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Original Article

Expression of vascular endothelial growth factor (VEGF) under hypoxia in placenta with intrahepatic cholestasis of pregnancy and its clinically pathological significance

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Abstract: The expression of vascular endothelial growth factor (VEGF) under hypoxia in the placenta with intrahepatic cholestasis of pregnancy (ICP) was observed, and mechanisms of ICP fetal distress were discussed. Methods: Different culturing times were established in hypoxia incubator, and protein expressions of VEGF in placental tissue were observed using immunohistochemical S-P method. Results: After 4 h hypoxic culture, VEGF protein expression in ICP group was higher than the normal group with significant difference ($P < 0.05$). With the extension of hypoxic exposure, VEGF protein expression in both groups was suppressed, but no distinction in-between. Regression analyses indicated a noticeable effect of CG on VEGF expression, the higher the CG was, the lower the VEGF protein expression was ($P < 0.05$). Conclusions: Short term hypoxia induces up-regulation of VEGF expression in ICP placenta, and this adaptive change is probably a protective mechanism of fetus in ICP.

Keywords: Vascular endothelial growth factor, intrahepatic cholestasis of pregnancy, placenta, hypoxia, immunohistochemistry

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is an obstetric complication occurs in late pregnancy with the clinical features of pruritus and increase of serum bile acid and CG in late pregnancy. Because ICP probably causes adverse perinatal outcomes: premature delivery (19-60%), fetal distress (22-41%), meconium-stained amniotic fluid (27%), fetal bradycardia (14%), and fetal death (0.4-4.1%), it has attracted more and more clinical attention [1]. The development of ICP probably involves gene, hormone, and environmental factors, but the exact cause is still unrevealed [2]. Vascular endothelial growth factor (VEGF) is an essential angiogenic factor, which plays an important role in the formation of placental angiogenesis and fetal growth [3, 4]. Recently, vascular endothelial injury and roles of various growth factors in ICP have attracted increasing attention [5]. A number of *in vitro* studies indicate that hypoxia (2-5% O_2) promotes proliferation [6], migration [7], and formation of capillary network [8] in endothelial cells. The present study aims to study the variation of VEGF protein expression after hypoxic culture, and verify whether VEGF is involved in the ICP clinical pathological process.

Materials and methods

Clinical data

25 ICP patients had cesarean delivery from September 2013 to December 2014 in our hospital are randomly selected, while 27 gravidas had cesarean delivery in full-term pregnancy for scar uterus, and social reasons etc. are selected as a normal group. ICP diagnostic criteria refer to "ICP treatment guidelines (obstetrics group, obstetrics and gynecology branch of Chinese Medical Association)" in "Chinese Journal of Obstetrics and Gynecology" volume 46, 2011: Blood CG ≥ 10.75 μmol/L or the total cholic acid increased ≥ 10 μmol/L. Samples in both groups were single births, had no other complications of pregnancy, placenta calcifica-

Table 1. Comparison of general information of gravidas and infants in both groups

Groups	*n*	Ages (y)	Pregnancy days	Birth weight (g)	Neonatal length (cm)	BPD (mm)	Apgar	PT (s)
ICP	25	27.40±3.04	257.84±15.35	2957.60±610.00	48.04±2.44	91.04±4.45	9.32±0.56	11.30±1.05
Normal	27	27.11±2.56	275.19±6.27	3458.15±297.54	50.48±0.81	94.67±2.59	9.44±0.58	10.72±1.09
t		0.37	-5.26	-3.714	-4.77	-3.63	-0.79	1.953
p		0.71	0.00	0.00	0.00	0.00	0.43	0.06

Table 2. Comparison of VEGF expression in both groups under the same hypoxia condition

Groups	*n*	Placenta tissue		Villus hypoxia 4 h		Villus hypoxia 12 h		Villus hypoxia 24 h	
		-	≥+	-	≥+	-	≥+	-	≥+
ICP	25	2	23	5	20	10	15	12	13
Normal	27	1	26	13	14	9	18	13	14
t		0.44		4.54		0.25		0.00	
p		0.51		0.03		0.62		0.991	

tion and intrauterine growth restriction were excluded by B ultrasonic examination, had no preoperative uterine contractions. Fasting blood before surgery were sampled for CG, total bile acid, coagulation testing for both groups. The study procedures complied with ethical standard established by committee of human trials in our hospital, the sampled patients were informed and consented with the grouping.

Placenta culture

A piece of 3 × 1 × 1 cm^3 placental tissue near the umbilical cord and close to cotyledons was taken under sterile conditions after delivery and rinsed extensively in sterile saline to remove blood, and excised out decidua and large placental blood vessels. 1/3 of the tissue was taken and fixed with 4% formaldehyde and embedded in routine paraffin, and the remaining 2/3 of the tissue was operated on aseptic table. Specific procedures: cut the chorionic villi into pieces of about 10 mg in 4°C saline. The villi tissue was seeded in 25 cm^2 glass petri dishes, 400 mg each dish, 5ml DMEM medium (Gibco, Life Technologies Co., CA, USA) was added, and preincubated in CO_2 thermostat incubator of 37°C, 5% CO_2/95% air for 24 hours.

After 24 hours, proper amount of 0.25% trypsin-EDTA was added into cultured tissue, and digested at 37°C incubator for l hour. Hank's was added to terminate the digestion. Undigested tissue fragments was removed, supernatant was taken after 3 min centrifugation in 1500 rpm, 0.1 mol/ml PBS was added twice to wash and counted with 0.04% trypan blue within three minutes. It was found that viable cells was > 90% after cultured under room temperature and oxygen for 24 hours, which indicated tissue viability for the next step of villus hypoxic culture.

Villus hypoxic culture

The placental villi were equally put into the three Petri dishes containing culture medium, and the Petri dishes were placed in a sealed box (25 × 12 × 8 cm^3). Hypoxic gas mixture (5% CO_2/3% O_2/92% N_2) was filled into the culture box from air intake with a gas flow rate of 5 L/min, continued for 2 min, and discharged the gas from the vent. And then the Petri dishes were put into the incubator under 37°C CO_2, and cultured for 4 h, 12 h, 24 h respectively. The tissue with each hypoxic duration was put into vials after culture and frozen at -80°C for further use.

Immunohistochemistry to evaluate VEGF expression

All villi tissue after hypoxic culture was fixed with 4% formaldehyde and embedded in routine paraffin, and stained by S-P immunohistochemistry, then the distribution and the intensity of positive staining of the two groups of placental tissue was observed under optical microscope. VEGF kit and second antibody were both purchased from Guangzhou Shenda Biological Products and Technology Co., Ltd., and manipulated strictly accordance with the kit instructions. Phosphate buffer (PBs) was employed instead of the primary antibody as a negative control. Scoring according to the proportion of positive cells and colored shades: cells without staining or no positive cells

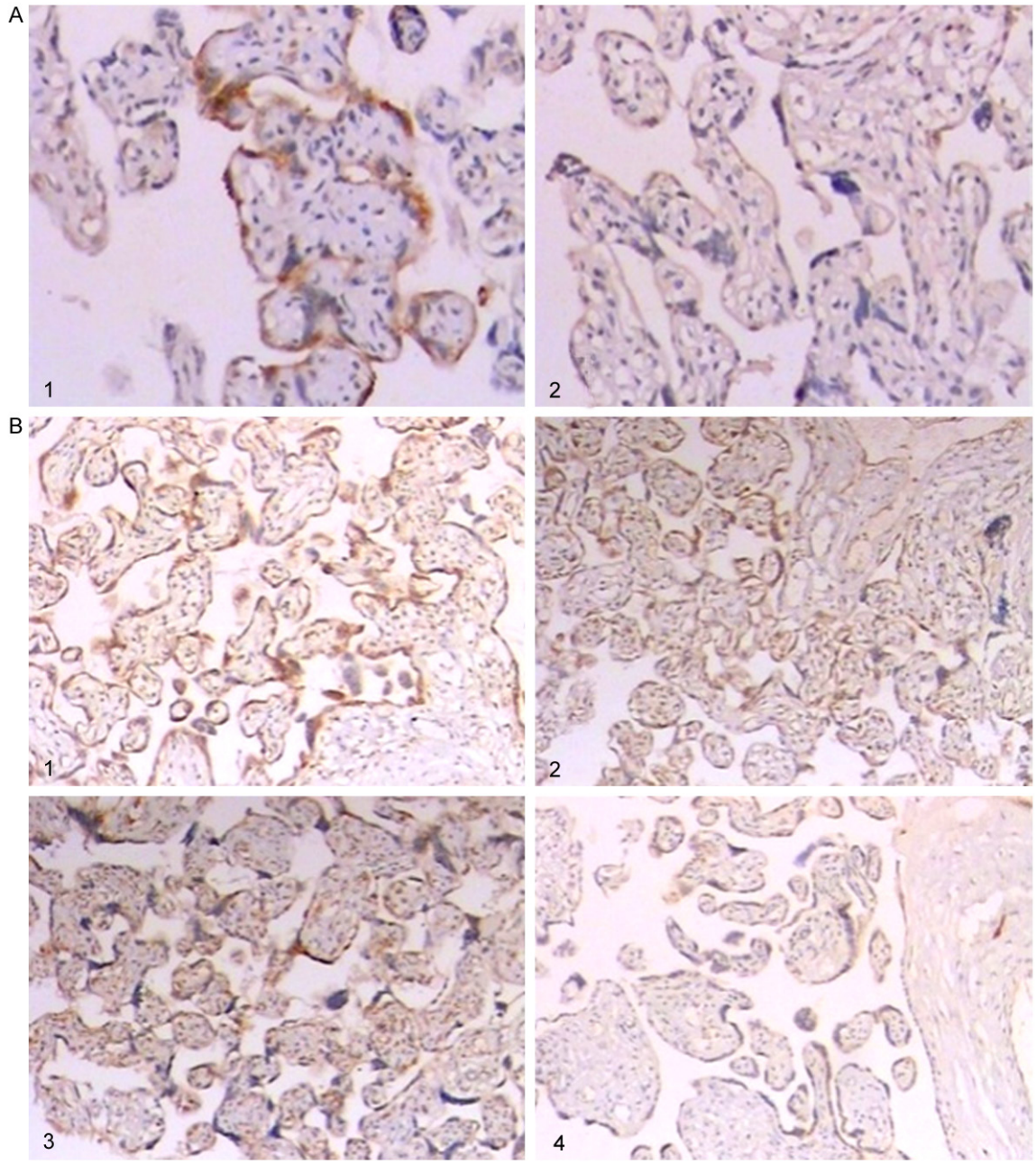
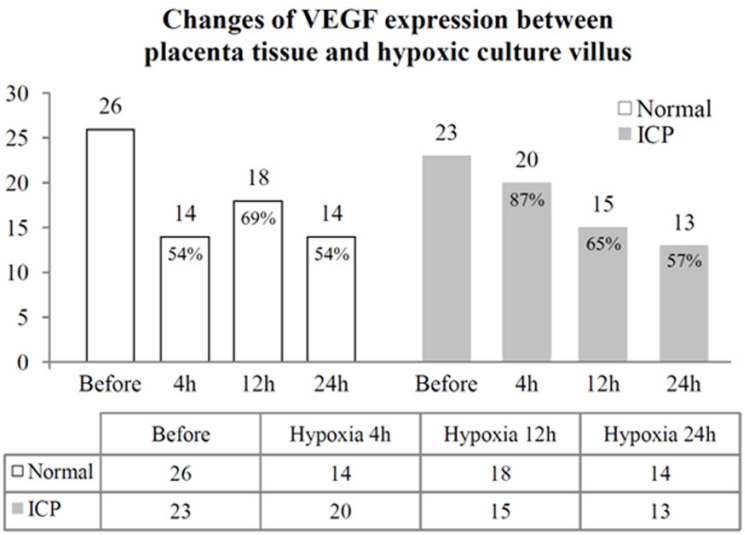


Figure 1. A: 1. Intensive expression of VEGF in ICP group after 4 h hypoxic culture. 2. Moderate expression of VEGF in normal group after 4 h hypoxic culture. There was statistic difference between these two groups ($P < 0.05$). B: 1. ICP group after 12 h hypoxic culture. 2. Normal group after 12 h hypoxic culture. 3. ICP group after 24 h hypoxic culture. 4. Normal group after 24 h hypoxic culture. There was no difference between 1 and 2, 3 and 4.

accounted as 0, cells stained into light brown or 1%~25% positive cells as 1, cells stained into brown or positive cells 26%~50% as 2, cells stained tan or 51 to 75% positive cells as 3, cells stained tan or positive cells > 75% as 4; a positive rating was determined based on scoring results: 0~1 as (-), 2~3 as (+/-), 4~5 as (+), 6~7 as (++). Scoring (+) and above are considered as a positive result.

Statistics

SPSS l3.0 statistical software was used for statistical analysis, measurement data was denot-



	Before	Hypoxia 4h	Hypoxia 12h	Hypoxia 24h
Normal	26	14	18	14
ICP	23	20	15	13

Figure 2. Comparison of VEGF expression change between the two groups before/after hypoxic culture.

ed by ($\bar{x}\pm s$), t test and x^2 test were used for inter-group comparison, spearman rank correlation analysis and regression analysis are used on the relevant data, $P < 0.05$ was considered as statistically significance.

Results

Comparison of general information of gravidas and infants in both groups

Age, Apgar score, prothrombin time (PT) of gravidas in both groups had no statistically significance. Gestational weeks in ICP group were less than the normal group which had statistically significance; growth and development indicators (birth weight, length, biparietal diameter) of the two fetal groups had statistical significance, and ICP group was lower than the normal group (P = 0.00). Correlation analysis showed that fetal growth and development indicators associated with gestational age (r = 0.78, $P = 0.00$) **Table 1**.

Comparison of VEGF expression before/after hypoxia

VEGF distributions in placenta of ICP group and the normal group were generally consistent, which were mainly in cytoplasm of trophoblast cells, vascular endothelium and villous stroma cells. After hypoxic culture for 4 hours, the expression of VEGF protein in ICP group was 87% higher than before hypoxia, with a significant difference ($P < 0.05$). With prolonged hypoxia, the expressions of VEGF in both groups were inhibited. After hypoxic culture of 12/24 hours, VEGF expression in ICP group decreased significantly ($P < 0.05$) than before hypoxia, but had no difference than the normal group after 12/24 hours hypoxia (**Table 2**; **Figures 1**, **2**).

Regression analysis showed that the determination coefficient of placental VEGF against CG and pregnancy days was 0.9 (F = 101.19, $P = 0.000$), which indicated that CG and the number of pregnancy days might affect the expression of VEGF in placenta. It can be seen from standard regression coefficient (Beta) that, Beta of CG was -1.173, Beta of pregnancy days was -0.295, comparing to the number of pregnancy days, CG has a more significant effect ($P < 0.05$) on the placental VEGF.

Discussions

Sepulveda et al [9] first reported the potential role of bile acids in triggering fetal asphyxia in ICP. Bile acids has a function of concentration-dependent vasoconstriction which can induce vasospasm placenta villus surface, increase vascular resistance villus vein, reduce oxygenated blood flow, and lead to fetal hypoxia and a variety of complications, such as fetal distress, premature delivery, etc. Our research shows that CG had a noticeable effect on VEGF expression, a high level of CG is accompanied by low expression of VEGF.

Hypoxia can up-regulate the expression of VEGF [10-12]. In the design of this study, we assume: hypoxic culture for 4 hours simulates hypoxia induced by irregular uterine contraction when a labor origination; culture for 12 hours is equivalent to normal incubation period of most gravidas; culture for 24 hours is equivalent to a longest time in the first stage of labor. Every intensive contraction is an acute hypoxia, and an accumulation of repeated hypoxia

results in more hypoxia and ischemia at the ICP placenta, which may bring injury to trophoblast cells and endothelial cells, adversely affects the substance exchange of placenta, and reduces the acute hypoxia tolerance of ICP placenta. The present research shows that hypoxia four hours, the expression of VEGF after 4 hours hypoxia in ICP group is 87% higher than before hypoxia, while the VEGF expression after 4 hours hypoxia in normal group only increases by 54%, suggesting that ICP placenta is more likely to adapt a short-term change of hypoxia, and subsequently more easily compensates transient hypoxia.

Regression analysis shows that pregnancy days also affects VEGF expression in placental tissue. We believe ICP patients should be terminated pregnancy timely. ICP fetal death occurs quickly so that is not easy to diagnose by prenatal care, when the blood bile acid or CG beyond the normal range, ICP patients should be admitted to hospital to infanticipate. Once on the emergence of abnormal fetal movement, variation and disappearance of fetal heart rate baseline or meconium-stained amniotic fluid, the ICP patient should be terminated of pregnancy timely. We believe that the timely termination of pregnancy before obvious clinical manifestations of hypoxia of ICP fetus is very important. High level of bile acids has a certain impact on the ICP placental function, but if closely monitored pregnancy and timely termination of pregnancy can improve the prognosis of ICP fetus.

Transient hypoxia up-regulates VEGR expression to temporarily maintain the normal blood supply to ICP placenta. When the high CG level and uterine contractions exceeds the reserve force of the fetus-placenta units, acute fetal distress and even fetal death will occur. Based on the above experiment, we believe ICP fetal death is a result of CG induced long-term placental vasoconstriction associated with acute hypoxia. In summary, decreased expression of VEGF ICP patient placenta may lead to placental ischemia and hypoxia, causing placental blood and material exchange barriers, which is likely to be one of the mechanisms of ICP inducing pathophysiological change.

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