The Effect of Advanced Glycation End Products on Primary Culture of Neonatal Rat Kidney Cells

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Abstract The advanced glycation end products (AGEs) may play an important adverse role in process of diabetic nephropathy; however, the pathological mechanism can not be explained. This study aimed to investigate the effect of AGEs on primary cultured neonatal rat kidney cells and discussed the functional mechanism. The kidney cells were isolated from 3-day-old rats for *in vitro* primary culture, and the 4–6th generations of the cells culture were treated with AGEs at different concentrations (0, 1.2, 2.5, 5, 10, 20 mg/ml) and different times (6, 12, 18, 24 h). Cell proliferation was determined by methyl thiazolyl tetrazolium (MTT) method, and the enzyme-linked assay kit evaluated the extracellular concentration alteration of lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAG), and the intracellular concentration alteration of reduced glutathione (GSH) and superoxide dismutase (SOD) influenced by AGEs. The results suggest that, along with higher concentration and longer action time of AGEs, the cell livability, the intracellular concentration of GSH, and the SOD activity are gradually decreased, however, the concentrations of LDH and NAG in culture solution are significantly increased ($P<0.001$), compared with the control group. There is a significant concentration-effect relationship between the concentration and action time of AGEs. Our findings support that AGEs can significantly damage primary cultivated kidney cells, Moreover, the effect of AGEs on kidney cell is dose and time-dependent. Therefore, we conclude that kidney cells are sensitive to AGEs and the changes of cell permeability and antioxidant capacity induced by AGEs might be linked to the pathogenesis of diabetic nephropathy.

Key words advanced glycation end products; aeonatal rat kidney; primary cell culture; diabetic nephropathy

With the increasing quantity of the patients who suffer from diabetes year after year, diabetic complications, such as atherosclerosis, cardiac dysfunction, retinopathy and nephropathy, are more and more remarkable, which has become the principal threat to the health and lives of diabetes patients. High blood glucose is a major pathological symptom of diabetes, which not only affects the normal metabolisms of cells and tissues directly, but also damages their structure and function indirectly by the non-enzymatic reactions to form advanced glycation end products (AGEs) with various intrinsic proteins, which were described to play an important role in diabetes pathogenesis [1]. Thus AGE-induced damages to cells and tissues as well as their pathogenesis have become an important focus of diabetic study. Kidney is one of the main target organs affected by AGEs, and present several studies have been carried out to identify AGE-related kidney injury, but its pathogenesis is still unclear[2,3]. Similar to kidney cells *in vivo* in morphology and function, the primary cultured neonatal rat kidney cells are the ideal material for *in vitro* study of AGE-related injury. The goal of this study was to investigate the effect of AGEs on cell growth and cell biochemical exponents by treating primary cultured neonatal rat kidney cells with different concentrations of AGEs at different times. In addition, we also presumed the mechanism of the AGE-induced damages to kidney cells, and present some new data for the pathogenesis of diabetic nephropathy.

1 Materials and Methods

1.1 Materials

Dulbcco's modified Eagle's medium and trypsinase

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were from Gibco (NY, USA). Bovine calf serum was obtained from Dingguo Biotechnology (Beijing, China). Trititaed thymidine was from Shisheng Cell Bio-technology (Shanghai, China). The followed kits were purchased from Nanjing Jiancheng Bio-engineering Institute (Nanjing, China): superoxide dismutase (SOD), lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), reduced glutathione (GSH) kit.

1.2 Preparation of AGEs

After a high-pressure sterilization process, D-Hank's buffer (pH 7.2) was used to prepare AGEs. Briefly, BSA (50 mg/ml) was sterilized using 0.22 μm filter membranes, and then incubated with 0.1 mol/L glucose in D-Hank's buffer (pH 7.2) containing 800 U/ml of benzylpenicillin and streptomycin at 37 ℃ and in the dark. Then incorporated sugars were removed by dialysis against phosphate-buffered saline. The AGEs incubated for 16 weeks were used in our study. The formation of AGEs was confirmed by a change in color to brown and positive fluorescence [4]. Control BSA was exposed to 37 ℃ for the same time interval and in the same buffer without glucose.

1.3 Cell culture

Three-day-old neonatal rat's kidneys were isolated for the primary cultures. The kidney cell suspensions were incubated in Dulbecco's modified Eagle medium (DMEM) containing 20% bovine calf serum, aqueous benzylpenicillin (800 U/ml), and streptomycin (800 U/ml) under a humidified atmosphere of 5% CO_2 at 37 ℃. Cells were passaged once every two days. Cells used in this experiment were at 4 th to 6 th passage. which were loaded in 24-well and 96-well plates in the concentration of 1×10^5 cells/ml. After the cells attached to the plate's surface, they were treated with different concentrations of AGEs.

1.4 Experimental groups

Cells were loaded in 24-well and 96-well plates in the concentration of 1×10^5 cells/ml. After the cells attached to the plate's surface, they were treated with different concentrations of AGEs. Six groups of AGEs, whose concentrations are 0, 1.25, 2.5, 5, 10, and 20 mg/ml respectively, were used to treat kidney cells for 6, 12, 18, and 24 h, then both the cells and cell culture solution were collected.

1.5 MTT cell viability assay

After adding 10 μl of MTT labeling reagent to each well, plates were incubated for 4 h. Solubilization solution (100 μl) containing 10% sodium dodecyl sulfate (SDS) in 0.01 mol/L HCl was added to each well and the wells were incubated for another 24 h. The absorbance was then measured with a spectrophotometer (Shanghai Anting Scientific instruments Co.Ltd, Shanghai, China) at a test wavelength of 550 nm. The optical density (OD) was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength.

1.6 Biochemical measurements

The contents of LDH, NAG, GSH and SOD in cell or medium were measured using the colorimetrical assay kits according to their respective kit manuals. The formulation for calculating the leakage rate of LDH:

Leakage rate of LDH=extracellular LDH activity/(extracellular LDH activity + intracellular LDH activity) ×100%

1.7 Statistical analysis

Data was analyzed using SPSS 11.5 software. Average values and standard deviation were calculated. The experimental groups are compared using *t* test, and *P* value<0.05 was considered to be statistically significant.

2 Results

2.1 Effect of AGEs on neonate rat kidney cell proliferation

Table 1 showed that AGEs inhibited significantly the proliferation of the neonate rat kidney cells when compared with the control (0 mg/ml AGEs). Moreover, with the extension of time and increase of AGEs concentration, the inhibition of cell proliferation became more obvious. The inhibition of cell proliferation by AGEs was in a concentration-dependent manner.

2.2 Effect of AGEs on the LDH leakage rate of neonate rat kidney cells

It is well-known that the higher LDH leakage rate in the cell culture solution indicates the higher membrane permeability of kidney cells and the more serious cell damage. Our results showed that with the extension

Table 1 Effect of AGEs at various concentrations and time points on the proliferation of the neonate rat renal cells ($\bar{x}\pm s$, n=8)

AGEs (mg/ml)	MTT (OD) 6 h	MTT (OD) 12 h	MTT (OD) 18 h	MTT (OD) 24 h
0	1.130±0.003	1.239±0.004	1.384±0.060	1.511±0.038
1.25	1.127±0.003	1.234±0.004	1.239±0.005**	1.248±0.048**
2.5	1.125±0.004	1.202±0.021*	1.185±0.042**	1.059±0.047**
5	1.118±0.007*	1.174±0.012**	1.164±0.013**	0.959±0.027**
10	1.105±0.008**	1.152±0.011**	1.083±0.032**	0.839±0.013**
20	1.098±0.018**	1.107±0.005**	0.935±0.068**	0.767±0.032**

Compared with the control group, *P<0.01, and **P<0.001.

Table 2 Effect of AGEs at various concentrations and time points on the LDH leakage rate of neonate rat renal cells ($\bar{x}\pm s$, n=8)

AGEs (mg/ml)	LDH (%) 6 h	LDH (%) 12 h	LDH (%) 18 h	LDH (%) 24 h
0	18.22±0.11	19.35±1.44	19.68±0.61	20.37±0.71
1.25	18.43±0.25	19.86±0.88	20.99±0.80	22.92±0.82**
2.5	18.70±0.30*	20.29±0.65*	22.83±0.74**	24.70±0.84**
5	19.14±0.54*	21.22±0.30**	23.67±0.71**	27.21±1.02**
10	19.30±0.68**	22.06±0.80**	25.66±1.32**	30.04±1.19**
20	19.84±0.72**	24.27±0.74**	28.01±1.50**	34.25±1.73**

Compared with the control group, *P<0.01, and **P<0.001.

Table 3 Effect of AGEs at various concentrations and time points on the NAG content of neonate rat renal cells ($\bar{x}\pm s$, n=8)

AGEs (mg/ml)	NAG (U/L) 6 h	NAG(U/L) 12 h	NAG (U/L) 18 h	NAG (U/L) 24 h
0	7.20±0.47	7.39±0.51	7.88±0.56	8.26±0.41
1.25	8.05±0.42	8.68±0.75*	9.94±0.53**	10.76±1.15**
2.5	9.15±0.90*	10.42±0.94**	13.34±0.50**	14.89±1.25**
5	11.02±0.50**	13.78±1.02**	16.94±1.03**	17.27±1.12**
10	12.35±1.02**	15.62±0.69**	19.75±0.73**	20.36±0.70**
20	14.23±1.13**	17.89±0.84**	21.88±0.74**	24.94±1.26**

Compared with the control group, *P<0.01, and **P<0.001.

of action time and the increase of AGEs concentration, the cell LDH leakage rate increase gradually in each group treated with AGEs compared with the control group, and the effects was markedly concentration-dependent (P<0.001, table 2).

2.3 Effect of AGEs on the NAG content of neonate rat kidney cells

NAG is the most sensitive marker for testing renal cell damage. Intracellular NAG will greatly leak out into the culture solution if the kidney cells are damaged. Therefore, higher NAG content in the culture solution, more seriously the kidney cell injury will occur. Our results showed that with the extension of action time and the increase of AGEs concentration, the NAG content increase gradually in each experimental group. There was a significant difference between the groups treated with AGEs and the control group (P<0.001, table 3).

2.4 Effect of AGEs on the GSH content of neonate rat kidney cells

The results of GSH content were shown in table 4. All the AGEs at the concentrations of 0, 1.25, 2.5, 5, 10, and 20 mg/ml lowered the GSH content of neonate rat kidney cells. The GSH content decreased markedly with the increase of AGEs concentration and the extension of action time, compared with the control group (P<0.001, table 4).

2.5 Effect of AGEs on the SOD activity of neonate ratkidney cells

Table 5 showed that all the AGEs of different concentrations lowered the SOD activity of neonate rat kidney cells. There was a significant negative correlation between SOD activity and the concentration and action time of AGEs as compared with the control group (P <0.001, table 5).

Table 4 Effect of AGEs at various concentrations and time points on the GSH content of neonate rat renal cells ($\bar{x}\pm s$, n=8)

AGEs (mg/ml)	GSH (μmol/10^5 cells) 6 h	GSH (μmol/10^5 cells) 12 h	GSH (μmol/10^5 cells) 18 h	GSH (μmol/10^5 cells) 24 h
0	1.558±0.074	1.535±0.068	1.513±0.048	1.486±0.052
1.25	1.506±0.094	1.471±0.052*	1.305±0.082**	1.263±0.066**
2.5	1.433±0.069	1.395±0.063**	1.209±0.053**	0.973±0.088**
5	1.415±0.082*	1.250±0.061**	1.164±0.013**	0.838±0.099**
10	1.320±0.056**	0.113±0.049**	0.993±0.087**	0.703±0.093**
20	1.271±0.060**	0.981±0.087**	0.875±0.071**	0.595±0.098**

Compared with the control group, *P<0.01, and **P<0.001.

Table 5 Effect of AGEs at various concentrations and time points on the SOD activity of neonate rat renal cells ($\bar{x}\pm s$, n=8)

AGEs (mg/ml)	SOD (U/mg) 6 h	SOD (U/mg) 12 h	SOD (U/mg) 18 h	SOD (U/mg) 24 h
0	11.64±0.59	11.25±0.56	11.15±0.55	10.97±0.64
1.25	11.07±0.47	10.15±0.66*	10.05±0.57*	9.58±0.91**
2.5	10.52±0.57*	9.74±0.73**	9.44±0.57**	9.02±0.54**
5	9.71±0.44**	9.05±0.82**	8.68±0.74**	8.03±0.65**
10	9.36±0.69**	8.80±0.81**	8.01±0.92**	7.39±0.83**
20	8.45±0.56**	8.52±0.75**	7.69±0.72**	6.51±0.45**

Compared with the control group, *P<0.01, and **P<0.001.

3 Discussion

The clinical researches have shown that diabetes can cause the pathological changes most if not of all organs and tissues, including heart, brain, blood vessels, eyes, nerves, kidney, and so on, which harm human health and cause death or deformity of patients[5]. To explain the complicated pathogenesis of diabetic complications, there appear several hypotheses, including non-enzymatic glycosylation theory, polyol pathway theory, diacylglycerol and protein kinase C theory, oxidative stress theory, and carbonyl stress theory. Therefore, the experimental demonstration to these hypotheses is very urgent. In this present study, we provided basic experimental data for the pathogenesis research of diabetic nephropathy and presumed the possible mechanism of kidney cell damages induced by AGEs. Moreover, this study was the first to show the damage of AGEs on primary cultured kidney cells.

Because cells *in vitro* were away from the effect of body fluid and nervous system, they were affected by less interference factors, more easily targeted and observed, and more feasibly measured for morphological and physiological changed and maintained in the constant experiment environment. Thereby we selected the primary cultured kidney cells as experimental materials; moreover they were very ideal for the research of diabetic nephropathy symptoms at the cellular level, because they share both the biological activity and characteristics with the kidney cells *in vivo*.

Our results showed that with the extension of action time and the increase of AGEs concentration, the cell viability decreased gradually, proving that AGEs affected the normal growth of kidney cells seriously. When the cells were damaged, the cytoplasmic membrane permeability increased, and some of the cellular enzymes leaked out, thus the measurements of these enzymes such as LDH, NAG, GSH, SOD can indicate the damage status of cells. In this study, obvious increases of LDH and NAG content in the culture medium showed that cell damage became more and more serious with the extension of action time and the increase of AGEs concentration. GSH content and SOD activity are two important markers of cells antioxidant capacity. As GSH content and SOD activity decrease, cell scavenging ability decreases, causing the increase of intracellular active oxygen content[6]. The active oxygen can destroy the cellular membrane structure and result in the increase of membrane permeability and the leakage of intracellular enzymes. We found intracellular GSH content and SOD activity decreased markedly after treated with the

AGEs in a concentration-dependent manner. It was presumed AGEs can catalyze lipid peroxidation and produce massive active oxygen, while the scavenging of intracellular active oxygen produced in the respiration process mainly depends on GSH and SOD. So, inhibition of cell activity and damage of kidney cells by AGEs may be due to the decreases of GSH content and SOD activity and the followed massive active oxygen injury to mitochondria and cellular membrane.

AGEs can also cause renal fibrosis by inducing over expression of various growth factors, and massive AGEs accumulation in the diabetic conditions can damage renal glomerulus. In our experiment, with the extension of action time and the increase of AGEs concentration, damage to the kidney cells became more and more serious. As a result, it will lead to the damage of renal tissue and the failure of renal function and eventually the formation diabetic nephropathy[7,8]. Our results indicated that the pathway by which AGEs damage the kidney cells may be via the decrease of renal cell antioxidant capacity and the changes of cellular membrane permeability, and this will be possibly one of the mechanisms, which lead to the diabetic nephropathy.

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糖基化终产物对原代培养乳鼠肾脏细胞损伤的影响

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摘要 糖基化终产物(AGEs)在糖尿病肾病的发生发展过程中起着重要的作用, 但目前其作用机制还不太清楚。通过体外乳鼠肾脏细胞的原代培养, 探讨AGEs对肾细胞的损伤作用及可能的作用机制。取出生3天的SD大鼠的乳鼠肾脏进行体外原代细胞培养, 并取传代到4~6代的细胞进行实验研究。分别用不同浓度的AGEs (0、1.2、2.5、5、10、20 mg/ml), 不同的作用时间(6、12、18、24 h)作用于体外培养的肾细胞, 用MTT法检测AGEs对肾细胞的增殖情况, 用酶试剂盒法检测AGEs对肾细胞培养液中乳酸脱氢酶(LDH)、β-N-乙酰氨基葡萄糖苷酶(NAG)的含量, 以及肾细胞内还原型谷胱甘肽(GSH)和超氧化物歧化酶(SOD)的含量。实验结果表明随着AGEs作用肾细胞时间的延长和浓度的增加, 细胞存活率、细胞内GSH含量和SOD活性均逐渐下降, 而细胞培养液中LDH和NAG的含量则逐渐升高, 与正常培养的对照组细胞相比差异非常显著($P<0.001$), 并且AGEs对细胞的作用与其浓度和作用时间呈显著的量效关系。实验结果说明AGEs对原代培养的肾细胞有明显的损伤作用, 并随着AGEs作用浓度的增加和作用时间的延长对肾细胞的损伤越来越严重, 实验结果也表明肾细胞对AGEs的作用很敏感, 其损伤细胞的途径和作用机制可能是由于改变了肾细胞膜的通透性和降低肾细胞抗氧化能力, 该实验研究也进一步提示了AGEs是导致糖尿病肾脏并发症发生的重要原因之一。

关键词 糖基化终产物; 乳鼠肾细胞; 原代细胞培养; 糖尿病肾病

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