WITHDRAWN: Dexamethasone reduces podocyte injury through the PTEN-PI3K/Akt signaling pathway

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Research

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The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Objective: To study the role of Akt and its downstream molecules in the PTEN-PI3K/Akt signaling pathway, namely,GSK3β and Bad, in the Dexamethasone(DEX)-mediated regulation of PAN-induced glomerular podocyte injury and to elucidate the molecular mechanism of podocyte injury regulation.

Methods: Glomerular podocytes (MPC5) were cultured in vitro and divided into four groups: the control group, PTEN silencing group (siPTEN group), puromycin group (PAN group), and puromycin group + DEX group (PAN+ DEX group). The cells in each group were treated for 8h, 24h, and 48h, and then, the experiment was carried out. The cells in the control group were cultured in RPMI 1640 with 0.02% DMSO, the cells in the siPTEN group were used to construct a silencing kit, the podocytes in the PAN group were treated with puromycin (final concentration of 50μg/ml), and the podocytes in the DEX+PAN group were pretreated with 0.1μmol/L DEX followed by PAN (final concentration of 50μg/ml). An inverted phase contrast microscope was used to observe the morphological changes in the podocytes and the changes in the cell body area in each group, laser confocal microscopy was used to detect the expression and distribution of the PTEN protein, and flow cytometry was used to detect and analyze the apoptosis rate and mitochondrial membrane potential of each group of podocytes. Western blot was used to detect the expression of the PTEN, P-Akt, Akt, P-GSK3β and GSK3β proteins in each group of podocytes, and transmission electron microscopy was used to observe the changes in the morphology and structure of each group of podocytes.

Results: After PAN was used to injure the podocytes, the expression of the PTEN protein decreased, the rate of apoptosis increased, and the flux of autophagy was inhibited. DEX treatment reversed the changes described above.After PAN was used to injure the podocytes, the expression of p-Ak and p-GSK3β decreased, and DEX reversed these effects on the expression of p-Akt and p-GSK3β in the podocytes. Compared with the control group, in the PAN group, the mitochondria gradually swelled and rounded, mitochondrial cristae arrangement became disordered, mitochondrial autophagy was inhibited; DEX reversed the changes described above after the PTEN gene was silenced.

Conclusion: This study confirmed that PAN can inhibit podocyte autophagy and induce podocyte damage. DEX can reduce the PAN-induced suppression of podocyte autophagy, enhance podocyte autophagy, and ameliorate podocyte damage. The protective mechanism may be through the upregulation of PTEN expression, which is achieved by inhibiting the activation of the PI3K/Akt signaling pathway.

Introduction

Although research on the mechanism of glomerular podocyte injury continues to advance, the prevention and treatment of glomerular podocyte injury and proteinuria are still difficult problems in clinical treatment. Recent studies have found that glomerular podocyte damage is an important event in the early stage of chronic kidney disease, and the identification effective and feasible methods for the treatment of

podocyte damage is the key to solving this problem[1-2].Our previous studies have found that the PI3K/Akt signal transduction pathway plays an important role in podocyte injury[3]. Phosphatase and tensin homolog (PTEN) is an important enzyme with lipid and protein phosphatase activities.PTEN is also a tumor suppressor. One of the typical functions of PTEN is the inhibition of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway and the inhibition of AKT and its downstream signaling pathways through its lipid phosphatase activity[4-6].Therefore, some scholars refer to this pathway as the PTEN-PI3K/AKT signal transduction pathway. However, there is no report on the mechanism by which the PTEN-PI3K/Akt signal transduction pathway is involved in glomerular podocyte damage. Therefore, in this study, we propose that the PTEN-PI3K/Akt signal transduction pathway regulates glomerular podocyte injury.If this hypothesis is verified, it will not only explain how glomerular podocyte damage promotes the occurrence and development of proteinuria, which is a current scientific problem,but also elucidate the exact mechanism of action and identify the precise point of action.Moreover,new targets in the PTEN-PI3K/Akt signaling pathway through which to inhibit glomerular podocyte damage could be identified,and this would answer the current question of how glomerular podocyte damage promotes the occurrence and development of proteinuria.

Methods

1. Podocyte culture and treatment

Immortalized mouse podocytes were cultured as previously described in detail[3]. All the experiments were performed under serum-free conditions. Under these conditions, the cells remained viable but in a nonproliferative state. To induce differentiation, the podocytes were cultured at 37°C for 14days. Podocytes in the logarithmic phase of growth were selected, and the podocytes were counted after trypsinization. The podocyte suspension was adjusted to a concentration of 5×10^4 cells/well for inoculation. These cells were cultured in an incubator at a constant temperature of 37°C and in 5% CO_2. To knock down PTEN, differentiated podocytes were transfected with Lipofectamine 2000 transfection reagent (Life Technologies, USA). To overexpress PTEN, we infected podocytes with an adenovirus expressing mouse PTEN (Biowit Technologies, ShenZhen, China) for 48h.

2. FITC-Annexin V and PI double staining flow cytometry to detect the podocyte apoptosis rate

The cells were washed twice with PBS solution, trypsinized without EDTA for 1-2 minutes, gently removed from the lower wall of the culture flask, collected into a 10-ml centrifuge tube, and centrifuged at 1000 rpm for 5minutes; then, the supernatant was removed. The buffer was prepared according to the instructions of the kit, and FITC-Annexin V and PI were added to the buffer solution at a final concentration of 1 μg/ml The lower layer of the cells was transferred to the flow tube, 500μl of cells suspended in buffer were added, and the labeling solution was added to the flow tube. Then, the reaction was allowed to continue for 10minutes in the dark. Next, the cells were analyzed by flow cytometry, and the relevant computer software was used to calculate the apoptosis rate of the podocytes.

3. Indirect immunofluorescence staining to detect the expression of the PTEN

The cell slides were fixed with precooled acetone at -20°C for 10minutes, washed 3 times with PBS for 5minutes each time, blocked with 0.3% Triton X-100 and 5% BSA for 30minutes at room temperature, and incubated with mouse anti-rabbit PTEN as the primary antibody (4°C,overnight). Then, the slides were incubated with a FITC-labeled secondary antibody (room temperature,45minutes). The control group was only incubated with the antibody diluent and washed 3 times with PBS for 5minutes each time, and then, the secondary antibody was added (PBS diluted 1:200). TRITC-labeled goat anti-rabbit IgG was incubated at room temperature for 45minutes, the slides were washed with PBS 3 times for 10minutes each time, and the anti-fluorescence attenuation mounting plate was applied in the dark prior to inspection.

4. Western Blot to detect the expression of the Pink1, Parkin and LC3-B proteins in podocytes

Cells were cultured in a six-well plate and washed with PBS three times after drug treatment. Then, RIPA cell lysis buffer was added to the cells, and the cells were placed on an ice shaker for lysis for 20minutes and collected. The cells were centrifuged at 12000g/min at 4°C for 10minutes, and the precipitate was discarded. The total protein concentration was determined by the BCA method, and the concentrations of the samples were adjusted to the same loading concentration. The proteins were denatured in a 100℃ water bath for 10 minutes, and the samples were stored in aliquots at -80℃. SDS polyacrylamide concentrate gels (5%), as well as 12% SDS polyacrylamide separation gels, were prepared.Total protein (20μg) was added to each well, and an 80-V current was applied for 15minutes until the bromophenol blue reached the separation gel;then,120V was applied for approximately 120-150minutes until the bromophenol blue marking line was near the bottom of the gel. A current of 350mA was used in the wet transfer method to transfer the proteins to PVDF films for 1h, and the Tanon 5200(Tianneng) chemiluminescence imaging system was used to visualize the proteins. The exposed images were analyzed by ImageJ image analysis software.

5. Transmission electron microscopy to detect the mitochondria in each group of cells

The podocytes of each group were collected by centrifugation at various time points, washed repeatedly with sucrose washing solution, and fixed with 1% osmic acid solution for 2h. The alcohol was dehydrated and put in 100% acetone. Epon 812:100% acetone (1:1) was added, and the cells were semisoaked for 1-3h. Epon 812 was added, and the cells were fully soaked at 35°C for approximately 2h, polymerized, and sliced. The cells in each group were double stained with uranyl acetate and lead citrate. The changes in the autophagosomes were carefully observed under an electron microscope.

6. Statistical methods

All the data were statistically processed with SPSS 26 statistical software. Measurement data were expressed as X±S, single-factor analysis of variance was used for comparison among multiple groups, t-test was used for comparison between two groups, and $p<0.05$ indicated that the difference was statistically significant.

Results

1. Morphological changes in the podocytes in each group

The relative area of the podocytes was observed under an inverted microscope. The podocytes of the control group differentiated well and showed a star-like shape. The cell body and nucleus of these cells were significantly larger than those of the proliferating cells. There were obvious dendritic protrusions from the cell body, and the adjacent cells formed interconnections. The PAN stimulation group exhibited foot process retraction at 8 h, and the cell body area was significantly reduced. At 24 h, the foot processes of the PAN-stimulated group were significantly retracted, and the cell body area decreased to 43% of that observed in the control group. At 48 h, the cell body area of the PAN-stimulated group decreased to 25% of that of the control group, and some podocytes showed a loss of foot processes and loss of intercellular connections. The cell area of the DEX intervention group was significantly larger than that of the PAN stimulation group at 8 h, 24 h and 48 h, which was statistically significant ($p<0.05$). See Figure 1-2.

2. Changes in the podocyte apoptosis rate in each group

The PI and Annexin V-FITC double staining apoptosis kit was used to detect the apoptosis rate of podocytes by flow cytometry. The results showed that the apoptosis rate of the podocytes in the PAN stimulation group at 8 h did not change significantly compared with that of the podocytes in the normal control group. At 24 h and 48 h, the apoptosis rate of the podocytes in the PAN stimulation group was significantly higher than that in the control group; at 24 h and 48 h, the apoptosis rates of the podocytes in the DEX treatment group were significantly lower than those in the PAN stimulation group. See Figure 3-4.

3. Changes in PTEN expression and distribution after PAN injury and DEX treatment

Immunofluorescence staining showed that in the control group, PTEN was evenly distributed in the nuclear membrane, cytoplasm and cell membrane; however, in the PAN stimulation group, the distribution of PTEN was abnormal, and there was a lack of PTEN distribution in the cell membrane and cytoplasm. The distribution of PTEN in the cell membrane and cytoplasm of the DEX treatment group was approximately normal at each time point, and the abnormal distribution and loss of PTEN observed in the PAN stimulation group were significantly improved in the DEX treatment group; however, some of cells did not fully return to normal, and there was a small amount of coarse particle-like distribution in the cytoplasm. See Figure 5.

4. Changes in the expression level of the PTEN protein

Under normal circumstances, the PTEN protein is expressed at a certain level in podocytes. Compared with the control group, the PAN stimulation group exhibited significantly reduced expression of the PTEN protein ($p<0.01$); compared with the PAN stimulation group, the DEX treatment group exhibited significantly increased protein expression that and tended toward normal levels ($p<0.05$).See Figure 6-7.

5. PI3K p85 protein expression changes

The results of western blotting showed that the expression of the PI3K p85 protein in the PAN-stimulated group was lower than that in the normal control group at 8 h and showed a downward trend over time. PI3K p85 protein expression continued to decrease at 24 h, and its protein expression could not be detected at 48 h ($p<0.01$). After DEX treatment, the expression of the PI3K p85 protein in the 8 h group was not significantly different from that in the PAN stimulation group, and it was higher than that in the PAN stimulation group at 24 h and 48 h ($p<0.05$). See Figure 8-9.

6. p-Akt (ser473) and total Akt protein expression changes

Taking total Akt as the internal reference and the blank group as the normal control group, the expression of p-Akt (ser473) decreased after PAN (50 μg/ml) stimulation of podocytes, and its expression was significantly lower than in the control group ($p<0.05$). DEX treatment significantly inhibited the abovementioned effects of PAN. After DEX treatment, the expression of p-Akt (ser473) was significantly higher than that in the PAN stimulation group, while LY294002 (25 mmol/L) pretreatment for 1 h basically inhibited the phosphorylation level of Akt in the podocytes ($p <0.05$). See Figure 10-11.

7. p-GSK3β (ser9) and total GSK3β protein expression changes

Taking total GSK3β as the internal reference and blank group as the normal control group, the expression of p-GSK3β (ser9) was significantly reduced after PAN (50 μg/ml) stimulation of podocytes; after DEX treatment, it was found that the effects of PAN described above could be significantly inhibited. After DEX treatment, the expression of p-GSK3β (ser9) was significantly higher than in the PAN stimulation group, and the phosphorylation level of GSK3β in podocytes was basically suppressed after pretreatment with LY294002 (25 mmol/L) for 1 h ($p<0.01$). See Figure 12-13.

8. Bad protein expression changes

The results of western blotting showed that the expression of the Bad protein in the PAN stimulation group was not significantly different from that in the control group at 8 h ($p>0.05$), but it increased significantly at 24 h ($p<0.05$) and showed an upward trend with time; at 48 h, Bad expression was significantly increased ($p<0.01$). There was no significant difference in the expression of the Bad protein in the DEX treatment group compared with that in the PAN stimulation group at 8 h ($p>0.05$), but it was significantly lower than that in the PAN stimulation group at 24 h and 48 h ($p<0.05$). See Figure14-15.

9. Changes in the expression of the LC3B protein

To evaluate the role of autophagy in TAC on PAN-induced podocyte damage, western blot was performed to analyze the proteins in podocytes treated with PAN, DEX and 3-MA. The western blot results showed that 3-MA and PAN could significantly inhibit podocyte autophagy, and the expression of the LC3B protein significantly decreased ($P<0.05$). After DEX treatment, autophagy was activated in podocytes, and the expression of LC3B significantly increased. See Figure 16-17.

10.The expression of related proteins after silencing PTEN and overexpression of PTEN

To evaluate the regulatory effect of the PTEN gene on the downstream PI3K/AKT signaling pathway and its effect on autophagy, we constructed a podocyte model of PTEN gene silencing and overexpression and performed western blot analysis on the podocyte proteins of each group. The results showed that after PTEN gene silencing, the expression of the downstream signaling pathway protein AKT increased, podocyte autophagy was significantly inhibited, and the expression of the LC3B protein significantly decreased ($P<0.05$). After the PTEN gene was overexpressed, the expression of the downstream signaling pathway protein AKT decreased, podocyte autophagy was activated, and the expression of LC3B significantly increased. See Figure 18.

11. Changes in autophagosome number in the podocytes of each group by transmission electron microscopy

The podocytes in the control group rarely contained isolated autophagosomes with double-layer or multilayer membranes. The number of autophagosomes in the podocytes in the PNA group was significantly reduced. The number of autophagosomes in the cytoplasm of the podocytes in the 3-MA group was significantly reduced; compared with that in the PNA group and 3-MA group, the number of autophagosomes in the cytoplasm of the podocytes in the DEX group significantly increased, and the difference was statistically significant ($P< 0.05$). See Figure 19.

Discussion

Podocytes are the largest intrinsic cell in the kidney.Early podocyte damage is reversible.Once the damaging factor is removed,the cytoskeleton proteins are repaired, and the foot processes still form a cross-connected pattern to perform their functions; however, if the damaging factor continues to act on podocytes,the podocyte damage irreversible[7-8].The worst consequence is that the proliferative capacity of the podocytes is limited. Once damage occurs, podocytes cannot continue to perform their functions through self-compensation, forming a vicious circle and accelerating the damage of other podocytes, ultimately leading to the occurrence and development of proteinuria. However, the mechanism of proteinuria development and glomerular podocyte damage is not yet fully understood.

The PTEN gene is a phosphatase gene that is homologous to phosphatase and tensin; the PTEN gene is located on human chromosome 10. As an upstream inhibitor of the PI3K/Akt signal transduction pathway, the most important substrate of PTEN is phosphatidylinositol(3,4,5)-triphosphate(PIP3).PIP3 is the product of phosphatidylinositol-3' kinase (PI3K) and mediates the activation of AKT (also known as PKB, protein kinase B), and PTEN dephosphorylates PIP3 to maintain a low level of PIP3, thereby inhibiting the PI3K/AKT pathway. Therefore, some scholars refer to this pathway as the PTEN-PI3K/AKT signal transduction pathway. In recent years, researchers have found that in addition to playing an important role in the field of tumors, PTEN is also involved in the progression of kidney disease[9-11]. Studies have found that in animal models of diabetic nephropathy, the expression of PTEN in glomerular

mesangial cells and podocytes is significantly downregulated, suggesting that PTEN may play an important role in glomerular sclerosis; in addition, as the expression of PTEN in the kidney is downregulated, podocytes are damaged, and urine protein levels gradually increase[12-14],suggesting that PTEN may be a protective gene in the kidney. The PI3K/Akt signaling pathway is widely present in eukaryotic cells and participates in many physiological and pathological processes, such as cell growth, differentiation and proliferation. Studies have shown that in some disease states, stabilizing the PI3K/Akt signaling pathway can effectively alleviate damage to podocytes and reduce the incidence of proteinuria[15-16].In recent years, studies have found that there are functional receptors for glucocorticoids in podocytes,and glucocorticoids can directly act on podocytes to protect them[17-20]. Glucocorticoids can mediate the activation of the PI3K/Akt pathway, inhibit cell apoptosis through a variety of mechanisms, and promote cell survival. Huber et al. showed that CD2AP can bind to PI3K p85 in podocytes and activate the PI3K/Akt signaling pathway[21-22].Our previous studies also found that DEX can resist PAN-induced podocyte apoptosis by regulating the PI3K/Akt signaling pathway.

In this study, we found that after PAN damaged podocytes, the expression of the PTEN protein decreased, the rate of apoptosis increased, and the flux of autophagy was inhibited. The distribution of PTEN in podocytes was abnormal, and there was a loss of PTEN distribution in the cell membrane and cytoplasm.After DEX treatment, the above changes could be reversed; furthermore, after PAN damaged podocytes, the expression of the PI3K p85, p-Ak, p-GSK3β and LC3B protein decreased, and the expression of the Bad protein increased. DEX treatment could reverse the changes described above; studies have also found that PTEN may be a kind of protective gene in the kidney[23-27]. High expression of PTEN can negatively regulate the PI3K/Akt pathway, inhibit the activation of Akt, improve the phenotype of renal podocytes, and reduce damage to renal podocytes[28].In early DN, as glomerular damage increases, the expression of PTEN gradually decreases, suggesting that PTEN has a certain correlation with glomerular damage[29-32]. To confirm the exact mechanism of PTEN in podocyte injury, after silencing the PTEN gene, we found that the expression of the downstream signaling pathway protein AKT increased,podocyte autophagy was significantly inhibited, and the expression of the LC3B protein significantly decreased. After the PTEN gene was overexpressed, the expression of the downstream signaling pathway protein AKT decreased, podocyte autophagy was activated, and the expression of LC3B significantly increased. In addition, under a transmission electron microscope, we observed that when the PTEN gene was silenced, the mitochondria of the podocytes gradually swelled and became rounded,the mitochondrial cristae arrangement was disordered, and mitochondrial autophagy was inhibited. Treatment of the podocytes with DEX reversed the above changes.This finding is consistent with relevant research results around the world.

The innovative point of this study is the elucidation of the relationship between podocyte injury and the PTEN-PI3K/Akt signaling pathway and the confirmation of the expression of Akt and its downstream molecules in the PTEN-PI3K/Akt signaling pathway, namely,GSK3β and Bad, during podocyte injury.The significance of this study, to a certain extent, is that it helps to reveal that the PTEN-PI3K/Akt signaling pathway is a potential molecular mechanism by which dexamethasone protects against podocyte

damage, providing a theoretical basis for the clinical use of dexamethasone to prevent early podocyte damage.

Declarations

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Availability of data and materials

All of the data and materials are available.

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Contributions

Ren-qi and Yu-shengyou were in charge of the experiment.Zeng-huasong and Ren-qi analysed the data and wrote the manuscript.Xia-huimin designed the experiment.Ren-qi and Yu-shengyou contributed equally to this work, they are the co-first author.

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Ethics declarations

The study was approved by the ethics committee at the Guangzhou women and children's Medical Center and was conducted in accordance with the Protocol of Helsinki.

Consent for publication

All authors have given their consent for the manuscript to be published.

Conflict of interest

No potential conflict of interest was reported by the authors.

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Figures

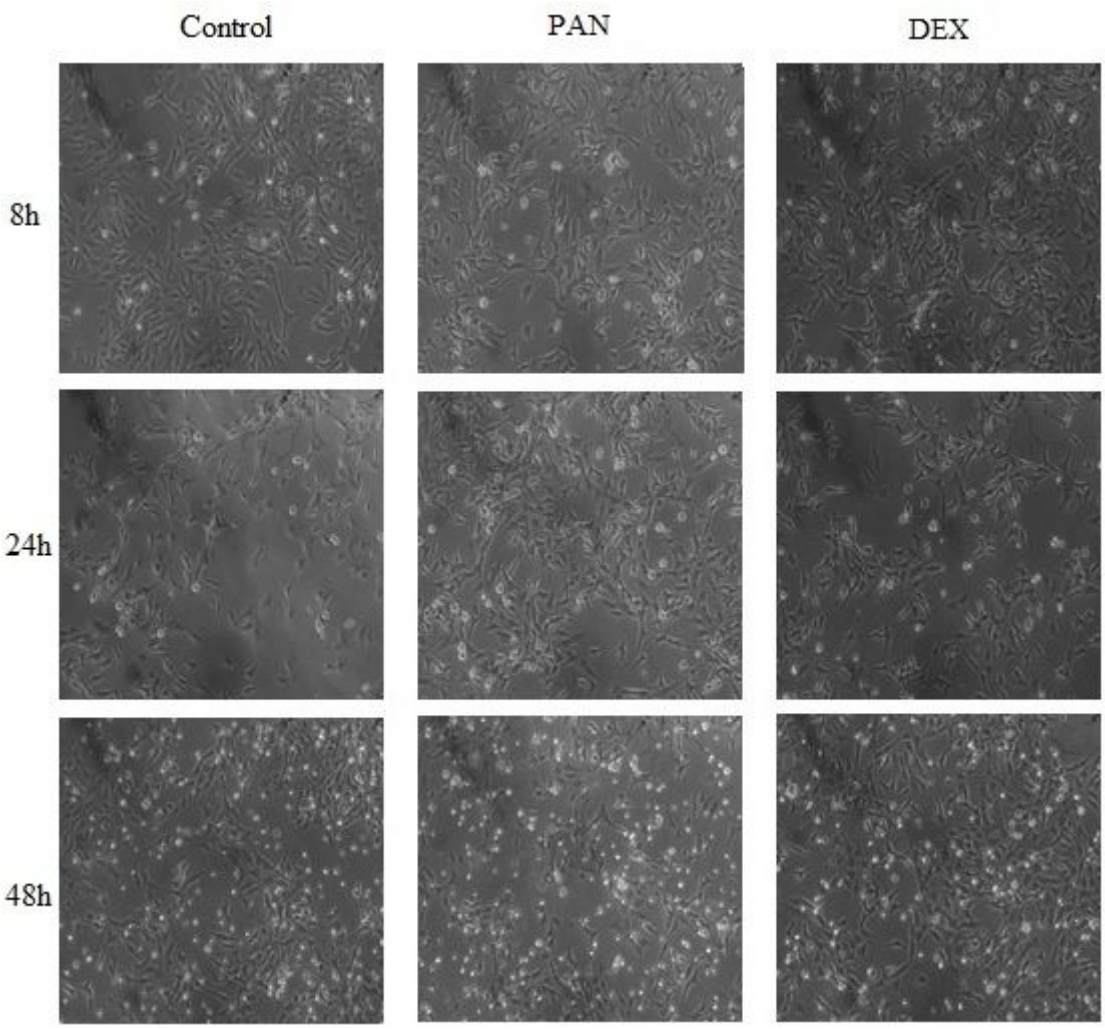

Figure 1

Cell body area and morphological changes at different time points in each group of podocytes

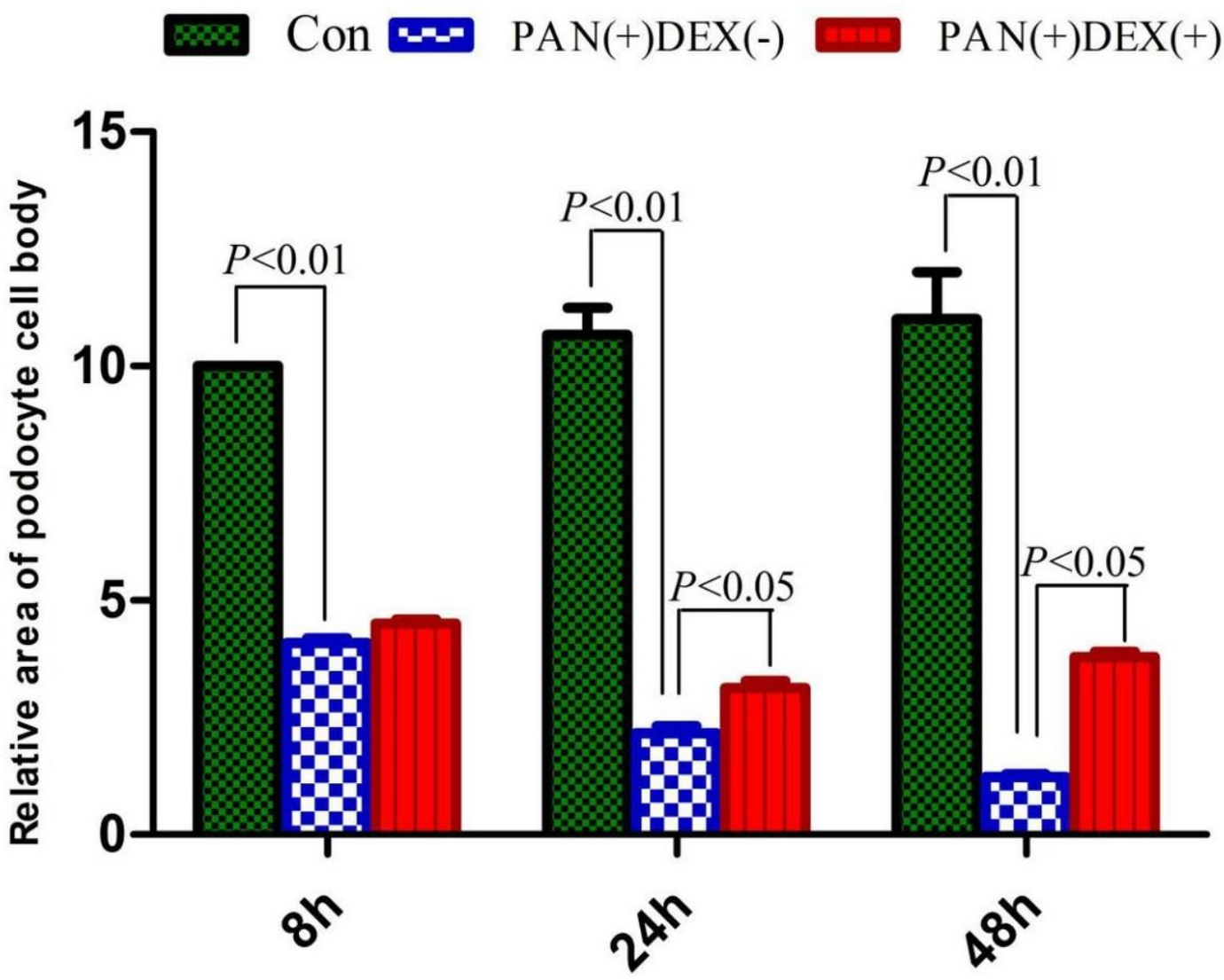

Figure 2

Histogram of the relative cell body area of each group of podocytes at different time points

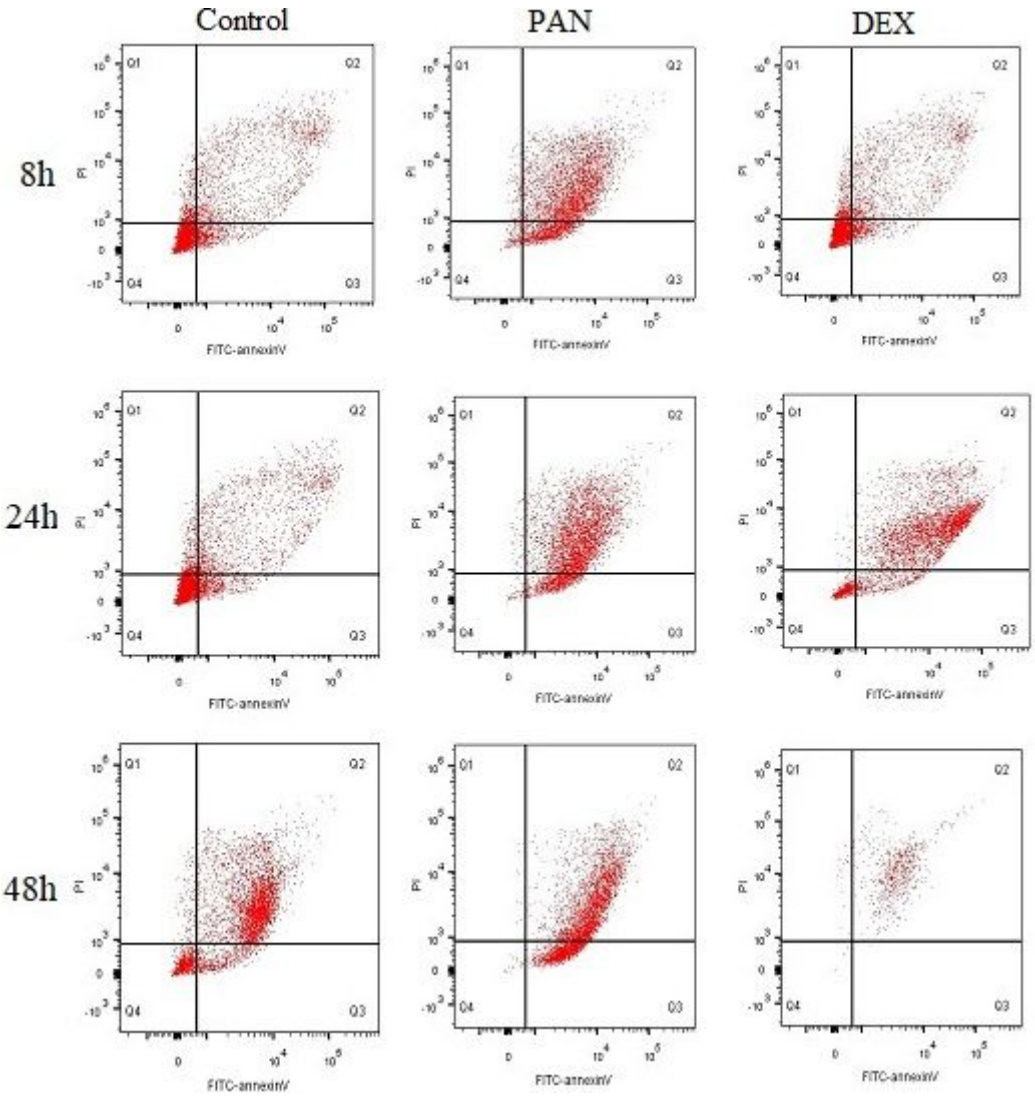

Figure 3

Changes in apoptosis rate of each group of cells at different time points

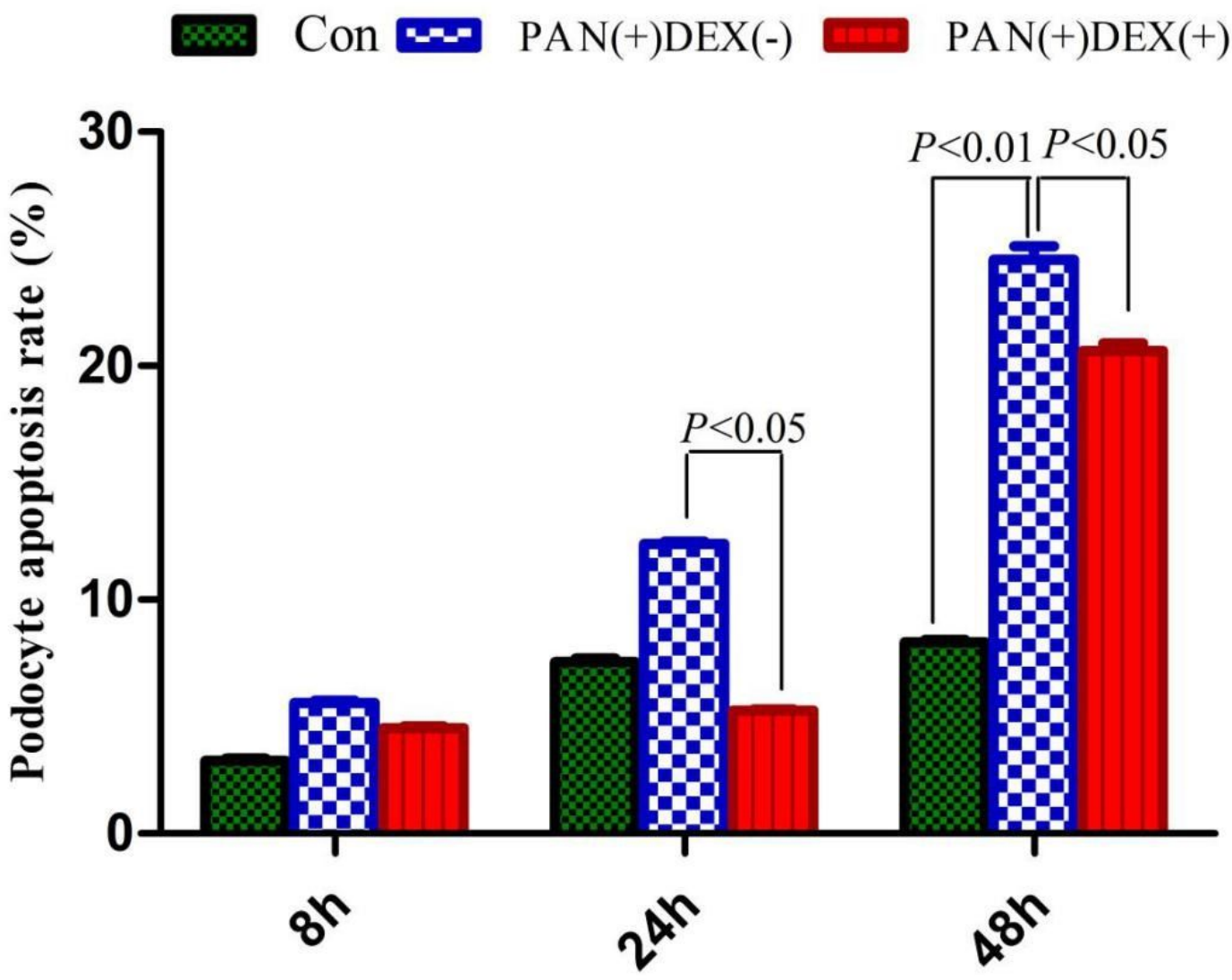

Figure 4

Histogram of apoptosis rate of each group of cells at different time points

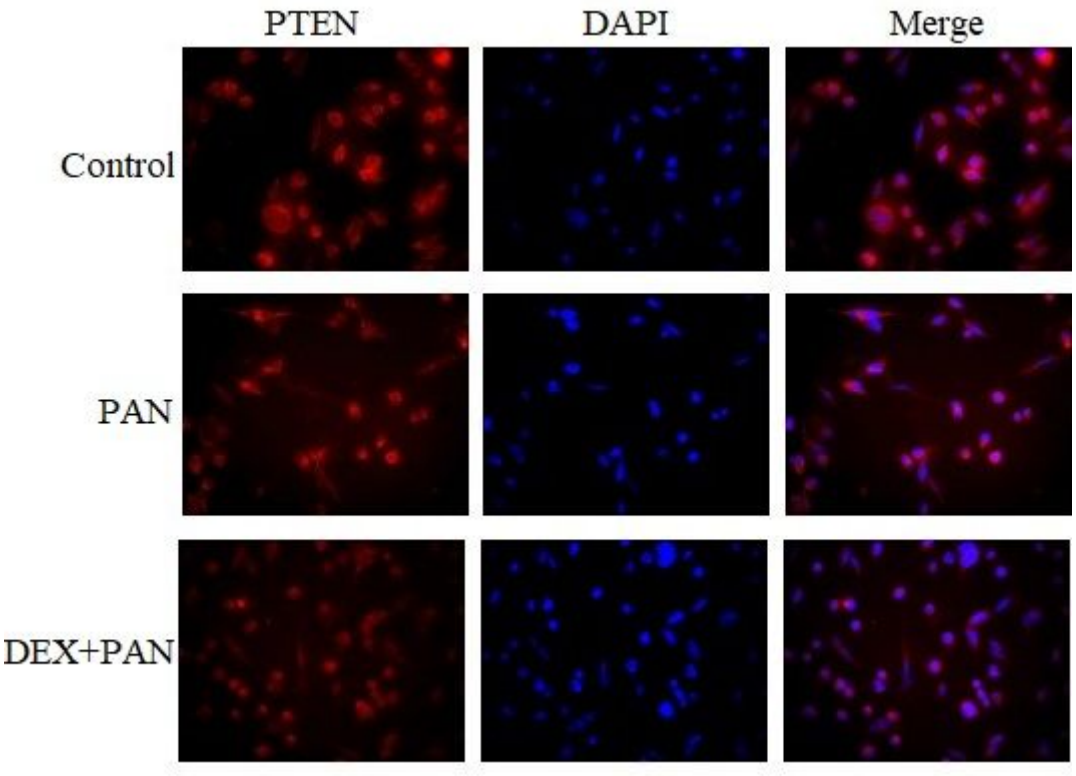

Figure 5

The distribution of PTEN in podocytes at each time point in the control group, PAN group and DEX group

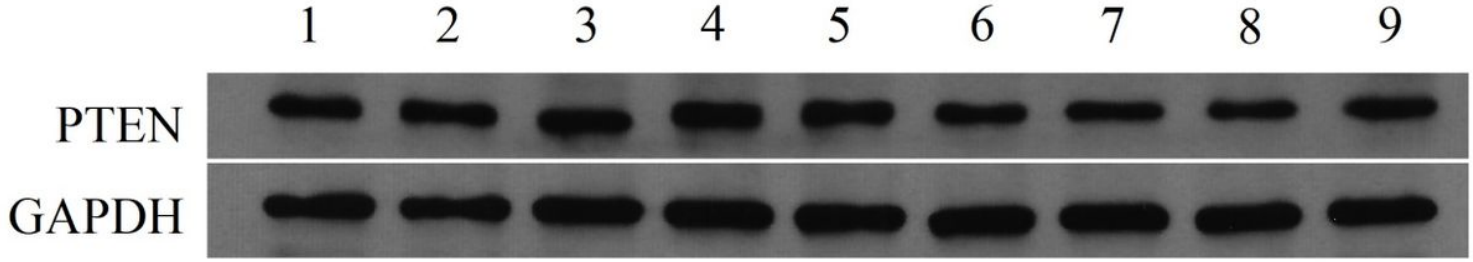

Figure 6

PTEN western blot results at each time point in the control group, PAN stimulation group and DEX group
Note: 1, 4, 7 are the control group, 2, 5, 8 are the PAN group, 3, 6, and 9 are the DEX group

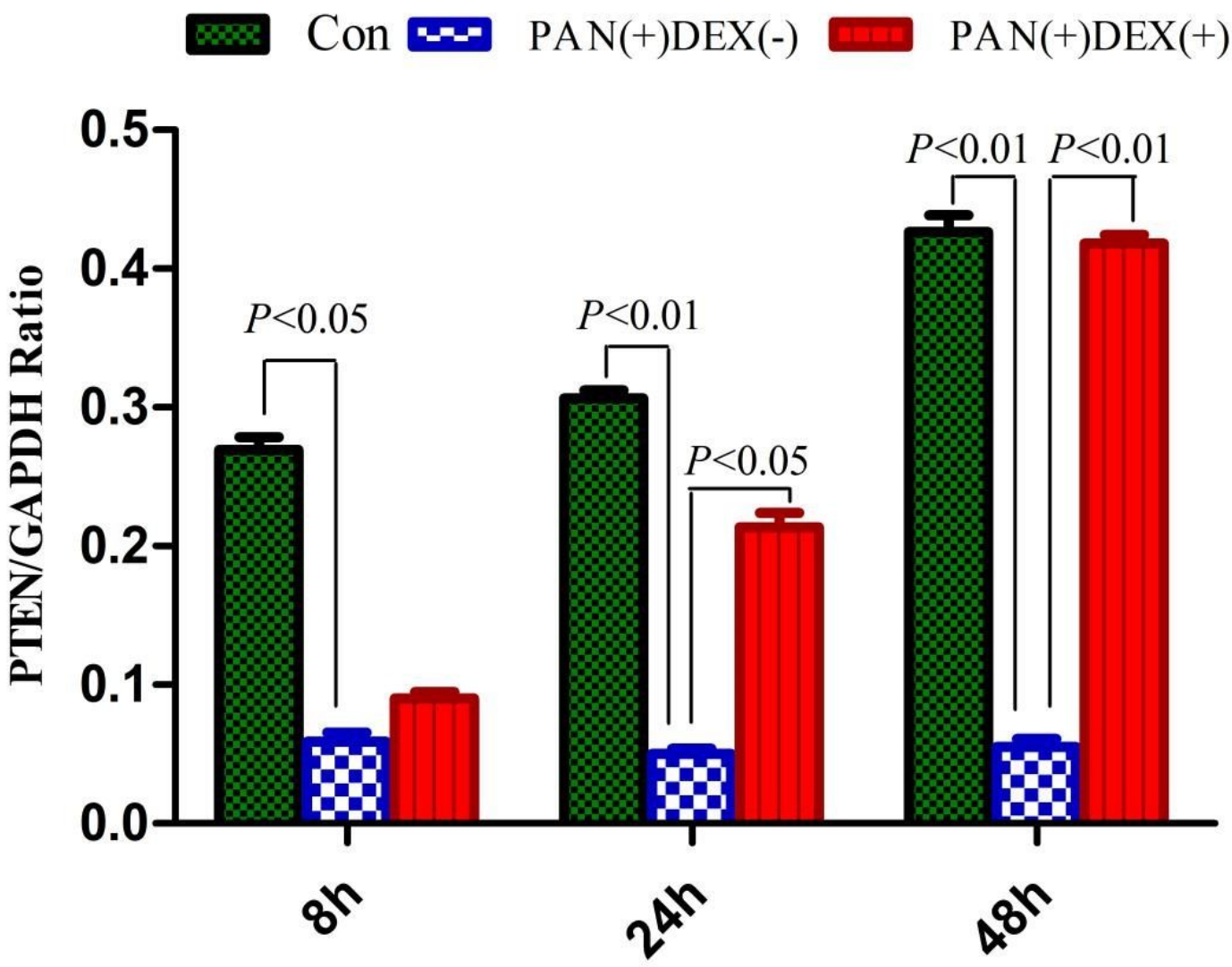

Figure 7

Changes in PTEN protein expression at each time point in the control group, PAN stimulation group and DEX intervention group

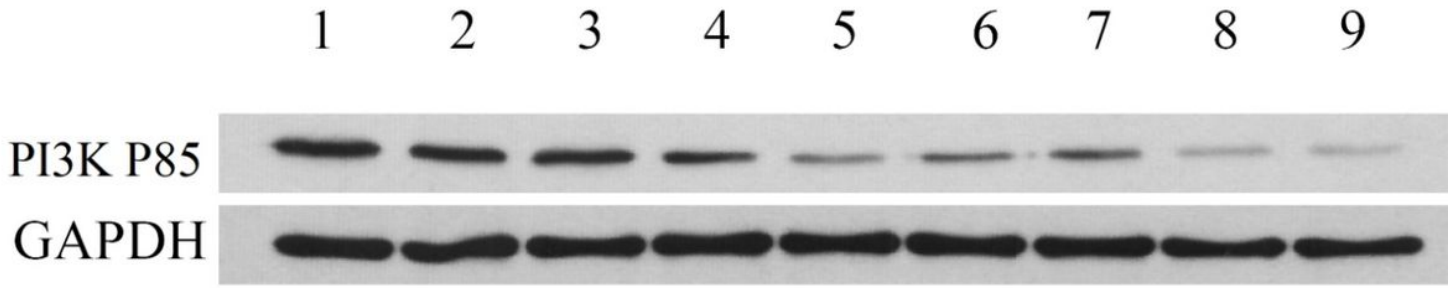

Figure 8

Western blot of PI3K p85 podocytes in the control group, PAN stimulation group and DEX intervention group at each time point Note: 1, 4, 7 are the control group, 2, 5, 8 are the PAN group, 3, 6, and 9 are the DEX group; control group: add 0.02% DMSO; PAN group: PAN (50μg/ml); DEX group: PAN (50μg/ml) and DEX (0.1μmol/l).

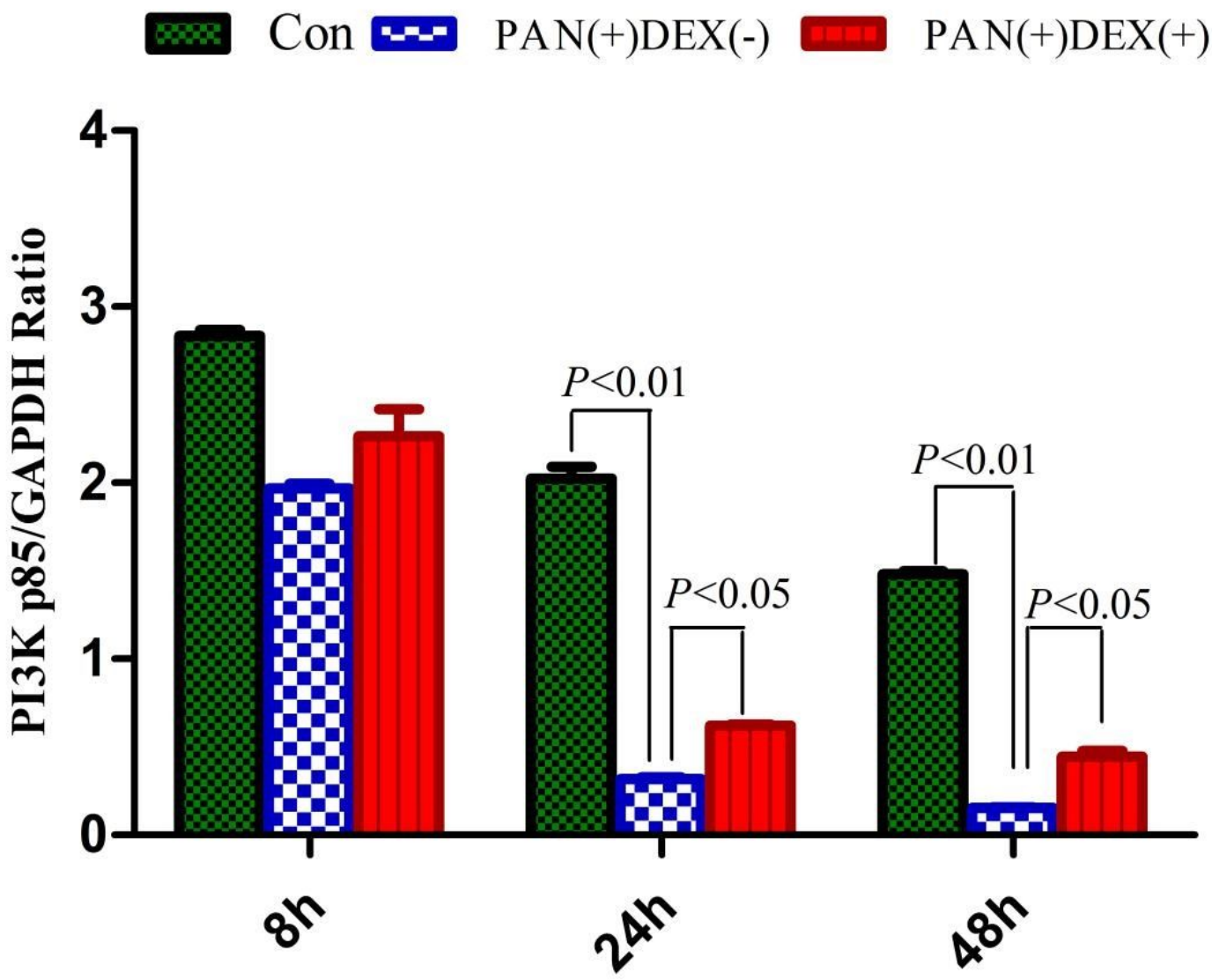

Figure 9

Changes in the expression of PI3K p85 protein in podocytes at each time point in the control group, PAN stimulation group and DEX intervention group

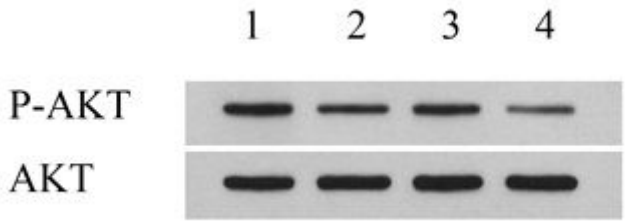

Figure 10

Western blot results of podocyte p-Akt(ser473)/Akt in each experimental group Note: 1. Normal control group; 2. PAN group; 3. PAN+DEX group; 4. LY294002 group

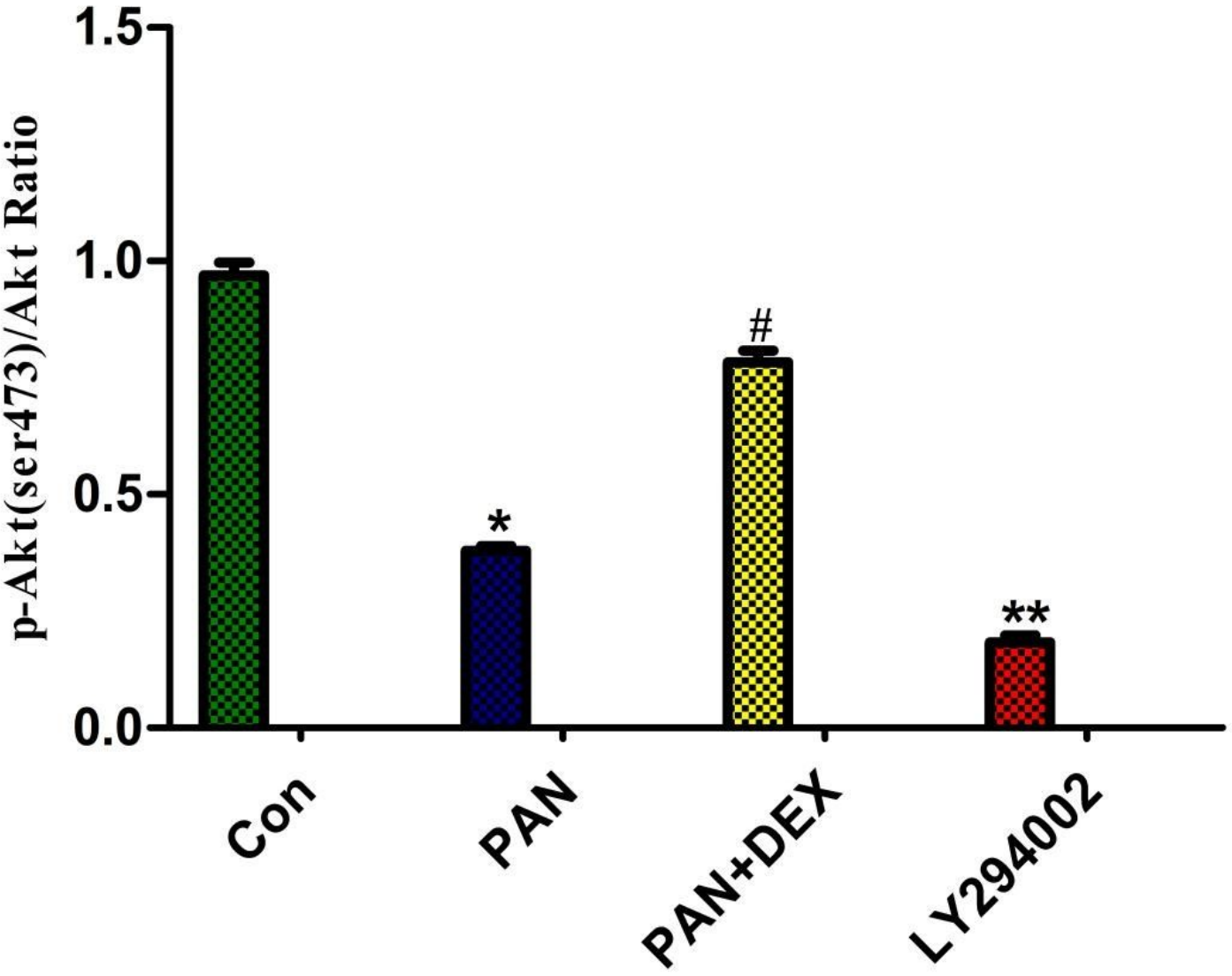

Figure 11

Changes in p-Akt(ser473)/Akt expression in podocytes of each group Note: Con is the control group, PAN group: PAN(50μg/ml), PAN+DEX group: PAN(50μg/ml)+DEX(0.1μmol/L), LY294002 pretreatment group: LY294002 pretreatment 1h +PAN(50μg/ml)+DEX(0.1μmol/L) stimulate podocytes for 15min, *p<0.05 vs control,#p<0.05 vs PAN group, **p<0.01 vs DEX group.

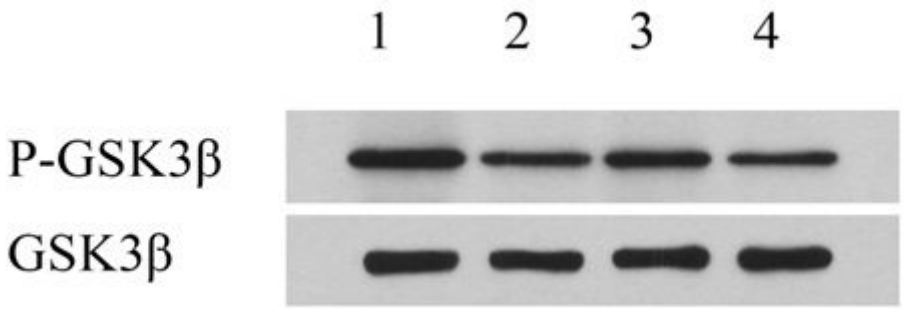

Figure 12

Western blot results of podocytes p-GSK3β(ser9)/GSK3βin each experimental group Note: 1. Normal control group; 2. PAN group; 3. PAN+DEX group; 4. LY294002 group

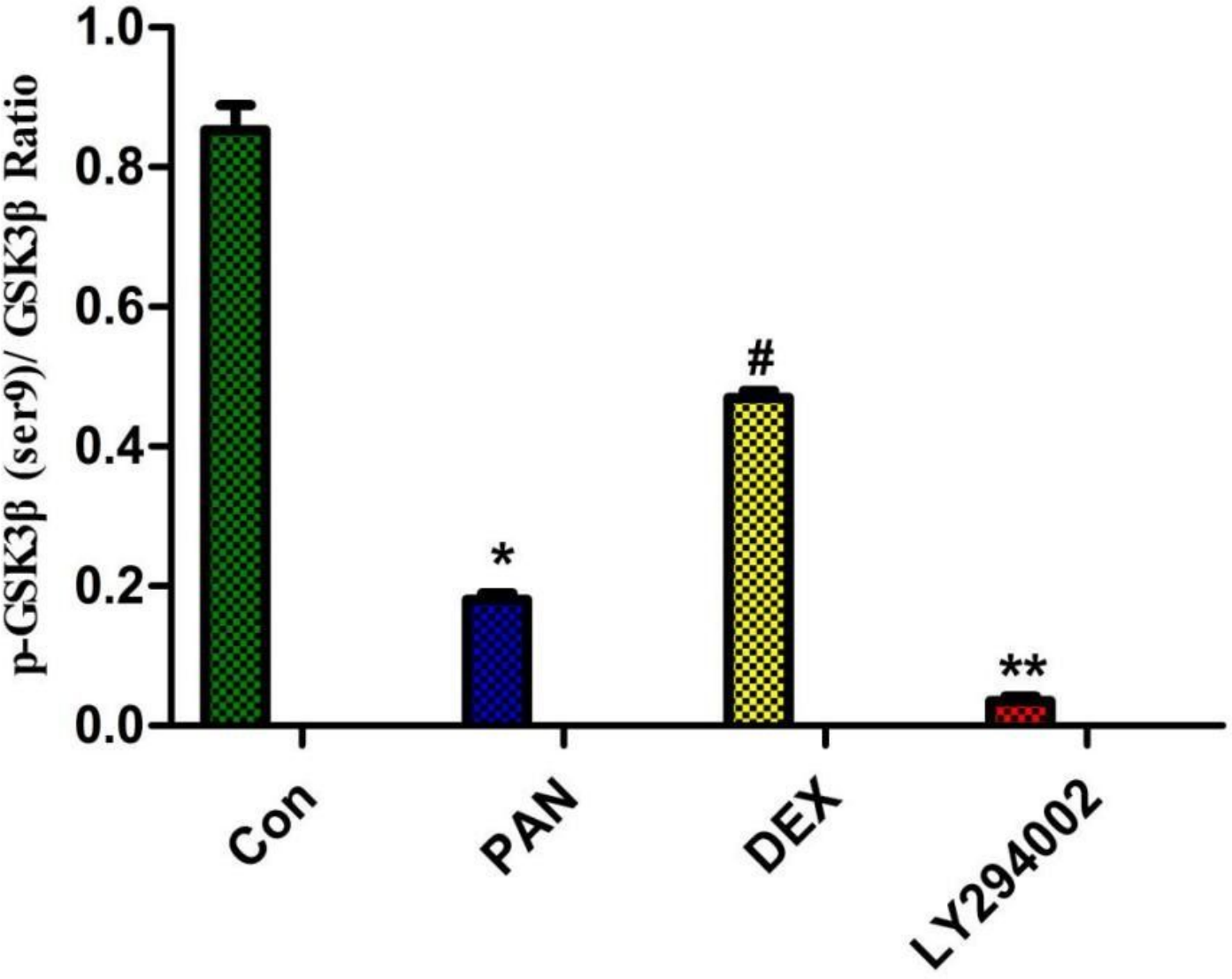

Figure 13

Changes in expression of p-GSK3β(ser9)/GSK3βin podocytes in each experimental group Note: Con is the control group, PAN stimulation group: PAN(50μg/ml), DEX intervention group: PAN(50μg/ml)+DEX(0.1μmol/L), LY294002 pretreatment group: LY294002 pretreatment for 1h +PAN(50μg) /ml)+DEX(0.1μmol/L) stimulate podocytes for 30min, *p<0.05 vs control,#p<0.05 vs PAN stimulation group, **p<0.01, vs DEX intervention group.

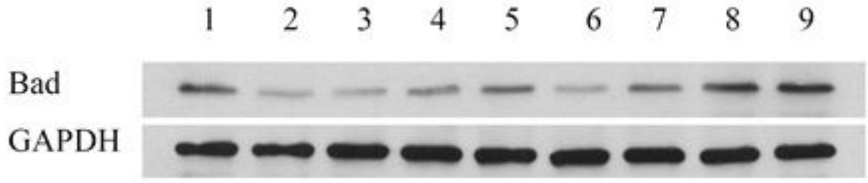

Figure 14

Bad at each time point in the control group, PAN group and DEX group Note: 1, 4, 7 are the control group, 2, 5, 8 are the PAN group, 3, 6, and 9 are the DEX group; control group: add 0.02% DMSO; PAN group: PAN (50μg/ml); DEX group: PAN (50μg/ml)+ DEX (0.1μmol/ l).

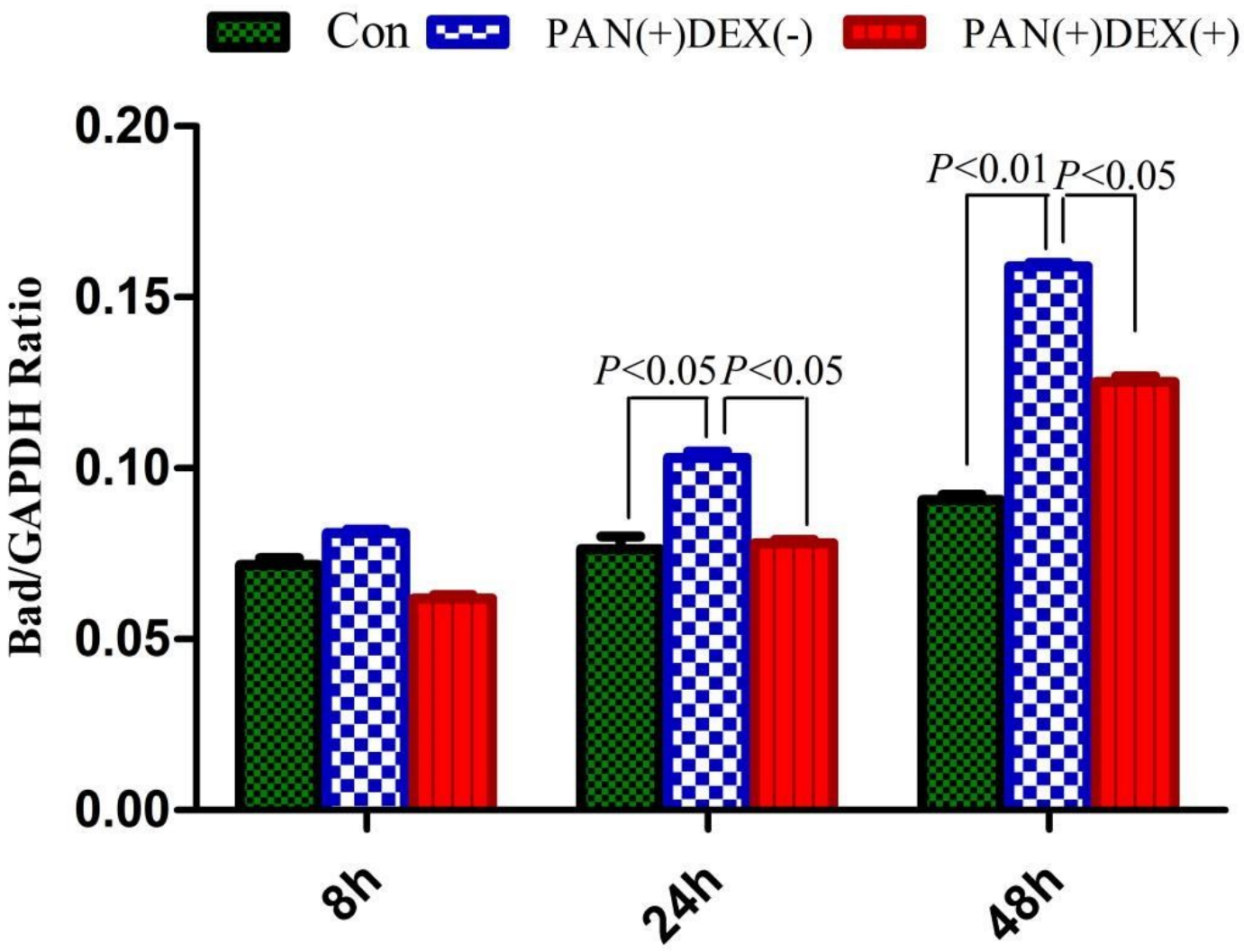

Figure 15

Podocyte Bad protein expression changes at each time point in the control group, PAN group and DEX group

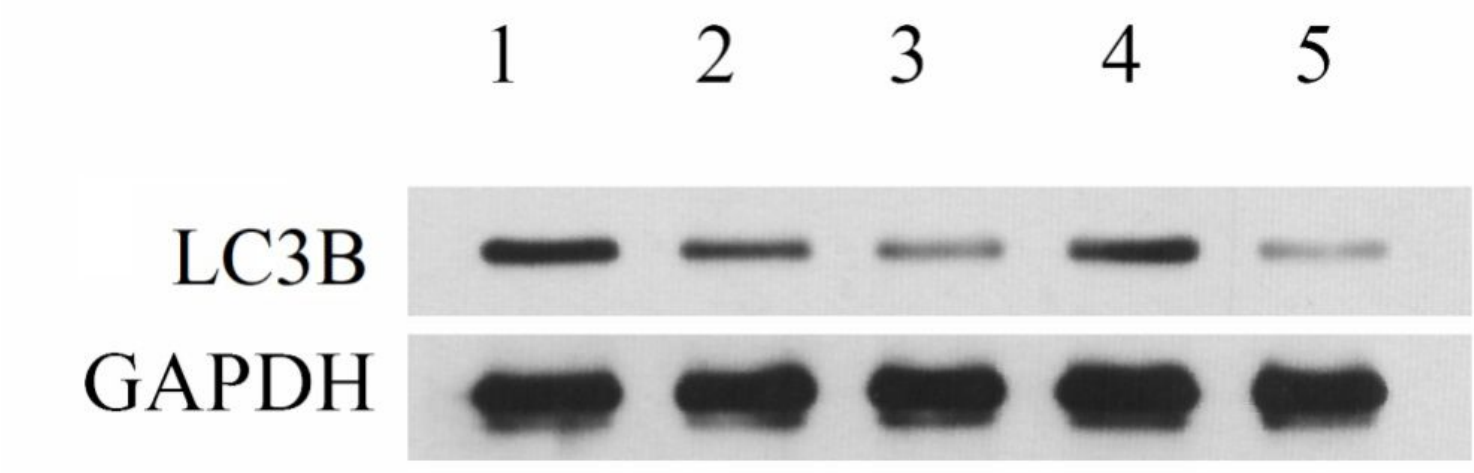

Figure 16

Western blot of LC3B protein of podocytes in each group Note: 1 is the control group, 2 is the DEX group, 3 is the PAN group, 4 is the DEX+PAN group, and 5 is the 3-MA group

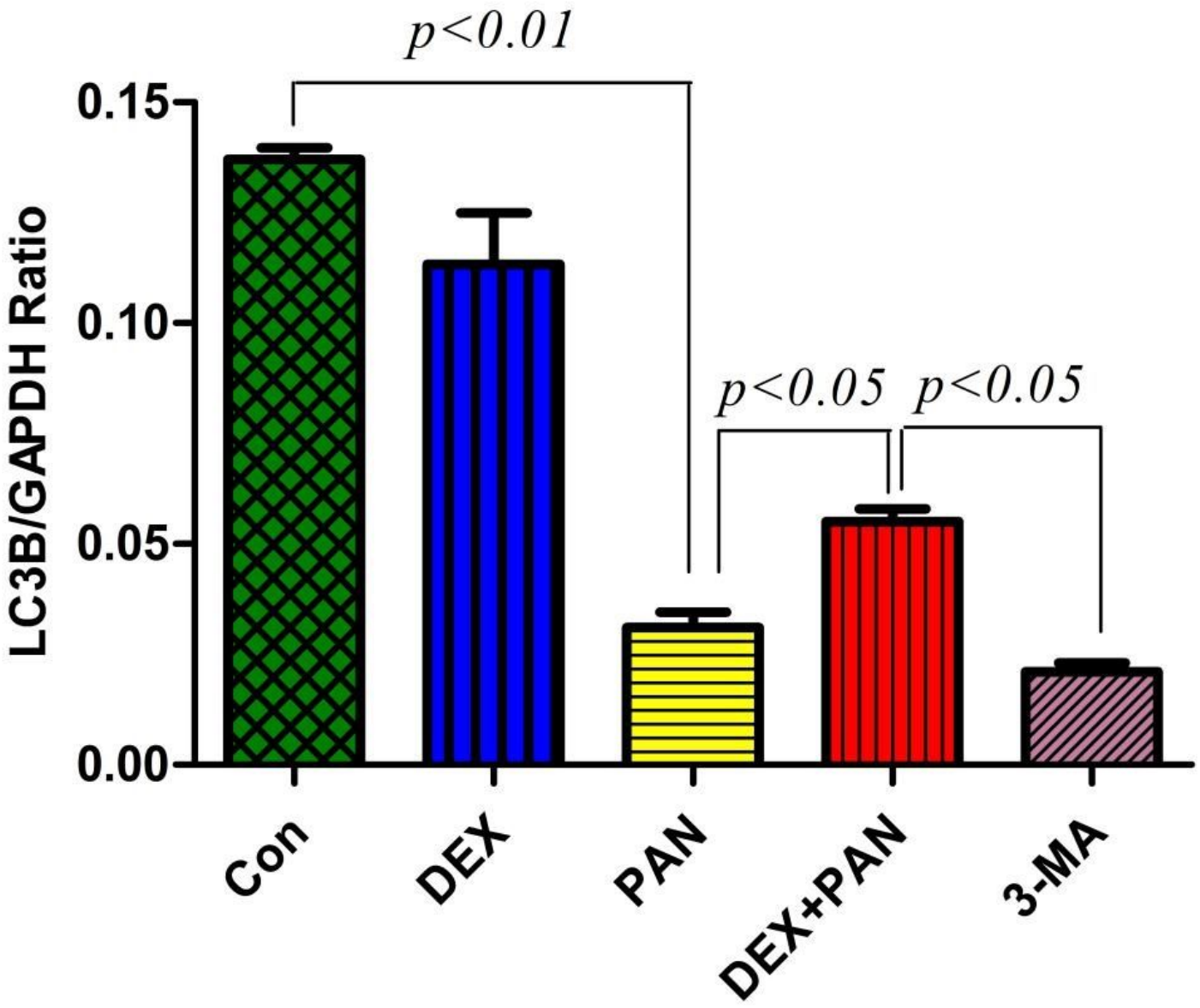

Figure 17

Changes in LC3B protein expression of podocytes in each group

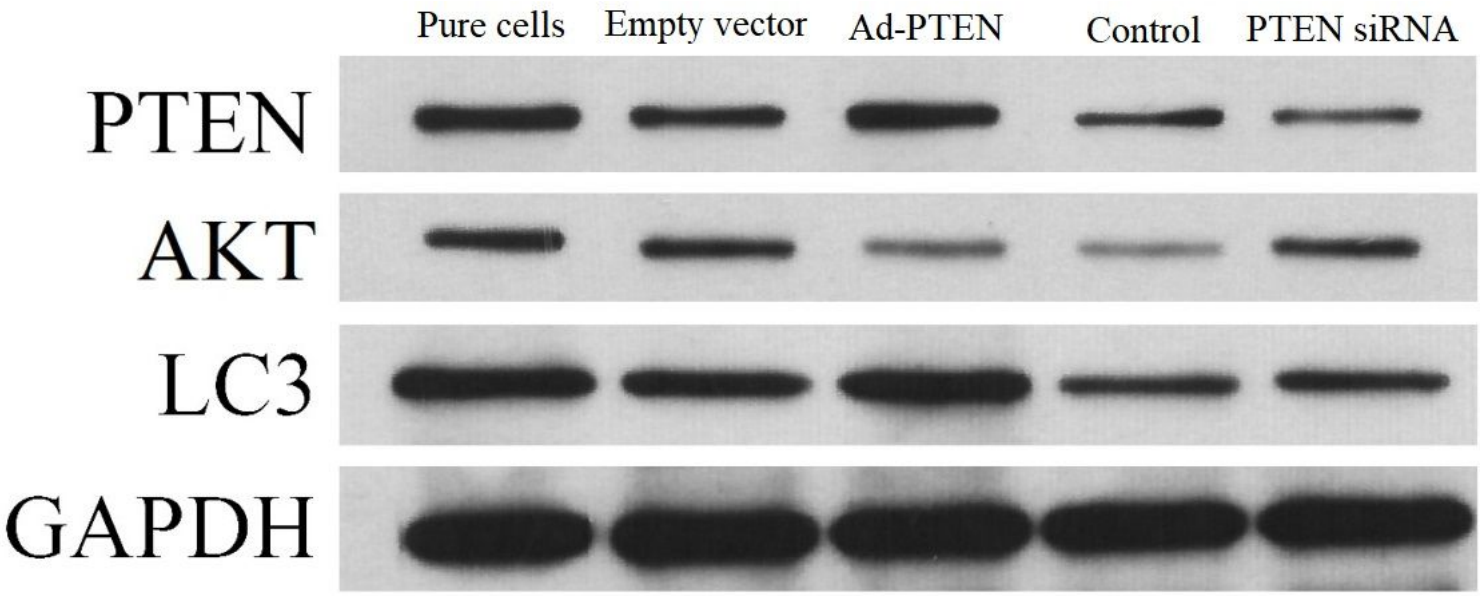

Figure 18

Western blot of related gene proteins after PTEN silenced and PTEN overexpression in each group of podocytes

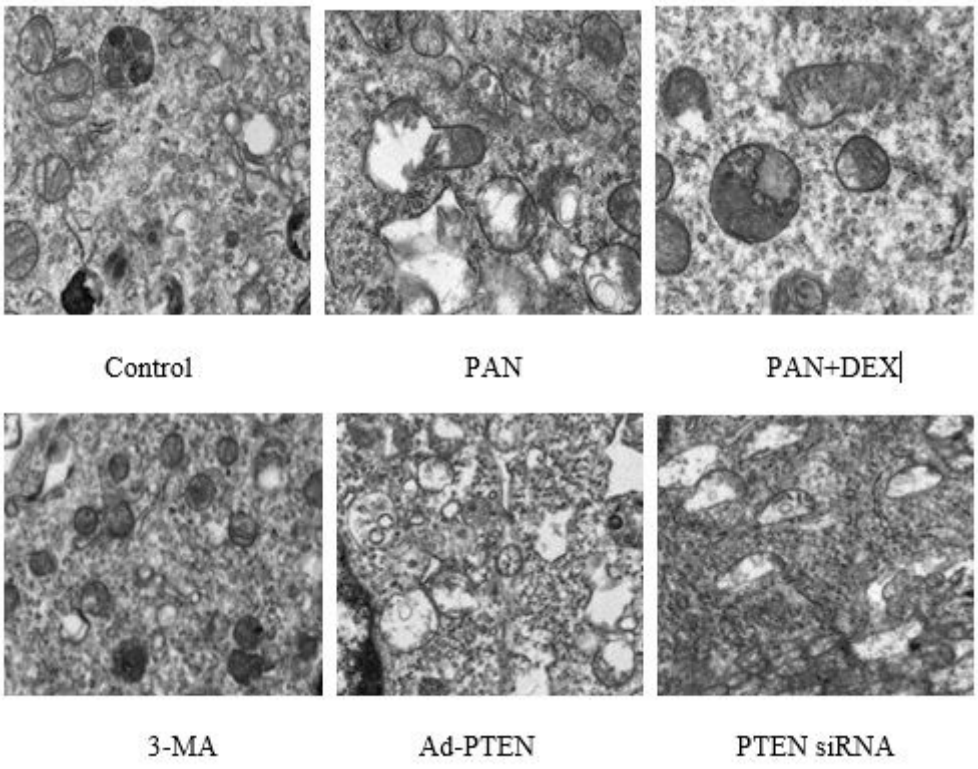

Figure 19

Changes in podocyte autophagosome expression under transmission electron microscope