Preeclampsia umbilical cord serum-induced changes in occludin expression in endothelial cells

Xia Zhang[1], Yaling Feng[1,2], Jianjuan Xu[1], Liyi Cai[1], Lingqing Hu[1], Qian Xu[1], Min Liu[1]

[1] Department of Gynecology and Obstetrics, Wuxi Maternal and Child Health Hospital, Nanjing Medical University, Wuxi 214002, China
[2] Department of Gynecology and Obstetrics, Baoan People's Hospital, Shenzhen 518101, China
13600182648@163.com & fengyl523@sina.com

Abstract: Objective: To investigate preeclampsia umbilical cord serum-induced changes in the expression of Occludin in human umbilical vein endothelial cells (HUVEC). Methods: With respect to the types of culture media used, well-grown HUVECs were divided into three groups, namely, fetal bovine serum group (Group A), normal full-term pregnancy umbilical cord serum group (Group B) and preeclampsia serum group (Group C). After 24 h growing, cell viability was assessed by MTT assay, while the expression level of Occludin was determined by both Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and Western blotting. Results: After 12 h growing in various media, obvious inhibition on cell proliferation was observed in Group C, while the serum used in Group A and B did not exhibit significant effects on HUVEC proliferation. Group C showed slow cell proliferation 24 h later. Both mRNA and protein expression levels of the tight junction protein, Occludin, in Group C were lower than those in Group A and B; and there were no significant differences between Group A and B ($P > 0.05$). Conclusions: Damages to the tight junctions may probably be one of the micro-molecular mechanisms for hyperpermeability induced by endothelial dysfunction in the patients with preeclampsia.
[Zhang X, Feng YL, Xu JJ, *et al.* **Preeclampsia umbilical cord serum-induced changes in occludin expression in endothelial cells.** *Life Sci J* 2014;11(3):279-283]. (ISSN:1097-8135). http://www.lifesciencesite.com. 41

Keywords: Preeclampsia； Human Umbilical Vein Endothelial Cell； tight junction； Occludin

1. Introduction

Preeclampsia, a pregnancy-specific syndrome, is one of the leading causes of maternal and perinatal morbidity and mortality (Redmancwg,2005) and generalized arteriolar spasm is the pathological changes underlying preeclampsia. As a result of such arteriolar spasm, vascular endothelial injuries induced by increased peripheral resistance would increase the endothelial permeability, leading to development of various clinical manifestations, such as edema, proteinuria, hypertension, pachyemia, or even damages to several target organs (e.g. kidneys, heart, liver, brain and etc.) in severe cases, resulting in multi-organ failures which are life-threatening to both mother and children. The tight junctions are the critical structure for the regulation of epithelial and endothelial permeability. Currently, the regulating mechanism underlying the micro-structural changes of tight junctions associated with pathological changes of preeclamptic vessels has yet been reported. In the present study, the changes of Occludin expression were investigated, in order to elaborate the micro-molecular mechanism underlying the increased permeability induced by preeclamptic endothelial damages.

2. Material and Methods

Subjects

In 5 in-patients (Gestational age ≥ 34 weeks) who were diagnosed as severe preeclampsia and admitted in Department of Obstetrics, Wuxi Maternal and Child Health Hospital between September 2011 and December 2012, umbilical cord serum was collected to culture human umbilical vein endothelial cells (HUVEC), which were taken as the preeclampsia serum group (Group C)(all of the enrolled subjects met the diagnostic criteria for severe preeclampsia stated in ACOG Practice Bulletin 2002 and ACOG Guidelines 2004), while HUVECs grown in the umbilical cord serum collected from 5 full-term normal pregnant women (without medical or surgical complications, C-section was performed due to breech presentation or contracted pelvis) were taken as the normal full-term pregnancy serum group(Group B). Immediately after delivery of placentas at C-section, a sterile syringe was inserted into the umbilical vein to collect the umbilical vein serum, which was then placed in a sterile centrifuge tube, before being grown at 37℃ for 4 h. The sample was then centrifuged at 4000 rpm/min for 30 min, before being stored at -20℃ for reservation. The subjects enrolled in the two groups had neither primary hypertension nor a history of heart, liver, kidney or endocrine diseases. There were no signs of preterm birth, premature rupture of fetal membranes or infection. The subjects all underwent C-section under epidural anesthesia. The protocol of sample collection has been approved by the Ethics

Committee and written consents have been obtained from all the subjects enrolled.

Materials

HUVECs were purchased from Changsha Yingrun Biotechnologies Inc. (China). After 24 h of HUVEC attachment, the culture media were changed to the corresponding media designated to each group. In the control group (Group A), HUVECs were grown in basal culture medium + 15% FBS; in the normal full-term pregnancy serum group (Group B), HUVECs were grown in basal culture medium + 15% umbilical cord serum collected from normal full-term pregnant subjects; in the preeclampsia group (Group C), HUVECs were grown in basal culture medium + 15% preeclamptic umbilical cord serum. The cells were grown in an incubator at 37℃ and 5%CO2 for 24 h, before being used for various laboratory assays. The prepared samples in every group were placed in triplicate wells, and the serums used in each of the 3 groups should be prepared 4 h before hand.

Reagents and Devices

Trizol Reagent(Invitrogen, USA), AMV (Progema, USA), RNasin (progema, USA), SYBR GREEN I Master Mix (Applied Biosystems, UK), Protease inhibitor cocktail (Pierce，USA), Rabbit Anti-Human β-actin (Santa Cruz, USA), PVDF membrane (Amersham, Sweden) and 200 μg/ml Goat Anti-Human Occludin polyclonal IgG antibody (Santa, USA); DMEM (Invitrogen, USA) and HRP-conjugated Mouse Anti-Rabbit IgG (Santa Cruz, USA); acrylamide, methylene bisacrylamide, sodium dodecyl sulfate (SDS), tetra-methyl ethylenediamine (TEMED), ammonium persulfate (AP), propyl sulfonate, tris (hydroxymethyl) aminomethane and etc. (Sigma-Aldrich, USA)

LKB-III Ultramicrotome (Sweden); low-temperature high-speed Universal 16R centrifuge (Hettich); Image scanner; PDQuest Advanced 2-D Analysis Software (Amersham Biosciences Inc Sweden) and etc.; LP115 pH meter (Metter-Toledo GmbH, Germany); ELISA reader (Thermo Labsystems).

Semi-quantitative RT-PCR evaluation of Occludin expressions

After total RNA isolation by using one-step method with Trizol (Invitrogen, USA) reagent, the extracted total RNAs were pretreated with DNAse I and reverse transcribed in accordance with the instruction of RT-PCR kits provided by the manufacturer (Fermentas, USA). The primers were designed by using Primer Express 3.0 software (PE Biosystems, USA) and provided by Sangon Co., Ltd. (China). The primer pair adopted was Occludin, which had primer sequence of 5'-GTGGAAAGAGTTGACAGT-3' (sense strand) and 5'-CAGCCATGTACTCTTCA CT-3' (anti-sense strand) and the amplified product had a length of 484bp. GAPDH was used as the internal control, which had primer sequence of 5'-ACCACAGTCCATGCCATCAC-3' (sense strand) and 5'-TCCACCACCCTGTT GCTGTA-3' (anti-sense strand) and the amplified product had a length of 452bp. PCR Amplification was performed with the following cycling conditions: initial denaturation at 95 °C for 5 min; 30 cycles of 20s denaturation at 94°C, 25s Occludin/GAPDH primer annealing at 58°C and 30s elongation at 72°C; then the final elongation at 72°C for 3 min. The amplified products were stored at 4 °C. 5 μL amplified products of target gene and equal amount of amplified products of internal control were analyzed by electrophoresis on 1.5% agarose gel containing 0.5mg/L ethidium bromide. The images were documented by using DOC1000 gel documentation system; while the relative mRNA expression was calculated as the ratio of absorbance corresponding to PCR product against that corresponding to GAPDH.

Determination of Occludin level by using Western blot

Total amount of protein in the monolayer adherent cells was isolated. After determination of the protein content, the volume of solution containing 50 μg of protein was calculated as the sample loading quantity. 50μg of total proteins obtained from the specimens in various group were subjected to 6% SDS-PAGE, respectively, to separate the proteins, which were then transferred to NC filter via electrical transcription. The membranes were blocked with 5% skimmed milk. Diluted (1:500) goat anti-human polyclonal occludin antibody (the primary antibody) was added into the membrane overnight. The Rabbit anti-Human β-actin (1:200) was taken as the internal control. After the membrane was washed, HRP-labeled mouse anti-rabbit IgG (1:1000) was added and then incubated at room temperature for 1 hour. Subsequently, the membranes were rinsed and freshly-prepared ECL agent was added for chemiluminescence-based immunodetection. After X-ray film exposure, the light intensities were scanned and measured by the imaging system and the results of scanning were statistically analyzed.

Procedures of MTT assay

Well-grown HUVECs at their growth log phase were selected and seeded into a 96-well cell culture plate at a density of 1*104 cells per well (volume of culture media at each well was 200μL. After 24 hours of HUVEC attachment, the culture media were changed to the corresponding media designated to each group. The cells were continued to be grown in an incubator at 37℃ and 5%CO2 for 24 h. MTT

assays were conducted on 12h, 24h and 48h post trans-infection.

The survival and viability of the cells studied were assessed by MTT colorimetric assay. Succinate dehydrogenase (SDH) in the mitochondria of viable cells, especial for those highly proliferative ones, was able to reduce exogenous pale-yellow methyl thiazolyl tetrazolium (MTT) to bluish purple crystals (Formazan), which were precipitated within the cells and in the peripheral regions. In contrast, such phenomenon would not be observed in the dead cells. At the pre-determined time intervals, 20μL of MTT solution (5mg/ml) was added into each wells before the cells in the various groups were incubated at 37℃ with 5% CO2 for 4 hours. The culture supernatants in each hole were carefully removed (avoid drawing of purple crystals). 150μL of DMSO was added into each well, and the plate was then oscillated for 10 minutes for thorough dissolution of the crystals. At 490nm, the absorbance of each well was determined by using an ELISA reader; and the results were documented. The cell growth curves were obtained by plotting the corresponding absorbance (y-axis) against time (x-axis).

Statistical Analysis

The data were statistically analyzed by using SPSS 13.0 software. All the results were expressed in the form of mean ± SD ($\bar{x}$ ±s) and tested by One-Way ANOVA analysis. α=0.05 (two tailed) was taken as the level of significance, and $P < 0.05$ was considered statistically significant.

3. Results

Occludin mRNA expression level

The technique of semi-quantitative RT-PCR evaluation was adopted. The calculated ratio of Occludin absorbance against GAPDH product absorbance were 0.53±0.13, 0.50±0.21 and 0.59±0.09 in the blank control group (Group A), the trial control group (Group B) and the preeclampsia serum group (Group C), respectively; and the inter-group differences were all statistically insignificant. (Figure 1) (P >0.05).

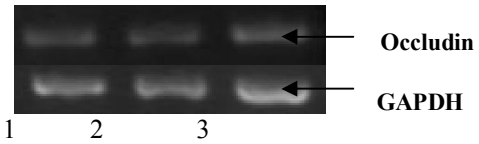

Figure 1. RT-PCR analysis of Occludin
Note: 1, 2 and 3 represented the blank control group (Group A), the trial control group (Group B) and the preeclampsia serum group (Group C), respectively.

Western blot analysis of Occludin expression

By using goat anti-human polyclonal Occludin antibody (1:500) as the first antibody and β-actin as the housekeeping protein, the expression levels of Occludin in HUVECs in the different groups were assessed; and the results were analyzed by gel imaging analyzer to obtain the relative absorbance corresponding to the expression level of Occludin. From the results of western blot analysis, the relative expression levels of Occludin were 0.44±0.012, 0.26±0.021 and 0.55±0.075 in the blank control group (Group A), the trial control group (Group B) and the preeclampsia serum group (Group C), respectively; and the inter-group differences were all statistically insignificant. (Figure 2) (P >0.05).

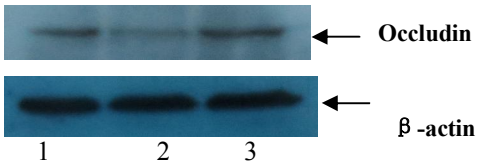

Figure 2. Results of Western blot analysis of Occludin
Note: 1, 2 and 3 represented the blank control group (Group A), the trial control group (Group B) and the preeclampsia serum group (Group C), respectively.

MTT assay

MTT assays were conducted in 3 groups. The absorbance in each well were measured at OD490nm on 12h, 24h and 48h post culturing, respectively, for the cells in all 3 groups. The results were tested by repeated measures ANOVA. The results showed that the absorbance of cells in Group A (the blank control group) was 1.94±0.12, 1.85±0.13 and 2.12±0.07 on 12h, 24h and 48h, respectively; while that in Group B (the trial control group) was 1.89±0.08, 1.89±0.04 and 2.05±0.10, that in Group C (the preeclampsia serum group) was 2.26±0.04, 1.28±0.18 and 1.55±0.03. The serums used as the media for Group A and B had no effects on the proliferation of HUVECs. In contrast, the serum-treated cells in Group C showed obvious inhibition on cellular proliferation on 12 hours post culturing; and the proliferation was slowed down on 24 hours post culturing (As shown in Figure3).

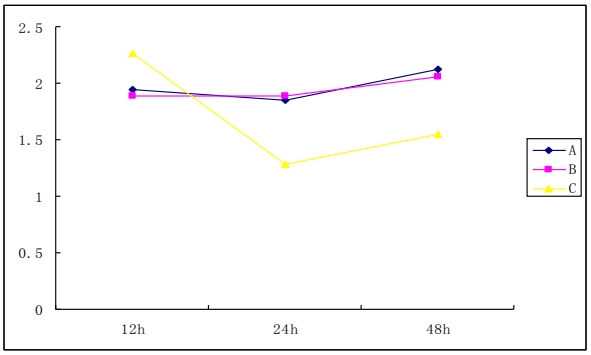

Figure 3: Results of MTT assays on serum-treated HUVEC-12

Note: The blank control group (Group A), the trial control group (Group B)and the preeclampsia serum group (Group C)

4. Discussions

As a pregnancy-specific syndrome, preeclampsia was the most common type of gestational hypertension (Redmancwg,2005) and the leading cause of maternal and perinatal morbidity and mortality (Sibaib,2005). The most underlying pathological component of preeclampsia was development of extensive vascular endothelial injuries, which would lead to increased endothelial permeability and leakage of body fluid and protein, and thus being clinical presented with edema, proteinuria, hypertension and pachyemia. Umbilical cord vein endothelial cell was the most common model of endothelial cells. The vascular endothelial cell (VEC), which played a part in the biological composition of the blood vessels, was sensitive to blood stream, pressure, inflammatory signals and any changes in hormonal levels in blood circulation; with abilities of reacting to various types of stimulations and maintaining constant internal/external environment, VEC played a significant role in different pathophysiological processes (Mandang, 2007; Yu,2006; Wang,2006; Wang,2008). Since the umbilical cord serum contained many essential bioactive substances, such as proteins, carbohydrates, lipids, hormones, serum enzymes, rarely-known hematopoietic stem cell growth factors and etc., umbilical cord serum culturing became a new method of growing HUVEC (human umbilical vein endothelial cells). The serum constituted partial internal environment for the growth and physiological functioning of umbilical cord vein. Currently, in vitro growth of endothelial cells in umbilical cord serum was an important technique to be used in studies on the associations between endothelial cytobiology and several diseases (Qiao, 2008; Mizuno, 2006). The vascular endothelial injuries and dysfunctions in the preeclamptic patients may be associated with the serum factors inducing cytotoxicity to human vascular endothelial cells (Roders, 2002; Rinehart,1999). Such cytotoxic factors may directly and/or indirectly damage the endothelial cells, resulting in a serial of pathological changes. In the present study, the umbilical cord serum collected from preeclamptic patients were successfully used to grow HUVEC cell lines, so as to simulate, in a more vivid manner, the internal environment of the patients with preeclampsia. Being a convenient and economical sampling method that avoids the blindness of single-gene interference or knockout, umbilical cord serum could be used as a novel cellular modeling tool for in vitro studies on preeclampsia.

Tight junction is a critical structure for the regulation of endothelial permeability. As a narrow strand-like structure acting as fences and barriers of the cells, tight junctions were protein complexes formed by interactions between the trans-membrane and peripheral proteins. These junctions were consisted of many different proteins, and Occludin was one of the major types. For a long time, the studies on preeclampsia mainly focused on identification of the genes predisposing preeclampsia, the inflammatory mediators released during oxidative stress-induced metabolic disorder and the immunologic factors mediating immune disorder; but the regulatory mechanism underlying the micro-structural changes of tight junctions associated with pathological changes of preeclamptic vessels has never been reported in China and rarely been reported abroad. In the present study, reflected by the significant lowered mRNA and protein expression levels of Occludin in the preeclamptic group when compared with the FBS and normal full-term pregnancy serum groups; such findings were another evidence indicating the association between the damages to tight junctions and increased permeability, suggesting the existence of factors cytotoxic to vascular endothelial cells in the umbilical cord serum and the presence of consequent endothelial injuries. This result was consistent with previous results (Wang Yuping,2002.), in which in vitro growing of HUVECs in preeclamptic umbilical cord serum was found to be associated with the increased permeability of monolayer endothelium.

Therefore, it was reasonable to propose that the damages to vascular endothelial cells may further increase endothelial permeability in the preeclamptic patients via disruption or degradation of tight junctions. Stabilization of the tight junctions could provide a potential approach for prevention and management of preeclampsia; further in vivo laboratory studies and clinical trials should be conducted to verify this hypothesis.

Acknowledgements:

Foundation item: Fund of China Postdoctoral Science Foundation (No.: 20110490960). Authors are grateful to the Department of Science and Technology, Government of China for financial support to carry out this work.

Corresponding authors Adress:
The Affiliated Wuxi Hospital for Maternal and Child Health care of Nanjing Medical University,48Huai Shu street,Wuxi,214002,China
Email addresses:
13600182648@163.com. (Yaling Feng)
fengyl523@sina.com (Jiangjuan Xu),

References

1. Redmancwg, Sargentil. Latest advances in understanding preeclampsia.Science， 2005， 308（5728）:1592-1594.
2. Sibaib, Dekkerg, Kuphermincm. Preeclampsia. Lancet，2005，365（9461）：785-799.
3. Mandang S, Manuelpillai U, Wallace EM. Oxidative stress increases placental and endo thelial cell activin A secretion. J Endocr inol, 2007, 192(3): 485- 493.
4. Yu Peng and Chen Xiao-hu. The relation between vascular endothelium cells function and essential hypertension. Chinese Journal of the Practical Chinese with Modern Medicine，2006，19(19): 2272-2273.
5. Wang Lin-hua and Chen Ming. Endothelial Function in Essential Hypertension. Advances In Cardiovascular Diseases,2006, 27(1): 60-63
6. Wang He and Ma Li. Advances in the studies on vascular endothelial cell dysfunction and its management. The Journal of Practical Medicine, 2008, 24(10): 1665-1667.
7. Qiao C, Xu W, Zhu W, et al. Human mesenchymal stem cells isolated fr om the umbilical cord. Cell Biol Int,2008, 32(1): 8- 15.
8. Mizuno N, Shiba H, Ozeki Y, et al. Human autologous se-rum obtained using a completely closed bag system as a substitute for foetal calf serum in human mesenchymal stem cell cultures. Cell Bio l Int, 2006, 30(6): 521- 524.
9. Roders GM, Taylor RN, Roberts JM. Preeclam psia is associated w ith a serum factor cytotoxic to hum an endothelial cells. Am J Obstet Gynecol 2002, 159: 908.
10. Rinehart BK, Terrone DA, Lagoo- Deen adayalan S et al.Expression of the placental cytokines tumor necrosis factor alpha, interleukin- 1 beta and in terleuk in- 10 is increased in p reeclampsia. Am J Obstet Gyneco, l 1999, 181 (4): 915.
11. Yuping Wang, Yang Gu,. Neil Granger, et al. Endothelial junctional protein redistribution and increased monolayer permeability in human umbilical vein endothelial cells isolated during preeclampsia. Am J Obstet Gynecol,2002,186 (2): 214-220.

2/13/2014