



EFFECT OF TROGLITAZONE ON MORPHOLOGICAL CHANGES AND PPAR- γ GENE EXPRESSION IN 3T3-L1 ADIPOCYTE CELLS

Efek Troglitazone terhadap Perubahan Morfologi dan Ekspresi Gen PPAR- γ di Dalam Sel Adiposa 3T3-L1

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ABSTRACT

3T3-L1 cells are extensively used as a model to study adipogenesis. However, one major concern is the prolonged period of time it takes the cells to differentiate into adipocytes form. To induce this differentiation, the adipogenic induction media is required. In this study, troglitazone, a hypoglycemic agent was added to adipogenic induction media and observed in order to determine the morphological changes and peroxisome proliferator-activated receptor gamma (PPAR- γ) gene expression in 3T3-L1 differentiation. It is generally known that PPAR- γ plays an important role as a transcription factor in adipocyte differentiation. Based on Oil Red O Staining, adipogenic induction with or without troglitazone changed the 3T3-L1 pre-adipocytes into mature round fat cells characterized by red droplet lipids. This cell also had a high absorbance level and degree of droplet accumulation of $P \leq 0.05$ in each group. In addition, cells treated by troglitazone had the highest PPAR- γ mRNA level (1.9 fold) than those treated by adipogenic induction media without troglitazone or cells un-treated at all.

Keywords: 3T3-L1, adipocyte, differentiation, PPAR- γ , troglitazone

ABSTRAK

Sel 3T3-L1 adalah jenis sel yang banyak digunakan dalam studi adipogenesis. Namun, salah satu kelemahan sel tersebut adalah lamanya waktu yang dibutuhkan bagi sel pre-adiposa untuk berdiferensiasi menjadi sel adiposa. Selain itu, dibutuhkan pula media induksi khusus untuk mengubah sel menjadi sel adiposa. Pada penelitian ini, kami mengobservasi fungsi troglitazone, sebagai antidiabetes terhadap perubahan morfologi dan ekspresi gen peroxisome proliferator-activated receptor gamma (PPAR- γ). Telah diketahui bahwa PPAR- γ berperan penting sebagai factor transkripsi dalam diferensiasi sel adiposa. Berdasarkan pewarnaan ORO, induksi sel pre-adiposa 3T3-L1 dengan media induksi dengan dan tanpa troglitazone merubah sel preadiposa menjadi sel berbentuk bulat yang dikarakterisasi dengan akumulasi droplet lemak. Nilai absorbansi sel adiposa juga menandakan adanya perbedaan yang signifikan antara kelompok sel yang diberi troglitazone dan tidak, dan sel tanpa diberi media induksi. Sementara, pada kelompok sel yang diberi troglitazone memiliki ekspresi mRNA PPAR- γ (1,9 kali) tertinggi jika dibandingkan dengan sel yang diberi media induksi tanpa troglitazone, dan tanpa media induksi sama sekali.

Kata Kunci: 3T3-L1, adiposa, diferensiasi, PPAR- γ , troglitazone

INTRODUCTION

The most commonly *in vitro* model cell lines used in the study of adipocyte is 3T3-L1 (Gregoire et al. 1998; Zebisch et al. 2012; Aoyagi et al. 2014; Morrison and McGee 2015). These cells are derived from the disaggregation of a 17-19 day old Murine Swiss mouse embryo. Generally, to convert the 3T3-L1 from their fibroblast phenotype into adipocytes, adipogenic agents such as insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and fetal bovine serum (FBS), at concentrations of 1 $\mu\text{g}\cdot\text{mL}^{-1}$, 0.25 μM , and 0.5 mM, respectively are needed (Ruiz-Ojeda et al. 2016; Mehra et al. 2007). The process spends about 12-14 days until the cells could be seen as adipocyte characterized by spherical shape, with dramatical changes in cell morphology, cytoskeletal components, level and type of extracellular matrix (ECM) component, and expression of numerous genes (Ali et al. 2013; Zhang et al. 2014).

One of the pivotal regulators in adipocyte differentiation is *Peroxisome proliferator-activated receptor gamma* (PPAR- γ). PPAR- γ is the transcriptional factor belonging to the type II nuclear hormone receptor family and mainly expressed in insulin responsiveness tissues, such as white and brown tissue, and to a lesser extent in immune cells (Siersbæk et al. 2010; Kawahara et al. 2013; Lee and Ge 2014; Lasar et al. 2018). The PPAR- γ regulates gene transcription by binding the peroxisome proliferator response element on the DNA with the AGGTCANAHHTCA sequence as obligate heterodimers using the retinoic acid receptor (RXR). This then induces different sets of genes in various tissues that in turn have distinct downstream biological effects (Deng et al. 2011; Vinayagam and Xu 2015). The effects of PPAR- γ depend on its specific ligands which play a role in the regulation of lipid metabolism and glucose homeostasis. One of such roles is as a synthetic hypoglycemic agent, in which troglitazone is reported as potent ligand (Camp et al. 2000; Zhu et al. 2016).

troglitazone is an insulin-sensitizing agent capable of enhancing the hyperglycemia and hyperinsulinemia by increasing their insulin responsiveness and

sensitivity in obese, type 2 diabetic, and glucose intolerant patients. *In vivo* studies found that troglitazone ameliorates peripheral glucose intake, while decreasing the serum insulin level and gluconeogenesis in rodent models, whilst *in vitro* studies showed its potential to enhance glucose uptake in 3T3L1 and F442A adipocytes by increasing *glucose transporter isoform 4* (GLUT4) and GLUT4 mRNA levels (Taher et al. 2015).

Therefore, the objective of this study was to evaluate the *in vitro* influence of two adipogenic cell culture media. The aim was clearly to define the most potent adipogenic growth media in 3T3-L1 cell differentiation. The quantitative real time - polymerase chain reaction (RT-PCR) was used to quantify the PPAR- γ as specific marker genes, and to ensure reproducible and accurate quantitative expression measures, and normalize the expression level of target genes with the reference gene.

MATERIALS AND METHODS

Place and time of research

Research was held in Mammalian culture laboratory, Center of Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology, Serpong, Indonesia. Its duration was six months (from June to December 2018).

Materials

Mouse 3T3-L1 fibroblast cells were obtained from Korea Research Institute of Bioscience & Biotechnology, South Korea.

Reagents

Dulbecco's Modified Eagle's Medium 12 (DMEM-F12), IBMX, amphotericin B, isopropanol and chloroform were obtained from Sigma Aldrich, USA. Fetal Bovine Serum (FBS) and Trypsin-EDTA (0.5%), were purchased from GIBCO™, penicillin-streptomycin was purchased from Thermo Fisher Scientific, while troglitazone and human insulin were obtained from Santa Cruz. Dexamethasone was acquired from Bioreagent, GENEzol™ Reagent from Biotline, while SensiFAST CDNA Synthesis Kit, and SensiFAST™ SYBR® No-ROX One-Step Kit were both purchased from Biotline. Primer PPAR γ , Beta actin, and GLUT4 were procured from Integrated DNA Technologies.

Cell culture and differentiation

3T3-L1 preadipocytes were grown and passed into DMEM-F12 containing 10% FBS and 1% penicillin-streptomycin (DMEM-F12 complete). For adipocyte differentiation, post-confluent cells were placed in two different adipogenic differentiation media with troglitazone and the other without it for a period of 2-days. In the first medium, we adopted the procedure described by Zebisch et al. (2012) (DMEM-F12 complete with 0.25 µM Dexamethasone, 0.5 mM IBMX, and 1 µg·mL⁻¹ insulin). For comparison, we added 5 µM troglitazone to this common medium. Other 3T3-L1 pre-adipocyte cells were also incubated with the DMEM-F12 and used as a negative control group. After 2 days, each

medium from those three groups were changed by adipogenic induction medium II (DMEM-F12 complete with only 1 µg·mL⁻¹ insulin). The cells were incubated again for two days, and replaced with only DMEM F-12 until the twelfth day. The flowchart of 3T3-L1 differentiation protocol using different media with or without troglitazone can be seen in Figure 1.

Oil Red O staining.

During differentiation time, morphological changing was observed by Oil red O staining. Sampling was carried out four times, the first was before induction was given (day 1), second was at day 4 or one day after induction with media containing troglitazone, and the last was done at the

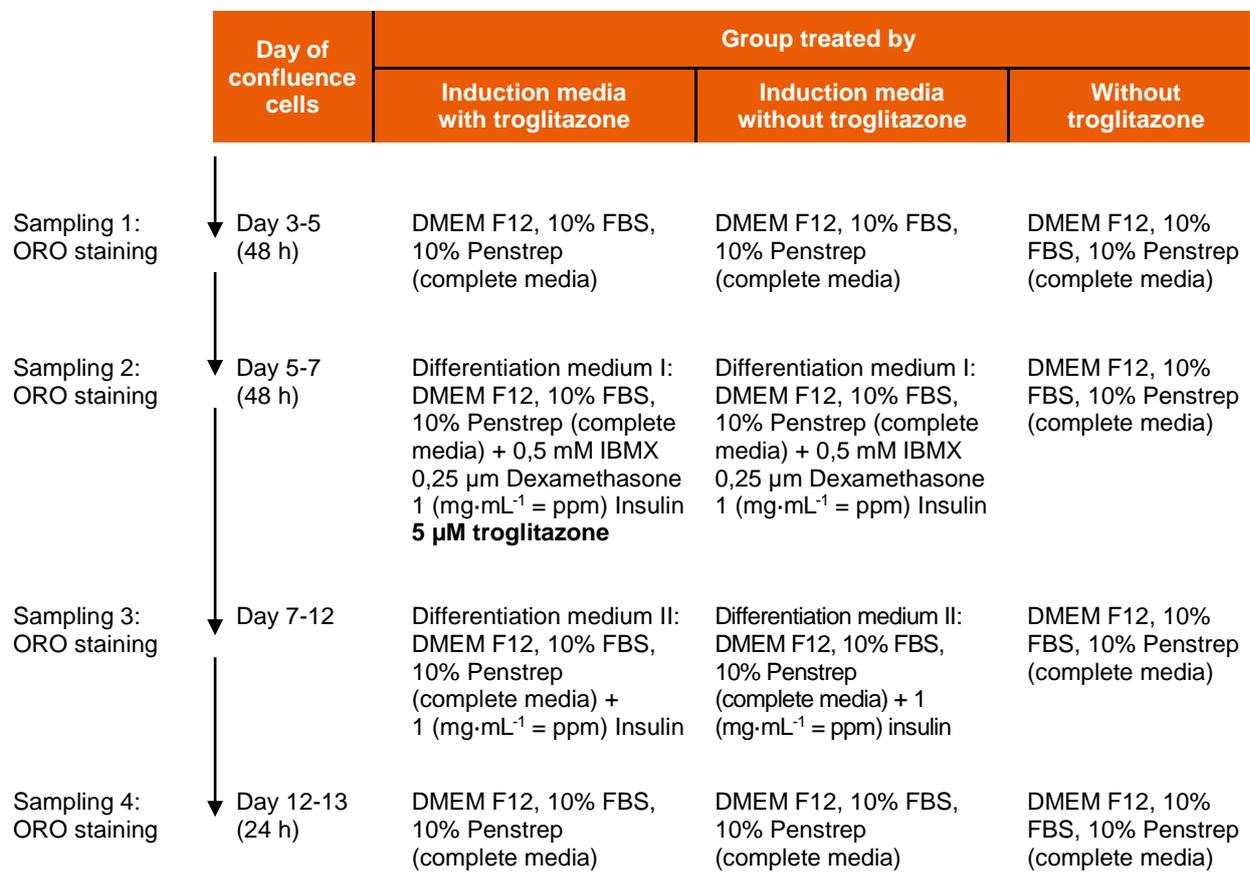


Figure 1. The flowchart of 3T3-L1 differentiation protocol using different medium with or without troglitazone.

Table 1. Sequence of primers used in this research

Type of primers	Sequence
Primer forward PPAR-γ	: 5'-GCATGGTGCCTTCGCTGA-3'
Primer reverse PPAR-γ	: 5'-TGGCATCTCTGTGTCAACCATG-3'
Primer forward beta actin	: 5'-CTCTGGCTCCTAGCACCATGAAGA-3'
Primer reverse beta actin	: 5'-GTAAAACGCAGCTCAGTAACAGTCCG-3'

end of differentiation (day 12). The cells were washed with phosphate-buffered saline (PBS) and fixed into a solution of 4% formaldehyde in 0.1 M phosphate buffer, with a pH value of 7.4 for 15 min at room temperature. It was then washed 3 times with deionized water. A mixture of Oil Red O (0.6% Oil Red O dye in isopropanol) and water at a 6:4 ratio were layered on the cells for 10 min. It was photographed and the cell absorbance was measured on a 510-nm wavelength.

Quantitative real time PCR

Total RNA was isolated using GENEzol™ reagent and the reverse was transcribed with SensiFAST CDNA Synthesis Kit according to the manufacture's instruction. A cDNA was then amplified with PPAR- γ and β -actin primer as reference gene. Quantitative real time PCR was performed on Eco Illumina 4.1 system using SensiFAST™ SYBR® No-ROX One-Step Kit in a final volume of 20 μ l. The conditions of real time PCR were as follows: 45°C for 10 sec, pre-denaturation at 95°C for 10 sec, denaturation at 95°C for 5 sec, annealing at 63°C for 10 sec, and extension 72°C for 5 sec. These steps were repeated for 40 cycles. In addition, a melting curve was built with a temperature range of 55-95°C at the end of the amplification. The primer sequences used in this step were presented in Table 1. All primers were designed by SnapGene and synthesized by Integrated DNA Technologies.

Gene expression and statistical analysis

PPAR- γ gene expression was measured by comparative CT method ($\Delta\Delta$ CT) and real time PCR using Eco system instrument. Beta actin expression was used as internal control, and before using $\Delta\Delta$ CT method for quantification, a validation experiment was performed for primer efficiency investigation. In addition, for the statistical analysis, all results are presented as mean \pm SEM. Differences between the groups were determined using the one-way analysis of variance (NOVA), with Post Hoc comparison by Tukey's Multiple Comparison Test. A value of $P \leq 0.05$ was statistically significant. All analysis was performed using GraphPad Prism 5.0 for Windows.

RESULTS AND DISCUSSION

Naturally, adipocyte differentiation requires hormone and growth factor that act by using specific receptors to transduce external growth and differentiation signals through a cascade of intracellular event. However, identification of agents or molecules that modulate this transcription process also provides insight into the signal transduction pathways involved. These agents and molecules then play important roles in modulating adipocyte differentiation by permitting the morphological changes and adipocyte specific gene expression that accompany differentiation (Vishwanath et al. 2013; Morrison and McGee 2015).

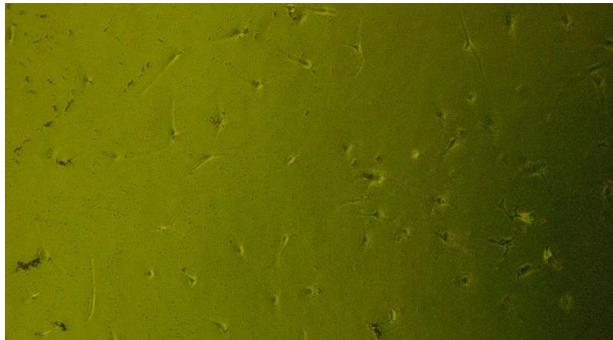
In this present study, we observed the effect of troglitazone on 3T3-L1 pre-adipocytes and its effect on PPAR- γ gene expression. Troglitazone, a hypoglycemic agent of Thiazolidinediones (TZDs) which happens to be anti-diabetic improves peripheral insulin sensitivity, leading to a reduction in the blood glucose and insulin level, and the preservation of pancreatic function (Ciaraldi et al. 2002; Alvim et al. 2015).

On the current study, we found that the addition of this anti-hyperglycemic in common adipogenic media was capable to stimulate 3T3-L1 pre-adipocyte into adipocyte cells. It was characterized by the size and morphological changes in 3T3-L1 pre-adipocyte after troglitazone treatment. Generally, cells differentiated by adipogenic induction media with or without the injection of troglitazone were large and had red oil droplets arranged in loose cluster. In contrast the un-treated cells were small, with compact clusters of oil droplets (Figure 2). However, cells stained with Oil Red O staining showed that cells treated with troglitazone had the highest absorbance level and degree of droplet accumulation compared to other groups. Statistical analysis figure shows that there were significant differences between each group in our study ($P \leq 0.05$) (Figure 3).

Treatment using insulin-sensitizing agent troglitazone also increased the transcriptional activation of PPAR- γ expression in 3T3-L1 adipocytes. Cells treated by troglitazone had a higher PPAR- γ mRNA level (1.9 fold) than those treated by adipogenic induction media without

troglitazone (1.4 fold). In contrast, 3T3-L1 cells un-treated at all with adipogenic induction media only had 1.0 fold mRNA level (Figure 4). However, PPAR- γ mRNA level

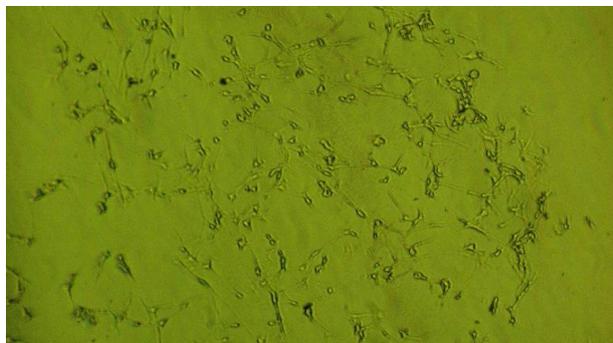
was found significantly different only between cells treated with adipogenic media plus troglitazone and non-treated at all with adipogenic media ($P \leq 0.05$).



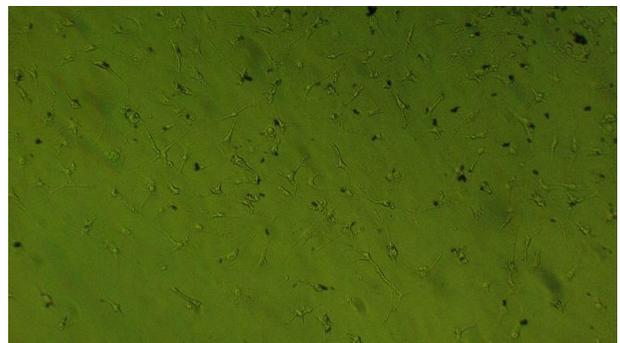
Day 1. Non-induction cells



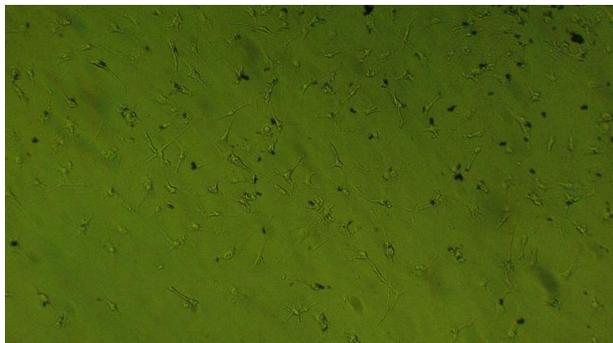
Day 1. Non-induction cells



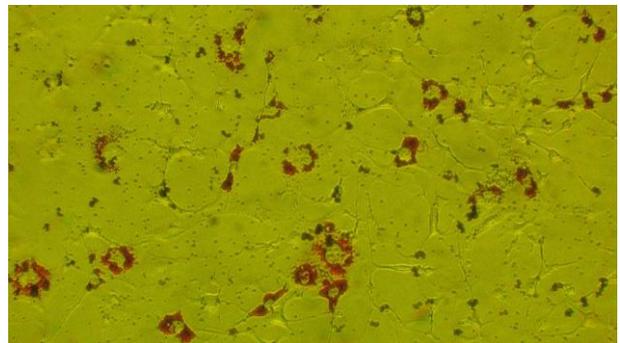
Day 4: 1 day after induction with media without troglitazone



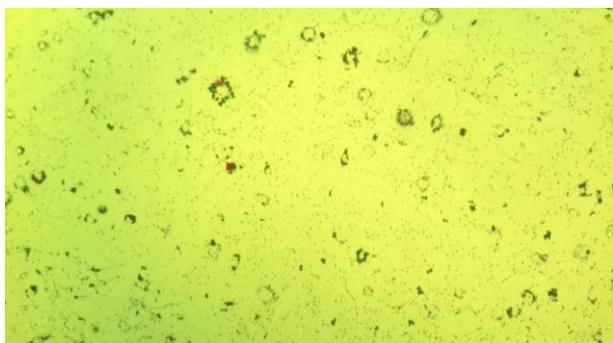
Day 4: 1 day after induction with media containing troglitazone



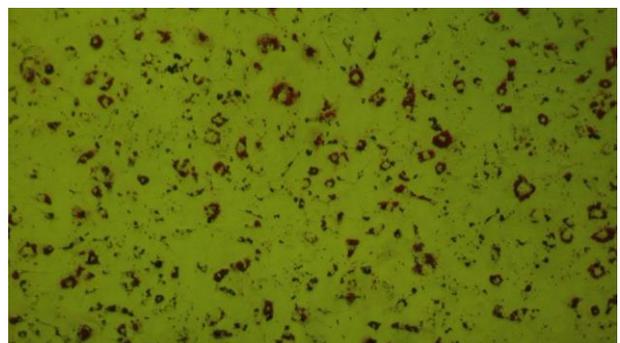
Day 6: 1 day after induction with 1 ppm insulin



Day 6: 1 day after induction with 1 ppm insulin



Day 12: End of differentiation time



Day 12: End of differentiation time

Figure 2. Oil red O staining result to the cells treated without induction media (left) and with induction media plus troglitazone (right) (Scale bar: 100 μ m).

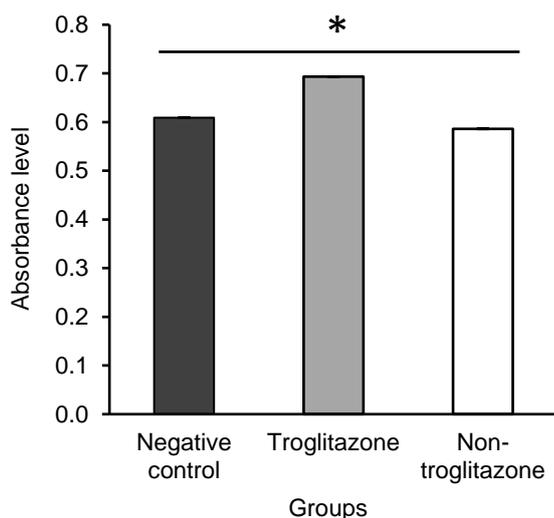


Figure 3. Absorbance level of 3T3-L1 adipose cells treated with no induction media (negative control), induction media with troglitazone, and without troglitazone. (* $P \leq 0.05$ for each group)

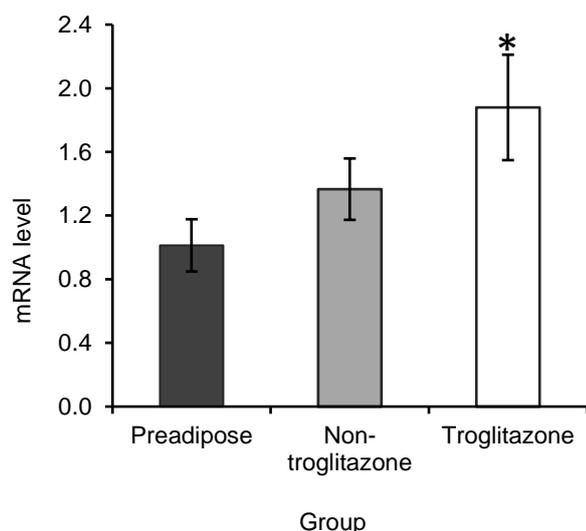


Figure 4. Effect of troglitazone on PPAR- γ mRNA levels in 3T3-L1 adipose cells. (* $P \leq 0.05$ compared to the negative control)

This possibly happened because cells treated only by adipogenic induction media contained insulin. Insulin act as key hormones involved in the control of adipocyte differentiation and fat metabolism in mature cells. Its anabolic effects (antilipolytic action, stimulation of glucose uptake) lead to fat accumulation and adipose tissue development. Moreover, this hormone also promotes change in the expression of important genes in the metabolic pathways of glucose and fatty acids in adipocytes, such as PPAR- γ (Rieusset et al. 1999).

Additionally, replenishment of the adipogenic induction media containing insulin with troglitazone caused the highest expression of PPAR- γ than the other cells. Troglitazone acts as peroxisome proliferator-activated receptor- γ agonist. Activation of this transcriptional factor requires a co-activator such PGC-1 to activate the signal transduction on some gene targets which is functional in adipocyte differentiation and, hence, lipid accumulation (Martinez et al. 2010; Lee and Ge 2014). In addition, PPAR- γ , together with C/EBP, are regarded as the master regulator of fat cell differentiation which increase efficacy and kinetics of adipocyte and suppress TNF α -mediated inhibition of adipocyte differentiation (Hamm et al. 2001; Min et al. 2014; Ghoniem et al. 2015). Meanwhile, other *in vitro* studies also found that the presence of troglitazone in 3T3-L1 adipocyte cell also caused these cells to become insulin responsive, with an absolute rate of insulin-stimulated glucose uptake increasing under those conditions (Kang et al. 2013)

CONCLUSION

In conclusion, this study demonstrates the ability of troglitazone in adipogenic induction media to transform 3T3-L1 preadipocytes into adipocytes on the histological and molecular level. Additionally, further research is required to observe this antidiabetic effect to other adipogenesis transcriptional factor such as, C/EBP or downstream genes regulated by PPAR- γ , such as GLUT4.

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