSCs exert their regenerative effects through differentiation into specific cell types, but recent studies suggest that their ability to stimulate regenerative effects is mainly induced via paracrine effects [3,4,8,21]. This theory is substantiated by several reports that MSCs secrete various growth factors and cytokines including VEGF, IGF-1, HGF, adrenomedullin (AM), and PGE2 [3–5,8,21,22]. In this study, we first confirmed that chorion MSCs as well as amnion MSCs secreted significant amount of these soluble factors, which would contribute to accelerating regenerative effects. Compared with chorion MSCs, amnion MSCs secreted significantly larger amounts of HGF and bFGF. However, amnion MSCs secreted less IGF-1 compared to chorion MSCs. We assume that these differences in the cytokine expression profile might reflect the angiogenic and cytoprotective properties of amnion and chorion MSCs, as we observed difference in the effect on endothelial cells and cardiomyocytes in our conditioned-medium analysis. However, the actual function of amnion or chorion MSC-derived cytokines should be further investigated *in vivo* because both human amnion and chorion MSC transplantation similarly induced angiogenesis in the hindlimb ischemia model.

Previous reports have shown that PGE2 is a major modulator of the MSC-induced anti-inflammatory response [13]. In this study, a noteworthy finding was a distinctly high concentration of PGE2 in amnion MSCs in comparison with chorion MSCs, particularly when co-cultured with CD4+ T cells. Because of their high PGE2 production, human amnion MSCs might be a better cell source from an immunosuppressive point of view. In fact, we proved for the first time that human amnion MSCs, but not chorion MSCs, improved the pathological situation of an acute GVHD model. Because our previous study demonstrated that human amnion MSCs markedly inhibited differentiation as well as proliferation of Th1/Th17 cells [6], human amnion MSCs could effectively suppress Th1/Th17 immunity and improve outcome in GVHD.

The merit of using FMs lies in that they are free form ethical concern and that a large number of MSCs can be obtained considering the size of FM. As more than one or ten million MSCs per gram of the amnion or chorion could be obtained, more than 10^9 or 10^{10} MSCs could theoretically be obtained at passage 3 within one month, respectively. Now we are planning to initiate clinical studies with human amnion MSCs in acute GVHD and Crohn's disease, and we need more than 10^{10} MSCs for the treatment of one patient. We are convinced that human FM-MSCs are an attractive source for cell therapy because of their easy availability compared with other somatic, embryonic stem, and iPS cells.

In conclusion, both amnion and chorion MSCs have angiogenic, cytoprotective, and immunomodulatory effects. Because of high PGE2 production and immunosuppressive properties, human amnion MSCs have the advantage for the treatment of immune-related diseases. In addition, since a large number of MSCs could be obtained from FMs, human amnion and chorion MSCs would be a useful cell source for regenerative medicine.

Author Contributions

Conceived and designed the experiments: KY AT TS HO JY MHS KK TI. Performed the experiments: KY KH MO SI SO HT KO SK JY TI. Analyzed the data: KY KH MO TI. Contributed reagents/materials/analysis tools: KY KH MO TI. Wrote the paper: KY KH MO TI.

References

1. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276: 71–74.

2. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, et al. (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279–4295.

3. Ishikane S, Ohnishi S, Yamahara K, Sada M, Harada K, et al. (2008) Allogeneic injection of fetal membrane-derived mesenchymal stem cells induces therapeutic angiogenesis in a rat model of hind limb ischemia. Stem Cells 26: 2625–2633.
4. Ishikane S, Yamahara K, Sada M, Harada K, Kodama M, et al. (2010) Allogeneic administration of fetal membrane-derived mesenchymal stem cells attenuates acute myocarditis in rats. J Mol Cell Cardiol 49: 753–761.
5. Tsuda H, Yamahara K, Ishikane S, Otani K, Nakamura A, et al. (2010) Allogenic fetal membrane-derived mesenchymal stem cells contribute to renal repair in experimental glomerulonephritis. Am J Physiol Renal Physiol 299: F1004–1013.
6. Ohshima M, Yamahara K, Ishikane S, Harada K, Tsuda H, et al. (2012) Systemic transplantation of allogenic fetal membrane-derived mesenchymal stem cells suppresses Th1 and Th17 T cell responses in experimental autoimmune myocarditis. J Mol Cell Cardiol 53: 420–428.
7. Tsuda H, Yamahara K, Otani K, Okumi M, Yazawa K, et al. (2013) Transplantation of allogenic fetal membrane-derived mesenchymal stem cells protect against ischemia-reperfusion-induced acute kidney injury. Cell Transplant.
8. Ishikane S, Hosoda H, Yamahara K, Akitake Y, Kyoungsook J, et al. (2013) Allogeneic Transplantation of Fetal Membrane-Derived Mesenchymal Stem Cell Sheets Increases Neovascularization and Improves Cardiac Function after Myocardial Infarction in Rats. Transplantation.
9. Portmann-Lanz CB, Schoeberlein A, Huber A, Sager R, Malek A, et al. (2006) Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. Am J Obstet Gynecol 194: 664–673.
10. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315–317.
11. Nakagawa O, Ogawa Y, Itoh H, Suga S, Komatsu Y, et al. (1995) Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. J Clin Invest 96: 1280–1287.
12. Yamahara K, Sone M, Itoh H, Yamashita JK, Yurugi-Kobayashi T, et al. (2008) Augmentation of neovascularization [corrected] in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. PLoS One 3: e1666.
13. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105: 1815–1822.
14. Kikuchi-Taura A, Taguchi A, Kanda T, Inoue T, Kasahara Y, et al. (2012) Human umbilical cord provides a significant source of unexpanded mesenchymal stromal cells. Cytotherapy 14: 441–450.
15. Wei JP, Zhang TS, Kawa S, Aizawa T, Ota M, et al. (2003) Human amnion-isolated cells normalize blood glucose in streptozotocin-induced diabetic mice. Cell Transplant 12: 545–552.
16. Pan HC, Yang DY, Chiu YT, Lai SZ, Wang YC, et al. (2006) Enhanced regeneration in injured sciatic nerve by human amniotic mesenchymal stem cell. J Clin Neurosci 13: 570–575.
17. Bailo M, Soncini M, Vertua E, Signoroni PB, Sanzone S, et al. (2004) Engraftment potential of human amnion and chorion cells derived from term placenta. Transplantation 78: 1439–1448.
18. Rossi D, Pianta S, Magatti M, Sedlmayr P, Parolini O (2012) Characterization of the conditioned medium from amniotic membrane cells: prostaglandins as key effectors of its immunomodulatory activity. PLoS One 7: e46956.
19. Kim SW, Zhang HZ, Guo L, Kim JM, Kim MH (2012) Amniotic mesenchymal stem cells enhance wound healing in diabetic NOD/SCID mice through high angiogenic and engraftment capabilities. PLoS One 7: e41105.
20. Lee JM, Jung J, Lee HJ, Jeong SJ, Cho KJ, et al. (2012) Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. Int Immunopharmacol 13: 219–224.
21. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, et al. (2006) Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 12: 459–465.
22. Gnecchi M, He H, Liang OD, Melo LG, Morello F, et al. (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11: 367–368.