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Research Paper

Effects of pioglitazone on proliferation and differentiation of human preadipocytes

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Abstract

Objective: To explore the effects of thiazolidinediones (TZDs) pioglitazone on proliferation and differentiation of human preadipocytes. **Methods**: Omental adipose tissue biopsies were obtained from 15 patients who were undergoing elective open-abdominal surgery. The primary culture and differentiated induction of human preadipocytes were performed, and the human preadipo-cytes were treated with pioglitazone at different concentrations at proper moments. Dynamic morphological changes of the human preadipocytes were observed, and their proliferation and differentiation were assessed with Colorimetric MTT Assay and Oil Red O Staining. **Results**: After 24 hours and 72 hours with pioglitazone, 0.1 μmol/L (μmol/ml) pioglitazone increased the MTT values of the human preadipocytes by 25.3% and 34.8%, respectively ($P < 0.05$), while 1 μmol/L pioglitazone by 27.4% and 26.6% ($P < 0.05$), compared with the control group without pioglitazone. The human preadipocytes with pioglitazone cumulated more adipose in the endochylema than those without pioglitazone obviously. 0.1 μmol/L pioglitazone increased the differentiation degree of the human preadipocytes differentiated for 8-10 days by 44.81% and 1 μmol/L pioglitazone by 53.76% ($P < 0.05$). **Conclusion**: Thiazolidinediones pioglitazone may significantly promote the proliferation and differentiation of the human omental preadipocytes.

Keywords: pioglitazone; human preadipocytes; proliferation and differentiation; insulin sensitivity

INTRODUCTION

In modern life-style, the prevalence of obesity has increased rapidly in the past few decades in the world. Due to its high correlation with insulin resistance [1-3], it is increasingly seen as a public health problem requiring concerted action. On cellular level, increasing amount and volume of adipocytes induced by unceasing differentiation of adipocytes result in obesity. Differentiation of adipocytes will affect glucolipid metabolism, even influence the development of metabolic disease. TZDs pioglitazone is used as insulin sensitizer to treat diabetes for improvement of insulin resistance in clinic, but it will cause accumulation of adipose and body weight gain, this inducing contradiction on therapy [4,5]. To explore the mechanism of action of pioglitazon, and provide theoretical evidences for its clinical application, our experiment group studied its effects on the proliferation and differentiation of human preadipocytes.

MATERIALS AND METHODS

Sample

Omental adipose tissue biopsies were obtained from patients who were undergoing elective open-abdominal surgery. None of the patients had diabetes, coronary heart disease or severe systemic illness. The patient group included 15 participants, 8 women (aged 58 ± 15 years, BMI 24.22 ± 3.12 kg/m^2) and 7 men (aged 53 ± 12 years, BMI 21.77± 2.63 kg/m^2).

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Major drugs and reagents

Active compound of pioglitazone was kindly provided by Jiangsu Province Heng-rui medicine corporation. Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12), D-Hank'S were purchased from JRH company. Fetal bovine serum (FBS) and Penicillin-streptomycin stock solution were purchased from Gibco Company. Bovine serum albumin (BSA),3-(4,5-Dimethylthiazolyl) -2,5-diphenyl tetrazolium bromide(MTT) were purchased from AMRESCO company. Triiodothyronine(T_3),1-methyl-3-isobutyl xanthine (IMX), and Oil red O were purchased from Sigma company . Collagenase Type II was purchased from Worthington Company. Human insulin was from Lilai Company. Dexameth(DXM) was production of Nanjing No.3 drug manufactory. Dimethyl sulfoxide (DMSO) was production of Shanghai Lingfeng reagent company. Cell culture plates were purchased from Corning Company.

The primary culture and differentiated induction of human preadipocytes

Adipose tissue samples were dissected from other tissues and minced in small pieces. Preadipocytes were isolated by collagenase(Type II,1 mg/ml) digestion for 90 minutes at 37℃. Then the adipose tissue was filtered through a sterile nylon mesh with a size of 80-mesh screen, and then centrifuged at 2500 *rpm* for 10 min at room temperature. The pellet was incubated in erythrocyte lysis buffer consisting of 0.154 mol/L NH_4 Cl, 10 mmol/L $KHCO_3$ and 0.1 mmol/L EDTA for 10 min at room temperature. After an additional centrifugation step, the fraction was resuspended in growth-promoting media consisting of DMEM/F12 medium and supplemented with 10% fetal bovine serum and 100 μg/ml penicillin. Cells were inoculated into 96-well and 12-well culture plates and kept at 37℃ in 5% CO_2. We changed the culture fluid in 24 h, and used the proposal of insulin (0.5 μmol/L)+ DXM(0.1 μmol/L)+ T3(0.2nmol/L)+IMX (0.2 mmol/L) to induce the differentiation of human preadipocytes. Then we stained the differentiated preadipocytes with oil red O and major lipid droplets stained to salmon pink may be seen inside the adipocytes, which confirmed that the primary culture of the human preadipocytes was successful.

The proliferation of human preadipocytes (Colorimetric MTT Assay)

After having been cultured in growth-promoting media for 24 h, the human preadipocytes inoculated in 96-well plates were serum-deprived and exposed to the DMEM/F12 medium with pioglitazone at different concentrations (0.1 μmol/L,1 μmol/L and 10 μmol/L) or without pioglitazone (as control group) for 24 h and 72 h before MTT assays. Then the cells were incubated with 10 μl MTT (0.5 mg/ml) for 4 h at 37℃. After removal of MTT, 100 μl of DMSO were added to the cells, and optical density was measured at 550 nm using a microplate reader. An empty well without cell was treated the same way and used as a blank.

Assessment of preadipocyte differentiation (Oil Red O Staining)

After having been cultured in growth-promoting media for 20 h, the human preadipocytes inoculated in 12-well plates were serum-deprived and exposed to the differentiating media (DMEM/F12 medium supplemented with some differentiation-promoting reagents as above mentioned) with pioglitazone at different concentrations (0.1 μmol/L, 1 μmol/L and 10 μmol/L) and without pioglitazone (as control group) for 8-10 days before Oil Red O Staining. The culture fluids were changed every 2-3 days. After 8-10 days' differentiation, cells were fixed with 10% formaldehyde in phosphate buffer saline, and their triglyceride content was stained with 1% Oil Red O in 60% isopropanol. We washed the cells with water repeatedly, observed the cells under inverted phase contrast microscope and took photographs. Then their Oil red O content was dissolved in 100% isopropanol and the optical density of this extraction was measured at 490 nm.

Statistical analysis

Quantitative data were expressed as mean ± SD. Statistical analysis was performed by 0ne-way ANOVA and $P < 0.05$ was regarded as statistically significant. All statistical tests were two-sided.

RESULTS

Verification of morphology

The inoculated preadipocytes were initially like round. Several hours later, cells adhered, in inequality of size. After 2-3 days'culture , they became spindle and stereo (***Fig 1***). The preadipocytes that propagated in growth-promoting media proliferated rapidly, most of which were spindle and in homogeneous distribution,with fairly equal morphor and size, lined up closely and parallelly or swirled , and no lipid droplet was seen inside the cells(***Fig 2***). During the differentiation-promoting culture, the proliferation of preadipocytes was inhibited, the morphology became polygonal or round gradually, and the volume was scale-up (***Fig 3***). With different

sizes, round lucency lipid droplets, most of which were scattered, could be seen inside the cells obviously after 8-10 days' differentiation (***Fig 4***). Along with the differentiating culture process, the amount of cells which appeared the lipid droplets increased and so was the content of lipid droplets, and reached a differentiated peak after 16–18 days and more than 80% preadipocytes could differentiate to mature adipocytes at last. The amount of cells which lipid droplets and the content of the lipid droplets in the groups with pioglitazone were significantly more while the volume was smaller than those in the control group without pioglitazone (***Fig 5***). The differentiated preadipocytes were stained with oil red O and major lipid droplets stained to salmon pink could be seen inside adipocytes (***Fig 6***). The adipocytes would disintergrate gradually after having been cultured in the differentiating medium for more than 25 days.

Effects of pioglitazone on proliferation of the human preadipocytes

The result of MTT assay showed that after 24 hours and 72 hours, 0.1 μM pioglitazone increased

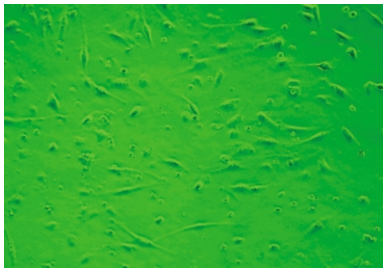


Fig 1 Cultured for 2-3 days(×100)

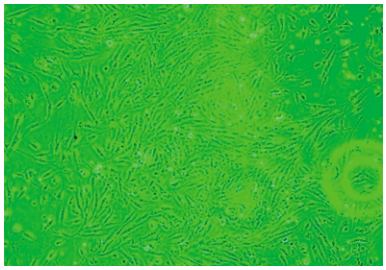


Fig 2 Propagated in growth-promoting medium for 8-10 days(×100)

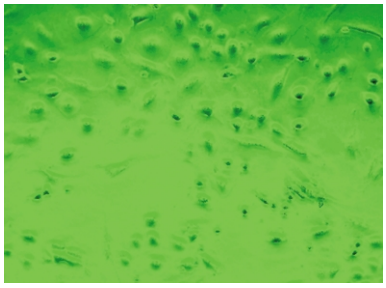


Fig 3 Cultured in differention medium for 5-6 days(×100)

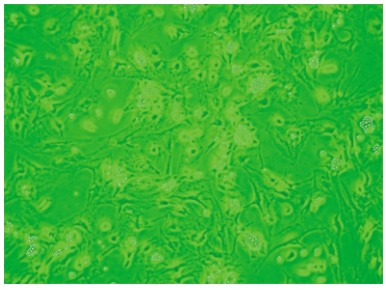
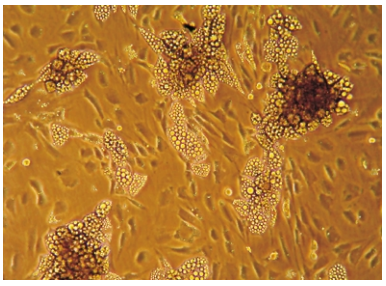
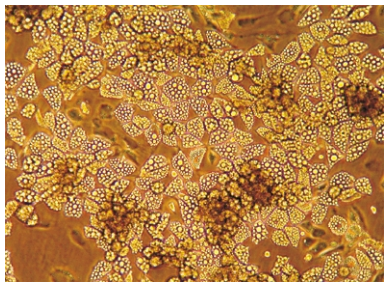


Fig 4 After 8-10 days' differentiation (× 100)

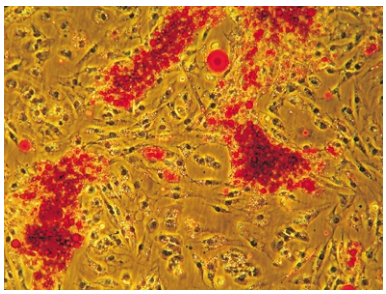


control group

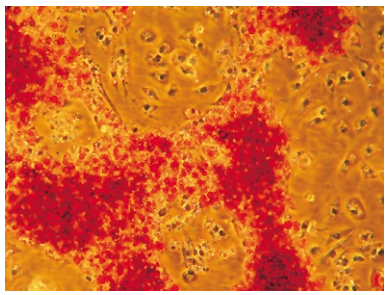


1 μM pioglitazone group

Fig 5 After 16-18 days' differentiation(× 200)



control group



1 μM pioglitazone group

Fig 6 Oil red O staining after 13 days' differentiation(× 200)

the MTT value of the human preadipocytes by 25.3% and 34.8%($P < 0.05$), while 1μmol/L pioglitazone by 27.4% and 26.6% ($P < 0.05$), compared with control group without pioglitazone. There was no significant statistical difference between these two groups (P_{24h}=0.844,P_{72h}=0.376). The effect of 10 μmol/L pioglitazone on the MTT value of the human preadipocytes had no statistical significance (P_{24h} = 0.558,P_{72h}=0.202). It indicated that 0.1 μmol/L and 1 μmol/L pioglitazone might significantly promote the proliferation of the human preadipocytes(***Tab 1***).

Tab 1 **Effects of pioglitazone on the proliferation of the human preadipocytes** ($\bar{x} \pm s$)

Groups	*n*	MTT value after 24 h	*n*	MTT value after 72 h
control group	15	0.099 ± 0.014	14	0.109 ± 0.014
0.1 μmol/L pioglitazone	14	0.124 ± 0.025*	12	0.150 ± 0.062*
1 μmol/L pioglitazone	15	0.123 ± 0.018*	14	0.137 ± 0.023*
10 μmol/L pioglitazone	12	0.095 ± 0.013	10	0.091 ± 0.018

Compared with control group without pioglitazone, *$P < 0.05$.

Effects of pioglitazone on differentiation of the human preadipocytes

The result of Oil Red O Staining showed that, compared with control group without pioglitazone, 0.1 μmol/L pioglitazone may increase the differentiation degree of the human preadipocytes differentiated for 8-10 days by 44.81% and 1μmol/L pioglitazone by 53.76%($P < 0.05$). There was no significant statistical difference between these two groups (P = 0.349). While the effect of 10μmol/L pioglitazone on the differentiation of the human preadipocytes had no statistical significance(The increased rate was 12.96%,P =0.225). It indicated that 0.1 μmol/L and 1 μmol/L pioglitazone might significa ntly promote the differentiation of human preadipocytes(***Tab 2***).

Tab 2 **Effects of pioglitazone on the differ entiation of the human preadipocytes** ($\bar{x} \pm s, n = 7$)

Groups	differentiation degree(OD)
control group	0.248 ± 0.032
0.1 μM pioglitazone	0.357 ± 0.038*
1 μM pioglitazone	0.383 ± 0.066*
10 μM pioglitazone	0.281 ± 0.055

Compared with control group without pioglitazone, *$P < 0.05$.

DISCUSSION

Adipose tissue is a huge energy storage system in body, which plays an important role in the maintenance of the energy metabolic balance of the organism [6-9]. On cellular level, obesity is caused by increasing amount and volume of adipocytes induced by unceasing differentiation of adipocytes. Abnormal differentiation of adipocytes will affect glucolipid metabolism, induce obesity and insulin resistance, and then influence the development of metabolic diseases. Therefore, studies in the world have recently focused on the regulation of the adipocyte differentiation and its relationship with mechanism of obesity and insulin resistance. Because proliferation and differentiation are two courses during the growth of cells, it is significant to study the effects of drugs on proliferation and differentiation of adipocytes for prevention and cure of those metabolic diseases which have a close relationship with obesity and insulin resistance, such as type 2 diabetes, hypertension, lipids metabolic disturbance and atherosis.

The peroxisome proliferator-activated receptor-γ (PPAR-γ) is the master regulator of adipocyte differentiation , and its synthetic ligands, thiazolidinediones (TZDs) are a new class of antidiabetic drugs that improve insulin action[10,11], mainly including rosiglitazone and pioglitazone. As shown as the study, it may promote the expression of PPAR-γ and adipocyte differentiation, accelerate glucose transport, and increase insulin sensitivity of adipocytes [10,11]. But it will cause more accumulation of adipose and body weight gain finally [4], while there is tight relativity between obesity and insulin resistance [1-3], which induces contradiction on therapy[5]。

Our experiment results showed that 0.1 μmol/L and 1 μmol/L pioglitazone might significantly promote the proliferation and differentiation of the human omental preadipocytes,but not in a dose-dependent manner. From the figures we may observe that preadipocytes with pioglitazone cumulated more lipid droplets but the volumes were relatively smaller than those without pioglitazone obviously. Animal studies [12] had discovered that TZDs might promote the expression of PPAR-γ and generate relatively small adipocytes with higher insulin sensitivity. Moreover, Sewter et al[13] reported that PPAR-γmRNA was highly expressed in subcutaneous preadipocytes, compared with o mental preadipocytes. This could cause a redistribution of body fat from the omental to the subcutaneous depots. So we may conclude that pioglitazone promoted the expression of PPAR-γ and stimulated differentiation of human preadipocytes, which spur adipocytes to shrink so as to elevate the insulin sensitivity. And this effect was extremely stronger in subcutaneous adipose tissue than in omental adipose tissue,therefore induced the redistribution of body fat from the omental to the subcutaneous adipose tissue, so as to improve central obesity and increase insulin sensitivity. Meanwhile, distribution of body fat may affect expression of

adipocytokines. A domestic study has reported that adiponectin mRNA was much lowerly expressed in human omental adipose tissue than in subcutaneous depot [14], so the redistribution of body fat might increase the expression of adiponectin, a kind of adipocytokines which was proven to be able to increase insulin sensitivity [15-17]. This conclusion was confirmed by Hiroshi et al [18] who used pioglitazone to treat Japanese patients with type 2 diabetes. A clinical trial also demonstrated that pioglitazone increased subcutaneous body fat, but not visceral fat [19]. Besides, there was a report that TZDs had prodifferentiating effects on abdominal subcutaneous preadipocytes while they had no such effects on omental preadipocytes[20].

At present, domestic studies on adipocytes were mostly limited on 3T3 cell strain. Our experiment group had set up the primary vitro culture method and the differentiating proposal of the human preadipocytes, so our experimental results may reflect the real situation of human body. Our study indicated that 0.1 μmol/L and 1 μmol/L pioglitazone might significantly promote the proliferation and differentiation of the human omental preadipocytes, though the effect didn't increase if the drug concentration was elevated. Pioglitazone would cause cumulation of adipose and body weight gain finally, but it might induce a redistribution of body fat, cause central obesity, and elevate the expression of adiponectin so as to increase insulin sensitivity. Therefore, it may be used for the treatment of diabetes to improve insulin sensitivity. But it seemed no use to increase drug dose for better curative effects. Further studies are neede d to explore the concrete mechanism.

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