Pigment epithelium-derived factor inhibits high glucose induced oxidative stress and fibrosis of cultured human glomerular mesangial cells

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ABSTRACT

الأهداف: تقييم ما إن كان عامل الصبغة الطبقية يمنع خلايا البشر المسراقية من الإصابة بالتليف والإجهاد التأكسدي الناتج من ارتفاع الجلوكوز.

الطريقة: أجريت الدراسة في مختبر أمراض الغدد الصماء لمستشفى رمنين لجامعة وهان، وهان، الصين خلال الفترة من ديسمبر 2009م إلى يونيو 2010م. تم علاج أثر الخلايا البشرية المسراقية الكبيبية بتراكيز الجلوكوز المختلفة بلغت 5.6، و15، و30 نانومول/لتر و 5.6 نانومول/لتر من الجلوكوز و 24.4 نانومول/لتر من المانيتو لمدة 24 و 48 ساعة. ولدراسة أثر عامل الصبغة الطبقية وخلايا البشر المسراقية تم حقن جلوكوز عالي يبلغ 30 نانومول/لتر بتراكيز مختلفة لعامل الصبغة الطبقية بلغت 5، 10، 40، 100، 160 نانومول/لتر لمدة 48 ساعة.

النتائج: يخفض عامل الصبغة الطبقية بشكل إحصائي من انقسامات عامل النمو بيتا، وبروتين ماتريكس خارج الكلية (فايبروستين والكولاجين الرابع) الناتج من ارتفاع الجلوكوز في خلايا البشر المسراقية. كما أن عامل الصبغة الطبقية يقلل من تكاثر نوع الأكسجين في خلايا البشر المسراقية الناتج من ارتفاع الجلوكوز.

خاتمة: تشير النتائج إلى الدور الوقائي لعامل الصبغة الطبقية ضد أمراض الكلية السكري بشكل جزئي عبر نشاط مضاد الأكسدة والتليف.

Objectives: To evaluate whether pigment epithelium-derived factor (PEDF) could prevent human mesangial cells (HMCs) from elevated glucose-induced oxidative stress and fibrosis.

Methods: The study took place in the Endocrinology Laboratory of Renmin Hospital of Wuhan University, Wuhan, China from December 2009 to June 2010. The HMCs were treated with different concentrations of dextroglucose (5.6, 15, and 30 mmol/l), and 5.6 mmol/l D-glucose+24.4 mmol/l D-mannitol (osmotic control) for 24 and 48 hours. To examine the beneficial effect of PEDF, HMCs were also incubated with high glucose (30 mmol/L) in the presence of different concentrations of PEDF (5, 10, 40, 100, and 160 nmol/l) for 48 hours.

Results: The PEDF significantly inhibited the overexpression of transforming growth factor-beta 1, and extracellular matrix proteins (fibronectin and collagen IV) induced by the elevated glucose in HMCs. The PEDF also impeded high glucose-induced reactive oxygen species generation in HMCs.

Conclusion: These results suggest that PEDF by virtue of its anti-oxidative and anti-fibrogenic properties may have a therapeutic potential in diabetic nephropathy.

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Diabetic nephropathy (DN) is a leading cause of end-stage renal disease worldwide. A structural hallmark of this disease is the thickening of the glomerular basement membrane and mesangial matrix expansion. Although the pathogenesis of diabetic nephropathy is complex and remains unclear, high glucose is presumed as an initiating factor. Hyperglycemia causes oxidative stress and leads to increased levels of transforming growth factor-beta (TGF-β) and increased production of extracellular matrix (ECM) proteins, such as fibronectin (FN).[1,2] This increased production in glomerular mesangial cells has been implicated in the development of DN.[3,4] Growing evidence indicated that oxidative

stress mediated by hyperglycemia-induced generation of reactive oxygen species (ROS) contributes significantly to the development and progression of diabetic vascular complications, including nephropathy, via induction of ECM overproduction.[5-7] Wang et al[8] reported that injection of adenovirus pigment epithelium-derived factor (PEDF) significantly reduced the production of ECM protein in the diabetic kidney. The PEDF is a glycoprotein that belongs to the superfamily of serine protease inhibitors, which assumed beneficial effects on diabetic retinopathy by acting as an endogenous antioxidant,[9-11] although it was first purified from the conditioned media of human retinal pigment epithelial cells, as a factor that possesses potent neuronal differentiating activity in human retinoblastoma cells . The PEDF is expressed in a broad range of human fetal and adult tissues, including the kidney.[12-14] Previous studies have shown that decreased PEDF levels in the kidney are implicated in diabetic nephropathy.[15] Moreover, research conducted on the systemic administration of an adenovirus expressing the PEDF gene has shown a beneficial role on the prevention of nephropathy, which drastically reduced the albuminuria, and ameliorated glomerular hypertrophy in the streptozotocin (STZ)-induced diabetic rat model.[8] Although PEDF inhibited the expression of FN in diabetic kidney, the protective effect of PEDF in diabetic kidney and its mechanism of action have not been demonstrated. Based on the causative role of hyperglycemia on oxidative stress in DN, and the anti-oxidative and anti-fibrogenic activities of PEDF, it is logical to hypothesize that PEDF protects the renal structure and function from diabetic injury, via its anti-oxidative and anti-fibrogenic activities. We have tested this hypothesis in the present study. The purpose of study is to provide theoretical basis for the clinical application of PEDF in the future.

Methods. The study took place in the Endocrinology Laboratory of Renmin Hospital of Wuhan University, Wuhan, China from December 2009 to June 2010. The human glomerular mesangial cells (HGMCs) were purchased from Xiangya Central Laboratory of Central South University (Changsha, China). The Ethics Committee of Renmin Hospital of Wuhan University confirmed that the ethics approval was not needed. This study was carried out according to the principles of Helsinki Declaration.

Cell culture. The HGMCs were cultured in Dulbecco Modified Eagle's Medium (DMEM) containing glucose (5.6 mmol/L) with 15% fetal bovine serum (FBS), 100 microgram/ml streptomycin, 100 unit/ml penicillin, 2 mmol/l glutamine at 37°C, in a humidified 5% CO_2 atmosphere. Cells of passages from 3-6 were used in the experiments. After reaching an 80% confluence, cells were quiescenced with a medium containing no FBS for 12 hours, and then exposed to different concentrations of glucose, or in the presence or absence of recombinant human PEDF (Peprotech, Princeton, USA).

Determination of the mRNA levels of TGF-β1, FN, and collagen IV by real-time polymerase chain reaction (RT-PCR). The total RNA was extracted from cultured HMC's using TRIzol reagent (Invitrogen Inc, Carlsbad, CA, USA) according to the manufacturer's protocol. Primers specific for TGF-β1 (forward,5'-GCAACAATTCCTGGCGATAC-3'; reverse, 5'-CTAAGGCGAAA GCCCTCAAT-3'), FN (forward, 5'-CGCCGAATGTAGGACAAGAAG -3'; reverse, 5'-AAGCACGAGTCATCCGTAGGTT-3'), collagen IV (forward, 5'-AACA GCAACGAACCCTAGAAAT-3'; reverse, 5'-AATGGGCAAACAGTATGGAAG-3'), β-actin (forward, 5'-GTCCACCGCAAATGCTTCTA-3'; reverse, 5'- TGCTGTCACCTTCACCGT TC-3') were used for RT-PCR. Total RNA, oligo-dT primer, and reverse transcriptase were used for RT-PCR in a final volume of 20 microliter, and the reaction was conducted at 42°C for 20 minutes (min), followed by a denaturation at 95°C for 5 min. The RT-PCR reaction mixture (total volume 25 microliter) consisted of the following reagents: 12.5 microliter of SYBR Green Mix (Toyobo, Osaka, Japan), 2.5 microliter of plus solution (plus solution is the reaction solution contained in the kit of the SYBR Green Mix), 1 microliter (5 pmol/microliter) of each of the forward and reverse primers, 5.5 microliter of ddH_2O, and 2.5 microliter of complementary DNA template. All reactions were performed in triplicate. The average CT (threshold cycle) of fluorescence units was used to analyze the messenger ribonucleic acid (mRNA) levels. The data were normalized to housekeeping gene β-actin to account for reverse transcription efficiencies. Quantification was calculated as follows: mRNA levels (percent of control) = $2^{-\Delta\Delta CT}$(where Δ means delta), with $\Delta C_T = C_{T,\ \text{target gene}} - CT_{\beta\text{-actin}}$, and $\Delta\Delta C_T = \Delta C_T - \Delta C_{T\text{control}}$.[16]

Measurements of TGF-β1 and FN by enzyme-linked immunosorbent assay (ELISA). The protein levels of TGF-β 1 and FN in cultured cells were quantified spectrophotometrically at a wavelength of 450 nm using the TGF-β 1 ELISA kit (Boster, Wuhan, China), and the FN ELISA kit (USCNLIFE, Wuhan, China) according to the protocols of the manufacturers.

Intracellular ROS measurement. Changes in intracellular ROS levels were determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology, Haimen, China).[17] The HGMCs ($1x10^6$ cells/ml) were isolated, and directly treated with 10 μM DCFH-DA, dissolved in one ml phosphate buffer saline (PBS [pH; 7.4, in which the contents were: NaCl - 8 g/L; KCl - 0.2 g/L;

Na_2HPO_4 - 1.44g/L; and KH_2PO_4 - 0.24 g/L]) at 37 °C for 20 min according to the manufacturer's instructions. The DCFH-DA was deacetylated intracellularly by non-specific esterase, which was further oxidized by ROS to yield the fluorescent compound 2,7-dichlorofluorescein (DCF).The DCF fluorescence was detected by fluorescence-activated cell sorter (FACS) scan flow cytometer at an excitation wavelength of 488 nm, and at an emission wavelength of 525 nm. The ROS level was represented by the DCF intensity.[17]

Data were analyzed and expressed as means ± standard deviation. Statistical analysis used the Student's t test and ANOVA with post hoc test. Statistical difference was considered significant at $p \leq 0.05$.

Results. ***The effect of high glucose concentration on the ROS generation in HMC.*** The results showed that the ROS generation is increased (p=0.003) in a proportional manner with the glucose concentration (**Figure 1**). Of note, there is no statistically significant difference (p=0.235) between the values of the 24 and 48 hours treatment with high glucose in ROS generation (**Figure 1**).The addition of mannitol of 24.4 mM had no significant effects on ROS production (p=0.178), suggesting that osmotic stress was not involved in ROS generation.

High glucose stimulated TGF-β1 secretion by HMC. The TGF-β1 generation is increased (p=0.004) in a proportional manner with the concentration of the glucose at both time points (24 and 48 hours, **Figure 2**) but osmotic control (mannitol 24.4 mM) has no effect (p=0.208).

High glucose induced overexpression of FN and collagen IV in a dose dependent manner. The results showed that whenever the glucose concentration is increased, the mRNA and protein of FN and collagen IV are increased (p=0.004) as well (**Figure 3**). Mannitol (24.4 mM) osmotic control did not affect the FN and collagen IV production (p=0.224), indicating that high glucose-induced FN and collagen IV overproduction was not due to enhanced osmotic stress.

PEDF inhibited high glucose-induced overexpression of FN and collagen IV in HMC. **Figure 4** shows that at low doses (5-40 nmol/l), PEDF decreased FN and collagen IV secretion in a dose dependent manner in HMCs, cultured in high-glucose containing medium (p=0.001) .

PEDF decreased high glucose-induced TGF-β1 secretion in HMC. As TGF-β1 is the key modulator of ECM formation in the mesangium leading to mesangial expansion, we suspected that PEDF may prevent ECM accumulation, such as FN and collagen IV overproduction via inhibition of TGF-β1 expression. As we have expected, PEDF at concentrations 40-160 nmol/l significantly downregulated TGF-β1 expression in a dose-dependent manner (p=0.005) (**Figure 5**).

PEDF impeded high glucose-induced ROS generation in HMC. Since excessive production of ROS is associated with high glucose-induced renal damage, and have been reported to cause ECM accumulation via TGF-β1 activation,[6] we went on further to examine the effects of PEDF on ROS generation in HMCs. Interestingly, PEDF at the concentration of 40-160 nmol/l significantly down regulates (p=0.001) the ROS production in a dose-dependent manner (**Figure 6**).

Discussion. The findings in this study has shown that ROS production, TGF-β1 secretion, FN, and collagen IV expression as index of fibrosis are increased in HMCs exposed to high glucose. However, these effects were inhibited by PEDF. These findings demonstrated that PEDF prevented the high glucose-induced ECM protein accumulation (TGF-β1, FN, and collagen IV) in HMCs through its anti-oxidative properties. It is generally accepted that overproduction of ROS is a direct consequence of hyperglycemia, and that ROS is a leading cause of the progression and development of diabetic complications, including diabetic nephropathy.[18,19] The ROS activate signal transduction cascade and transcription factors, leading to upregulation of fibrogenic gene and protein involved in glomerular mesangial expansion and fibrosis.[6] Our results have confirmed that ROS generation, TGF-β1, and extracellular matrix (ECM) expression were increased in a proportional manner with glucose concentration.

Previous studies have shown that PEDF is a multifunctional protein with demonstrable neurotrophic, antitumorigenic, antiangiogenic, antiatherogenic and anti-vasopermeability activities.[12,20-23] A few studies have shown that the PEDF is implicated in the pathogenesis of diabetic nephropathy.[8,15,24-26] Mesangial matrix expansion is the most prominent pathological feature of diabetic nephropathy, which is characterized by the accumulation of ECM proteins, such as FN and collagen IV. As mesangial cells are important cells in maintaining renal functions, for they are capable of producing ECM proteins and regulating GFR through their contractility, we used HMCs as a model to determine if PEDF is a potent inhibitor of ECM production. The results showed that high glucose significantly increased FN and collagen IV secretion, PEDF blocks the high glucose-induced FN and collagen IV overproduction in a dose dependent manner, which is beneficial in preventing ECM accumulation in diabetic nephropathy.

It is believed that TGF-β is involved in the pathogenesis of early stage diabetic nephropathy. Accumulated evidence have shown that overexpression of TGF-β

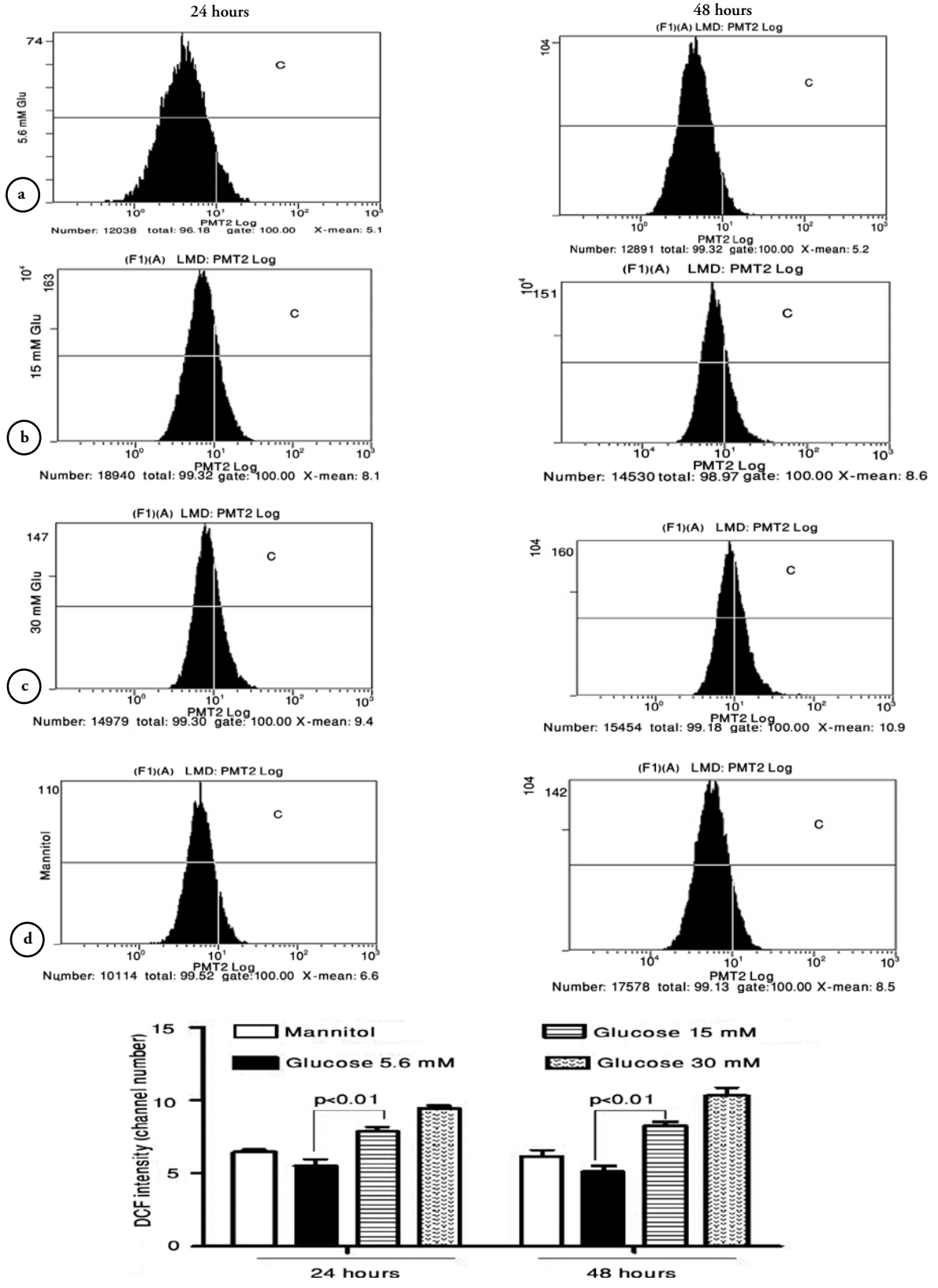

Figure 1 - Flow cytometric results expressed as 2,7-dichlorofluorescein (DCF) fluorescence intensity of: **a-d**) the effect of different concentrations of glucose and mannitol, and **e**) histogram, on the effect of reactive oxygen species generation by the human mesangial cells cultured for a period of 24, and 48 hours (n=3-5).

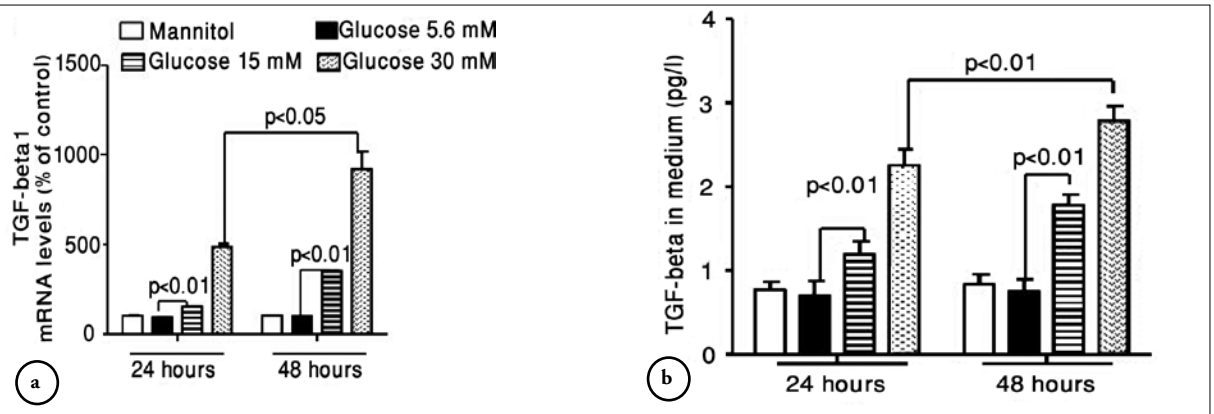

Figure 2 - Effect of different concentrations of glucose and mannitol on **a**) transforming growth factor-beta1 (TGF-β1) messenger ribonucleic acid (mRNA) and protein, **b**) expressions by the human mesangial cells (HMC) cultured for a period of 24, and 48 hours (n=3-5). The mRNA and protein levels of TGF-β1 were determined by real-time polymerase chain reaction, and enzyme-linked immunosorbent assay.

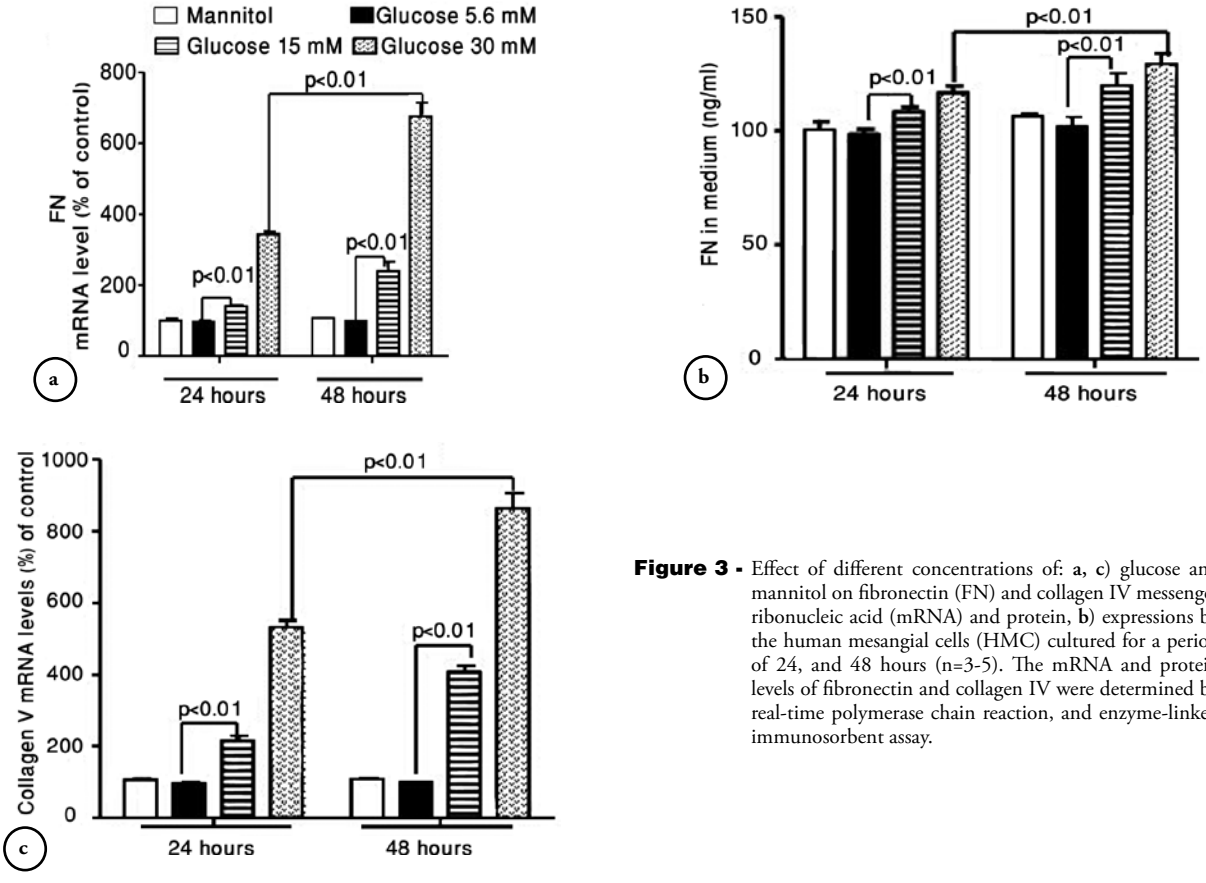

Figure 3 - Effect of different concentrations of: **a, c**) glucose and mannitol on fibronectin (FN) and collagen IV messenger ribonucleic acid (mRNA) and protein, **b**) expressions by the human mesangial cells (HMC) cultured for a period of 24, and 48 hours (n=3-5). The mRNA and protein levels of fibronectin and collagen IV were determined by real-time polymerase chain reaction, and enzyme-linked immunosorbent assay.

induced by elevated glucose causes glomerular basement membrane thickening, and mesangial matrix expansion via stimulation of matrix protein expression.[27-29] On the other hand, inhibition of TGF-β expression abolishes the above pathological changes.[29,30] As discussed previously, high glucose significantly increased FN and collagen IV secretion, PEDF blocks the high glucose induced FN and collagen IV overproduction in a dose dependent manner, which is beneficial in preventing ECM accumulation in diabetic nephropathy. The results showed that TGF-β1 expression was up-regulated in the HMC incubated with high glucose, and PEDF

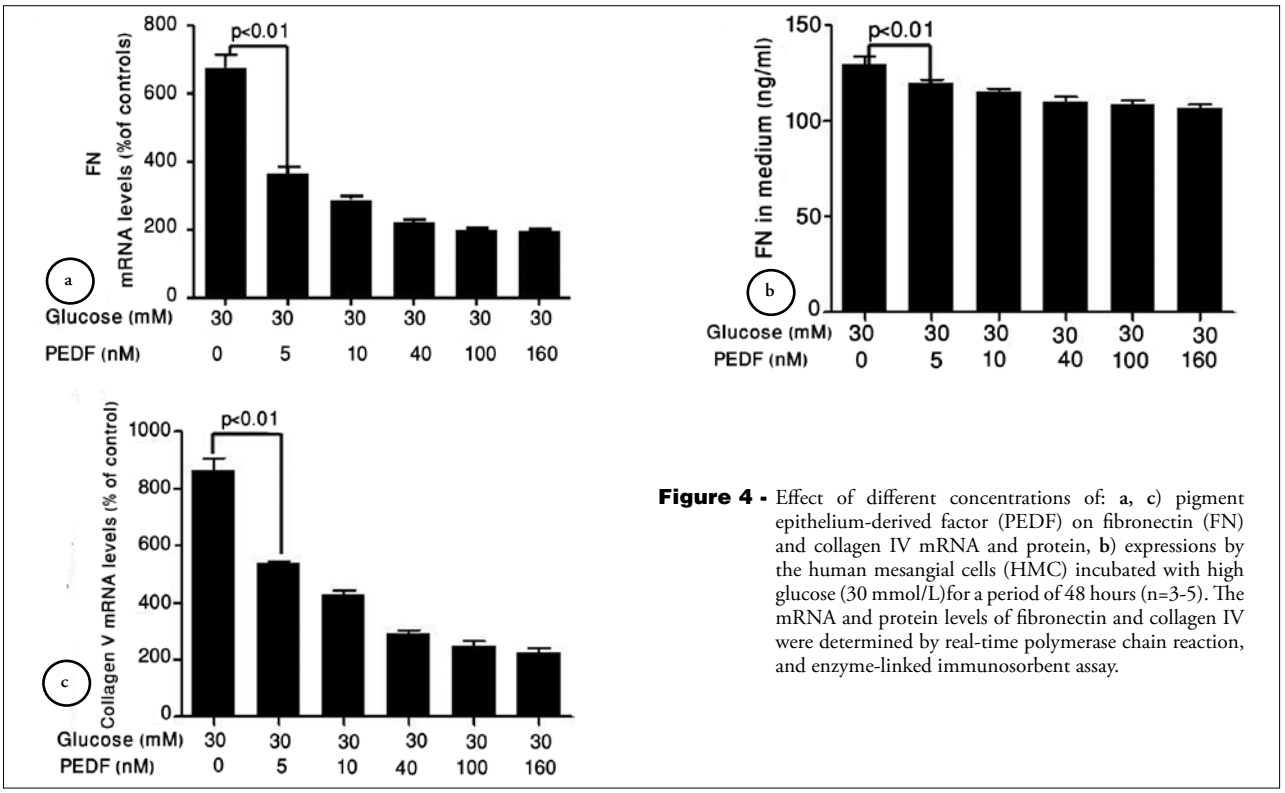

Figure 4 - Effect of different concentrations of: **a**, **c**) pigment epithelium-derived factor (PEDF) on fibronectin (FN) and collagen IV mRNA and protein, **b**) expressions by the human mesangial cells (HMC) incubated with high glucose (30 mmol/L)for a period of 48 hours (n=3-5). The mRNA and protein levels of fibronectin and collagen IV were determined by real-time polymerase chain reaction, and enzyme-linked immunosorbent assay.

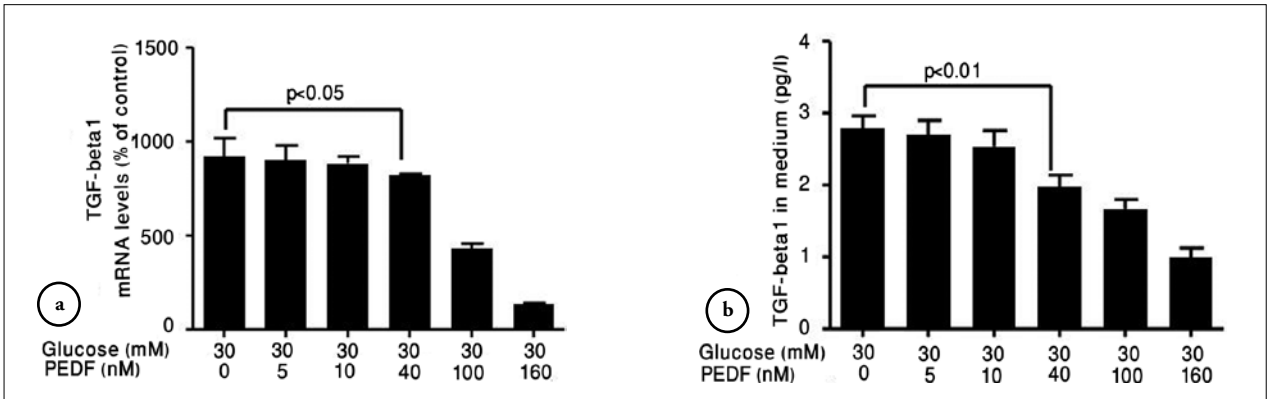

Figure 5 - Effect of different concentrations of pigment epithelium-derived factor (PEDF) on: **a**) transforming growth factor-beta1 (TGF-β1) messenger ribonucleic acid (mRNA) and protein, **b**) expressions by the human mesangial cells (HMC) incubated with high glucose (30 mmol/L) for a period of 48 hours (n=3-5). The mRNA and protein levels of fibronectin and collagen IV were determined by real-time polymerase chain reaction, and enzyme-linked immunosorbent assay.

significantly blocked the high glucose-induced TGF-β1 overexpression, suggesting that PEDF may act as an endogenous inhibitor of TGF-β1 expression via a paracrine or autocrine regulation in normal kidney, and exogenous PEDF treatment may be beneficial via inhibition of TGF-β1 expression. However, our results cannot exclude the possibility that PEDF may also block the function of TGF-β in the induction of ECM production secretion, that is, via inhibition of TGF-β1 receptor, or the post-receptor signaling pathway.

It has been proposed that the activation of 4 major biochemical pathways implicated in diabetes (increased advanced glycation end products (AGE) formation, activation of protein kinase C isoforms, and increased flux through the polyol and hexosamine pathways)[31] is attributed to oxidative stress as a unifying mechanism. Therefore, it is well-recognized that oxidative stress is not only implicated in DN, but also plays a pivotal role in its development and progression. Oxidative stress represents the imbalance between ROS production

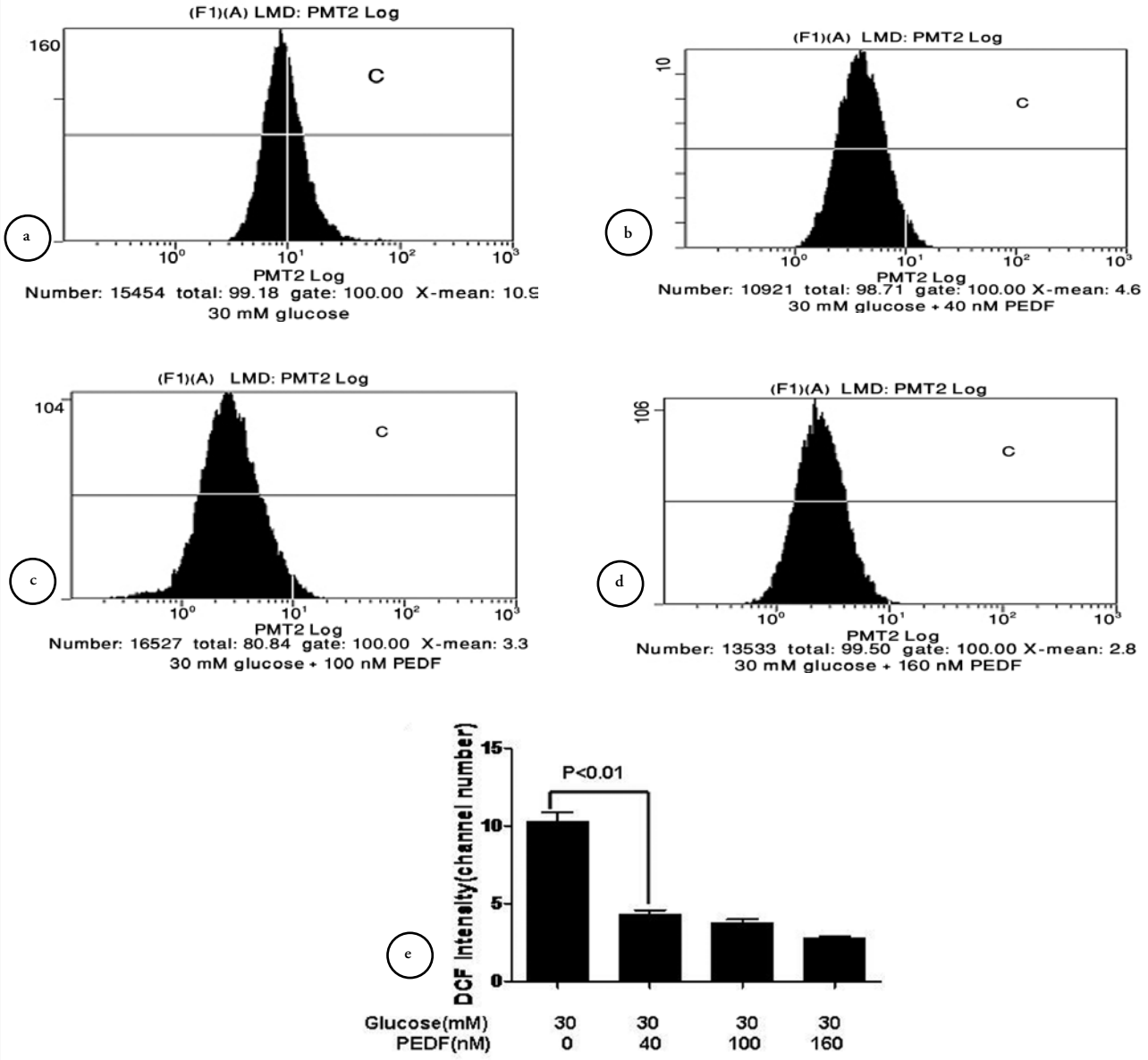

Figure 6 - Flow cytometric results expressed as 2,7-dichlorofluorescein (DCF) fluorescence intensity of: **a**-**d**) the effect of different concentrations of pigment epithelium-derived factor (PEDF), and **e**) histogram on the effect of reactive oxygen species generation by the human mesangial cells (HMC) incubated with high glucose (30 mmol/L) for a period of 48 hours (n=5).

and antioxidant capacity, and it occurs if the ROS production exceeds the antioxidant capacity. It has been reported that ROS overproduction induced by elevated glucose trans-activates TGF-β1, FN, and collagen IV gene expression, and addition of antioxidants abolishes those profibrotic gene upregulations.[32-35]

The present study showed that ROS generation in the HMC incubated with high glucose was significantly increased compared with that cultured on normal glucose containing media. This study showed that re-treatment with PEDF have suppressed the ROS production. Taken together, it is suggested that PEDF protect the diabetic kidney from ECM accumulation via its anti-oxidative activities. Ide et al[36] reported that PEDF could inhibit the AGE-induced inflammatory and thrombogenic gene expressions in human cultured mesangial cells by suppressing NF-$_K$B activation via inhibition of ROS generation. Yamagishi et al[37] reported that AGEs-bovine serum albumin (BSA) significantly increased intracellular ROS generation in human umbilical vein endothelial cells (HUVECs), which was completely inhibited by PEDF;PEDF, or an anti-oxidant

N-acetyl-L-cysteine significantly restored the decrease in elicited endothelial nitric oxide synthase (eNOS) mRNA levels in AGEs-exposed human umbilical vein endothelial cells. Tsao et al[38] demonstrated that PEDF can protect ARPE-19 cells from H_2O_2-induced cell death via induction of extracellular regulated protein kinase (ERK)1/2 phosphorylation. The PEDF decreased ROS generation in AGE-exposed endothelial cells by suppressing triphosphopyridine nucleotide oxidase activity via down-regulation of mRNA levels of $p22^{PHOX}$ and $gp91^{PHOX}$.[39] Amano et al[40] revealed that PEDF protects against high glucose, or H_2O_2-induced pericyte apoptosis and dysfunction through its anti-oxidative properties via glutathione peroxidase (GPx) induction.

In summary, our data provide the evidence suggesting that PEDF is an endogenous anti-oxidative and anti-fibrogenic factor in the kidney, PEDF may play an important role in preventing mesangial ECM overproduction, and pathological growth factor upregulation in the kidney by eliminating ROS generation, and the subsequent signaling pathways. We propose that PEDF and its derivatives, by virtue of its anti-oxidative and anti-fibrogenic properties, may have a therapeutic potential in diabetic nephropathy.

The limitation of this research is that the mechanism of PEDF inhibiting ROS generation is not studied. This is our future research focus.

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Ethical Consent

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.