The establishment of insulin resistance model

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Abstract. Establishment of insulin resistance model of HepG2 cells in vitro is aimed at studying puerarin responsible for the hypoglycemic effect. HepG2 cells induced by palmitate and glucosamine were used to establish the model of insulin resistance. Simultaneously, the effect of the two inducing methods was compared. From the test results of MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) and glucose uptake, it was significantly found insulin resistance of HepG2 cells when it was treated with palmitate under the concentration of 0.125mM. The hypoglycemic effect of puerarin testing by insulin resistance model of HepG2 cells indicated that HepG2 cells induced by low concentration of Palmitate can be used to produce stable and reliable insulin resistance. The establishment of insulin resistance model have a certain value for in vitro screening hypoglycaemic herbal medicine.

1 Introduction

Type 2 diabetes accounts for over 90% of diabetic cases, the occurrence of which are closely associated with the development of insulin resistance, followed by the progressive decline of pancreatic-β cell function that results in relative deficiency of insulin secretion [1]. Type 2 diabetes is primarily featured by insulin resistance ,which occurs when the insulin-responsive tissues, mainly skeletal muscle, adipose tissue, and the liver, cannot respond to insulin properly [2]. Insulin resistance plays a key role in the pathophysiology of metabolic syndrome which is a constellation of common metabolic disorders that is associated with diabetes mellitus [3-4]. Simultaneously, insulin resistance is also correlated with several other chronic diseases, such as dyslipidaemias, cardiovascular diseases, neurodegenerative disorders and cancers [5-6], also including obesity, atherosclerosis, hypertension, and hepatic steatosis [7-8]. HepG2 cell is a table hepatocellular carcinoma cell line derived from human fetal liver tumor cells, which is very similar to liver cells [9]. Insulin resistance is manifested by free fatty acid production, increased hepatic glucose production, and decreased skeletal muscle uptake of glucose. Therefore, this experiment was conducted using the HepG2 cells to establish hepatic insulin resistance model by Palmitate and for the preliminary screening of herbal medicine.

2 Materials and methods

2.1 Main materials

The Hepg2 cell line were bought from Procell of Wuhan city, DMEM(Dulbecco's modified Eagle's medium):(HyClone), MTT (Biotopped), DMSO (Sangon Biotech), palmitic acid (PA, Sigma), Insulin from bovine pancreas **(**Sigma), fetal bovine serum (FBS, Gibco), Glucosamine (Beyotime Biotechnology), BSA (Roche), Puerarin (Nanchang Wante).

2.2 Main instruments

IX-71 type inverted optical microscope (Olympus, Janpan), carbon dioxide incubator (Thermo scientific forma, USA), forma class-II/A2 biological safety cabinet (Forma, USA), 5804R type centrifuge (Eppendorf, Germany), multiscan MK3 type full automatic microplate reader (Thermo lab systerms, USA), milli-Q water purification systems (Millipore, Shanghai)**.**

2.3 Methods

2.3.1 Cell culture

HepG2 cells were cultured in DMEM low glucose medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg / mL streptomycin. Cells were incubated at 37°C in a humidified incubator supplied with 5% CO_2. When the cells grew to 80% confluence, passing the cells with 0.25% trypsin.

2.3.2 Determination of cell viability in different concentrations of different inducing agents

For the development of insulin resistance by high glucose and palmitate, HepG2 cells were seeded in 96-well plates at a density of 2×10^4 cells/well [1] and grown for 24 hours to reach 80%-100% confluence. The cells in 96-well plates were divided into control group and model group. The model group was incubated for 24 hours with low glucose complete medium containing 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM concentrations of palmitate, the control group an equal volume of the vehicle for 24 hours. Another model group of cells were inducing by 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM concentrations of high glucose medium for 24 hours. After 24 hours, the cells were washed twice with PBS, each well was added fresh complete medium with 20μl MTT and incubated for 4 hours to add. After in the incubator 4 h, each well had 150 ul DMSO, by MTT method detection of cell growth.

2.3.3 Optimization time, concentration and detection of cell viability

A low concentration inducer was beneficial to the growth of the cell. Through many experiments, the cell density was adjusted accordingly and the cell growth status is better under the density of 5×10^4 cells/well. HepG2 cells were seeded in 96-well plates at a density of 5×10^4 cells/well and grown for 24 hours to reach 60% confluence. Palmitate was added to the medium to reach 0.125 mM, 0.25 mM, 0.50 mM. High glucose was added to the medium to reach 20 mM, 30 mM, 40 mM. Each of the 96-well plates was corresponding to the corresponding inducer and the cell viability was detected by

MTT in 6, 12, 24 and 36 hours respectively.

2.3.4 Repeated experiments to determine the induction time and concentration

When HepG2 cells were in the logarithmic phase, they were seeded in 96-well plates at a density of 5 $\times$ 10^4 cells/well and grown for 24 hours to reach 50% confluence. Cells incubated in 0.05 mM, 0.1 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM by Palmitate for 24, 48, 72 hours. The cell viability was detected by MTT.

2.3.5 Establishment of insulin resistance model and the hypoglycemic effect of Puerarin

HepG2 were maintained in DMEM low glucose medium. When subcultured at 80% confluence, HepG2 cells were seeded in 96-well plates at a density of 5 $\times$ 10^4 cells/well and grown for 24 hours to reach 70%-80% confluence. Cells were induced by palmitate in the concentration of 0.125 mM for 24 hours, with PBS washed twice, then respectively set blank group, control group, model group. Each control group and model group were added to puerarin, rosiglitazone, metformin. Blank groups were divided into without DMSO and DMSO in the same medium. Absorbance at 550 nm was then determined using a microplate reader. Puerarin concentration was 25 ug/ml, rosiglitazone20 ug/ml, metformin was 5 ug/ml. It was indicated that insulin resistance model was established successfully from glucose consumption difference between the control group and model group. The concentration of glucose is in direct proportion to the absorbance. Five microliters of the resulting supernatant was mixed with 195 μL of Glucose kit in a 96-well plate and incubated at 37 °C for 20min. Absorbance at 550 nm was then measured using a microplate reader.

3 Results

3.1 The results of absorbance and cell viability of HepG2 with palmitate and high glucose

The concentration of palmitate is 0.125-2 mM and high glucose is 20-70 mM effecting on cell proliferation after 24 hours. As shown in Table 1 and Table 2, Figure 1, Palmitate(PA) at low concentration has little effect on the cells, while high glucose concentration on cell survival rate obviously higher inhibited.

Table 1. The absorbance and Cell viability of HepG2 at A492 with palmitate for 24h(p<0.05).

Concentration(mM)	Absorbance	Cell viability
0	0.847±0.033	1
0.125	0.893 ±0.002	1.054 ±0.002
0.25	0.865 ±0.017	1.021 ±0.020
0.50	0.660 ±0.005	0.780 ±0.005
1	0.485 ±0.002	0.573 ±0.002
2	0.232 ± 0.006	0.274±0.006

Table 2. The absorbance and Cell viability of HepG2 cells at A492 with high glucose for 24h($p < 0.05$).

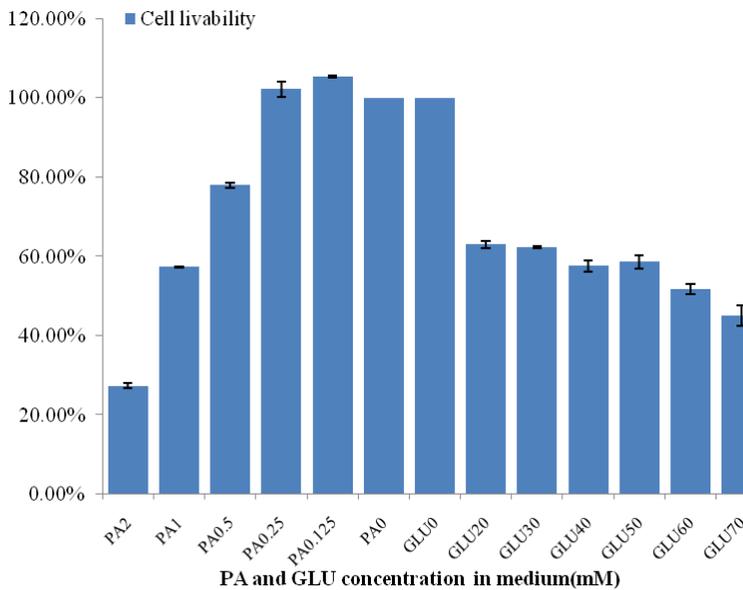| Concentration (mM)	Absorbance	Cell viability
0	1.277±0.02	1
20	0.805±0.008	0.630±0.008
30	0.796±0.002	0.623±0.002
40	0.735±0.014	0.576±0.014
50	0.750±0.017	0.587±0.017
60	0.662±0.013	0.519±0.013
70	0.575±0.027	0.451±0.027

Figure 1. Effects of cell viability with different concentration of High glucose and palmitate induced HepG2 cells. (PA0 was without palmitate, GLU 0 was without glucose, $p < 0.05$).

3.2 The results of optimization time and concentration and detection of cell viability

Figure 2 shows that the cell viability was gradually decreased with the increase of inducer concentration and induction time. The inhibitory effect of Palm Acid on cells was less than that in high glucose group(judged by both p between groups<0.05). Subsequent experiments with the palm acid as inducer, while the concentration of high glucose still need to make appropriate adjustments.
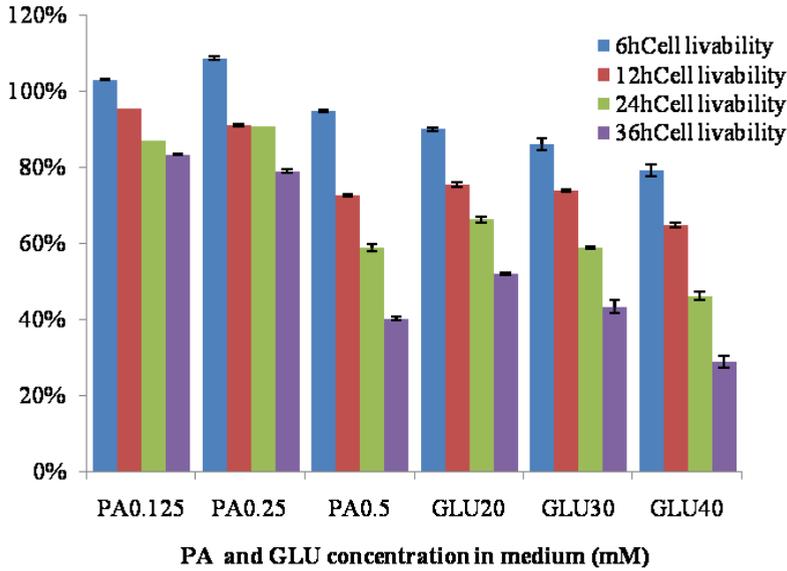
Figure 2. 6h, 12h, 24h, 36h, cell viability (Data were represented as means ±S.D. $p < 0.05$).

3.3 The results of the induction time and concentration

From the chart below(Figure 3**)**, cells in low concentration 0.05-0.125 mM were less inhibitory effect on cell growth, while in 0.25mM-2mM cell viability decreased significantly. High concentration is not conducive to the subsequent experiment. Under comprehensive consideration, subsequent induction agent was in low concentration of palmitate in the concentration of 0.125 mM for 24 hours.
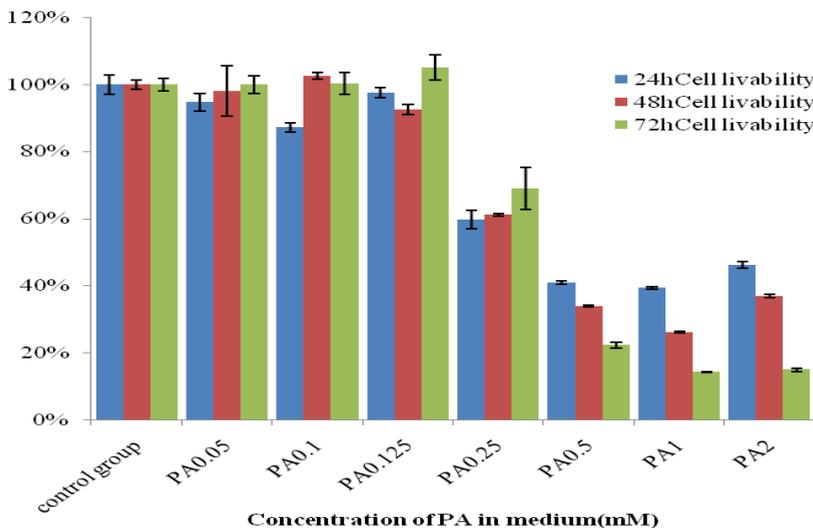

Figure 3. Detection of 24h, 48h, 72h, cell viability(Data were represented as means ±S.D. $p < 0.05$).

3.4 The standard curve of glucose(presented as the absorbance at 500 nm)

The linear regression of the concentration X was carried out in the light absorption value Y, and the standard curve regression equation was $Y=0.0552x+0.0565$, $R^2 =0.9997$. The linear range of the standard curve is 0.02~20 mmol/L.
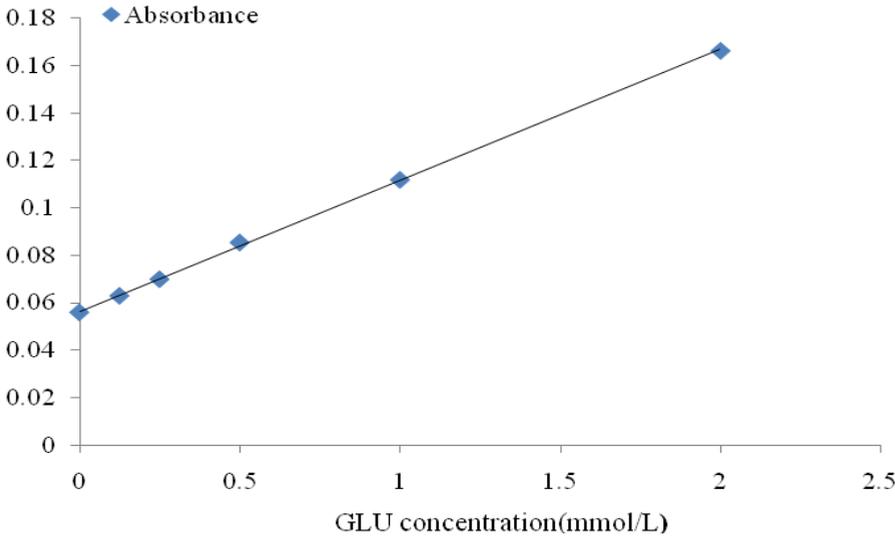

Figure 4. The standard curve of different concentration of glucose.

Table 3. Establishment of insulin resistance model and effect of Puerarin on hypoglycemia(Data were represented as means ±S.D.p<0.01).

Group	Glucose consumption(mM)	Glucose consumption difference (%)
control+ puerarin	1.585 ± 0.002	
Model+ puerarin	1.295 ± 0.002	18.297
control+ rosiglitazone	2.672 ± 0.003	
Model+ rosiglitazone	2.473 ± 0.001	7.478
control+ Metformin	2.129 ± 0.005	
Model+ Metformin	2.092 ± 0.004	1.378

3.5 The results of glucose uptake

The concentration of glucose was in direct proportion to the absorbance. Five microliters of the resulting supernatant was mixed with 195 μL of Glucose kit in a 96-well plate and incubated at 37 °C for 20 min. Absorbance at 550 nm was then measured using a microplate reader .
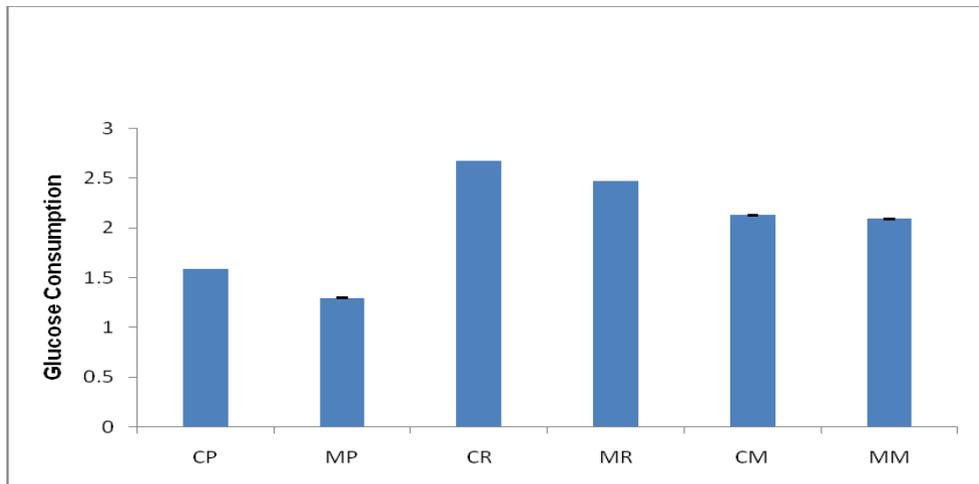
Figure 5. Insulin resistance model glucose consumption(mmol/L). Control+ Puerarin(CP), Model + Puerarin (MP), Model+ Rosiglitazone(MR), Control+ Metformin(CM), Model + Metformin(MM).

From the charts above, the results showed that glucose consumption of model groups under the same concentration and time of puerarin and rosiglitazone were significantly lower than that of the control group. To explain the resistance of cells induced by 24hour in the post - HepG2 cells.

4 Summary

It was found that low consentration of palmitate achieved better results on establishing insulin resistance model using HepG2 cells induced by palmitate and glucosamine. The insulin resistance model was used to verify hypoglycemic effect of puerarin, which showed that an effective and stable insulin resistance model was established successfully. Treatment of insulin resistance is a major strategy in the prevention and management of type2 diabetes[10-12]. The underlying mechanism about the phenomena of palmitate - treated HepG2 cells needs further research.

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