**Abstract**

**Introduction:** Blood glucose fluctuation is an important factor for the development of diabetic complications. Glucose fluctuation aggravated the renal injury in diabetic nephropathy. In the present study, our aim was to investigate the effects of blood glucose fluctuation on the glomerular mesangial cells and its related mechanism.

**Material and methods:** Mesangial cells were divided into four groups: the normal glucose group (NG) cells were incubated in normal glucose conditions (5.6 mmol/l); the high glucose group (HG) cells were treated with 25 mmol/l; the glucose fluctuation (FG) group received 5.6 mmol/l and 25 mmol/l glucose repeated 3 times; the mannitol group (MG) received 5.6 mmol/l glucose plus 24.4 mmol/l mannitol as a control. Cell viability and apoptosis were detected, reactive oxygen species (ROS) level, superoxide dismutase (SOD) activity and malonaldehyde (MDA) levels were measured. Phosphorylated ser/thr protein kinase (P-AKT, phosphor-Ser473), phosphorylated glycogen synthase kinase-3 \( \beta \) (P-GSK-3\( \beta \), phosphor-Ser9) and cleaved cysteinyl aspartate-specific proteinase-3 (cleaved caspase-3) levels were assessed using western blot.

**Results:** Data suggested that mesangial cells in the FG group show higher cell viability in 12 h, and lower cell viability from 48 h. The FG group showed cell apoptosis accompanied by a significant MDA level increase and SOD activity decrease in 48 h. More importantly, glucose fluctuation could aggravate oxidative stress in glomerular mesangial cells. Furthermore, the P-AKT level was lower, and increased P-GSK-3\( \beta \) and cleaved caspase-3 levels were higher in the FG group than in the HG group.

**Conclusions:** Glucose fluctuation aggravates mesangial cell apoptosis, which may be partly induced by activating oxidative stress and inhibiting the AKT signaling pathway.

**Key words:** glucose fluctuation, glomerular mesangial cells, apoptosis, P-AKT.

**Introduction**

Diabetic nephropathy is one of the most common diabetic microvascular complications and a leading cause of end-stage renal disease [1]. There are many factors contributing to the development of diabetic ne-
Glucose fluctuation increased mesangial cell apoptosis related to AKT signal pathway

Rat glomerular mesangial cells were bought from Boster (Wuhan, China). We selected the 5th–10th generation of mesangial cells in the present study. The cells were cultured in DMEM (5.6 mmol/l glucose) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin), at 37°C in 5% CO₂ for 24 h. The cultured cells were randomly divided into four groups: the normal glucose group (NG, 5.6 mmol/l glucose), the high glucose group (HG, 25 mmol/l glucose, and then normal glucose conditions incubated for the night), the glucose fluctuation group (FG, alternated 5.6 mmol/l glucose and 25 mmol/l glucose every 3 h, repeated 3 times, and then normal glucose conditions incubated for the night) and the mannitol group (MG, 5.6 mM glucose plus 24.4 mM mannitol as an osmotic pressure control).

Effects of fluctuation of blood glucose on mesangial cell viability

Cell viability was assayed by the CCK8 kit according to the manufacturer's protocol [20]. Briefly, mesangial cells were seeded onto 96-well plates and every well contained 5 × 10³ cells. Then they were grown to 90% confluency. The cells were cultured for 12, 24, 48 and 72 h. Then, we added 10 μl of CCK8 reagent into every well. After 1–4 h of incubation at 37°C, cell viability was assessed by scanning with a microplate reader at 450 nm. There were at least 6 duplicate wells in each group.

Mesangial cell apoptosis using flow cytometry

Mesangial cell apoptosis can be detected using flow cytometry analysis [20]. In brief, the mesangial cells were seeded onto 96-well plates at 37°C in a humidified incubator containing 5% CO₂ for 48 h. The cells were washed once in phosphate-buffered saline (PBS), collected by trypsin digestion with 0.25% trypsin (without EDTA), washed twice in phosphate-buffered saline (PBS), added to each well. Then, the cells were incubated in the dark for 15 min at 37°C and apoptosis was immediately analyzed.

Effects of fluctuating blood glucose on ROS levels in mesangial cells

The level of intracellular ROS was determined by flow cytometry using the peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as previously described [21]. In brief, mesangial cells were seeded in a 96-well culture plates and incubated with 10 μM DCFH-DA for 30 min at 37°C. Then, cells were harvested

Material and methods

Material

CCK8 was purchased from Dojindo (Kumamoto, Japan). Malondialdehyde (MDA) and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) to evaluate oxidative stress. 2′,7′-dichlorofluorescin diacetate (DCFH-DA), AKT, GSK3, caspase-3 and actin antibodies were obtained from Beyotime Institute of Biotechnology (Shanghai, China). P-AKT and P-GSK3β antibodies were purchased from Cell Signaling Technology, Inc (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM/High Glucose) was obtained from Thermo Fisher Scientific (Shanghai, China) as well as DMEM/Low Glucose.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Arch Med Sci 3, May / 2019 731
and washed three times with PBS, re-suspended in ice-cold PBS and placed on ice in a dark environment, and washed twice with DMEM deprived of serum to fully remove unbound ROS from the intracellular DCFH-DA. The fluorescent signal was recorded using fluorescence microscopy (488 nm filter; OLYMPUS IX-73, Japan).

**Effects of fluctuating blood glucose on MDA level and SOD activity in mesangial cells**

The MDA level and SOD activity were analyzed using specific reagents according to the protocols provided by the manufacturer. Briefly, mesangial cells were seeded into 96-well plates and every well contained $5 \times 10^3$ cells. Cultured cells were incubated for 48 h. Then, we collected the supernatant to determine the MDA level and SOD activity.

**Western blotting**

Glomerular mesangial cells were plated in 100 mm dishes for western blot analysis. After 48 h cells were lysed with cell lysis solution. Lysate was centrifuged at 14 000 g for 30 min at 4°C, and the supernatant was stored at −80°C. The protein was loaded per lane, separated by SDS–PAGE gel and transferred onto an NC membrane and incubated with the primary antibody (1 : 500). After washing, the membranes were incubated for 2 h with secondary antibody (1 : 200). The immunoblots were scanned using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

**Statistical analysis**

At least six samples were selected from each group for experimental statistical analysis. Image J analysis software was used to analyze the western blotting results. Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test using SPSS 13.0 computer software. $P < 0.05$ was considered statistically significant.

**Results**

**Mesangial cell viability under glucose fluctuation at different time points**

In order to elucidate the change of glomerular mesangial cells under glucose fluctuation at different time points, we examined glomerular mesangial cell viability using the CCK-8 method. As shown in Figure 1, mesangial cell viability in both the HG and FG groups was increased significantly in comparison with the NG group at 12 h, but there was no significant difference between the HG group and the FG group. However, mesangial cell viability was significantly decreased in the FG group compared with the NG and HG groups at 24 h, while no significant difference was found between the NF group and the FG group. Moreover, mesangial cell viability was decreased in the HG and FG groups at 48 h and 72 h; especially, the FG group had lower cell viability than the NG group. In general, these results indicated that fluctuating blood glucose obviously reduced mesangial cell viability from 48 h when compared with the HG group.

**Fluctuating blood glucose increased glomerular mesangial cell apoptosis**

Next, we detected glomerular mesangial cell apoptosis using flow cytometry. As shown in Figure 2, glomerular mesangial cell apoptosis in the FG group was significantly increased compared with either the NG group or the HG group, even though in the HG group it was higher than in the NG group. There was no significant difference between the NG group and the MG group. In general, these results indicated that fluctuating blood glucose obviously reduced mesangial cell viability from 48 h when compared with the HG group.

**Fluctuating blood glucose aggravated oxidative stress in glomerular mesangial cells**

It is reported that oxidative stress plays an important role in glomerular mesangial cell injury in high sugar conditions [22]. In addition, fluctuating blood glucose aggravated renal injury by increasing the generation of oxidative stress [23]. As shown in Figure 3, the level of intracellular ROS in the HG
Glucose fluctuation increased mesangial cell apoptosis related to AKT signal pathway

The effect of glucose fluctuation on P-AKT, P-GSK-3β and cleaved caspase-3 expression in glomerular mesangial cells

Previous studies indicated that high glucose accelerated the glomerular mesangial cell death via the AKT signaling pathway [24, 25]. To explore the effect of glucose fluctuation on the AKT pathway in glomerular mesangial cells, we examined P-AKT, P-GSK-3β and cleaved caspase-3 expression levels in glomerular mesangial cells exposed to glucose
Figure 3. Effects of glucose fluctuation on intracellular ROS level, MDA level and SOD activity on mesangial cells.

Data are shown as mean ± SD. *p < 0.05 vs. the NG group, †p < 0.05 vs. the HG group and ‡p < 0.05 vs. the FG group by one-way ANOVA.
fluctuation. As shown in Figure 4, there was no significant difference in the total AKT expression among the four groups. The level of P-AKT was lower in the HG group compared with that in the NG group, and the P-AKT level was lower in the FG group than that in the HG group. Moreover, high glucose could increase P-GSK-3β and caspase-3 activation in glomerular mesangial cells, but glucose fluctuation increase of P-GSK-3β and cleaved caspase-3 levels was more remarkable than stabilized glucose. These results indicated that glucose fluctuation accelerated glomerular mesangial cell apoptosis, which was linked to inhibiting decreased phosphorylation of AKT and increasing P-GSK-3β and cleaved caspase-3 levels.

Discussion

A previous in vitro study showed that glomerular mesangial cell apoptosis is a response to high glucose stimulation [26]. Furthermore, in an in vivo study, glomerular cell apoptosis could be found in the late phase of nephropathy [27]. In the present study, we chose a different time point to observe the mesangial cell vitality using the CCK8 kit, and we found that sugar fluctuation reduces mesangial cell vitality from 24 h, but has a significant reduction when compared to continuous high sugar stimulation from 48 h. Furthermore, we found that mannitol inhibited cell vitality at 72 h, so in the present study, we assessed the effect of glucose fluctuation on the mesangial cells and its related mechanism at 48 h. However, these results are different from a previous report [13]; we consider that this discrepancy may due to a different experimental protocol.

Overwhelming evidence indicated that high sugar induced cell apoptosis by increasing the generation of intracellular ROS in glomerular mesangial cells [5, 28]. Our previous study showed that fluctuating blood glucose increased intracellular ROS generation more than continuous high sugar; the SOD activity was obviously decreased and the MDA levels were increased in kidney subject to hyperglycemia in diabetic rats [23]. Furthermore, high sugar decreased the SOD activity and increased the MDA levels at 48 h compared with the normal glucose level, and blood glucose fluctuation markedly decreased their SOD activity and increased their MDA levels at 48 h. In addition, blood glucose fluctuation augmented apoptosis in pancreatic β cells through oxidative stress [20]. It is worth mentioning that our data suggested that glucose fluctuation accelerated cell apoptosis by oxidative stress-dependent mechanism, and this result was consist with the present studies [29–32]. Hence, partly, we concluded that glucose fluctuation increased glomerular mesangial cell apoptosis via an oxidative stress mechanism.

Figure 4. Effects of glucose fluctuation on P-AKT, P-GSK-3β and cleaved caspase-3 levels. A – Representative bands of P-AKT and AKT. B – Representative bands of P-GSK-3β and GSK-3β. C – Representative bands of caspase-3. D – Analysis for optical density (OD) value of P-AKT, AKT, P-GSK-3β, GSK-3β and cleaved caspase-3 protein expression

The data are shown as mean ± SD and are expressed as fold difference vs. the NG group, *p < 0.05 vs. the HG group and **p < 0.05 vs. the FG group by one-way ANOVA.
The study confirmed that blood glucose fluctuation obviously decreased P-AKT action and increased the level of P-GSK-3β and cleaved caspase-3 in glomerular mesangial cells compared to high sugar conditions. High glucose evokes an intrinsic pro-apoptotic signaling pathway in glomerular mesangial cells [6]. AKT activation was combined with protein, GSK-3β, which inhibits its kinase activity [33]. Studies have shown that P-GSK-3β was activated when there was inhibition of AKT phosphorylation in mesangial cells [34]. Especially, high sugar could decrease AKT activation and increased its downstream apoptosis related factors, such as GSK-3β and cleaved caspase-3, resulting in mesangial mesangial cell apoptosis [19]. In this study, glucose fluctuation could obviously decrease the P-AKT level and increased the expression of P-GSK-3β and cleaved caspase-3 in glomerular mesangial cells compared to high sugar conditions. On the basis of these results and previous work, mesangial cell apoptosis may be partly involved in inhibition of the AKT signaling pathway. These result demonstrate that the AKT signaling pathway may exert an important effect on glomerular mesangial cell apoptosis induced by unstable glucose stimulation.

In conclusion, blood glucose fluctuation increased mesangial cell apoptosis. This result may be partly induced by activating oxidative stress and inhibiting the AKT signaling pathway. These data provided important empirical evidence for us to understand the harm of blood glucose fluctuation.

Acknowledgments

Changjiang Ying, Shanshan Wang and Yan Lu contributed equally to this work.

This study was funded by the Department of Science & Technology, Xuzhou, Jiangsu, China (Grant number: KC17194).

Changjiang Ying has received research grants from the Department of Science & Technology, Xuzhou, Jiangsu, China.

Conflict of interest

The authors declare no conflict of interest.

References

1. Kahraman C, Kahraman NK, Aras B, et al. The relationship between neutrophil-to-lymphocyte ratio and albuminuria in type 2 diabetic patients: a pilot study. Arch Med Sci 2016; 12: 571-5.

2. Harris RD, Steffes MW, Bilous RW, et al. Global glomerular sclerosis and glomerular arteriolar hyalinosis in insulin dependent diabetes. Kidney Int 1991; 40: 107-14.

3. Kikkawa R, Koya D, Haneda M. Progression of diabetic nephropathy. Am J Kidney Dis 2003; 41 (3 Suppl 1): S19-21.

4. Nerlich AG, Schleicher ED, Wiest I, Specks U, Timpf R. Immunohistochemical localization of collagen VI in diabetic glomeruli. Kidney Int 1994; 45: 1648-56.

5. Kang BR, Frencher S, Reddy V, et al. High glucose promotes mesangial cell apoptosis by oxidant-dependent mechanism. Am J Physiol Renal Physiol Renal Physiol 2003; 284: F455-66.

6. Mishra R, Emancipator SN, Kern T, Simonson MS. High glucose evokes an intrinsic proapoptotic signaling pathway in mesangial cells. Kidney Int 2005; 67: 82-93.

7. Yamagishi S, Inagaki Y, Okamots T, et al. Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. J Biol Chem 2002; 277: 20309-15.

8. Gassler N, Elger M, Kranzlin B, et al. Podocyte injury underlies the progression of focal segmental glomerulosclerosis in the f/fα Zucker rat. Kidney Int 2001; 60: 106-16.

9. Lemley KV, Abdullah I, Myers BD, et al. Evolution of incipient nephropathy in type 2 diabetes mellitus. Kidney Int 2000; 58: 1228-37.

10. Nalysnyk L, Hernandez-Medina M, Krishnarajah G. Glycaemic variability and complications in patients with diabetes mellitus: evidence from a systematic review of the literature. Diabetes Obes Metab 2010; 12: 288-98.

11. Wang C, Song J, Ma Z, et al. Fluctuation between fasting and 2-H postload glucose state is associated with chronic kidney disease in previously diagnosed type 2 diabetes patients with HbA1c >/= 7%. PloS One 2014; 9: e102941.

12. Liu TS, Pei YH, Peng YF et al. Oscillating high glucose enhances oxidative stress and apoptosis in human coronary artery endothelial cells. J Endocrinol Invest 2014; 37: 645-51.

13. Sun J, Xu Y, Deng H, et al. Involvement of osteopontin upregulation on mesangial cells growth and collagen synthesis induced by intermittent high glucose. J Cell Biochem 2010; 109: 1210-21.

14. Rane MI, Song Y, Jin S, et al. Interplay between Akt and p38 MAPK pathways in the regulation of renal tubular cell apoptosis associated with diabetic nephropathy. Am J Physiol Renal Physiol 2010; 298: F49-61.

15. Ribback S, Cigliano A, Kroeger N, et al. PI3K/AKT/mTOR pathway plays a major pathogenetic role in glycogen accumulation and tumor development in renal distal tubules of rats and men. Oncotarget 2015; 6: 13036-48.

16. Maurer U, Preiss F, Brauns-Schubert R et al. GSK-3β at the crossroads of cell death and survival. J Cell Sci 2014; 127: 1369-78.

17. Paeng J, Chang JH, Lee SH, et al. Enhanced glycogen synthase kinase-3beta activity mediates podocyte apoptosis under diabetic conditions. Apoptosis 2014; 19: 1678-90.

18. Pap M, Cooper GM. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem 1998; 273: 19929-32.

19. Lin CL, Wang JY, Huang YT, et al. Wnt/beta-catenin signaling modulates survival of high glucose-stressed mesangial cells. Am Soc Nephrol 2006; 17: 2812-20.

20. Zhang Z, Li J, Yang L, et al. The cytotoxic role of intermittent high glucose on apoptosis and cell viability in pancreatic beta cells. J Diabetes Res 2014; 2014: 712781.

21. Zhang L, Pang S, Deng B, et al. High glucose induces renal mesangial cell proliferation and fibronectin expression through JNK/NF-kappaB/NADPH oxidase/ROS pathway, which is inhibited by resveratrol. Int J Biochem Cell Biol 2012; 44: 629-38.
22. Jiang T, Huang Z, Lin Y, et al. The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. Diabetes 2010; 59: 850-60.
23. Ying C, Zhou X, Chang Z, et al. Blood glucose fluctuation accelerates renal injury involved to inhibit the AKT signaling pathway in diabetic rats. Endocrine 2016; 53: 81-96.
24. Hiromura K, Monkawa T, Petermann AT, et al. Insulin is a potent survival factor in mesangial cells: role of the PI3-kinase/Akt pathway. Kidney Int 2002; 61: 1312-21.
25. Rovin BH, Wilmer WA, Lu L, et al. 15-deoxydelta-12,14-prostaglandin J2 regulates mesangial cell proliferation and death. Kidney Int 2002; 61: 1293-302.
26. Tsuboi N, Utsunomiya Y, Kawamura T, et al. Shedding of growth-suppressive gangliosides from glomerular mesangial cells undergoing apoptosis. Kidney Int 2003; 63: 936-46.
27. Franczyk-Skóra B, Gluba A, Olszewski R, et al. Heart function disturbances in chronic kidney disease – echocardiographic indices. Arch Med Sci 2014; 10: 1109-16.
28. Lee HB, Yu MR, Yang Y, et al. Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. J Am Soc Nephrol 2003; 14 (8 Suppl 3): S241-5.
29. Abdelzaher LA, Imaizumi T, Suzuki T, et al. Astaxanthin alleviates oxidative stress insults-related derangements in human vascular endothelial cells exposed to glucose fluctuations. Life Sci 2016; 150: 24-31.
30. Li W, Liu X, Yanoff M, et al. Cultured retinal capillary pericytes die by apoptosis after an abrupt fluctuation from high to low glucose levels: a comparative study with retinal capillary endothelial cells. Diabetologia 1996; 39: 537-47.
31. Saito S, Teshima Y, Fukui A, et al. Glucose fluctuations increase the incidence of atrial fibrillation in diabetic rats. Cardiovasc Res 2014; 104: 5-14.
32. Yin X, Zheng F, Pan Q, et al. Glucose fluctuation increased hepatocyte apoptosis under lipotoxicity and the involvement of mitochondrial permeability transition opening. J Mol Endocrinol 2015; 55: 169-81.
33. Jiang Y, Cheng DW, Levi E, Singh LP. IGF-1 increases laminin, cyclin D1, and p21Cip1 expression in glomerular mesangial cells: an investigation of the intracellular signaling pathway and cell-cycle progression. J Cell Biochem 2006; 98: 208-20.
34. Singh LP, Jiang Y, Cheng DW. Proteomic identification of 14-3-3zeta as an adapter for IGF-1 and Akt/GSK-3beta signaling and survival of renal mesangial cells. Int J Biol Sci 2007; 3: 27-39.