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Original Article

Fetoscopic laser coagulation of intertwin anastomoses reduces discordant placental autophagic activities in discordant twin growth

Yao-Lung Chang*, Tzu-Hao Wang, Shuenn-Dyh Chang, An-Shine Chao, Peter C.C. Hsieh

Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan, ROC

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ABSTRACT

Objectives: To investigate placental autophagic activity in cases of twin-twin transfusion syndrome (TTTS) after successful laser therapy and to evaluate the effect of intertwin anastomoses on discordant placenta autophagic activity in monochorionic twins with one twin exhibiting selective intrauterine growth restriction.

Materials and methods: Placenta samples were prospectively obtained from 11 cases of successful TTTS post-laser therapy with two living babies. Among these infants, five infants had selective intrauterine growth restriction (sIUGR), based on the definition of a birth weight below the 10th percentile. After protein extraction, western blot tests were used to determine the amount of placenta microtubule-associated protein 1A/1B-light chain 3 (LC3)-II protein in the two individual placenta territories of the twin pair. The LC3-II protein fold change ratio (FCR) in a twin pair was defined as the LC3-II protein fold value over β-actin of the smaller twin divided by the LC3-II protein fold value over β-actin of the larger twin.

Results: The LC3-II FCRs were not significantly different between TTTS with sIUGR and TTTS without sIUGR, after successful laser therapy.

Conclusion: The discordance of placenta autophagic activity in the monochorionic twin with sIUGR was reduced after laser coagulation of the intertwin anastomoses, which may result from the effect of correction of the discordant intertwin placenta perfusion.

Introduction

Autophagy, also known as type II programmed cell death, is a catabolic process used by eukaryotic cells to recycle long lived proteins and lipids, and to eliminate protein aggregates and organelles [1]. Autophagic activity is upregulated by starvation, growth factor deprivation, and hypoxia [2], and autophagic activity is increased in the placenta of singleton fetuses with intrauterine growth restriction (IUGR) [3].

Microtubule-associated protein 1A/1B-light chain 3 (LC3) proteins have been reported in human placenta [4,5]. During autophagy, autophagosomes engulf intracellular proteins and organelles. At the same time, the cytosolic form of LC3, also called LC3-I, conjugates to form phosphatidylethanolamine. This conjugated form of LC3, called LC3-II, can reflect autophagic activity and the autophagy-related process [6].

Twin-twin transfusion syndrome (TTTS) complicates approximately 20% of all monochorionic diamniotic twin pregnancies [7]. Laser therapy is the first-line treatment for all stages of TTTS diagnosed before 26 weeks [8]. The discordance in placental perfusion between the donor and recipient resulting from unbalanced intertwin flow can be reduced to some extent by coagulating the vascular anastomoses [9,10]. We have also found that autophagic activity was increased in the placental territory of the twin with selective intrauterine growth restriction (sIUGR) in monochorionic (MC) twins [11]. We suspected this finding may have resulted from relative hypoxia or hypoperfusion in the placental territory of the sIUGR fetus, compared to the placental territory of the appropriate-for-gestational age (AGA) co-twin. In this paper, we were interested to know what the placental autophagic activity would be like in infants with TTTS who were successfully treated by laser, which resulted in two live babies for whom part of the

* Corresponding author. Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Linkou Medical Center, 5, Fu-Shin Street, Kweishan, Taoyuan 333, Taiwan, ROC.
E-mail address: j12054@cgmh.org.tw (Y.-L. Chang).

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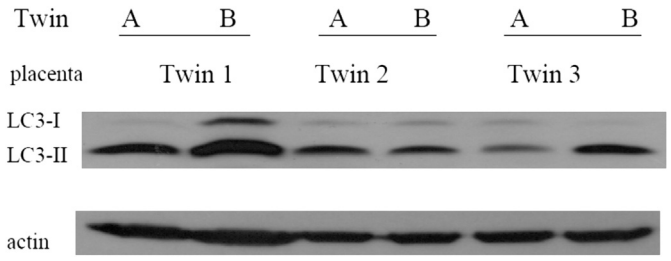

Figure 1. Western blot results of the LC3-I and LC3-II proteins in twin pairs. A = first twin; B = second twin; LC3 = microtubule-associated protein 1A/1B-light chain 3.

unbalanced blood flow between the two fetuses had theoretically been corrected by laser. We focused on determining whether the difference in autophagic activity between the two placental territories from the AGA and IUGR fetuses would be changed by the laser operation. Therefore, the purposes of this study were to investigate the effect of eliminating intertwin vascular anastomoses by laser operation in TTTS on placental autophagic activity, and to discuss the role of vascular anastomoses in causing discordant placental autophagic level in MC twins with sIUGR.

Materials and methods

The placentas were collected prospectively from women with TTTS treated by successful laser therapy with two live babies after delivery at the Chang Gung Memorial Hospital (Taoyuan, China). They were sufficiently intact to be studied after birth. Laser therapy was deemed successful when there was resolution of the polyhydramnios–oligohydramnios sequence after the operation and two viable babies after delivery, and when the gross placenta examination detected no evidence of residual anastomoses. All twins were delivered by cesarean section. A twin pregnancy with sIUGR was defined as an estimated fetal weight below the 10th percentile in one twin [12]. The diagnosis of TTTS was based on the ultrasound findings defined by Quintero et al [13]. In a twin pair, the smaller twin had the lower birth weight and the larger twin had the higher birth weight. In TTTS with sIUGR, the twin with sIUGR had a birth weight below the 10th percentile and the AGA twin did not have IUGR. This study was approved by the local institutional ethics committee.

Placenta collection

Two or three pieces of placenta (0.5 cm × 0.5 cm × 0.5 cm) from each placental territory were obtained approximately midway from the vascular equator and individual cord insertion and from the middle thickness part between the maternal and fetal surfaces. The sampled specimens in the MC placenta of the two twins were freshly collected. The vascular equator of TTTS was defined as the border in the middle of the avascular zone on the chorionic fetal surface when there was no intertwin vascular anastomoses or on the anastomosis points where the twin–twin communicating vessels met (i.e., the anastomoses had been coagulated by laser) [14]. Areas that contained calcification and infarction were avoided. The placental specimens were briefly rinsed with ice-cold phosphate-buffered saline (PBS) to wash off blood. The placental tissue was then frozen in liquid nitrogen, and stored at −70°C.

Western blot analysis

In this study, proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-Cl, 150 mmol/L sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) at pH 7.5]. The RIPA buffer contained 1 mmol/L phenylmethylsulfonyl fluoride. The protein extract was later quantified with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). One hundred micrograms of placental proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose membrane. These proteins were probed with primary antibodies against human LC3-I and LC3-II (1:2000; Novus Biologicals, Littleton, CO, USA) at 4°C overnight. The membrane was washed three times with Tris-buffered saline and Tween 20 (TBST), and then probed with a secondary antibody for 2 hours. The proteins were stained by enhanced chemiluminescence (WBKLS0500, Pierce 32209, or Santa Cruz sc-2048; Millipore, Santa Cruz, CA, USA) and the LC3-II protein content was normalized by β-actin as the fold change (Figure 1).

The LC3-II fold change ratio (FCR) in a twin pair was defined as LC3-II protein fold change over β-actin of the smaller (i.e., sIUGR) twin divided by the LC3-II protein fold change over β-actin of the larger (i.e., AGA) twin, which represents the autophagic activity of the sIUGR twin's placenta territory by using the AGA twin's placenta territory autophagic activity as the internal control. In this study, all smaller twins were donor twins.

Statistical analysis

Statistical analysis was conducted with SPSS for Windows software, version 11.0 (SPSS Inc, Chicago, IL, USA). Two-sample Student *t* test or Mann–Whitney *U* test was used for between-group comparison for the continuous variables. A probability value of less than 0.05 was considered statistically significant.

Results

Clinical characteristics

In this study period, there were 11 pairs of TTTS postlaser therapy, which included five infants with sIUGR. All placentas were sufficiently intact for study. The two groups with and without

Table 1
Characteristics of TTTS with and without sIUGR.

	TTTS with sIUGR (n = 5)	TTTS without sIUGR (n = 6)	p
Gestational age at delivery (wk)	32.3 ± 2.4	35.1 ± 0.8	0.022
Maternal age at delivery (y)	31.2 ± 5.0	33.2 ± 2.8	0.425
Gestational age at laser therapy (wk)	21.2 ± 2.9	18.8 ± 2.6	0.174
Birth weight of the larger twin baby (g)	1915 ± 483	2217 ± 249	0.141
Birth weight of the smaller twin (g)	1183 ± 497	2142 ± 258	<0.001
LC3-II FCR	0.92 ± 0.25	1.13 ± 0.27	0.225

The data are presented as the mean ± the standard deviation.
FCR = fold change ratio [defined as the LC3-II protein fold change of the smaller (sIUGR) twin over β-actin divided by the LC3-II protein fold change of the larger (i.e., appropriate-for-gestational age) twin over β-actin]; LC3 = microtubule-associated protein 1A/1B-light chain 3; sIUGR = selective intrauterine growth restriction; TTTS = twin-twin transfusion syndrome.

sIUGR, were comparable in maternal age, gestational age at laser therapy, and birth weight of the recipient. However, the TTTS with the sIUGR twins were delivered significantly earlier. The mean birth weight of the donor twin was less than the weight of the twin without sIUGR (Table 1).

Placental LC3-II protein expression

The level of placental LC3-II proteins were not significantly different between the smaller (sIUGR) twin and larger (AGA) twin in TTTS with (1.04 vs. 1.05, respectively; $p = 0.39$) and TTTS without sIUGR (1.01 vs. 1.89, respectively; $p = 0.32$). The LC3-II FCRs consequently showed no significant difference between TTTS with and without sIUGR (Table 1).

Discussion

In this study, after having delivered successful laser therapy, placental autophagic activity was not significantly different between the sIUGR (i.e., smaller) twin and the AGA (i.e., larger) co-twin in TTTS with or without sIUGR. We previously found the phenomenon of discordant placenta autophagic activity in the MC twin with sIUGR: the autophagic activity was increased in the placenta territory of sIUGR fetuses, especially in an sIUGR twin with abnormal umbilical artery Doppler flow [11]. However, in this study, after eliminating intertwin vascular anastomoses, the discordant placenta autophagic activity was not present between the AGA and sIUGR fetuses.

The twins with sIUGR were delivered earlier than those without sIUGR out of consideration for prompt delivery of the sIUGR twin. Placentas from late gestation fetuses delivered by cesarean section had lower levels of LC3-II, compared to early and midgestation fetuses [15]. However, the AGA twin was the control for the sIUGR twin in each twin pair and the two fetuses in a twin pair were born at the same gestational age; therefore the effect of different gestational ages at delivery between the two groups of TTTS would not influence the FCRs of placenta LC3-II.

In MC twins, unique intertwin vascular anastomoses exist; therefore, the discordant intertwin perfusion in TTTS may be partly caused by unbalanced transfusion via vascular anastomoses. There are reports that demonstrate that applying a laser to coagulate the intertwin anastomoses could to some degree reverse the intertwin discordant umbilical venous perfusion [9,10]. According to our previous study, discordant placental autophagic activity was detected in MC twins with sIUGR, especially when the sIUGR twin presented with abnormal umbilical artery Doppler [11], which indicates less placental perfusion [16]. Therefore, a reduction in the difference in intertwin placenta autophagic activity between sIUGR twins and AGA twins after lasering the intertwin vascular anastomoses may be because of the effect of minimized intertwin discordant placental perfusion. In our present case series, we chose infants with successful operation results in which the anastomoses had been completely coagulated, and we found no significant discordant autophagic activity in each twin pair's individual placental territory whether they presented with or without sIUGR. Autophagic activity is stimulated by starvation and oxidative stress [17]; therefore, our finding suggested that eliminating the intertwin anastomoses in TTTS may reverse the stress condition in the sIUGR twins.

In conclusion, using a model of TTTS treated by laser operation, we found the discordance in placental autophagic activities between the AGA and sIUGR twins could be reduced by eliminating the intertwin vascular anastomoses. This finding may be because laser therapy improved the discordance of intertwin placental perfusion to some extent, which decreased the intertwin autophagic activity discrepancy.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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