

行政院國家科學委員會補助專題研究計畫成果報告

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以三維影像重建及體外共同培養模式研究早期絨毛間胎兒循環
The study of intervillous circulation in the first trimester with *in vivo*
3D interrogation and *in vitro* co-culture model

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計畫主持人：謝豐舟

共同主持人：施景中

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- 赴國外出差或研習心得報告一份
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以三維影像重建及體外共同培養模式研究早期絨毛間胎兒循環

The study of intervillous circulation in the first trimester with *In-vivo* 3D interrogation and *in-vitro* co-culture model

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一、中文摘要

古典觀念認為由滋養層細胞間相通而成的絨毛間空腔，自胚胎非常早期即開始發生作用養育胚胎。晚近，這個觀念開始為哈斯丁等人懷疑，哈斯丁觀察在子宮完全切除標本當中的胚胎，他們發現在12週之前對比顯影劑無法進入此絨毛間空腔中，因此他們最後結論早期的胚胎乃泡在由母親循環所產生的細胞外液中。滋養層細胞封住母體螺旋動脈後，在12週前才逐漸將動脈末稍開放。由於目前學術界對這領域的討論仍莫衷一是，遂興起吾人深究的意願。

立體超音波為新近發展之全新領域，可提供較傳統平面超音波其它許多不同的好處。新一代的彩色能量超音波有比傳統彩色都卜勒超音波四倍以上的靈敏度，本研究即利用彩色能量超音波的優點，輔以三維影像重建，對12週之前的胚胎實施檢查，將往昔所謂的滋養層細胞邊血流，精確加以定位，確定其為在胎盤絨毛間空腔中，亦或僅是靠近胎盤而未進入絨毛間空腔中因此三維彩色能量超音波可謂研究絨毛間循環的發育的最佳工具。在吾人系列且謹慎的觀察下，於六至十二周的早期懷孕永遠都可以三維彩色能量超音波顯示，由母親螺旋動脈的分枝進入蛻膜組織中，並形成絨毛間隙的循環。這與目前主流的哈斯丁看法大相徑庭，而且三維彩色能量超音波的結果亦可提供對懷孕預後的預測。

另外，我們亦進一步採取體外模式去研究絨毛間循環的發育。培養的效果雖不如預期，但以免疫螢光對原位組織觀察滋養層細胞及蛻膜在著床後互動的型態，卻直接證實早期子宮螺旋動脈的栓塞其實只在部分血管發生，以印第安墨水對早期懷孕的子宮做灌流，亦可發現墨水可暢流無阻直達絨毛間隙的循環，也因此直接證實絨毛

間隙的循環於第一週產期確實存在。

關鍵詞：滋養層細胞、立體超音波、細胞培養、免疫螢光。

Abstract

Classic concept indicates the intervillous space, which is formed by the intercommunicating lacuna within trophoblasts, begin to function at early gestation and nourish the early embryo. Recently, this concept has been challenged by the Hustin's observation on the "en bloc" embryo within the hysterectomy specimen. He found that the barium sulfate could not enter the intervillous space before 12 weeks gestation. He finally concluded that the early embryo is bathed within extracellular fluid produced from maternal circulation. The trophoblasts obliterated the distal end of spiral arteries, and the plug gradually open at the end of first trimester.

Three-dimensional (3D) ultrasound is a new technique offering various possible advantages over conventional two-dimensional ultrasound. The new generation of color Doppler - power Doppler ultrasound has 4-times sensitive to the conventional Doppler US. We attempt to use the power Doppler interrogation for the early embryos to search the existence of intervillous circulation before 12 weeks gestation weeks. By using the 3D reconstruction of the power Doppler signals, we are able to display the intervillous flow by 12 weeks of gestation. The abnormal 3D power Doppler signals can also apply to predict the abnormal outcome of the pregnancy.

We adopt the *in-vitro* model for assessment of

the intervillous circulation. We collect trophoblasts and decidual explants from therapeutic abortion ranging 8-12 weeks, and co-culture them in a physiological condition. We fail to isolate and culture the trophoblast, thus we shift our work to investigate the immunofluorescence of the trophoblast. The results are rather exciting. We found in most cases, the trophoblast only form plug in a small numbers of vessels. By using Indian ink perfusion of the pregnant uterus (after hysterectomy due to gynecologic reason), we did find the ink directly pass from transformed spiral artery and into the intervillous space (first-trimester uterus). We therefore proved the existence of first-trimester intervillous circulation.

Keywords: trophoblast, three-dimensional ultrasound, cell culture, immunofluorescence.

二、緣由與目的

There are debates existing in the developmental process of the intervillous circulation. In the human, the blastocyst adheres to the surface of the endometrium and then embeds itself into the underlying stroma between the glands. From the blastocyst, there is a differentiation of the peripheral layer of the primitive syncytiotrophoblasts and an inner layer of cytotrophoblasts. Trophoblastic cells invade the maternal tissue from proliferating tips of anchoring villi and the cytotrophoblastic shell. The intermediate trophoblasts also plug the distal tips of maternal spiral arteries, migrate in a retrograde fashion within the vessels, and eventually become incorporated into the wall of the spiral artery. The uteroplacental vasculature, including the intramyometrial segment of spiral arteries, the subjacent myometrium circulation (radial and arcuate arteries), and main uterine arteries are adapted to nourish the embryo-placental compartment.

Classic concept indicates the intervillous space, which is formed by the intercommunicating lacuna within trophoblasts, begin to function at early gestation and nourish the early embryo. Recently, this concept has been challenged by the Hustin's

observation on the "en bloc" embryo within the hysterectomy specimen. He found that the barium sulfate could not enter the intervillous space before 12 weeks gestation. He finally concluded that the early embryo is bathed within extracellular fluid produced from maternal circulation. The trophoblasts obliterated the distal end of spiral arteries, and the plug gradually open at the end of first trimester. Premature opening of the trophoblastic plug in the distal end of spiral arteries would result in the disruption of materno-trophoblastic border and eventually the embryo death.

Three-dimensional (3D) ultrasound is a new technique offering various possible advantages over conventional two-dimensional ultrasound. The new generation of color Doppler - power Doppler ultrasound has 4-times sensitive to the conventional Doppler US. We attempt to use the power Doppler interrogation for the early embryos to search the existence of intervillous circulation before 12 weeks gestation weeks. With the adjunct of 3D reconstruction, we may able to differentiate the location of power Doppler signals either within the intervillous space or only adjacent to placenta. The origin of flow signals and the developing uteroplacental circulation can also depicted. Therefore the 3D power Doppler can be the best way to detect the existence of intervillous circulation.

Besides, we adopt the in-vitro model for assessment of the intervillous circulation. We collect trophoblasts and decidual explants from therapeutic abortion ranging 8-12 weeks, and co-culture them in a physiological condition. The morphological interactions between the trophoblasts and decidual explants are observed with histological block (either with light microscope or scanning EM) in a temporal sequence. The development of intervillous circulation then can be observed in extrauterine situation.

三、研究方法

3D power Doppler is potentially the best way to examine the intervillous circulation. First of all, the

power Doppler is about four times sensitive than conventional color Doppler ultrasound. Thus it is possible to detect any significant blood flow in more early gestation. Furthermore, the origin of signal can be traced through three-dimensional reconstruction, rather than simply depicted by the sectional planes. We can directly confirm the sporadic signals within the echogenic rims of gestational sac in connection with their intramyometrial spiral arteries, and produce a typical "coral" shape of vascular network composed of intervillous circulation and the transformed spiral arteries, radial arteries, arcuate arteries and uterine arteries.

Power Doppler Ultrasound

The ultrasound we used in performing image acquisition is the new generation ultrasound capable of power Doppler interrogation. Currently the ultrasound HDI-3000 (Advanced Technology Laboratories, Bothell, WA, US) can meet our requirement in this sensitive examination. The scanning transducers include 4-2 MHz curvilinear transabdominal arrays and 7-5 MHz curvilinear transvaginal arrays.

We use the new generation of Doppler ultrasound equipped with updated 3D software to examine the developing placenta. We enroll those pregnancies ranging (1) before first trimester (12 weeks' GA) (2) 13-18 weeks' GA (early second-trimester). The correct dating of embryos are based upon the information of last menstruation as well as judged by fetal CRL (crown-rump length) in first- trimester, and BPD (biparietal diameter), AC (abdominal circumference) in second-trimester.

3D reconstruction

We conduct two types of reconstruction. The first is the built-in method using maximal intensity projection (MIP) and producing multislicing cube to display the angioarchitecture of developing placenta. The second method incorporates a workstation which is capable of processing both gray-scale and color flow information. For this purpose, we decide to add the investment to buy the new generation of 3D workstation (Compact 3D®;

Tom-Tec, Munich, Germany) couple to the original ultrasound unit (HDI 3000).

Isolation and co-culture of trophoblast

We initially try to isolate the trophoblast from the third-trimester placenta. Third trimester chorionic placental villi were obtained from Cesarean section at 37-40 weeks. All incubations were carried out in the standard condition as the literature cited. However, although we try several conditions, the abundant RBCs in the tissues always prohibited the trophoblast plating. We then try to use the Percoll gradient to isolate the trophoblast in the specific gravity. Nonetheless, although we try several gradient, we still cannot purify the trophoblast. Finally we give up to isolate and co-culture the trophoblast.

Immunocytochemistry

For identification of the migrating interstitial trophoblast (iCTB) and endovascular trophoblast (vCTB), we use a panel of antibodies for that purpose. Anti-pancytokeratin (CK 7, Dako Ltd, UK) was used to identify the migrating interstitial trophoblast (iCTB), and CD 56 (Santa Cruz, US) was used to identify the endovascular trophoblast. Besides, phalloidin was used in the identification of actin filaments that commonly present in the muscular media of the deciduas vessels. Hoechst 432 was used to recognize the nucleus of all kinds of cells. The antibodies concentrations were as follows: anti-pancytokeratin (CD7)- 1:200; CD 56- 1:100, Hoechst 432- 1:1000. The anti-pancytokeratin and CD 56 was conjugated with the secondary antibody of FITC (1:200); and Phalloidin was conjugated with rhodamin (1:200) for fluorescence identification. In some case, for comprehensive illustration of the corresponding anatomy structure, we use first antibodies to conjugated with DAB staining and counter-stain with Hematoxylin. The materials we use is also the surgical specimen from the first-trimester uterine curetage and the third-trimester placenta from term Cesarean section.

四、結果與討論

With optimizing the Doppler flow parameters,

2D power Doppler is able to display the blood flow signals within deciduas basalis (the future placenta) in the pregnancies ranging 6-12 weeks of gestation. By using 3D reconstruction, we are able to differentiate the overlapping signals either from maternal or fetal side. The flow can be separate into fetal flow signals and maternal circulation into the future placenta. The flow coming from maternal side becomes a fountain-like inward sinusoid entering chorion frondosum in most of the cases. It directly *in-vivo* proves the existence of intervillous circulation in the first trimester.

Of note is that, when patients experienced vaginal bleeding or other signs of threaten abortion, 3D power exhibits changes in 68% of patients. The decidual thickness is decreasing, and the intervillous circulation fuse to form more extensive flow signals around the gestational sac. In these patients with changes of 3D power Doppler, the clinical symptoms are also parallel. Moreover, patients with the changes of 3D power Doppler foretell the occurrence of abortion in 87% cases in this group.

We examine the iCTB with anti-pancytokeratin and found that iCTB has a transition from cell column to uterine myometrium. The iCTB in cell column is non-polar, non-motile rounded cell. When iCTB detach the cell column, the iCTB begins to differentiate into polarized, motile iCTB with actin upregulation. When iCTB reach myometrium, it begins to fully differentiate into stellate cell (non-polar, non-motile). The transition process is quite important for the normal implantation. As the endovascular CTB (vCTB), we observed the vCTB will adopt the endothelial cell characteristics, and also share some characteristics of malignant cell. vCTBs will migrate upstream the spiral arteries, in some areas, will become multilayering cells as frequently seen in the cancer cells. With TEM and SEM observation, we also disclose the apical surface of vCTB is specialized to a unique structure that can adhere other vCTBs. During observation of the transform spiral arteries, trophoblast plug the spiral artery is seldom seen. These basic works prove the existence of the first-trimester intervillous circulation.

五、計畫成果自評

The results obtained from 3D power Doppler were exciting. Not only we proved the existence of intervillous circulation in the first trimester, but also identified some adverse factors of implantation failure.

In cases of co-culture system, we fail to reproduce the *in vitro* trophoblast and deciduas co-culture in the literature (our initial plan). However, the immunofluorescence findings were even more illustrative and exciting. We directly prove that the trophoblast plugging the spiral arteries in the first trimester is only an occasional finding. In most of the cases, the endovascular trophoblasts in the spiral arteries show aggregation, but seldom totally obstruction. From the Indian-ink perfusion experiment, we also directly prove the existence of first-trimester intervillous circulation. The results certainly contradict to the mainstream of current concepts, and will impact on the reproductive immunology. All the above 3D power Doppler and immunofluorescence findings are so novel and we will submit these data to SCI journals.

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