·Basic Research·

Toxicity of endogenous peroxynitrite and effects of puerarin on transplanted retinal pigment epithelial sheets in the subretinal space in mice

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Foundation items: Supported by Hebei Province Science Foundation, China (No.07276101D-3); Clinical Science Project Fund of the Ministry of Health in Hebei Province, China (No. 03078); Foreign Studying Project Fund in Hebei Province, China (No. 07-03)

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Received: 2011-03-08 Accepted:2011-04-20

Abstract

• AIM: To evaluate the toxicity of endogeneous peroxynitrite on transplanted retinal pigment epithelial (RPE) sheets and the effect of puerarin on their survival in the C57BL/6 mice after RPE sheets have been transplanted into SD rats' subretinal space .

• METHODS: C57BL/6 mice eyes were used to culture RPE cells. Ninety-six SD rats were involved in the experiment. They were divided into control(block control), streptozotocin (STZ, negative control), untransplanted RPE (positive control) and transplanted RPE groups respectively. Diabetes was induced in SD rats by intra-peritoneal STZ injection in the latter three groups. Saline was injected into the subretinal space of 24 SD rats in the untransplanted RPE group and primary RPE sheets were injected into the subretinal space of 24 SD rats in the transplanted RPE group. Puerarin (45 mg/kg) was administrated into both untransplanted RPE and transplanted RPE groups of diabetic rats through intra-peritoneal injection route after RPE sheets transplantation. At 20,40,60 days after surgery, Western blotting analysis, DNA ladder and RT-PCR were used for determining the differences in expression of nitrotyrosine (NT, the foot print of peroxynitrite), apoptosis and iNOS mRNA in the control, STZ, untransplanted RPE and transplanted RPE groups respectively. HE staining was used for determining the RPE survival in the subretinal space of the transplanted RPE group.

• RESULTS: Apoptosis and expression of NT and iNOS mRNA were observed in STZ, untransplanted RPE and transplanted RPE groups, but were delayed in untransplanted RPE and transplanted RPE groups in a time-dependent manner compared with control and STZ groups ($P<0.01$). There were no differences between the two groups ($P>0.01$). NT, DNA ladder, iNOS mRNA were down-regulated, which were associated with the decrease of expression of peroxynitrite. Numerous pigmented cells emerged and increased in number in the subretinal space during the 60-day observation period after transplantation. On day 20, heavily pigmented cells were visible at the transplant site; On day 40, monolayer and multilayered transplant was visible in the subretinal space; On day 60, heavily pigmented monolayer and multilayered transplants with round apical profile were present along Bruch's membrane.

• CONCLUSION: Puerarin increased the 60-day survival of C57BL/6 mice RPE xenografts in the SD rats' subretinal space, which may be related to its direct inhibition of apoptosis of RPE cells and antagnism of damage of peroxynitrite to RPE cells.

• KEYWORDS: retinal pigment epithelial sheets; trans-pantation; oxidative; puerarin

DOI:10.3980/j.issn.2222-3959.2011.03.07

Hao LN, Zhang YQ, Shen YH, Li MQ, Yang T, Zhao ZH, Wang ZY, Wang YH, He SZ. Toxicity of endogenous peroxynitrite and effects of puerarin on transplanted retinal pigment epithelial sheets in the subretinal space in mice. *Int J Ophthalmol* 2011;4(3):250-254

INTRODUCTION

The retinal pigment epithelium (RPE) performs many important functions that are crucial to maintaining the integrity of the retina and choriocapillaris, including phagocytosis of the distal tips of photoreceptor outer segments, transport and isomerization of bleached visual pigments, and maintenance of the blood-outer retina barrier. The importance of these RPE functions in maintaining normal functions of the retina, can be demonstrated, for instance, in age-related macular degeneration (AMD), which is the leading cause of blindness in developed countries after age 55 [1]. The major limiting feature in visual recovery after submacular surgery for exudative AMD is the unavoidable removal of RPE beneath the fovea at the time of surgery. Thus, the rapid repopulation of the bare area of Bruch's membrane beneath the fovea by RPE transplantation is essential for recovery of central vision. In our previous study, we found that peroxynitrite played a crucial role in RPE cell damage and function loss in SD rats with streptozotocin (STZ)-induced diabetes [2]. Diabetic patients are more likely to have retinal fundus diseases [3-6]. Thus, our design is to explore the toxicity of endogenous oxidative agent (peroxynitrite in the present study) on transplanted RPE sheets and the effect of Chinese medicine puerarin on mouse (C57BL/6) RPE sheet survival after it is transplanted into the subretinal space of diabetic Sprague-Dawley (SD) rats.

MATERIALS AND METHODS

Materials Forty healthy C57BL/6 mice aged 2-3 weeks and 96 healthy male SD rats weighted around 250 g were used in this study. RPE were harvested. Freshly enucleated mouse eyes were cleaned of extraocular tissue. Scissors were introduced at the optic nerve into the vitreous cavity and the incision cut through the sclera, choroid, and retina and was extended toward the iris. Three additional radial incisions were made with a scalpel (size 10), and the tissue was cut into 3-mm squares. The retina was removed carefully and the remaining tissue was incubated with dispase 6.25U/mL (Invitrogen-Gibco) for 8 hours at 4℃. The loosened RPE sheets were separated from the remainder of the ocular tissue and placed on a drop of 250g/L gelatin with the apical surface of the RPE facing upward. Contamination of choroidal cells was avoided by identifying RPE sheets under a dissecting microscope during harvesting. The gelatin film containing the RPE sheet was then incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37℃ for 2 minutes to allow the gelatin to melt and encase the RPE sheet. The specimen was kept at 4℃ for 4 minutes for the liquid gelatin to solidify, covered with 20 μL of 100g/L gelatin and 300mmol/L sucrose in DPBS at 37℃, returned to 4℃ for 6 minutes, and then played in the laboratory overnight in CO_2-free medium (CFM; Gibco-Invitrogen) at 4℃. On receipt in the laboratory, the RPE-containing sheet was triturated into small microaggregates in DMEM and diluted to a desity of 4×10^9 cells/L for injection into the subretinal space. Cytokeratin staining before transplantation confirmed that all the cells were of epithelial origin. Ninety-six SD rats were randomly divided into control (block control), STZ (negative control), untransplanted RPE (positive control) and transplanted RPE groups respectively. SD rats in STZ, untransplanted RPE, and transplanted RPE groups received intraperitoneal STZ injections (45mg/kg) to establish the animal model of diabetes. Three days after STZ injection, rats with blood glucose levels greater than 16mmol/L were considered diabetic. Three days after the start of experiment, the rats in untransplanted RPE and transplanted RPE groups started to receive puerarin 140mg/kg per day. The rats in the control group received the same amount of saline. The blood glucose and weight of the animals were monitored weekly by tail vein blood measurements and scale (Data was not shown). The eyes of the animals were examined by slit-lamp and ophthalmoscope every other day.

Methods All surgical procedures were performed in right eyes of SD rats. Care and use of animals were adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Forty-eight animals in untransplanted RPE and Transplanted RPE groups underwent subretinal injectionss. Animals were sedated with intramuscular ketamine, and a sterile field was prepared around the right eye. A glass pipette was introduced through the pars plana and vitreous cavity to enter the subretinal space under direct visualization. A bleb neurosensory retinal detachment was created by injecting approximately 10μL of DMEM followed by 10μL of cell suspension which contained approximately 40000 RPE cells in microaggregates into the subretinal space of the eyes in the transplanted RPE group, and by injecting 20μL of DMEM into the subretinal space of the eyes in the untransplanted RPE group. The instrument was withdrawn, and the single sclerotomy was closed with a 7-0 coated Vicryl suture (Ethicon, Somerville, NJ). Two milligrams of dexamethasone (Anpro Pharmaceuticals, Arcadia, CA) and 20 mg of gentamicin sulfate (Elkins-Sinn, Cherry Hill, NJ) were injected subconjunctivally. Subcutaneous buprenorphine 0.005mg/kg (Reckitt and Colman Pharmaceuticals, Inc., Richmond, VA) was administered for postoperative pain control. RPE sheet was able to be delivered in 22 of 24 eyes (94%) without significant complications. Two eyes were excluded from further analysis because of retinal detachment.

Identification of transplanted RPE cells A modified

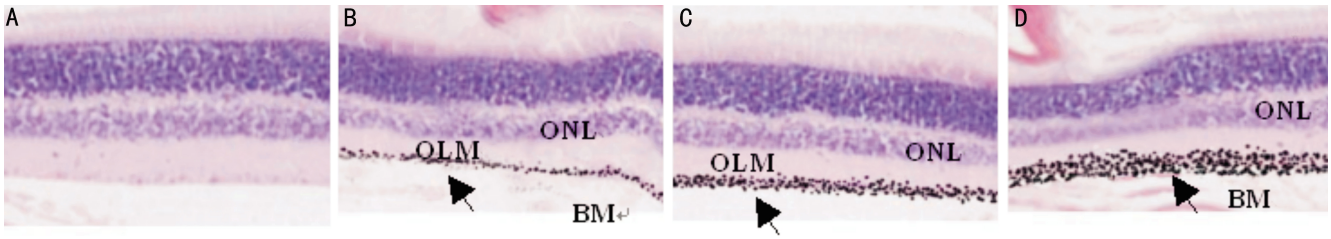

Figure 1 Pigmented cells at the transplant site (*arrow*) ONL: Outer nuclear layer; OLM: Outer limiting membrane; BM: Bruch's membrane A: Control;B: 20d;C: 40d;D: 60d

Barr-body staining technique was used to identify the transplant after surgery. Unstained sections were deplasticized and stained using a modified Guard's sex chromatin staining method. Briefly, tissue sections were treated sequentially in 950mL/L alcohol, 700mL/L alcohol, and Biebrich Scarlet for 2 minutes each. Sections were then placed in 500mL/L alcohol for 5 minutes and rinsed with 500mL/L alcohol, stained in fast green solution for 1 to 2 hours, and rinsed in 500mL/L alcohol for 5 minutes before dehydration in 700mL/L, 950mL/L, and pure ethyl alcohol for 2 minutes each. Sections were then placed in two changes of xylene for 2 minutes each and mounted (Permount; Fisher Scientific, Pittsburgh, PA). Sections were examined with an upright microscope at 100x under oil immersion, and cells were considered Barr-body positive if intranuclear red staining was observed. Histologic sections of retina, RPE, and choroid from SD rats served as positive and negative controls, respectively. For staining of the paraffin-embedded sections, the rats were euthanized at 20, 40, and 60 days after transplantation, and the eyes that received transplants were enucleated. The specimens were fixed with 40g/L paraformaldehyde (PFA) at 4℃ overnight and then embedded in paraffin. Consecutive sections (5μm in thickness) of the transplantation area mounted on slides (Superfrost; Fisher Scientific, Pittsburgh, PA) were used for transplant localization after overview staining.

Detection of nitrotyrosine RPE cells were prepared as described and the protein content of the supernatants was determined by the Bradford method. After proteins in the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 120g/L linear slab gel, these proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a semidry electrophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blot was stained at room temperature with 1:600 monoclonal mouse anti-nitrotyrosine (NT) and mouse anti-C3 antibody overnight at 4℃ respectively. After washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:1000), the blot was developed using the enhanced chemiluminescence Western blot analysis detection systerm (ECL Plus; Amersham Pharmacia Biotech, USA).

DNA ladder for apoptosis RPE cells and DNA ladder procedures were performed.

iNOS mRNA expression RT-PCR was performed using 2μg of total RNA of RPE cells for the first-strand synthesis followed by amplification in the presence of specific primers for iNOS (5′ -CGCCCTTCCGCAGTTCT-3′ and 5′ -TC CAGGAGGACATGCAGCAC-3′) and β-actin (5′ -GAG ACCTTCAACACCCAGCC-3′ and 5′ -GCGGGGCAT CGGAACCGCTCA-3′). The amplification consisted of 29 cycles of denaturation for 1 minute at 94℃, annealing for 1 minute at 60℃, and extension for 1 minute at 72℃.

Statistical Analysis Statistical analyses of the data were performed by computer (SPSS 16.0). The results were expressed as mean ±SD. Statistical significance was determined by a one-factor analysis of variance (ANOVA) followed by the Fisher *post hoc* test for multiple comparisons. $P<0.05$ was considered significant. All tests were repeated at least three times.

RESULTS

RPE Transplant in the Subretinal Space As time passed by, numerous pigmented cells were visible in the subretinal space on 20，40，60 days after surgery. Twenty days after surgery, a hyperpigmented patch was readily visible in the transplant bed. The inner retina and outer nuclear layers were intact and the choriocapillaris was patent. Forty days after surgery, monolayer and multilayered transplant was visible in the subretinal space. The transplant bed contained a pigmented multilayer along Bruch's membrane and a lightly pigmented layer, closer to the neurosensory retina. Sixth days after surgery, there was a heavily pigmented monolayer along Bruch's membrane, cells with rounder apical profile could be seen occassionally. The outer nuclear layer was intact, and photoreceptor outer segment length appeared to be normal in the transplant bed. Barr-body-positive cells were visible in the heavily pigmented monolayer along Bruch's membrane(Figure 1).

NT Expression With Western blot analysis, a faint expression of NT could be seen in the control group. Expression of NT in eyes in STZ group gradually increased

with time. Compared to the STZ group, expressions of NT in untransplanted RPE and transplanted RPE groups were down-regulated and associated with the decrease of expression of peroxynitrite in a time-dependent manner compared with control and STZ groups ($P<0.01$); Expression of NT in untransplanted RPE and transplanted RPE groups was up-regulated from 20 to 40 days, and then went back to a level that was comparable to 20 days by 60 days after STZ injection (Figure 2). Computer photo analysis indicated that there were no differences between the two groups and associated with the decrease of expression of peroxynitrite ($P>0.01$, Figure 2).

DNA Ladder for Apoptosis No DNA ladder band appeared in the control group. In the STZ group, however, distinct typical DNA ladder band was visible at time slices examined. Expression of DNA ladder band in the untransplanted RPE and Transplanted RPE groups became stronger from 20 to 40 days, and then attenuated at 60 days, which was with the changes in expression of peroxynitrite (Figure 3).

iNOS mRNA Expression There was no expression of iNOS mRNA in the control group, but there was distinct up-regulation of iNOS mRNA in the STZ group as time passed by. Expression of iNOS mRNA between untransplanted RPE and transplanted RPE groups gradually from faint to strong during the period of 20 to 40 days, then turn to weak at 60 days (Figure 4). Computer photo analysis indicated that there were no differences between two groups and associated with the decrease of expression of peroxynitrite ($P>0.01$, Figure 4).

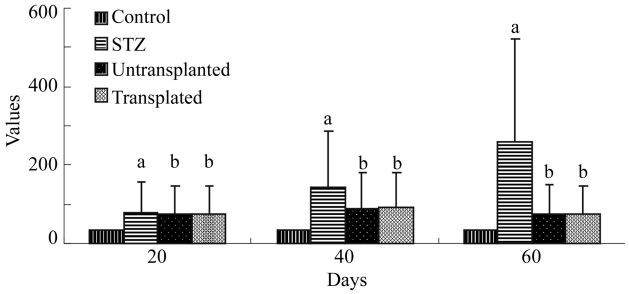

Figure 2 NT expression in delivery of mouse RPE sheets into rat subretinal space with Western blotting

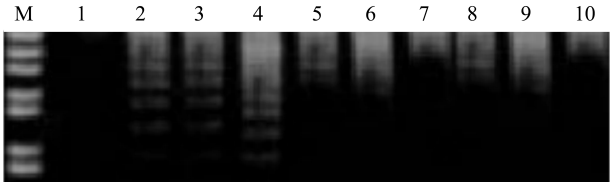

Figure 3 DNA ladder for apoptosis of transplanted RPE sheets M: Maker; 1: Control; 2-4: STZ at 20, 40, 60 days; 5-7: Untransplanted RPE at 20, 40, 60 days; 8-10: Transplanted RPE at 20, 40, 60 days

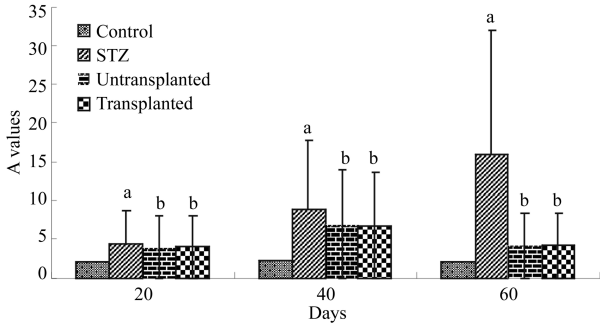

Figure 4 iNOS mRNA expression in transplanted RPE sheets (RT-PCR)

DISCUSSION

Approximately two decades ago an interest in RPE transplantation was fueled by the concept that primary RPE dysfunction is an early step in the pathogenesis of tapetoretinal degenerations, including retinitis pigmentosa. Subsequent genotyping studies of retinal degenerations demonstrate that the gene defect causing retinitis pigmentosa and similar retinal degenerations is nearly always caused by abnormalities in a gene expressed in photoreceptors, with the exception of mutations in the RPE65 gene that cause some cases of Leber's congenital amaurosis and the cellular retinaldehyde-binding protein (CRALBP) gene. Thus, it is unlikely that RPE transplantation alone is sufficient to help most patients with tapetoretinal degenerations. However, a major need for successful RPE transplantation exists in the management of AMD. Loss of the RPE precedes loss of choriocapillaris in patients with nonexudative AMD, and RPE transplantation may prevent or reverse choriocapillaris atrophy in these individuals. The rationale for RPE transplantation in exudative AMD is deceptively simple. In this disease, the native RPE is excised with the choroidal neovascular complex during submacular surgery, and removal of RPE leads to progressive choriocapillaris atrophy and photoreceptor loss, which might be prevented by RPE transplantation. In preliminary studies, RPE transplantation in patients with exudative or nonexudative AMD has not resulted in significant improvement in vision. In most studies, graft survival may be hampered by the lack of immune suppression and damage to the host Bruch's membrane with incomplete RPE repopulation [7]. Until now, despite an extensive body of peer-reviewed literature on RPE transplantation, some fundamental questions remain unanswered about the behavior of transplanted RPE. As we all know, the subretinal space is an immune -privileged site, but this immune privilege is relative, rather than absolute. Therefore, our design to study whether there was toxicity of host origin oxidative agent -peroxynitrite on transplanted RPE sheets and to explore the effect of Chinese medicine

puerarin on RPE sheets' survival.

In the present study, we observed behavior of transplanted RPE sheets at 20, 40, 60 days after surgery. At 20 days, there was a heterogeneous distribution of intracellular pigment at the transplant site, and the outer nuclear layer and outer limiting membrane appeared normal. At 40 days after surgery, the multilayered transplant was visible in the subretinal space. The transplant bed contained a pigmented multilayer along Bruch's membrane. The outer nuclear layer was intact. At 60 days after surgery, there was hyperpigmentation at the transplant site, which expressed a heavily pigmented multilayer along Bruch's membrane. We also observed the peroxynitrite-mediated protein nitration product, NT, was localized in RPE cells and decreased with the intervention of puerarin. We found that NT level increased greatly in STZ group. Expression of a small amount of NT in the control group provided physiological evidence for the existence of peroxynitrite. Purerarin could inhibit the expression of iNOS, therefore decrease formation of peroxynitrite [8]. It is likely that iNOS may contribute to oxidative stress by helping to develop more powerful oxidative agents such as peroxynitrite under pathological conditions, to up-regulate iNOS mRNA in RPE cells to over-produce NO, and to activate oxidant enzyme as well as increase the level of O_2^-. Excessive NO and O_2^- produce extra peroxynitrite which acts as a strong oxidant [9,10] to cause apoptosis.

Puerarin, a major isoflavonoid derived from the Chinese medical herb radix puerariae (kudzu root), has been reported to be useful in the treatment of many diseases. Chang *et al*[11] from Taiwan examined the detailed mechanisms underlying the inhibitory effects of puerarin on inflammatory and apoptotic responses induced by middle cerebral artery occlusion (MCAO) in rats. The authors found that the expressions of iNOS, ctive caspase-3 protein as well as the mRNA of tumor necrosis factor-alpha (TNF-alpha) in ischemic regions were all markedly inhibited by puerarin, which is a potent neuroprotective agent on MCAO-induced focal cerebral ischemia *in vivo*. This effect may be mediated, at least in part, by the inhibition of both HIF-1alpha and TNF-alpha activation, followed by the inhibition of inflammatory responses (*i.e.*, iNOS expression), apoptosis formation (active caspase-3), and neutrophil activation, resulting in a reduction in the infarct volume in ischemia-reperfusion brain injury. The other three Chinese authors [12-14] also found that puerarin significantly decreased the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling staining cells compared with the vehicle group. They verified the inhibitory effects of puerarin on angiopoiesis of endometriotic tissue and the regulatory effects of puerarin on tumor-related gene expression of endometriosis as well as reduced the occurrence of apoptosis and improved neurotrophic function of astrocytes, which may be related to its antioxidant effects during oxidative stress. Our results are consistent with these reports. In conclusion, we have demonstrated that puerarin is a potent RPE protective agent on peroxynitrite-induced RPE disfunction. Thus, puerarin treatment may represent a novel approach to improve survival of RPE after transplantation .

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