[UCI]I804:24011-200000241403

February, 2011
Thesis for Master Degree

Troglitazone induced cell cycle inhibition and apoptosis in human pharyngeal squamous carcinoma cells

Chosun University Graduate School
Department of Dentistry
Do Thi Anh Thu

2011년 2월

석사학위 논문

트로글리타존에 의한 두경부 암세포의

세포주기억제 및 세포사멸 연구

조선대학교 대학원

치의학과

도티안트

트로글리타존에 의한 두경부 암세포의

세포주기억제 및 세포사멸 연구

Troglitazone induced cell cycle inhibition and apoptosis

in human pharyngeal squamous carcinoma cells

2011년 2월 25일

조선대학교 대학원

치의학과

도티안트

Troglitazone induced cell cycle inhibition and apoptosis in human pharyngeal squamous carcinoma cells

지도교수 유 훈

이 논문을 약학 석사학위신청 논문으로 제출함

2010년 10월

조선대학교 대학원

치의학과

도티안트

Do Thi Anh Thu 의 석사학위 논문을 인준함

위원장	조선대학교 교 수	안 상 건	인
위원	조선대학교 교 수	방 일 수	인
위원	조선대학교 교 수	유 훈	인

2010년 11월

조선대학교 대학원

CONTENTS

CONTENTS vi

LIST OF FIGURES viii

국문초록 ix

ABSTRACT 1

1. Introduction 3

2. Materials and Methods 9

 2.1 Chemical 9

 2.2 Cell culture 9

 2.3 MTT assay 9

 2.4 Cell cycle analysis by Flow cytometry 10

 2.5 Apoptotic cells analysis 10

 2.6 Detection of DNA fragmentation by agarose gel electrophoresis 11

 2.7 Western blot analysis 11

 2.8 Determination of cytochrome C release from Mitochondria 11

 2.9 Real-time PCR 12

3. Results 13

3.1 PPARγ agonists effected on cell viability 13

3.2 Troglitazone induced apoptosis in FaDu cells 15

3.3 Effect of Troglitazone on cell cycle regulation 17

3.4 Troglitazone induced apoptosis via caspase cascades and mitochondrial pathway 22

3.5 Troglitazone induced apoptosis was PPAR gamma dependent. 26

3.6 Role of MAPK signaling in Troglitazone induced cell death 29

4. Discussion 31

5. References 37

ACKNOWLEDGEMENTS 42

LIST OF FIGURES

Figure 1. Effect of PPARγ and PPARα agonists on FaDu cell viability. 14

Figure 2. Cell morphology after treatment with Troglitazone 15

Figure 3. DNA fragmentation 16

Figure 4. Annexin-V binding assay (apoptotic cells analysis) 17

Figure 5. Cell cycle analysis 19

Figure 6. p27 expression 20

Figure 7. p21 expression 20

Figure 8. cyclin D1 and cdk4 expression 21

Figure 9. cyclin E1 and cdk2 expression 21

Figure 10. cyclin B1 and cdk1 (cdc2) expression 22

Figure 11. Cleaved form of caspase-3 expression 23

Figure 12. Cleaved form of caspase-7 expression 23

Figure 13. Inactive PARP protein expression 24

Figure 14. cytochrome C release 25

Figure 15. Expression of apoptosis related proteins 25

Figure 16. Gene expression of Bcl-2 family proteins 26

Figure 17. PPARγ protein expression 27

Figure 18. Gene expression of PPARs 28

Figure 19. Growth inhibitory of Troglitazone was PPARγ dependent 29

Figure 20. Expression of MAPK kinase proteins 30

국문초록

트로글리타존에 의한 두경부 암세포의

세포주기억제 및 세포사멸 연구

도티안트

지도교수 : 유훈

치의생명공학과

조선대학교 대학원

Troglitazone (Rezulin)은 thiazolidinedione 화합물로써 성인 인슐린 비의존성 당뇨병의 치료제였었으나 심각한 간세포 독성 때문에 2000년 이후 판매가 중지되었다. 최근 Troglitazone이 세포주기 조절과 세포사멸 유도와 관련된 신호경로를 변화시킴으로써 다양한 암세포들의 사멸을 일으키는 것으로 보고되었다. 본 논문에서는 사람의 FaDu 두경부 암세포를 사용하여 세포생존과 사멸기전에 관련된 Troglitazone의 세포영향을 연구하였다. Troglitazone은 세포사멸을 초래하며 농도 의존적으로 세포성장을 억제하였다 (IC_{50}=150 μM). 세포 성장의 억제는 G2/M에서 세포분열을 막는 것과 연관있으며다양한 세포주기 조절인자 및 억제자들의 발현양에 변화를 일으켰다. Western blotting 실험을

통해 Troglitazone이 세포주기에서 음성조절자인 p27^{Kip1}의 발현을 증가시키는 반면, cdc2 (cdk1), cdk2, cdk4, cyclin B1, cyclin D1, cyclin E1의 발현을 억제시키는 것을 확인하였다. 세포사멸은 caspase-3와 caspase-7의 활성을 유도하였다. Troglitazone은 세포질에서 분비되는 proapoptotic 단백질인 Bax, Bad뿐만 아니라 세포질에서의 cytochrome C농도를 증가시켰으며 미토콘드리아 경로에 의한 세포사멸을 PPARγ 의존적으로 유도하였다. 뿐만 아니라 Troglitazone 처리는 빠른 시간 내에 p38 활성 억제와 세포외 신호조절인산화효소(ERK)의 발현을 증가시켰다. 결론적으로, Troglitazone은 PPARγ 의존적으로 세포성장의 억제와 세포사멸의 유도를 통해 FaDu 세포의 성장을 억제시켰다.

ABSTRACT

Troglitazone induced cell cycle inhibition and apoptosis in human pharyngeal squamous carcinoma cells

Do Thi Anh Thu

Supervisor: Prof. Hoon Yoo, Ph.D

Department of Pharmacology and Dental Therapeutics

Graduate School of Chosun University

Troglitazone (Rezulin), a thiazolidinedione compound, was an antidiabetic agent that was originally approved to treat patient with adult-oneset non-insulin-dependent diabetes. However, Troglitazone was withdrawn from the market in 2000 due to its severe hepatotoxicity. Recent studies showed that Troglitazone caused cell death in a variety of tumor cell lines by altering signaling pathway associated with cell cycle regulation and apoptosis induction. In this study, we investigated the cellular effects of Troglitazone on cell survival and death mechanism in FaDu human pharyngeal squamous carcinoma cells. Troglitazone was dose-dependently toxicity on cell growth with IC_{50} at 150 μM and induced apoptosis. The growth inhibition was linked to the cell cycle arrest at G2/M checkpoint with two folds increase of DNA content at G2/M (from 14.34% to 28.03%). Cell cycle inhibition was further confirmed by altered expression of various cell cycle regulators and inhibitors. Western blotting studies showed that Troglitazone suppressed the expression of cdc2 (cdk1), cdk2, cdk4, cyclin B1, cyclin D1, cyclin E1, while upregulated the expression of $p27^{Kip1}$, a negative regulator of cell cycle. Apoptosis induction was confirmed by DNA fragmentation and the activation of

caspase-3 and caspase-7. Troglitazone increased the gene level as well as protein expression of pro-apoptotic protein Bax, Bad and cytochrome C release into cytoplasm, suggesting that Troglitazone induced apoptosis was related with mitochondrial pathway. Incubation of FaDu cells by Troglitazone led to the up-regulation of PPAR gamma (PPARγ) gene expression and this effect was PPARγ dependent. Further more, Troglitazone treatment up-regulated the expression of extra-cellular signal-regulated protein kinase (ERK) at early time points and inhibited p38 activation. Taken together, Troglitazone inhibited the growth of FaDu cells via the inhibition of cell growth and the induction of apoptosis in a PPARγ dependent manner.

1. Introduction

Squamous cell carcinoma of the head and neck affects more than 40,000 people each year in the U.S., and at least 13,000 people each year die of this disease. In many countries, oral cancers are one of the leading causes of cancer incidence, and a major cause of morbidity and mortality [1]. Unfortunately, the mortality rates for this disease have not improved in the past 40 years despite advances in the drug treatment and surgical reconstruction [3]. Head and neck cancer is a malignant tumor that appears in or around the throat, larynx (voice box), nose, sinuses, and mouth. There are five main types of head and neck cancer, named by the part of the body where they begin. The larynx is a tube-shaped organ in the neck that is important for breathing, talking, and swallowing. It is located at the top of the windpipe, or trachea. Meanwhile, the hypopharynx (also called the gullet) is the lower part of the throat that surrounds the larynx. A human epithelial cell line, FaDu, from a squamous cell carcinoma of the hypopharynx has been isolated and established from a patient from Calcutta, India [30]. About 95% of all cancers of the larynx and hypopharynx are of the squamous cell carcinoma type, meaning that they originated from the flat squamous cells in the lining of the organs. The vast majority of patients with head and neck squamous cell carcinoma (HNSCC) had a history of tobacco and alcohol use. The risk of oral cancer increased as a function of both intensity and duration time of each exposure, indicating that alcohol and tobacco act synergistically to promote cancer development.

Troglitazone is one of members of Thiazolidinediones (TZDs), the class of antidiabetic drugs which include Pioglitazone, Rosiglitazone, Ciglitazone [2]. Troglitazone (the drug Rezulin) was approved for the treatment of type II diabetes by the Food and Drug

Administration (FDA, 1997). Approximate two million patients were treated with Troglitazone from 1997 through the end of February 2000 until Troglitazone was withdrawn from the market, due to numerous reports of liver failure associated with Troglitazone using [21]. In addition to the insulin sensitization action, Troglitazone, one of the PPAR gamma (PPARγ) agonists, has been shown to suppress tumor development in several *in vitro* and *in vivo* models and was investigated as anti-cancer therapeutic drug for the most common types of cancers including, lung, breast, and colon [7, 10, 22, 36]. Among the proposed mechanisms for the anti-tumor effects of Troglitazone, apoptosis induction, cell cycle arrest, and differentiation have been extensively reported [2]. Interestingly, some of the observed anti-tumor effects were independent of PPARγ activation. Therefore, the principal PPARγ dependent and independent mechanisms need to be explored by its exertion on anti-tumor effects. PPARγ agonists bind with high affinity to the PPARγ, a subtype of Peroxisome Proliferator Activated Receptors (PPARs). Upon activation, PPARγ forms a hetero-dimer with the Retinoid X Receptor (RXR) and binds to PPAR Response Elements (PPRE) that regulates the transcription of select target genes [2]. There are more than seventy PPAR target genes with PPREs [2] and this list of PPAR regulated genes is likely continue to grow. Furthermore, the complexity of PPARγ regulated gene expression is enhanced by various co-activators and co-repressors that are recruited to the transcriptional complex by the activated PPARγ hetero-dimer. There can be numerous downstream consequences of binding to PPARγ, including altered lipid and glucose metabolism and changes in the expression of genes that control apoptosis.

Apoptosis is an essential part of life for multi-cellular organisms that plays an important role in development and tissue homeostasis. During development, many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpting organs and tissues. Apoptosis and proliferation are linked by cell cycle regulators, and

apoptotic stimuli affect both cell proliferation and death. Cells respond to the cell damage or stress by controlling the cellular proteins of cell cycle and apoptosis. In mammals, the cell cycle consists of five distinct phases: gap phase G0, in which cells remain in a quiescent or resting state; G1 and G2, during which RNA synthesis and protein synthesis occur; S phase during which DNA is replicated; and M-phase, in which cells undergo mitosis and cytokinesis. The five distinct phases of the cell cycle are controlled by specific cyclin/cdk complexes. The cyclin/cdk complexes in turn are negatively regulated by CIP/KIP and INK4 family members. Specific cyclin/cdk complexes are activated and thereby modulate a distinct phase(s) of the cell cycle. cyclin D/cdk4(cdk6) complexes initiate progression through G1 by phosphorylating substrates, such as members of the retinoblastoma (Rb) family of pocket proteins, and eventually lead to the activation of transcription of genes necessary for DNA synthesis and subsequent cell cycle progression. The cyclin E/cdk2 complex is important in the G1/S transition, where expression level is maximum at the restriction point; cyclin A/cdk2 is important during S-phase progression; and cyclin A/cdc2 (also known as cdk1) and cyclin B/cdc2 are important for progression through G2 and M. The regulation of cyclin synthesis and degradation, in addition to cdk activity levels, are tightly controlled and is a key to the ordered progression through the mammalian cell cycle [32].

PPARγ activation may reduce tumor development through the arrest of cancer cell proliferation, effects on cell cycle checkpoints or growth factor inhibition. Several studies demonstrated that PPARγ activation suppressed proliferation rates in certain cancer types [2]. For instance, PPARγ activated by ligand Troglitazone induced cell cycle arrest linking G1-phase through up-expression of the cyclin dependent kinase, p21 and p27 proteins, and the hypo-phosphorylation of retinoblastoma protein of Hepatocellular carcinoma cell lines [16]. Exposure to Troglitazone for 24 h caused G0/G1 cell cycle arrest in many cell lines [8, 16, 29,

36]. Cyclin D1 overexpression and/or amplification were common features of several human cancers, promoting G1 phase progression [6]. Troglitazone treatment not only decreased protein levels of cyclin D1, but also reduced proliferating cell nuclear antigen, pRb, cdk4 and increased the cyclin dependent kinase inhibitors p21 and p27 in a time dependent manner [8]. A family of negative regulators is the cdkIs. CIP (cdk-Interacting protein)/KIP (Kinase Inhibitor Protein) families includes p21 (CIP1/WAF1/SDI1), p27 (KIP1), and p57 (KIP2). They interact with and inhibit the kinase activities of cyclin E/cdk2, cyclin D/cdk4, cyclin D/cdk6, cyclin A/cdk2 and cyclin B/cdc2 complexes [32], resulting in cell cycle arrest. Troglitazone mediated growth inhibition was associated with decreased levels of G1 cdks (cdk 2, cdk4) and D type cyclins and increased p21 and p27 protein levels in MDA-MB-231 cells [10].

Caspase cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptosis signals [34]. Activation of apoptotic signaling is achieved by either an extrinsic or an intrinsic pathway. The extrinsic pathway is triggered by ligation of cell surface death receptors with their specific ligands and then followed by recruiting inactive caspase-8 to form a Death Inducing Signaling Complex (DISC), resulting in the activation of caspase-8. Active caspase-8 then cleaves and activates caspase-3 and caspase-7 [23]. The intrinsic pathway is mediated by diverse apoptotic stimuli such as growth factor deprivation, oxidants and DNA-damaging agents, which converge at the mitochondria. Release of cytochrome C from the mitochondria to the cytoplasm initiates a caspase cascade. Cytosolic cytochrome C binds to Apoptosis Protease Activating Factor 1 (Apaf-1) and pro-caspase-9, generating an intracellular DISC-like complex known as “apoptosome”. Within the apoptosome, caspase-9 is activated, leading to processing of caspase-3, -6 and caspase-7 [39]. Besides, apoptotic process is affected by many other signaling pathways including MAPK

pathway. The Mitogen Activated Protein Kinases (MAPKs) are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, such as stress [33]. At least three MAPK families have been characterized: Extracellular signal Regulated Kinase (ERK), Jun kinase (JNK/SAPK) and p38-MAPK. It has been known that ERKs are important for cell survival. ERKs are activated by the phosphorylation of a threonine and a tyrosine residue by the dual specificity MAPK kinases, MEK1 and MEK2, which relay Ras and Raf signal transduction to ERK1 and ERK2. ERK1 and ERK2 are activated by various growth factors and induce transition from the quiescent state into the cell cycle. However, ERKs are also involved in the stress response and cell death in some case. JNKs and p38-MAPK are also stress responsive and involved in apoptosis [33]. JNK has been implicated in pro-apoptotic or anti-apoptotic effect or no role in cell death. The role of the JNK pathway in apoptosis may depend on cell type, the nature of the death stimulus, the duration of its activation and the activity of other signaling pathways, including NF-κB and p53 [39]. p38-MAPKs are phosphorylated and activated by dual kinases MKK3 and MKK6 at threonine and tyrosine regions. Similar to JNK pathways, the involvement of p38-MAPK in apoptosis is also diverse [33].

The molecular basis for the anti-tumor actions of Troglitazone has been incompletely elucidated. Recently, Troglitazone has been shown to induce apoptosis in a wide variety of cells including both cancer cells and normal cells [18, 27, 29, 38]. This may occur through the mitochondrial pathway involved in cytochrome C release [21]. Mingyue Li et al demonstrated the apoptosic induction in human non small lung cancer cells by Troglitazone which was PPARγ and ERK dependent [24]. In contrast, Troglitazone and Rosiglitazone induced apoptosis in rat vascular smooth muscle cells through an ERK1/2 independent pathway [12]. Troglitazone induced apoptosis in HepG2 cells that preceded by activation of c-Jun N-terminal

protein kinase (JNK) and p38 mitogen activated protein kinase (p38 kinase), and increased levels of proapoptotic protein (Bad, Bax), cytochrome C release and of Bid cleavage [26].

In this study, Troglitazone was chosen to understand the mechanism of cell signal as well as the response of the cells in the cell cycle arrest and apoptosis phenomena when FaDu pharyngeal cancer cells was triggered by this drug.

2. Materials and Methods

2.1 Chemical

Troglitazone (Cayman Chemical Industry, USA) was dissolved in dimethy sulfoxide (DMSO) and then diluted to appropriate concentrations with culture medium. The final concentration of DMSO in the culture medium was less than 0.1 %. GW9662, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide were purchased from Sigma-Aldrich Chemical (St.Louis, MO, USA). Antibodies p21, PPARγ, cyclin D1, Cleaved caspase-3, cleaved caspase-7 were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA), p38, JNK1, β-actin, cyclin E1 from AbFrontier (Seoul, 120-750, Korea), Cleaved p85 from Epitomics Inc. (California, USA), cyclin B1, cdc2, cdk4, p27, Bax, Bad, Bcl-2, and second antibodies from Santa cruz biotechnology. Inc. (USA). All other chemicals were of the highest commercial grade available.

2.2 Cell culture

FaDu cells were cultured in MEM medium (Welgene Inc., Korea) containing 10 % fetal bovine serum (Welgene Inc., Korea), and 1X antibiotics (Invitrogen, USA). Cells were grown at 37 ℃ in an atmosphere of 95 % air and 5 % CO_2. The media was changed every 24 hours.

2.3 MTT assay

Cell proliferation was detected by 3-(4-, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) assay. Cells were seeded on a 96-well plate (2×10^4 cells/100 μL/well) for 24 hour until 70 to 80 % confluence. Cells were treated with various concentrations of Troglitazone (50, 100, 150, 200 μM), in the present of absence of inhibitor agents and using DMSO with similar concentration as control group. After 24 hours, 200 μl

MTT working solution 1X (stock solution 5 mg/ml in PBS, Sigma, USA) was added and incubated at 37 ℃ for 4 h. 200 μl of lysis buffer (10 % sodium dodecylsulfate, 0.1 N HCl) was added to each well, which was oscillated for 90 min until the crystals were dissolved completely. Absorbance (A) was detected with an enzyme calibrator at 540 nm. Cell viability = (A of study group/A of control group) × 100 %. The experiment was repeated three times and the data was analyzed using GraphPad Prism software, version 4.

2.4 Cell cycle analysis by Flow cytometry

Cells were seeded onto a 6-well plate (1.2 x 10^6 cells /dish) and allowed to grow to 80 % confluence for 16 hours. After treatment with or without Troglitazone, both floating and adherent cells were collected. Cells were suspended and then fixed with ice-cold 75 % ethanol (250 μl 1X PBS and 750 μl absolute ethanol) at -20 ℃ for 2 hours, followed by washing and staining with 250 μl PI solution (stock 20 μg/mL propidium iodide in PBS) in the presence of 200 μg/mL of DNase-free Rnase A for 15 min at 37 ℃ in the dark. DNA content was analyzed in Beckman-Coulter system (Olympus, USA) with excitation wavelength of 488 nm.

2.5 Apoptotic cells analysis

Cells were seeded and treated with Troglitazone. Using Trypsine to harvest and wash cells in PBS. Cells were suspend and stained with 5 μl Alexa Fluor® 488 annexin V and 2 μl PI working solution following supplied protocol of Vybrant® Apoptosis Assay Kit #2 (Invitrogen, USA). As soon as possible, cells were analyzed by flow cytometry by Beckman-Coulter system (Olympus, USA). After staining a cell population stains, apoptotic cells show green fluorescence, dead cell show red and green fluorescence and live cell show little or no fluorescence. These populations can be easily distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation.

2.6 Detection of DNA fragmentation by agarose gel electrophoresis

DNA fragmentation manifested as laddering in agarose gel was examined. After drug treatment, the cells pellet was lysed in lysis buffer (200 mM HEPES, pH 7.5, 2 % (v/v) Triton X-100, 40 mM NaCl and 20 mM EDTA, pH 8.0) on ice for 30 min, and treated with RNase A and proteinase K for 1.5 hours. Following DNA precipitation with isopropanol and ammonium acetate at 25 °C for 30 min, DNA was dissolved in TE buffer, electrophoresed in 1.2 % agarose gel for two hours under 50 voltages. Gel image was taken using Biorad UV photographer.

2.7 Western blot analysis

Troglitazone-treated FaDu cells were prepared in a cell lysis buffer. The protein concentration was determined by BCA Protein assay kit (Perbio Biotechnolody, USA). Protein samples (30, 50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes, immunoblotting were performed by incubating with primary antibodies (Cell signaling, USA) and horseradishperoxidase - labeled secondary antibodies (Santa Cruz Biotechnology) were added. Proteins on membrane were detected by Welgene detection kit (Welgene, Korea). Duration of film exposure was adjusted depending on the proteins detected. Density of protein bands were analyzed using Biorad image master software. The value was normalized with the band intensity of beta actin protein in the same lane. Control samples were used as standards (1 unit of density) to calculate the normalized band intensity of the others.

2.8 Determination of cytochrome C release from Mitochondria

Cytochrome C that released form mitochondria into cytoplasm was detected using BMS263 kit (Bender Medsystems, USA). Cells were seeded in 6-well plate with 5 x 10^5 cells/well. The cells were incubated with 75 or 150 μM of Troglitazone for 24 hours. Cells were harvested by Trypsine, and cell pellets were re-suspended in 200 μl lysis buffer and incubated for one hour at room temperature with gentle shaking. The cell lysates were

centrifuged at 1000 g for 15 min at 4 ^{o}C. The supernatant from the final centrifugation was used as the cytosolic extract. For cytosolic cytochrome C assessment, an ELISA kit was used in accordance with the manufacturer's instructions. After color development had stopped, the absorbance at 450 nm was measured on the micro-plate reader. The data was analyzed using Microsoft Excel to produce the ratio of cytosol cytochrome C level to the total protein.

2.9 Real-time PCR

Total RNA was isolated from cells using TRI reagent (Ambion, USA) following the supplied protocol. Contaminated gemomic DNA in total RNA was removed by RNase-free DNase I digestion. The reverse transcription was performed in a total volume 50 µl using 1 µg of total RNA, Oligo(dT)$_{12\text{-}18}$ Primer and SuperScript^{TM} II Reverse Transcriptase (Invitrogen, USA). Primer for genes of interest were designed using Vector NTI Advance software (InforMax, Inc.,USA) and tested to ensure amplification of single discrete band with no primer dimmers. Primer sequences were as follows: h-GAPDH forward, 5'-CTCTGACTTCAACAGCGACA-3', h-GAPDH reverse, 5'-TCTCTCTCTTCCTCTTGTGC-3', Bcl2 forward, 5'-AGGAGCTCTTCAGGGACGG-3', Bcl2 reverse, 5'-CCAGGTGTGCAGGTGCC-3', Bad forward, 5'-TGGGCAGCACAGCGCTA-3', Bad reverse, 5'-CCAGGTGTGCAGGTGCC-3', Bax, PPARα, PPARβ, PPARγ. Real-time PCR was conducted using LightCycler® FastStart DNA Master SYBR Green I kit (Roche, German) by a LightCycler 1.5 Instrument (Roche, German). Each reaction was run in triplicate a contain 1 µl of cDNA template along with Master mix in final reaction volume of 20 µl. Program for Relative Quantification are: Pre-incubation were 10 min at 95 ^{o}C for 1 cycle; Amplification were 10 s (denaturation) at 95 ^{o}C, 10 s (annealing) at 60 ^{o}C and 10 s (extension) at 72 ^{o}C for 45 cycles; Melting curve were 15 s at 65 ^{o}C for 1 cycle; Cooling were 30 s at 40 ^{o}C. PCR products were analyzed by LightCycler software 3.5.3 for the results

3. Results

3.1 PPARγ agonists effected on cell viability

To evaluate the cytotoxic effects of PPARs agonists on cultured FaDu cells, FaDu cells were treated with various concentrations of Troglitazone or Clofibrate for 24 hours and the cell viability was measured by MTT assay. Troglitazone, PPARγ agonist, decreased cell viability in a dose dependent manner, whereas Clofibrate, PPARα agonist did not affect the cell viability (Fig. 1). At 50 and 100 μM Troglitazone, only 10 to 20 % of FaDu cells died after 24 hours of treatment, whereas very few cell died (< 1 %) in the DMSO treated control. The half maximal inhibitory concentration (IC_{50}) occurred at concentration of 150 μM.

Cell morphology was observed after exposing FaDu cells to 75 μM and 150 μM Troglitazone for 24 hours. The morphology of Troglitazone treated FaDu cells changed into small and thin shape whereas DMSO treated control showed no morphological change (Fig. 2). In the dose of 150 μM, Troglitazone inhibited cell growth and induced cell death. Cells were detached and suspended and so the floating dead cell bodies were observed in medium. Compared with the control group, live cells were regular shape in morphology and grew fully in patches. Cells treated with 75 μM for 24 hours were sloughed off the dish.

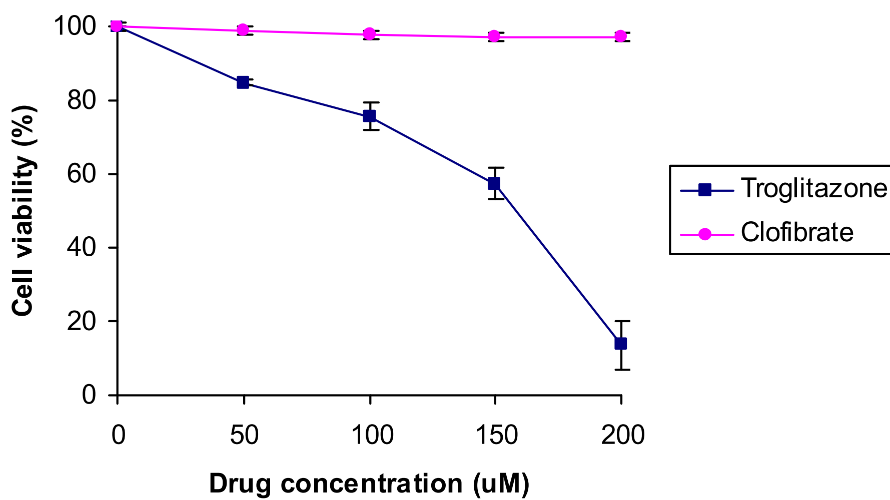
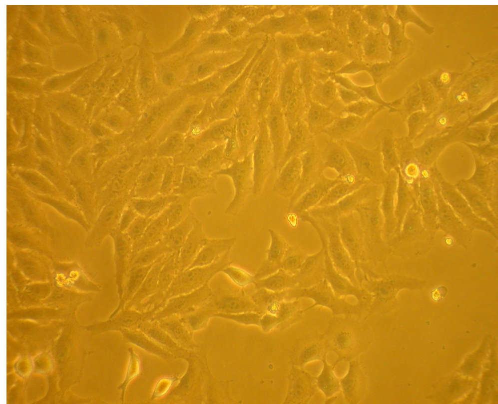
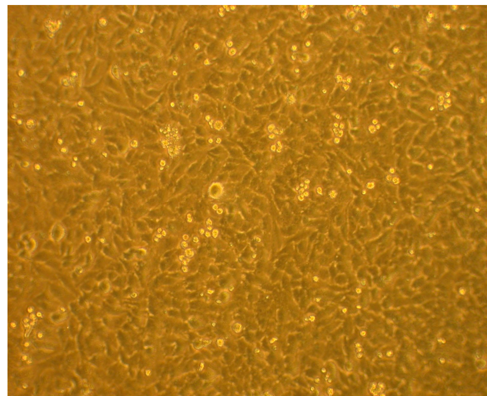


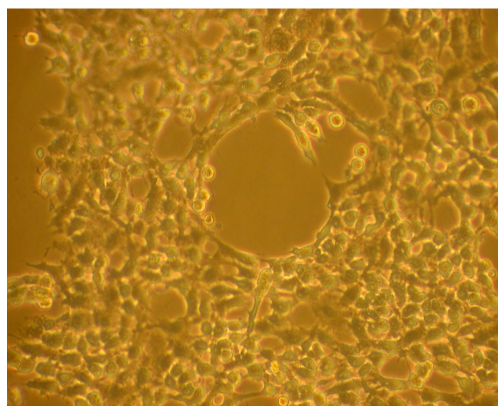
Fig.1. Effect of PPARγ and PPARα agonists on FaDu cell viability. Cells were treated with various concentrations of Troglitazone and Clofibrate for 24 hours. The cell death was assessed by MTT assay. Data are mean ± SD for 3 independent experiments.



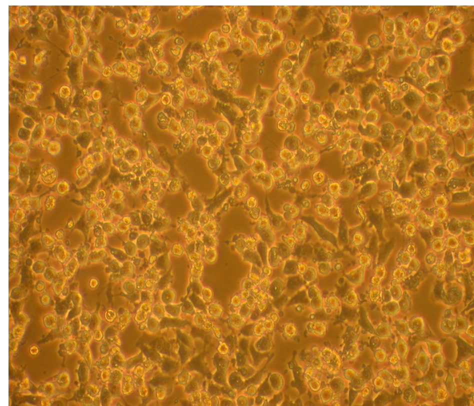
FaDu cells before treatment



FaDu cells 24 hours growing no treatment



FaDu cells treated with 75 μM Troglitazone for 24 hours



FaDu cells treated with 150 μM Troglitazone for 24 hours

Fig.2. Cell morphology study after treatment with Troglitazone 75 and 150 μM. There were significant differences in confluence between treatment group and control group, as shown by inverted microscopy (original magnification, X 100).

3.2 Troglitazone induced apoptosis in FaDu cells

Cell death occurring by apoptosis has several characteristic features, including blebbing of the cell surface, cell shrinkage, nuclear and DNA fragmentation, and the phagocytosis of the dying cells [21]. Loss of cell viability by Troglitazone may be mediated by induction of

apoptosis. To test this possibility, cells were treated with 150 μM Troglitazone for 24 hours and apoptosis evidence was collected.

One of markers on apoptosis is the caspase-activated deoxyribonuclease, an endonuclease that cuts DNA at sites exposed by histones and produces DNA fragments consisting of multimers of 182 bp. [11]. After treated with Troglitazone (75 μM and 150 μM), the genomic DNA of treated cells exhibited a characteristic ladder band after electrophoresis on agarose gel (Fig.3). To further confirm the induction of apoptosis by Troglitazone, cells were subjected to Cytometric analysis. Cytometric analyses of anexin V positive cells demonstrated a 40.51% increase in cells treated with Troglitazone as shown in right upper quadrants (Fig. 4). These data suggested that cell death by Troglitazone was largely attributed to apoptosis.

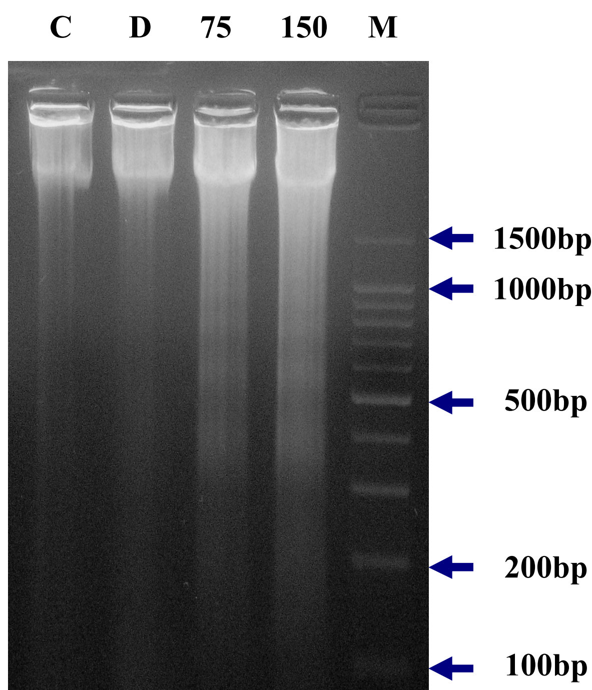
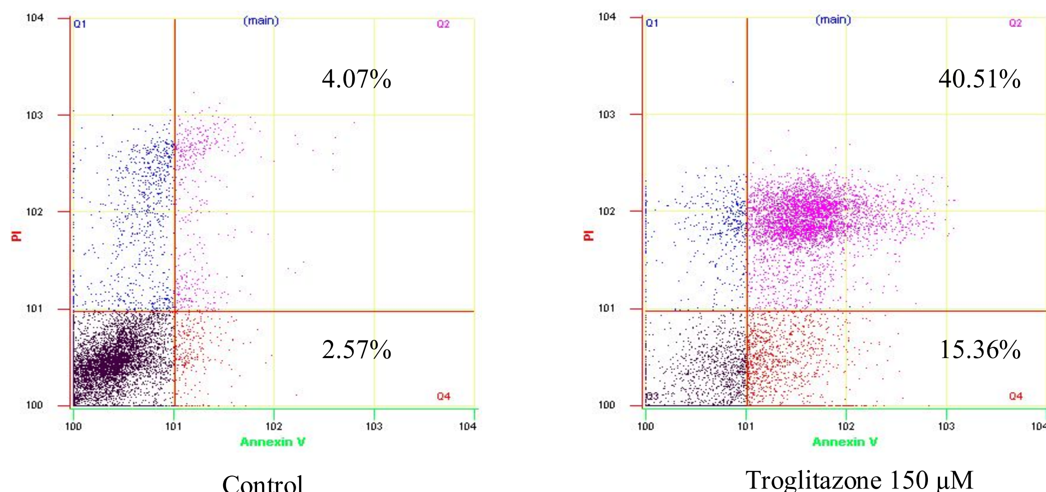


Fig.3. Agarose gel picture showing DNA fragmentation of FaDu cells after treatment with 75 μM and 150 μM Troglitazone for 24 hours. Lane C: the control, lane D: DMSO control, lane M: marker, lane 75 and 150: cells treated with 75 μM and 150 μM Troglitazone, respectively.



	Live cells	Early apoptosis	Late apoptosis
Control	85.28%	2.57%	4.07%
Troglitazone 150 µM	39.53%	15.36%	40.51%

Fig.4. Annexin-V binding assay. The percentage in right upper quadrants indicates the proportion of early/primary and late/secondary apoptotic cells, respectively.

3.3 Effect of Troglitazone on cell cycle regulation

To examine whether the inhibitory effect observed in the cell viability assay reflected a delay or arrest of cells, cells were treated with Troglitazone 75 µM and 150 µM for 24 hours. The cell cycle progression was evaluated by fluorescence activated cell sorting analysis after Propidium Iodide (PI) staining (Fig.5). In the FaDu cells treated with Troglitazone, the percentage of G1 phase decreased with the corresponding increase in S phase and G2/M phase. The number of cells at G2/M phase was dramatically increased from 14.34% to 28.03%, supporting that Troglitazone prolonged or arrested cells at G2/M phase.

Because the cell cycle is tightly regulated through a complex network of positive and negative regulatory molecules such as cyclin dependent kinase (cdks), cyclin and cdk inhibitors (cdkIs), we measured the expression of these molecules in protein level by western blotting. Protein extracts were prepared from the cells treated with 150 μM of Troglitazone at different time periods. Western blotting was performed using antibody against cdk4, cdk2, cdk1 (cdc2), cyclin D1, cyclin E1, cyclin B1, p27 and p21 inhibitor.

It is well established that p27 inhibits cyclin dependent kinase activation and formation of cdk/cyclin complexes [14]. In our study, after treating with 150 μM Troglitazone, p27 expression level was increased dramatically (Fig.6). p21 is the prototype of universal inhibitors of cdk enzyme activity. It is capable of inactivating cdk activity when expressed at high levels and inducing cell cycle arrest. The up-regulation of p21, an inhibitor of CIP/KIP inhibitors family, was in agreement with the low proliferation rate observed in FaDu cells. Troglitazone treatment dramatically increased p21 expression within 24 hours. However, the expression of p21 was maximal at 12 hours and then decreased at 24 hours (Fig.7).

The elevation of p27 and p21 inactivates cyclin D/cdk4, 6 and cyclin E/cdk2 complexes, resulting in cell cycle arrest. In our result, Troglitazone (150 μM) significantly altered the cyclin D1, cdk4, cyclin E1 and cdk2 levels in a time dependent manner. The level of cyclin D1 markedly decreased at 6 hours and disappeared after 24 hours (Fig.8). Under the same experimal condition, the amount of cdk4 and cdk2 changed relatively slowly and the overall expression was decreased after Troglitazone treatment (Fig.8, Fig.9).

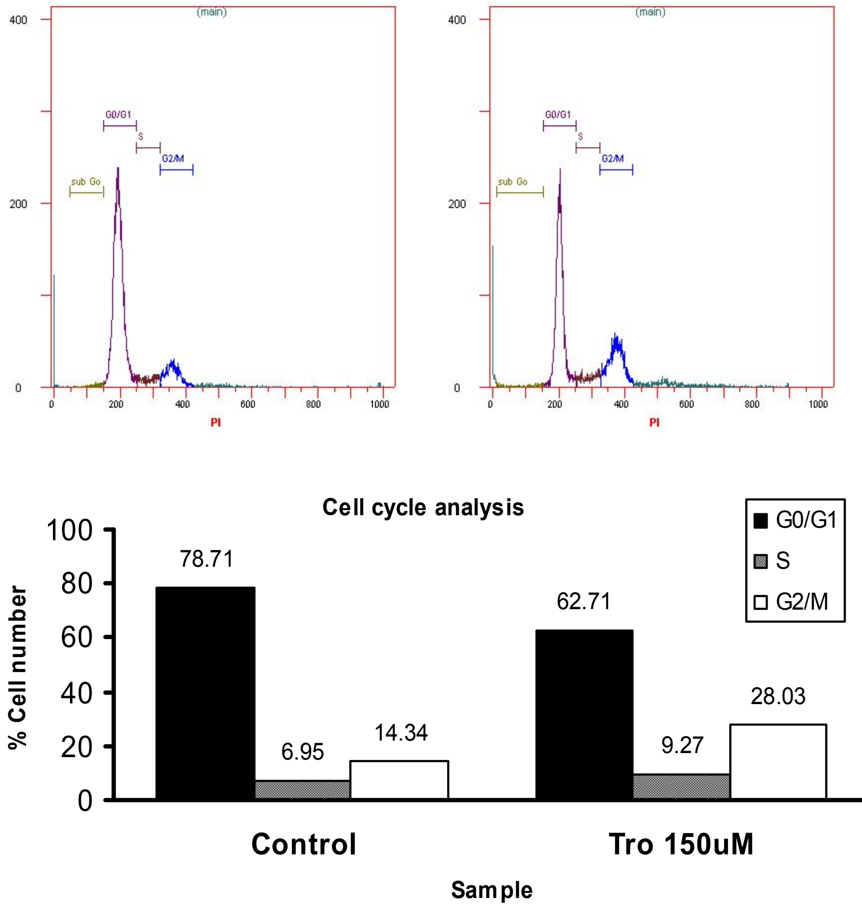


Fig.5. Histogram (upper) and % cell distribution (lower) of cell cycle in FaDu cells treated with Troglitazone. The cells were treated with 150 μM (data of 75 μM not shown) Troglitazone for 24 hours. The Troglitazone increased the G2/M phase proportion of cell cycle as analyzed by flow cytometry

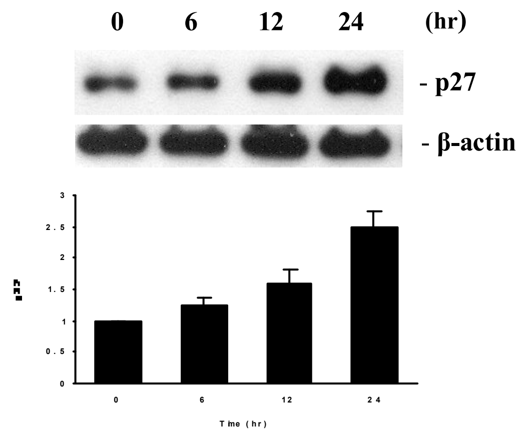


Fig.6. p27 expression after Troglitazone treatment in FaDu cells. The level of p27 protein after Troglitazone treatment was markedly increased time-dependently.

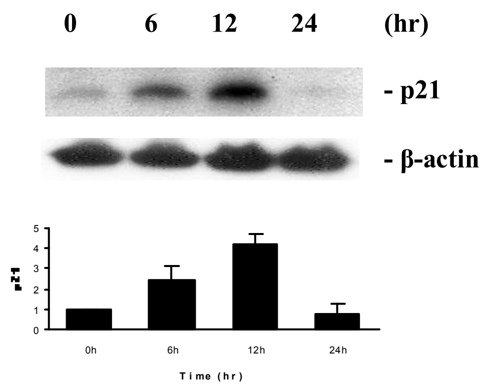


Fig.7. p21 expression after Troglitazone treatment.

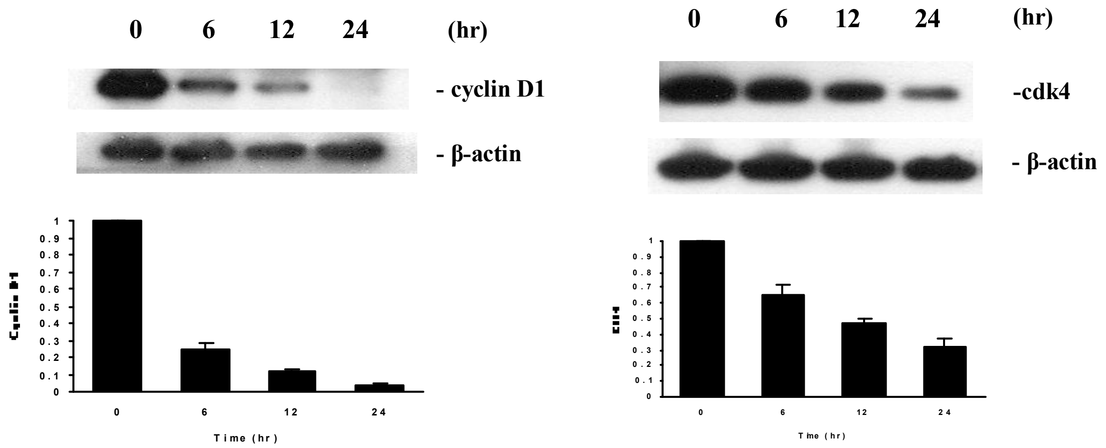


Fig.8. Expression of cyclin D1 and cdk4 after treated with Troglitazone (150 μM)

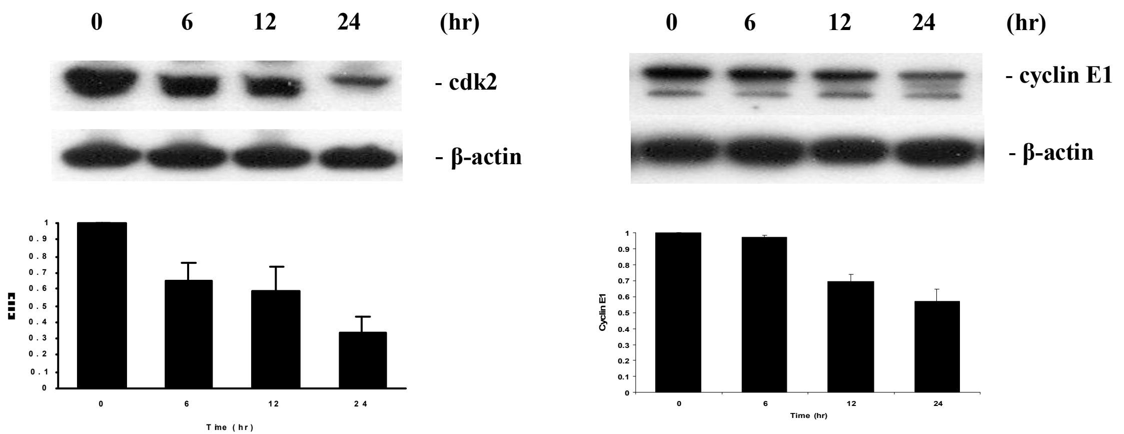


Fig.9. Expression of cyclin E1 and cdk2 after treated with Troglitazone (150 μM).

p53 and p21 are necessary to maintain G2 arrest following DNA damage, since tumor cells lacking these proteins enter into mitosis with accelerated kinetics. The mechanism of p53 dependent G2 arrest involves an initial inhibition of cyclin B1/cdc2 activity by p21 and a subsequent reduction of cyclin B1 and cdc2 protein levels [13]. In our data, the expression of

cyclin B1 and cdc2 were also decreased time-dependently after treated with Troglitazone (150 μM) (Fig.10). The result in the reduction of cdc2 and cyclin B1 suggests that interphase cdk/cyclin complexes drove cell cycle into and passed through the S phase, which is consistent with previous results of FACs analysis on cell cycle (Fig.5)

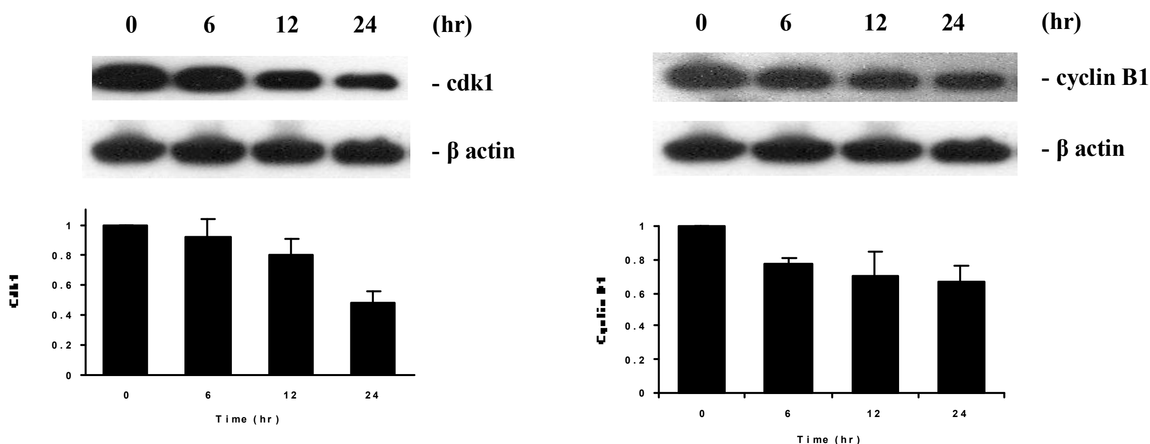


Fig.10. Expression of cdc2 and cyclin B1 after treated with Troglitazone (150 μM).

3.4 Troglitazone induced apoptosis via caspase cascades and mitochondrial pathway

A variety of apoptotic stimuli activate a unique set of cysteine proteases called caspases that cleave critical cellular proteins, leading to apoptosis. The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals [34]. Among the caspases, caspase-3 and caspase-7 have an executing and also amplificating roles in the apoptosis [24]. Our Western blot results showed that Troglitazone induced the cleavage of caspase-3, which was shown as a weak band (Fig.11). In addition, the cleavage of capase-7 was observed in a time-dependent manner in the cells treated with Troglitazone (Fig.12). The enzyme poly (ADP-ribose) polymerase (PARP) is an important

DNA repair enzyme and is one of the first proteins which are cleaved by caspase during apoptosis. The cleavage of PARP has been observed in almost all apoptotic models. The activation of caspase-7 suggested that it can subsequently cleave distinct cellular proteins such as PARP [poly (ADP-ribose) polymerase] [4]. In order to confirm it, we attempt to detect PARP protein. After Troglitazone treatment, the strong expression of inactive form (85kDa) was detected at 24 hours (Fig. 13).

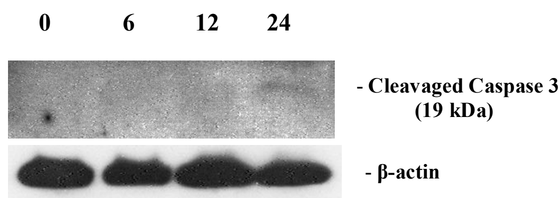


Fig.11. Cleaved form of caspase-3 after Troglitazone treatment

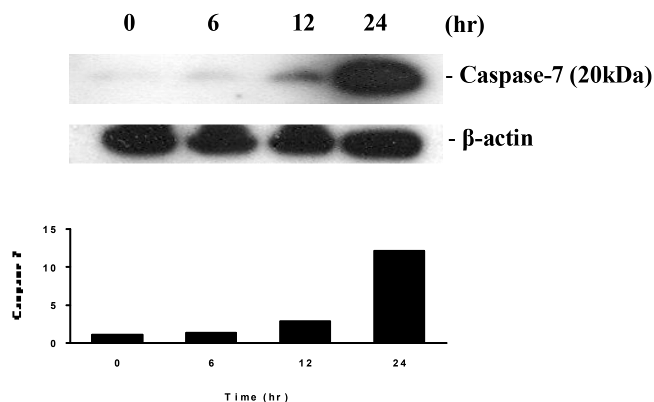


Fig.12. Cleaved form of caspase-7 after Troglitazone treatment.

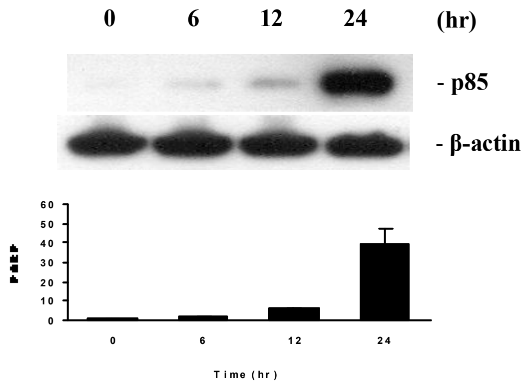


Fig.13. Expression of inactive PARP protein (p85)

The activation of caspase cascade was triggered apoptosis proteins related to mitochondrion-mediated procaspase-activation pathway [34]. The mitochondrial pathway of apoptosis begins with the permeabilisation of the mitochondrial outer membrane. The opening of the Permeability Transition (PT) pore triggers the dissipation of the proton gradient created by electron transport, causing the uncoupling of oxidative phosphorylation. The opening of the PT pore also causes water to enter the mitochondrial matrix, which results in swelling of the intermembranal space and rupturing of the outer membrane causing the release of apoptogenic proteins include cytochrome C. We investigated the release of cytochrome C from mitochondrial inner membrane into cytosol after treating FaDu cells with 75 μM and 150 μM Troglitazone (Fig. 14). PT pore dependent mitochondrial membrane permeabilisation is regulated by Bcl-2 family members, a family of apoptotic proteins. Some of these proteins (such as Bcl-2 and Bcl-XL) are anti-apoptotic, while others (such as Bad, Bax or Bid) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti-apoptotic Bcl-2 proteins [8, 28]. Therefore, we determined the levels of pro-apoptotic Bax, Bad

and their anti-apoptotic counterpart, Bcl-2 by immunoblotting analysis (Fig. 15).

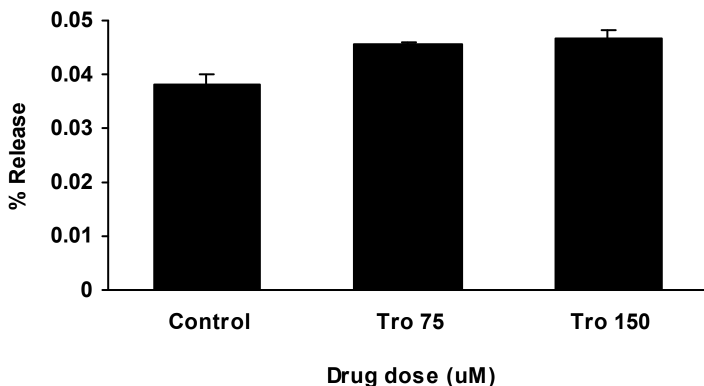


Fig.14. Cytochrome C release per total protein concentration. FaDu cells were treated with two concentration of Troglitazone, 75 μM and 150 μM.

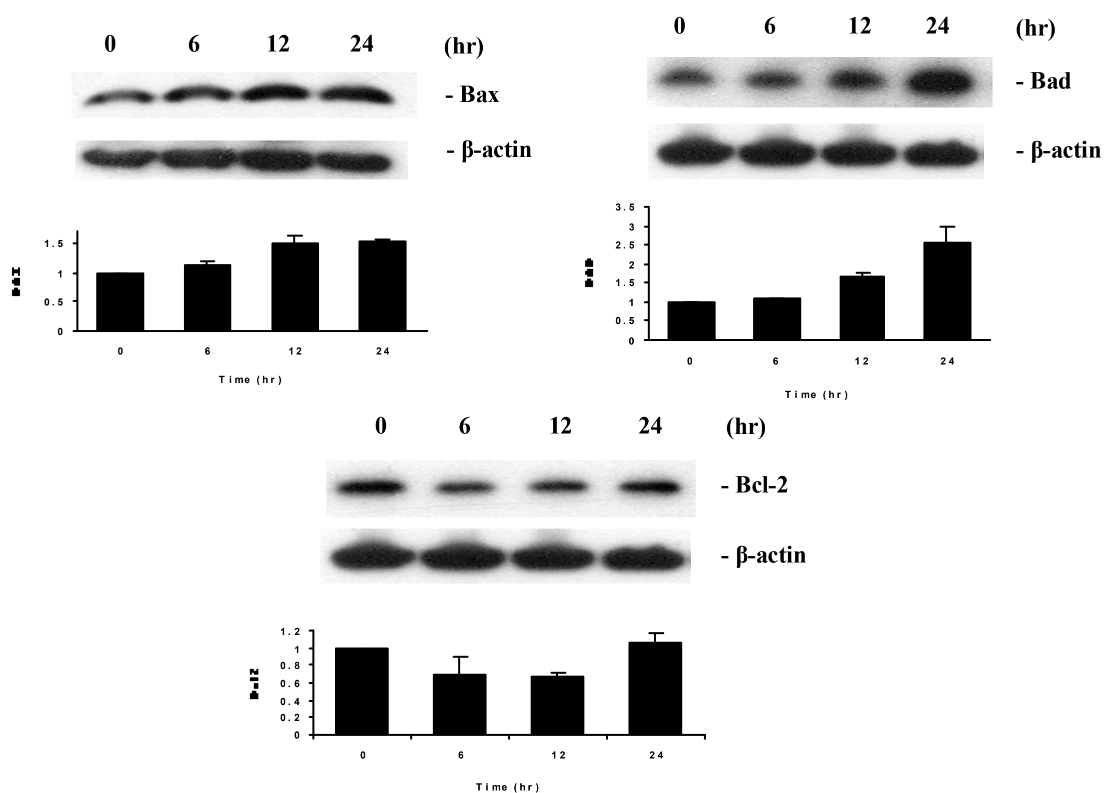


Fig.15. Western blots of apoptosis related proteins.

We found that Troglitazone significantly increased the expression of Bax and Bad proteins. The expression of Bcl-2 was decreased only by 12 hours. We also attempted to determine mRNA expression of three genes Bad, Bax and Bcl-2 (Fig.16). Gene expression of Bad or Bax increased approximately 3 times after treating with Troglitazone (150 μM) for 24 hours. In case of Bcl-2, there was only slight increase. The ratio of pro-apoptotic protein (Bad, Bax) to anti-apoptotic protein Bcl-2 was increased compared with control. These results supported the mitochondrial pathway in the process of programmed cell death by Troglitazone (Fig.16).

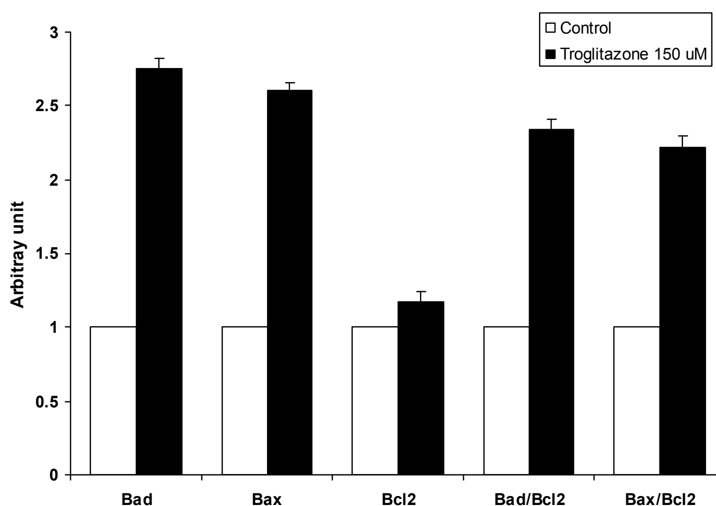


Fig.16. Effect of Troglitazone on Bcl-2 family gene expression in FaDu cells. mRNAs of Bcl-2 family member were determined by using a pair of corresponding primers correspondent.

3.5 Troglitazone induced apoptosis was PPAR gamma dependent.

The role of PPARγ in cell differentiation, cell cycle arrest and apoptosis attracts increasing attention. The chemo-preventive effect of PPARγ ligands on human cancers was dependent on PPARγ expression in the cancer cells such as both PPARγ mRNA and protein in the human gastric carcinoma cell line MGC803 [35]. We first examined whether Troglitazone

induces PPARγ expression in FaDu cells. Exposure to 150 μM of Troglitazone produced a time dependent increase of PPARγ (Fig.17). PPARγ protein, a molecular mass of approximately 55-57 kD, was expressed time dependently with the maximum intensity at 12 hours. The activation of PPARγ protein decreased at 24 hours, probably due to the degradation of mRNA gene. The induction of PPARγ expression by Troglitazone was further supported by the quantitative analysis of gene expression. Real-time PCR, used for mRNA quantification, allowed precise analysis of gene expression from low quantities of starting template. Troglitazone up-regulated PPARs family, particularly the PPARγ gene with 4 to 5 folds induction (Fig.18).

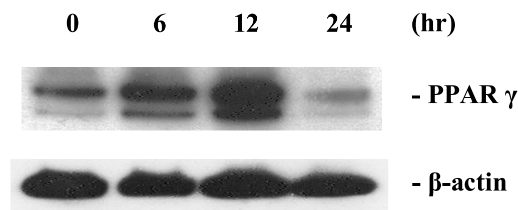


Fig.17. Effect of PPARγ agonist, Troglitazone (150 μM) on PPARγ expression in FaDu cells. PPARγ protein was determined by western blot analysis. Equal loading was confirmed by probing with antibodies against actin.

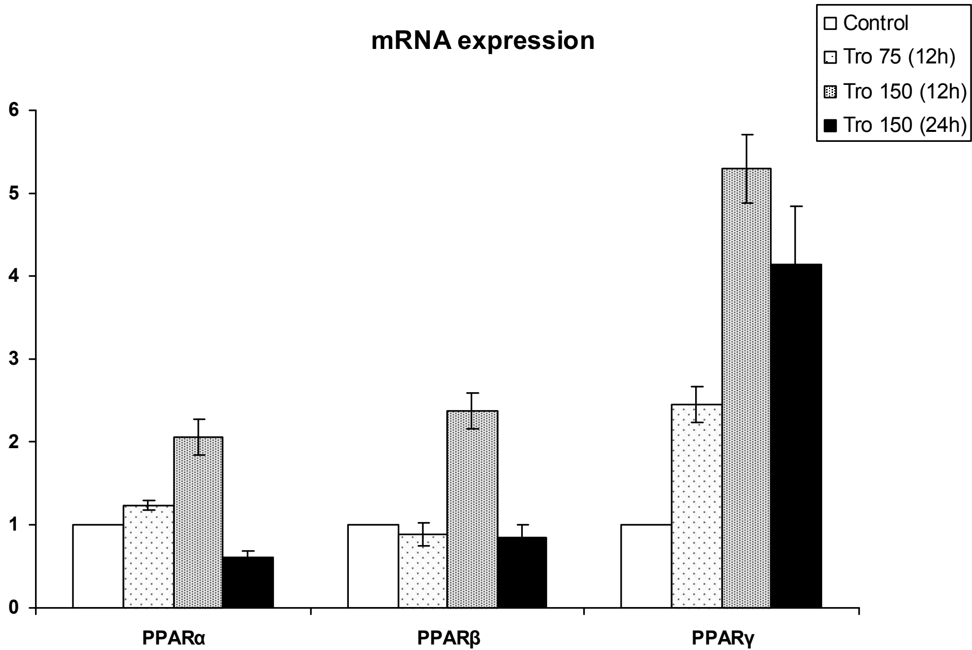


Fig.18. RT-PCR analysis for PPARs. Total RNA was extracted form the cells treated with Troglitazone and consequently subjected to RT-PCR using specific primers for PPARs and GAPDH. Data are means ± S.D of triplicate cultures.

To further determine whether Troglitazone induced cell death was through a PPARγ dependent mechanism, cells were treated with Troglitazone with or without the PPARγ antagonist, GW9662. As shown in Fig.19, the addition of GW9662 significantly prevented cell death induced by Troglitazone, indicating that the PPARγ activation was involved in the Troglitazone induced cell death.

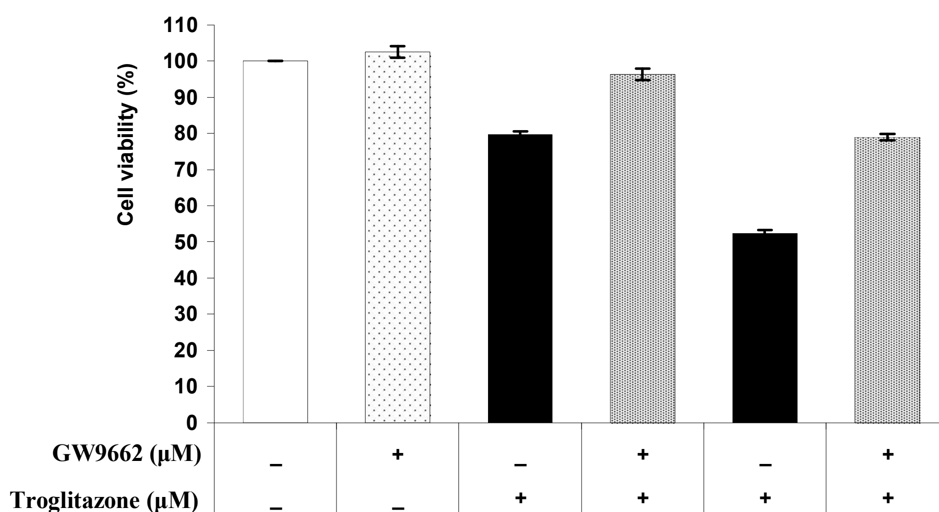


Fig.19. Growth inhibition of Troglitazone was dependent of PPARγ. FaDu cells were incubated with or without Troglitazone 75 or 150 µM (shown as black column), GW9662 20 µM (shown as dot column), or their combinations (shown as gray column). Cell viability assay was carried out at 24 hours. Values expressed as % control. Data presented are means ± S.D.

3.6 Role of MAPK signaling in Troglitazone induced cell death

Mitogen Activated Protein Kinase (MAPK) family members are crucial for the maintenance of cells [33]. However, the regulations of apoptosis by MAPKs are complex and often controversial. They may exert either anti-apoptosis or pro-apoptosis depending on the cellular context. Western blot was carried out to analyze MAPKs in the cells treated with Troglitazone. An early activation of ERK1/2 was observed, starting at 15 min, peaking at 45 to 60 min, and returning to basal levels at 2 hours (Fig.20), suggesting that Troglitazone was associated with ERK1/2 activation. The similar increase of ERK1/2, when induced by Troglitazone, was also observed in other cell lines such as human lung cancer cells [24, 25], human MCF7 breast carcinoma cells [7]. The expression of ERK indicated that ERK may

responsible for the Troglitazone induced cell death. In addition, Troglitazone induced p38 activation and prolonged its activity for 30 min, and then slowly decreased its expression time dependently (Fig.20). It is not clear yet how the activation of p38 in FaDu cell contributed to the induction of apoptosis. The activation of JNK1 was not changed much through 24 hours treatment (Fig.20). From these data, we suspects that ERK pathway may be the upstream signal in the apoptosis induction by Troglitazone in FaDu cells and further study is in progress.

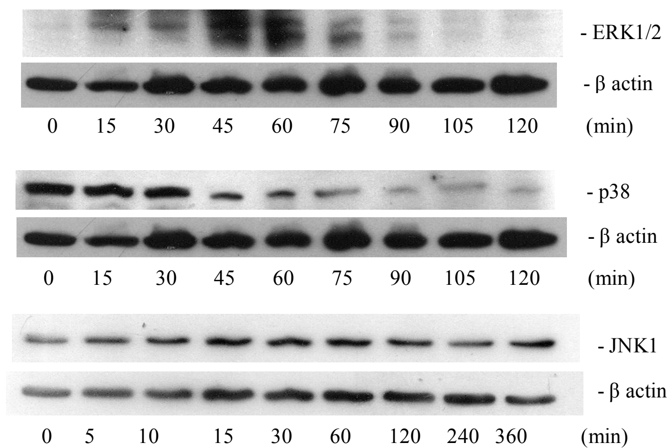


Fig.20. Effects of Troglitazone treatment on the expression of MAPK kinase proteins in FaDu cells.

4. Discussion

PPARγ agonists play important roles in fat and glucose metabolism in the liver, muscle, and adipose tissue and also differentiation of adipocytes [21]. Along with the insulin-sensitizing action of PPARγ agonists, Troglitazone was also shown to cause growth arrest and apoptosis of various normal and cancerous cells. These cells include hepatoma cells [27, 37, 38], colon cells [22], papillary thyroid carcinoid cells [17], human breast cancer cells [7, 10], human lung carcinoma cells [24, 25], human liver [18], human ovarian cancer cells [36] and vascular smooth muscle cells [12]. These reports showed different mechanisms of apoptosis, depending on the cell type, duration of drug treatment, dosage used, and the presence of other mitogenic factors. In this study, we investigated the toxicities of Troglitzone on FaDu human pharyngeal squamous carcinoma cells and their apoptosis mechanism with respect to the cell cycle regulation, caspase cascades, mitochondrial pathway and MAPK signaling.

Our data suggested the Troglitazone influenced FaDu cancer cells by suppressing cellular proliferation and induced apoptosis. The inhibition of FaDu cell growth by Troglitazone, which was assessed by MTT assay and flow cytometry suggested that the activation of PPARγ agonists down-regulated the cell growth. From studies with two different ligands, Troglitazone, a synthetic ligand of PPARγ and Clofibrate, a synthetic ligand of PPARα, Troglitazone showed the toxicity on FaDu cells dose dependently with the IC_{50} at 150 μM (Fig.1), but not Clofibrate.

Some recent observations hypothesized that thiazolidinediones (Troglitazone belong to this group) can induce differentiation of cancer cells and inhibit cancer growth in developmental and cancer models. Several mechanisms have been proposed, including

inhibition of phosphatidylinositol 3-kinase/Akt signaling, retinoblastoma protein (Rb) dephosphorylation, decreased cyclin D1 and Bcl-xl/Bcl-2 expression, up-regulation of p21 and p27, as well enhanced sensitivity to Tumor necrosis factor–Related Apoptosis-Inducing Ligand (TRAIL) induced cell death [9]. Our results support that the inhibition of cell growth by PPARγ activation was involved in the arrest of the cell cycle. In the cell cycle profile of FaDu cells treated with Troglitazone (150 μM), flow cytometry data showed that Troglitazone increased the population of cells in G2/M phase (Fig.5), causing G2/M phase arrest and blocking cells from entering G1 phase to initiate a cell cycle. The cell cycle is tightly regulated to ensure properly controlled proliferation. p27, a cell cycle inhibitor whose cell abundance increase in response to many anti-mitogenic stimuli, acts in G0 and early G1 to inhibit G1-cyclin cdks with primary target being cyclin E/cdk2 complexes. Our results showed the increase in p27 corresponded with the decreased level of cyclin/cdk complexes. The expression level of cyclin D1 dramatically decreased at 6 hour (Fig.8). PPARγ and cyclin D1 appear to have a reciprocal relationship. PPARγ ligands inhibit cyclin D1-mediated Rb phosphorylation at Ser807 and Ser811 and thereby maintain Rb in its active form, which prevents G1 to S phase transition [9]. Wang et al. showed that cyclin D1 inhibits the expression and promoter activity as well as transcriptional activity of PPARγ through a cyclin dependent kinase (cdk)- and Rb-independent mechanism [40]. It is well known that thiazolidinediones decrease cyclin D1 expression both directly via proteasomal degradation and indirectly by promoting proteasomal degradation of β-catenin [41]. Cdk2 and cdk4 were also significantly decreased (Fig.8 and 9).

However, p27 inhibitory effect on cell cycle was not strong enough to arrest cell cycle completely at G1/S or S phase although it may delay the G1/S and S phase transition. Cyclin B/cdc2 complex is also known for its function, passing G2/M checkpoint and completing cell division. In our study, the expression of cyclin B1 and cdc2 were time dependently decreased

(Fig. 10). Their inhibition resulted in the cell cycle arrest at G2/M phase. The data agreed with the result of FACs analysis, showing the inhibition of cell cycle progression by the increased cell population at G2/M phase about 2 folds (Fig. 5).

In the cell cycle inhibitor protein family, p21 is also known as the prototype of universal inhibitors of cdk enzyme activity. It is capable of inactivating cdk activity when expressed at high levels and inducing cell cycle arrest [4]. It was reported that the mechanism of p53-dependent G2 arrest was associated with an initial inhibition of cyclin B1/cdc2 activity by p21 and a subsequent reduction of cyclin B1 and cdc2 protein levels [13]. However, Rumi et al. showed that the p27 and p18 proteins were increased but p21 was decreased by troglitazone in HepG2 cells [19]. In another report, Motomura et al. demonstrated that Troglitazone increased p27 but not p21 or p18 protein levels in pancreatic carcinoma cell line [20]. Conversely, Kawa et al. reported that Troglitazone induced up-regulation of p21 but not p27 protein expression in a pancreatic carcinoma cell line [31]. These suggest that the activation as well as the expression of p21 in the cancer cells when treated with Troglitazone is quite controversial. In our studies, as showed in Figure 7, Troglitazone treatment caused a marked increase in p21 expression before 24 hours. The up-regulation of p21, an inhibitor of CIP/KIP inhibitors family was in agreement with the low proliferation rate observed in FaDu cells. The expression of p21 was increased by 12 hours and then followed by the decrease.

The cell death by Troglitazone treatment was mediated by apoptosis induction. The evidences of apoptosis were demonstrated by the increase of apoptotic cells, DNA fragmentation and activated caspases. Flow cytometry revealed the increased number of the apoptotic cells up to 40.51% of the cell population (Fig.4). Troglitazone also exhibited DNA fragmentation (Fig.3) and the typical morphological changes associated with apoptosis such as membrane bleeding, cytoplasmic shrinkage and apoptosis bodies (data not shown).

Clarifying the apoptosis-related signaling that is associated with Troglitazone-induced apoptosis is of primary importance. Multiple pathways are involved in apoptosis including mitochondrial pathway and caspase cascade. Caspase is a group of cysteine hydrolytic protease that specifically cleaves the peptide chain behind the residue base of the target protein aspartate. It is the key molecule in the inducing of apoptosis phenomenon. Caspase triggers a cascade reaction under the control of apoptosis signals. Caspases are classified as initiator and effector caspases [5]. Among them, caspase-3 is the most important terminal effector caspase in apoptosis, and plays an irreplaceable role. After initiation of the apoptotic process, caspase-3 transforms from a zymogen form to the activated form, and functions by hydrolyzing proteins essential for survival of many types of cells. The cleaved form of caspase-3 and caspase-7 were detected after treatment with Troglitazone at 24 hours (Fig.11, 12).

The enzyme poly (ADP-ribose) polymerase, or PARP, is an important DNA repair enzyme and was one of the first proteins identified as a substrate for caspases. The ability of PARP to repair DNA damage was prevented when caspase-3 cleaved the PARP [8]. The recent evidences showed that caspase-7 cleaves PARP more efficiently than caspase-3 in vitro [4]. In our results, the inactive form of PARP and the expression of activated caspase-7 were significantly increased in a dose dependent manner (Fig.12, 13). As PARP expression increased, apoptotic DNA ladder was appeared (Fig. 3).

Although the caspases represent a central point in apoptosis, their activation is regulated by a variety of other factors. Among them, Bcl-2 family plays a pivotal role in caspases activation. Bcl-2 family members are known to focus much of their response to the mitochondria level, which all belong to mitochondrial pathway. In Bcl-2 family, Bcl-2 and its closest homologues, Bcl-xL and Bcl-w, potently inhibit apoptosis in response to many cytotoxic insults. The presence of an anti-apoptotic molecule, Bcl-2 or Bcl-xL, inhibits the

activation of Bax. Bax is a cytosolic monomer in healthy cells, but it changes its conformation during apoptosis, integrates into the outer mitochondrial membrane, oligomerizes, and release pro-apoptotic proteins such as cytochrome C, which allows activation of caspase-9 [27]. In our study, we observed that activation of PPARγ by Troglitazone markedly increased Bax and Bad (Fig. 15), in both gene and protein expressions (Fig.16). The expression of Bcl-2 decreased time dependently until 12 hours and then slightly increased again at 24 hours. Some of controversial evidences suggest that the expression levels of Bcl-2 and Bax were not affect by Troglitazone [10, 17]. The expression as well as the activation of Bcl-2 may need further studies. Because the sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti- apoptotic proteins, the excess of pro-apoptotic proteins made cells more sensitive to apoptosis [8] which was confirmed by the result showed in Fig.16. Moreover, the release of cytochrome C after treating with Troglitazone (about 20%, Fig.14) confirmed that the apoptosis signaling was induced by Troglitazone through mitochondrial pathway and caspase dependent way.

Troglitazone is known as the PPARγ agonist with high affinity to the PPARγ. On ligand binding, PPARγ heterodimerizes with retinoid X receptor, translocates to the nucleus, and transactivates multiple genes involved in metabolism. The activated nuclear PPARγ/retinoid X receptor transcriptional complex binds to PPARγ response elements and recruits coactivator proteins, in a ligands pecific manner that is thought to provide biological specificity [9]. PPARγ agonists evoke both PPARγ dependent and PPARγ independent effects. Some reports have shown that PPARγ agonists are able to act by non-genomic mechanism so that its effect might be mediated by a PPARγ independent mechanism [36]. In our study, Troglitazone induced FaDu cell death PPARγ dependently as evidenced by the protection of cell death with the PPARγ antagonist GW9662 (Fig.19). GW9662 (2-chloro-5-nitrobenzanilide) is a potent

irreversible PPARγ selective antagonist with high affinity and selectivity and can fully abrogate PPARγ-dependent mechanism. Troglitazone also up-regulated PPARγ gene with 4 to 5 folds increase (Fig.18). All these results convinced that Troglitazone affected on the growth of FaDu cells through PPARγ action.

The importance of MAPK signaling pathways in regulating apoptosis during the condition of stress has been widely investigated. Many studies have supported the general view that activation of the ERK pathway delivers a survival signal that counteracts pro-apoptotic effects associated with JNK and p38 activation. We investigated whether the effect of Troglitazone on cell viability is associated with the activation of Mitogen Activated Protein Kinase (MAPK) in FaDu cell line. Troglitazone stimulated the expression of ERK1/2 occurred at early time point. This expression of ERK indicates that ERK was responsible for the Troglitazone induced cell death. This is in agreement with the results observed in other cell lines when treated with Troglitazone such as human lung cancer cells [24] or human MCF-7 breast carcinoma cells [7].

In conclusion, our study demonstrated that Troglitazone inhibited the growth of FaDu cancer cell line through cell cycle arrest and apoptosis induction. The signaling pathway was associated with mitochondrial pathway and probably mediated through ERKs pathway in a PPARγ dependent manner.

5. References

1. Bruce Brockstein, M.D. and Gregory Masters, M.D. Head and neck cancer. Kluwar Academic Publishers 2004, 1-13.
2. Carmelo Blanquicett et al., 2008. Thiazolidinediones as anti-cancer agents. *Cancer Ther* **6**(A), 25-34.
3. Charles J. Lin, BA et al., 2006. Head and neck squamous cell carcinoma cell lines: established models and rationale for selection. *Head Neck* **29**, 163–188.
4. Casaba Szabo. Cell death: The role of poly (ADP-ribose) polymerase. CRC Press LLC 2000.
5. DW Nicholson, 1999. Caspase stucture, proteolytic substrates, and function during apoptotic cell death. *Cell Death and Differentiation* **6**, 1028-1042.
6. Elizabeth A. Musgrove et al., 1994. Cyclin D1 induction in breast cancer cell shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. *Cell biology* **97**, 8022-8026.
7. Fen Yin et al., 2004. Signaling pathway involved in induction of GADD45 gene expression and apoptosis by troglitazone in human MCF-7 breast carcinoma cells. *Oncogene* **23**, 4614-4623.
8. Feng-guang Yang et al., 2005. Peroxisome proliferator-activated receptor γ ligands induce cell cycle arrest and apoptosis in human renal carcinoma cell lines. *Acta Pharmacologica Sinica* **26**(6), 753-761.
9. Fu-Sheng Chou et al., 2007. Effects of Thiazolidinediones on differentiation, proliferation, and apoptosis. *Mol Cancer Res* **5**(6), 523-530.
10. Hong-Nu Yu et al., 2008. Induction of G1 pharse arrest and apoptosis in MDA-MB-

231 breast cancer cells by troglitazone, a synthetic peroxisome proliferator-activated receptor γ (PPARγ) ligand. *Cell Biology International* **32**, 906-912.

11. Hugh J.M. Brady. Method in Molecular Biology, Volume 282: Apoptosis methods and protocols. Humana Press 2004.
12. Ioanna Gouni-Berthold et al., 2001. Troglitazone and rosiglitazone induce apoptosis of vascular smooth muscle cells through an extracellular signal-regulated kinase-independent pathway. *Nauyn-Schmiedeberg's Arch Pharmacology* **363**, 215-221.
13. J.A. Pietenpol and Z.A. Stewart, 2002. Cell cycle checkpoint signaling: Cell cycle arrest verus apoptosis. *Toxicology* **181-182**, 475-481.
14. J.Slingerland and M.Pagano, 2000. Regulation of the Cdk Inhibitor p27 and Its Deregulation in Cancer. *Journal of Cellular Physiology* **183**, 10-17.
15. Ju Young Jung et al., 2007. Role of mitogen-activated protein kinase (MAPK) in troglitazone-induced osteoblastic cell death. *Toxicology* **234**, 73-82.
16. Kaname Yoshizawa et al., 2002. Peroxisome Prolifertor-Activated Receptor γ Ligand Troglitazone Induces Cell Cycle Arrest and Apoptosis of Hepatocellular Carcinoma Cell Lines. *Cancer* **95**(10), 2243-2251.
17. Kazuyasu Ohta et al., 2001. Ligands for Peroxisome Proliferator-Activated Receptor γ Inhibit Growth and Induce Apoptosis of Human Papillary Thyroid Carcinoma Cells. *The Journal of Clinical Endocrinology and Metabolism* **86**(5), 2170-2177.
18. M Toyoda et al., 2002. A ligand for peroxisome proliferation activated receptor γ inhibits cell growth and induces apoptosis in human liver cancer cell. *Gut* **50**, 563-567.
19. MAK Rumi et al., 2001. Peroxisome proliferator-activated receptor γ ligand induced growth inhibition of human hepatocellular carcinoma. *British Journal of Cancer* **84**(12), 1620-1647.

20. Mao Song Lin et al., 2007. Activation of peroxisome prolifertor-activated receptor γ inhibits cell growth via apoptosis and arrest of the cell cycle in human colorectal cancer. *Journao of Digestive Diseases* **8**, 82-88.
21. Martyn T. Smith, 2003. Mechanisms of Troglitazone Hepatotoxicity. *Chemical Research in Toxicology* **16**(6), 679-687.
22. Masashi Kato et al., 2004. Induction of differentiation and peroxisome proliferator-activated receptor γ expression in colon cancer cell lines by troglitazone. *J Cancer Res Clin Oncol* **130**, 73-79.
23. Mels Sluyser. Application of apoptosis to cancer treatment. Springer, 2005.
24. Mingyue Li et al., 2005. Activation of Peroxisome Proliferation-Activated Receptor-γ by Troglitazone (TGZ) Inhibits Human Lung Cell Growth. *Journal of Cellular Biochemistry* **96**, 760-774.
25. Mingyue Li et al., 2006. Apoptosis Induced by Troglitazone Is Both Peroxisome Proliferator-Activated Receptor-γ- and ERK-Dependent in Human Non-small Lung Cancer Cells. *Journal of Cellular Physiology* **209**, 428-438.
26. Myung-Ae Bae and Byoung J. Song, 2002. Critical Role of c-Jun N-Terminal Protein Kinase Activation in Troglitazone-Induced Apoptosis of Human HepG2 Hepatoma Cells. *Morlecular Pharmacology* **63**(2), 401-408.
27. Myung-Ae Bae et al., 2003. Troglitazone but not rosiglitazone induces G1 cell cycle arrest and apoptosis in human and rat hepatoma cell lines. *Toxicology Letters* **139**, 67-75.
28. Phil Dash. Apoptosis. *www.sgul.ac.uk/dept/immunology/~dash*.
29. Ryuta Ymazaki et al., 2002. Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis in Association with Activation of Peroxisome Proliferator Activated Receptor γ in Rheumatoid Synovial Cells. *The Journal of Pharmacology and Experiental*

therapeutics **302**(1), 18-25.

30. S. R. S. Rangan, ScD, 1972. A new human cell line (FaDu) from a hypopharyngeal carcinoma. *Cancer* **29**, 117-121.
31. Shigeyuki Kawa et al., 2002. Growth Inhibition and Differentiation of Pancreatic Cancer Cell Lines by PPARγ Ligand Troglitazone. *Pancreas* **24**(1), 1-7.
32. T. Humphrey and G. Brooks. Cell Cycle Control: Mechanisms and protocols. Humana Press Inc., Totowa, NJ.
33. Teiji Wada and Josef M Penninger, 2004. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* **23**, 2838-2849.
34. Ting-Jun Fan et al., 2005. Caspase family proteases and apoptosis. *Acta Biochimica et Biophysica Sinica* **37**(11), 719-727.
35. Xiu-Mei Ma et al., 2009. Peroxisome proliferation-activated receptor-γ is essential in the pathogenesis of gastric carcinoma. *World J Gastroenterol* **15**(31), 3874-3883.
36. Yang Y-C et al., 2007. Perxisome proliferator-activated receptor-γ agonists cause growth arrest and apoptosis in human ovarian carcinoma cell lines. *Int J Gynecol Cancer* **17**, 418-425.
37. Yan-Ming Zhou et al., 2008. Troglitazone, a peroxisome peroxisome proliferator-activated receptor γ ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells. *World J Gastroenterol* **14**(4), 2168-2173.
38. Yui Yamamoto et al., 2001. Cytotoxicity and apoptosis produced by troglitazone in human hepatoma cells. *Life Science* **70**, 471-482.
39. Zhaoyu Jin and Wafik S. El-Deiry, 2005. Overview of cell death signaling pathways. *Cancer Biology and Therapy* **4**(2), 139-163.
40. Wang C et al., 2003. Cyclin D1 repression of peroxisome proliferator-activated

receptor g expression and transactivation. *Mol Cell Biol* **23**, 6159-6173.

41. Qin C et al., 2003. Peroxisome proliferator-activated receptor g agonists induce proteasome-dependent degradation of cyclin D1 and estrogen receptor a in MCF-7 breast cancer cells. *Cancer Res* **63**, 958-964.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my supervisor, Professor Hoon Yoo, who first brought me into the world of research and with whom I began to learn about the knowledge of molecule biology as well as the bioassay on cancer cells. I appreciate his encouragement, enthusiasm, guiding and supporting me through my Master's course. The excellence, scientific profession in his working will be the model for me to follow. It was a great privilege for me, and I believe, for all of those who have ever had an opportunity to work under his guidance. I cannot figure out appropriate words to express how thankful and admirable I am to my great mentor. I also wish to express my sincere thanks to all professors at College of Dentistry, Chosun University for their teaching, support and emotional encouragement during my graduate training.

In completing the course I have also been particularly impressive with the boundless helps of our lab members Le Thanh Do, Park Hyung Wook, Myong Seong Eun, with whom I have shared the hardship, daunting difficulties and excitements during the working time. Thank you for their excellent assistance and significant contribution, especially much respect to Le Thanh Do for his technical advices and enthusiastic helps.

I would like to convey my thanks to all teachers in ABC center who made my life in Korea so beautiful, who not only teach me Korean but also gave me chances to experience Korean culture, tours around Gwangju city as well as other cities in Korea. Thank you for their kindness and warm sentiments

Thank you to all my Vietnamese and Korean friends for their friendly helping during my stay in Korea.

Finally, my work would not have been possible without the unfailing support of my family. I would like to express my special gratitude to aunt Do Thi Ung Sao, who oriented my way on study, to my parents and my sister for their love, patience, constant encouragement, and enthusiasm which have sustained my endeavor to complete my work.

Korea, February, 2011

Do Thi Anh Thu

저작물 이용 허락서

학 과	치의학과	학 번	20097713	과 정	석사
성 명	한글: 도 티 안 트 영문: DO THI ANH THU				
주 소	광주 동구 서석동 조선대학교				
연락처	E-MAIL: anhthu303@gmail.com				
논문제목	한글: 트로글리타존에 의한 두경부 암세포의 세포주기억제 및 세포사멸 연구 영문: Troglitazone induced cell cycle inhibition and apoptosis on Human pharyngeal squamous carcinoma cancer cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. **저작물의** DB**구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함**
2. **위의 목적을 위하여 필요한 범위 내에서의 편집 · 형식상의 변경을 허락함.**
다만, 저작물의 내용변경은 금지함.
3. **배포 · 전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.**
4. **저작물에 대한 이용기간은** 5**년으로 하고, 기간종료** 3**개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.**
5. **해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는** 1**개월 이내에 대학에 이를 통보함.**
6. **조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음**
7. **소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송 · 출력을 허락함.**

2010 **년** 2**월** 25**일**

저작자: DO THI ANH THU (**서명 또는 인**)

조선대학교 총장 귀하