



**REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEř SCIENCE AND TECHNOLOGY
UNIVERSITY**

**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOENGINEERING**

**INVESTIGATION EFFECTS OF PTEROSTILBENE ON
DIFFERENTIATION AND ADIPOGENESIS IN 3T3-L1 CELLS**

**BİRGÜL GÜLNAR
MASTER OF SCIENCE**



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**SUPERVISOR
Assoc. Prof. Dr. Sibel ÖZDAŞ**

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I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all information that is not original to this work.

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Birgöl GÜLNAR

ABSTRACT

INVESTIGATION EFFECTS OF PTEROSTILBENE ON DIFFERENTIATION AND ADIPOGENESIS IN 3T3-L1 CELLS

Birgül GÜLNAR

Department of Bioengineering

Supervisor: Assoc. Prof. Dr. Sibel ÖZDAŞ

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Diabetes mellitus (DM) is a chronic illness that occurs when there is inadequate insulin secretion and/or insulin action defect. DM is usually characterized by high blood sugar (hyperglycemia) above normal blood sugar levels. Having consistently high blood glucose causes general vascular damage that affects the kidneys, nerves, heart, eye vessels and can lead to a variety of health problems. In etiology, diabetes is divided into three main groups as gestational diabetes (GDM), type 1 diabetes and type 2 diabetes. Plant extracts are very important sources in the search for alternative new drug active ingredients. Pterostilbene is known as a naturally dimethylated analogue of resveratrol and its main source is grapes and blueberries. In this study, it was aimed to evaluate the effects of Pterostilbene on differentiation and adipogenesis in 3T3-L1 cells.

For this purpose, Pterostilbene was treated to mature 3T3-L1 adipocytes at different concentrations and half-maximum inhibitory concentrations (IC_{50}) were determined by MTT. Based on the results, 5 and 7.5 μ M doses of Pterostilbene were chosen to apply to cells in subsequent experiments. In addition, Oil Red O was applied to determine the lipid accumulation and effects of Pterostilbene in preadipocytes and 3T3-L1 adipocytes, and it was observed that Pterostilbene treatment reduced lipid accumulation in adipocyte differentiation. The cytotoxic effect of Pterostilbene in 3T3-L1 adipocyte cells was confirmed by dead/live cell assay. Phase contrast microscopy and Giemsa staining, F-actin staining and DAPI staining were applied to investigate the effect of Pterostilbene on the morphology of 3T3-L1 adipocyte cells. According to the study result, adipocytes treated with a dose of 7.5 μ M on day 14 showed less intense lipid

deposition and a more spindle-like morphology compared to the other treatment group. Finally, the expression of *Glucose transporter-4 (Glut-4)* and *Adiponectin* associated with insulin resistance was evaluated by immunofluorescent staining and qRT-PCR. Especially on the 14th day, compared to the other treatment group, actin filaments were filamentous in adipocytes treated with Pterostilbene 7.5 μ M compared to the other treatment group, the filaments were similarly oriented as in preadipocytes, and chromatin condensation was observed to be quite high.

As a result of this study, it was observed that Pterostilbene induce apoptosis, reduce lipid accumulation but induce *Glut-4* and *Adiponectin* level, and may conduce to the reduction of insulin resistance in diabetes.

Keywords: Diabetes mellitus, Pterostilbene, 3T3-L1, Adipogenesis, Differentiation, Glucose transporter 4, Adiponectin.

ÖZET

3T3-L1 HÜCRELERİNDE PTEROSTILBEN'İN FARKLILAŞMA VE ADİPOGENEZ ÜZERİNDEKİ ETKİLERİNİN İNCELENMESİ

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Diabetes mellitus (DM), yetersiz insülin salınımı ve/veya insülin etki kusuru olduğunda ortaya çıkan kronik bir hastalıktır. DM genellikle normal kan şekeri seviyelerinin üzerinde yüksek kan şekeri (hiperglisemi) ile karakterize edilir. Sürekli olarak yüksek kan şekere sahip olmak, böbrekleri, sinirleri, kalbi, göz damarlarını etkileyen ve çeşitli sağlık sorunlarına yol açabilen genel damar hasarına neden olmaktadır. Etiyolojide diyabet, gestasyonel diyabet, tip 1 diyabet ve tip 2 diyabet olmak üzere üç ana gruba ayrılır. Bitki ekstreleri, alternatif yeni ilaç aktif bileşenlerinin araştırılmasında çok önemli kaynaklardır. Pterostilben, resveratrolün doğal olarak dimetillenmiş bir analogu olarak bilinir ve ana kaynağı üzüm ve yaban mersinidir. Bu çalışmada, Pterostilbenin 3T3-L1 hücrelerinde Pterostilben'in farklılaşma ve adipogenez üzerindeki etkilerinin araştırılması amaçlanmıştır.

Bu amaçla, Pterostilben, farklı konsantrasyonlarda olgun 3T3-L1 adipositlerine uygulanarak MTT ile yarı maksimum inhibitör konsantrasyonları (IC₅₀) belirlenmiştir. Çıkan sonuçlara dayanarak, sonraki deneylerde hücrelere uygulamak için Pterostilben'in 5 ve 7.5 µM'lık dozları seçilmiştir. Ek olarak, preadipositlerde ve 3T3-L1 adipositlerde lipid birikimini ve Pterostilben'nin etkilerini belirlemek için Oil Red O uygulanmış ve Pterostilben tedavisinin adiposit farklılaşmasında lipid birikimini azalttığı gözlenmiştir. Pterostilben'nin 3T3-L1 adiposit hücrelerinde sitotokik etkisi ölü/canlı hücre asay ile doğrulanmıştır. Pterostilben'nin 3T3-L1 adiposit hücrelerinin morfolojisi üzerindeki etkisini değerlendirmek için faz kontrast mikroskobu Giemsa boyaması, F-aktin boyaması ve DAPI boyaması uygulanmıştır. Çalışma sonucuna göre 14. günde 7.5 uM doz ile tedavi edilen adipositler, diğer tedavi grubuna kıyasla daha az yoğun lipid birikimi, daha iç benzeri bir morfoloji, ve preadipositlerdeki gibi

filamentler benzer şekilde yönlendiği gözlenmiştir. Aynı zamanda Pterostilben dozları tedavisi ile farklılaşan adipositler, zamana ve doza bağlı bir şekilde artan DNA yoğunlaşması sergilemiştir. Son olarak, insülin direnci ile bağlantılı *Glikoz taşıyıcı-4 (Glut-4)* ve *Adiponektin* ekspresyonu, immunfloresan boyama ve qRT-PCR ile değerlendirilmiştir. Glut-4 ve Adiponektin floresansı, Pterostilben dozları ile tedavi edilen adipositlerde tedavi edilmeyen adipositlere kıyasla doza bağlı olarak azalmıştır. Bununla birlikte, *Glut-4* ve *Adiponektin* mRNA ekspresyonu da tedavi edilen adipositlerde tedavi edilmeyen adipositlere kıyasla azalmıştır. Özellikle 14. günde diğer tedavi grubuna kıyasla Pterostilben'in 7.5 µM uygulanmış adipositlerde, aktin filamentleri filamentöz yapıda olup, preadipositlerdeki gibi filamentler benzer şekilde yönlendirilmiş ve kromatin yoğunlaşması oldukça yüksek olarak gözlenmiştir.

Bu çalışma sonucunda, Pterostilben'nin apoptozu indüklediği, lipid birikimini azalttığı, *Glut-4* ve *Adiponektin* düzeyini artırdığı ve diyabette insülin direncinin azalmasına katkıda bulunabileceği gözlenmiştir.

Anahtar Kelimeler: Diabetes mellitus, Pterostilben, 3T3-L1, Adipogenez, Farklılaşma, Glikoz taşıyıcı 4, Adiponektin.

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NOMENCLATURE

DM: Diabetes Mellitus
T1D, T1DM: Type 1 Diabetes Mellitus
T2D, T2DM: Type 2 Diabetes Mellitus
GLUT-4: Glucose Transporter-4
IGT: Impaired Glucose Tolerance
IDDM: Insulin Dependant Diabetes Mellitus
NIDDM: Non-Insulin Dependant Diabetes Mellitus
IAA: Insulin autoantibody
ICA: Islet Cell Antibody
Anti- GAD: Anti-glutamic acid decarboxylate
DERI: Diabetes Epidemiology Research
CHH: Canadian Heart Health
AFI: Amniotic Fluid Insulin
ODM: Diabetic Mothers
BMI: Body Mass Index
IGT: Impaired Glucose Tolerance
ADA: American Diabetes Association
FPG: Fasting Plasma Glucose
OGTT: Oral Glucose Tolerance Test
HbA1c: Glycosylated Haemoglobin
WHO: World Health Organization
A1C: Glycated Hemoglobin
IR: Insulin Receptor
FFA: Free Fatty Acid
IRS-1: Insulin Receptor Substrate-1
IRS-2: Insulin Receptor Substrate-2
PI3K: Phosphoinositide 3-kinases
PKB: Protein Kinase B
Glut 4: Glucose Transport -4
PTS: Pterostilbene
GRAS: Generally Recognized as Safe

FDA: Food and Drug Administration

PEPCK: Phosphoenol Pyruvate Carboxykinase

PKC: Protein Kinase C

PIP2: Phosphatidylinositol 4,5 bisphosphate

PDK1: Phosphoinositide Dependant Kinase-1

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

IF: Immunofluorescence



1. INTRODUCTION

Diabetes mellitus (DM) stands out a type of the metabolic illness with long-term hyperglycemia, which has metabolic consequences due to insufficiency in insulin secretion and/or insulin action. Nervous and renal system diseases, eye and cardiovascular diseases often occur with various complications due to hyperglycemia in diabetic patients (Cho et al., 2018). In etiology, it is classified into three major groups as diabetes (DM), gestational diabetes (GDM), type 2 DM (T2DM) and type 1 DM (T1DM). T2DM stands out as the most widespread type of diabetes among patients, constituting approximately 90% of the total DM (Saeedi et al., 2019).

The characteristic of T2DM disease is that there is a gradually developing insulin secretion defect, and insulin deficiency and insulin resistance are the basis of this (Hu et al., 2013; Xu et al., 2017). DM is increased worldwide prevalently. According to estimates, 578 million diabetics in 2030 and 700 million in 2045 are expected (Nam H Cho et al., 2018; Saeedi et al., 2019).

It causes the development of both systemic and local insulin resistance due to dysfunction in adipose tissue, changes in metabolic and endocrine secretions and chronic inflammation. In addition to this, there is a serious increase in DM disease mostly due to metabolic and endocrine dysfunctions in adipose tissue and also due to T2DM. (Guilherme et al., 2008). In order to protect and manage public health, DM control can be achieved by increasing insulin sensitivity of adipose tissue and reducing system complications (Raveendran et al., 2018). The fundamental problems in the treatment of T2DM patients are the toxic effects, the development of drug resistance mechanisms, and the high costs for existing drugs used for treatment (Patel et al., 2012). It is prominent to research new treatment approaches to facilitate the life and treatment of diabetes patients. Therefore, it is prominent to research new therapeutic approaches (accessible, natural, low- toxicity and cost-effective targeting molecules) in potential signaling pathways for diabetes and alternative anti-diabetic agents (Ye, 2013). They are known as important sources in the approaches of new alternative drug active components to be obtained from medicinal plants (Bilal et al., 2018).

Resveratrol (3,5,40-trihydroxystilbene) is a natural phytoalexin and natural dimethylation of resveratrol forms Pterostilbene (3,5-dimethoxy-40-hydroxystilbene; PTS). PTS is an antimicrobial molecule in the structure of phytoalexin, which is synthesized by plants against microbes by de novo method. PTS is an antifungal agent 10 times more potent than resveratrol (Langcake and Pryce, 1977). Furthermore, one hydroxyl and two methoxy groups in the structure of PTS provide more cellular uptake and lipophilic properties with a higher potential than resveratrol, which has three hydroxyl groups in its molecule. The molecular structures of resveratrol and PTS are two geometric isomers, trans and cis, and naturally occurring molecular structures are mostly found in the trans form. The cis- or trans-form of resveratrol can be either glucose-bound or free (Mattivi et al., 1995).

The region where PTS was essentially isolated from *Pterocarpus santalinus* which is the heartwood of red sandalwood and is synthesized in plants and occurs as a secondary metabolite (Spath and Schlager, 1940). In addition, PTS is mainly found in blueberries and other berry-like fruits (Aiyer et al., 2012), grapes (Adrian et al., 2000), *Guibourtia tessmanii*, a flowering plant genus of the legume family and the Indian Kino tree (*Pterocarpus marsupium*) (Fuendjie et al., 2002).

When the molecular structures of PTS and resveratrol are compared, it has been noticed that due to their structural similarities, they have similar biological properties in anti-inflammatory, antioxidant, anti-cancer, anti-diabetic activities and immune regulation activities (Bhakkiyalakshmi et al., 2016). The heartwood, flowers and leaves of the *P. marsupium* have long been used in Ayurvedic medicine for their medicinal characters (Maurya et al., 2004). Gum resin has been used as the sole herbal product by Ayurvedic practitioners in the treatment of diabetes. The antihyperglycemic activity observed after oral administration of PTS and marsupin phenolics from *P. marsupium* tested in rats with streptozotocin-induced hyperglycemia was first demonstrated in 1977 by Manickam et al (Manickam et al., 1997). Equivalent to these findings, it has been reported that hyperinsulinemia and hypertriglyceridemia were significantly prevented, and serum glucose levels were severely reduced as a result of the previous treatment with a *P. marsupium* extract (Grover et al., 2005). Based on these biological effects of Pterostilbene, this study purposed to evaluate the effects of Pterostilbene on differentiation and adipogenesis in 3T3-L1 cells.

2. LITERATURE REVIEW

2.1 Diabetes mellitus (DM)

In a healthy person, blood glucose value ranges are evaluated in two ways. These are fasting blood glucose and postprandial blood glucose value ranges. In healthy people, fasting blood glucose ranges between 70-100 mg/dL and the upper limit for postprandial blood glucose is 140 mg/dL. DM stands for a metabolic illness characterized by a deficiency in insulin action and/or synthesis (Baena-Díez et al., 2016). Having consistently high blood glucose creates general vascular damage that affects the kidneys, nerves, heart, eye vessels and that may lead to a variety of health concerns (Organization, 2009). Insulin insufficiency results in prolonged hyperglycemia because it interferes with glucose, lipid, and protein metabolism, among other things (Beverley and Eschwège, 2003; Committee, 1997; Kumar and Clark, 2002; Lindberg et al., 2004)

The progression of severe diabetes adverse conditions such as retinal disease that causes visual impairment or loss of vision, (Bearse et al., 2004; Hove et al., 2004) , dysfunction or damage of one or more nerves that typically results in tingling, numbness, pain and muscle weakness in the affected area (Moran et al., 2004; Seki et al., 2004) , disease or dysfunction of one or several peripheral nerves that causes weakness, and damages to blood vessels or tissue (Control and Prevention, 2003; Wallace et al., 2002). As a consequence, diabetes is associated with a broad spectrum of medical disorders. Diabetes is the most prevalent disease among endocrine-based disorders. It is predicted that 300 million humans worldwide will have diabetes by 2030 (Amos et al., 1997; King et al., 1998).

Type-1 diabetes mellitus (T1DM) and Type-2 diabetes mellitus (T2DM) are known as the most common types of diabetes today. However, it is possible for Diabetes Mellitus to manifest itself in different ways, except for type 1 and type 2 varieties (Canada, 2011; Maahs et al., 2010). The terms Insulin Dependent Diabetes Mellitus and Non-insulin-dependent diabetes mellitus are often used to indicate IDDM and NIDDM, respectively, in the context of etiology. Other types of diabetes including gestational diabetes, as well as genetic impairments in β -cell activity and insulin secretion, exocrine pancreatic disorders, endocrinopathies, and drug- or chemical based infections (congenital rubella, CMV, etc.), are rare. Both IDDM and NIDDM have been referred to as "adolescent-onset diabetes".

2.1.1 T1DM

There are patients who have been diagnosed with T1DM according to the etiology, who has inadequate insulin secretion or no endogenous insulin and hence who need insulin injection to survive. There are two major types of clinical T1DM: type 1a and type 1b. T1DMa is a type of diabetes that consists as a consequences of the destruction of pancreatic β cells by the immune system, which is thought to cause insulin deprivation. T1DMb (idiopathic, approximately 10% of T1DM) is idiopathic or non-autoimmune diabetes without proof of autoimmunity. TDMa is defined by the existence of insulin autoantibody (IAA), anti-glutamic acid decarboxylate (anti-GAD), islet cell antibody (ICA), and anti-tyrosine phosphatase antibodies (IA-2). Approximately 10% of T1DM are type 1b diabetes which is idiopathic or non-autoimmune diabetes without evidence of autoimmunity (Maahs et al., 2010; Tan and MacLean, 1995). These antibodies are indicative of autoimmune destruction of the β cell. T1DMb has no known basis in etiology. Without evidence of autoimmunity, some type 1b patients have persistent insulinopenia and are likely to ketoacidosis (Silverman, 1998).

2.1.2 T2DM

T2DM, which is among the most prevalent types of DM, is characterized by insulin resistance as well as insulin production disorders (Panagiotopoulos, 2013). T2DM is called the inability of cells to respond properly to insulin and, accordingly, the failure of cells to develop insulin resistance (Tripathy, 2012). In the later stages of the disease, this kind of diabetes, also known as NIDDM or "adult-onset diabetes", is caused by inadequate of insulin. Excess body weight and an insufficiency of physical activity in daily life is one of the major causes of this type of DM (WHO, 2013). T2DM is a disorder that can be treated with or without insulin-containing medications (Tripathy, 2012). Low blood sugar can be caused by insulin and some oral medicines (Irwin, 2010). In individuals with T2DM who are obese, weight loss surgery stands out as a viable treatment option (Picot, 2009).

2.2 Epidemiology of DM

If we explain the percentage of T1DM and T2DM cases worldwide, T1DM accounts for approximately 3-5% of all diabetes cases, while T2DM accounts for more than 90-95% of diabetic patients' cases (Shaw and Zimmet, 1999; Zimmet, 2001; Zimmet et al., 2001). Obesity, lack of exercise, and genetics are all factors that conducted to the occurrence of T2DM in persons over the age of 40 (Eriksson et al., 1991; Zimmet et al., 1990). According to current data from

numerous nations, T2DM is becoming more prevalent in teenagers and even children (Glaser, 1997; Scott et al., 1997). T1DM is less prevalent among children in some countries than T2DM (Cockram, 2000). Diet treatment, exercise, and hypoglycemic medications are prevalently used to manage the illness (Miller et al., 2001; Zimmet, 2001).

The probability of diabetes-related death is at least twice as high in diabetics, and it has therefore been estimated that 592 million diabetics will die by 2035 (IDF, 2014). The expense of diabetes treatment will rise in the future on a global scale (Association, 2013; *IDF DIABETES ATLAS*, 2013). More than 150 million people in China are showing symptoms of Diabetes Mellitus (BBC, March 25, 2010). According to the results of the researches, 42.5 years is the average age of onset of diabetes. It is anticipated that 1 million people a year suffer from diabetes (Gale, 2010). Diabetes symptoms are caused by a sedentary lifestyle, high-calorie and high sugar diet, lack of exercise, genetic disposition (Kleinfeld, 2006).

2.2.1 Epidemiology of T1DM

The models of T1DM and epidemiology are influenced by biological, demographical, cultural, geographical and other variables in human communities (Maahs et al., 2010). According to reports, T1DM is increasing at a pace of 2-5 % worldwide with a frequency of one in 300 people aged 18 in the United States. By investigating the risk factors for T1DM, it is possible to increase the quality of life and treatment options in diabetic individuals, and to identify environmental and genetic factors as well as clinical data. Among the reasons why epidemiological studies of T1DM are necessary is a better understanding of its clinical care, role, reason, prevention and treatment in pregnant women and their babies. (Tan and MacLean, 1995).

However, despite the fact that the age of onset of T1DM is more common in the 0-14 age range than in the 15-29 age group in most societies. For children aged 0-4, 5-9, and 10-14 in the United Kingdom, well-established data at high levels (e.g., 98.6 percent in Scotland) demonstrate that the incidence rises with age: 15.3, 24.4, and 31.9 Per 100,000 / year in children aged 0-4, 5-9 (Rangasami et al., 1997). Aside from that, in many communities, there is a significant male overabundance in young people (Gale and Gillespie, 2001).

The highest prevalence occurs around puberty in the majority of populations, with the biggest peaks appearing a bit sooner in girls (9-12 years) than in boys (approximately 12–13 years) in

most cases. While historically more frequent in European populations than in non-European populations, ethnic discrepancies are likely to be lessening in high-occurrence countries, particularly among long-term immigrants, as the world's population becomes more diverse. (Group, 2006). It is worth noting that the incidence among South Asian children is comparable among white children. Lower rates were found when comparing Asia with Leicestershire, England (Raymond et al., 2001).

The initial group of people who took part in the DERI (Diabetes Epidemiology Research International) research had an IDDM rate of less than 15%. In the second group, there are no significant geographic differences when considering the prevalence of DM in Canada. According to the research conducted by CHH (Heart Health of Canada), the general prevalence of self-reported DM in adults aged 18-74 is 5.1%. T1DM patients became more prevalent as they became older. Data on diabetic deaths: Between January 1982 and December 1984, 321 people with diabetes died on PEI, accounting for around 2% of total fatalities. Diabetes is not cited as a contributory factor in 41.7 % of fatalities and not at all in 41.1 %. Cerebral vascular disease and myocardial infarction were known to be the two most prevalent causes of death in these 321 diabetic individuals, with or without an indication for diabetes. According to the Canadian Heart Health Survey, the most common causes among adults with diabetes are hypertension, obesity, a sedentary lifestyle, and high cholesterol (hypercholesterolemia).

2.2.2 Epidemiology of T2DM

According to global data, around 382 million people worldwide suffer with diabetes as of 2013, with T2DM accounting for roughly 90% of cases. In addition, they make up 8.3% of the adult male and female population with T2DM in equivalent proportions (Shi and Hu, 2014; Vos et al., 2012).

2.2.3 Epidemiology of Gestational DM

The idea that metabolic events in the uterus may affect long-term postnatal development was investigated (Silverman, 1998). A prospective study was conducted between 1977 and 1983. Children of mothers with T1DM, T2DM and gestational diabetes were participated in this study. Amniotic fluid insulin (AFI) was used to evaluate fetal beta cell function between 32 and 38 weeks of pregnancy. Children were subjected to cognitive tests, anthropometric measurements, and modified glucose tolerance tests for annual assessment after birth.

Macrosomia status is used for much larger-than-average infants born to diabetic mothers (ODM), and macrosomia normally disappears at one year of age. Large cognitive abnormalities were not substantially different from national estimates in cohort research. Abnormal maternal metabolism, on the other hand, has been associated with psychomotor development and poor intellectual performance.

Obesity occurs again periodically in childhood; and 14-17 years of age, the mean BMI was 20.9 ± 3.4 kg/m² in control groups versus 24.6 ± 5.8 kg/m² in ODM. Conditions associated with obesity in adolescence are: maternal weight, AFI concentration and gender. According to the results, 36% of ODMs had impaired glucose tolerance (IGT). These perturbations were associated with high AFI in the uterus.

Abnormal maternal metabolism was linked to offspring's impaired psychomotor and intellectual development, IGT and also obesity, proving the theory. Immoderate insulin production in uterus, as measured by AFI concentration, is an indicator of both IGT and obesity throughout young generation. Through this experiment, a long-term prospective assessment of the extent to which pregnant women and children are affected by diabetes was provided. Researchers have discovered a link between indices of fetal and maternal metabolism during pregnancy and their metabolic, physical and psychological development from birth to adolescence. T2DM is on the rise among children worldwide, and among ethnic groups, children are more prone to develop the disease, according to the results of the available reports (Panagiotopoulos, 2013).

2.3 Geographical variation of T1DM

It is predicted by the International Diabetes Federation (IDF) that almost 96,000 new T1DM patients are diagnosed in children and adolescents aged 0-15 years worldwide each year. The USA, Brazil, India, China, Russian Federation, United Kingdom, Saudi Arabia, Germany, Algeria and Nigeria, which are the top ten countries with the newest cases, account for more than 60% of all new patients of T1DM. In the study, which included children from different countries, it was found that the prevalence of T1DM differs approximately 400 times between countries. Based on age-adjusted incidence rates, one concludes that incidence rates have ranged from 0.1 per 100,000 per year in the regions of China and Venezuela to 37.8 in Sardinia and 40.9 in Finland (Group, 2006). There are also varying incidences within countries: For example, China, which stands out with an incidence of 0.13-0.61 per 100,000, has a 12-fold

range according to the region, but has a medium and high prevalence in European countries, moderate in African countries, and low in Asian countries. Kuwait stands out as an important exception among all these countries.

2.4 Diversity of age, gender and ethnicity in T1DM

T1DM is likely to occur at any age, but it is uncommon within one year after birth. In many societies, the incidence rises consistently with age until maturity, and excess among those aged under 15 years than between the ages 15 to 29. However, population-based incidence data are not available for people over 35 years of age. In general, there is a male overabundance among young adults.

2.5 Temporal variation in T1DM

It has been revealed that T1DM is more common in autumn and winter. Worldwide, T1DM has become more common, with an annual increase of about 3.0%. It is notable that the extent increases more in low incidence countries. It stands out as the youngest age group with the most significant increase in children aged 0-4 years (Forouhi and Wareham, 2019).

2.6 Etiological factors in T1DM

Environmental variables have a larger role in the development of islet autoimmunity to illness, presumably because better living conditions have lowered microbe exposure, resulting in greater autoimmune. Despite study, no single environmental factor has been confirmed to cause T1DM. Dietary and nutritional factors such as immunizations, toxins, viral infections, medications, intrauterine variables, exclusive breastfeeding and delayed delivery of cow's milk are all associated with early social mixing. As a result of some genetic and observational studies, vitamin D deficiency has been associated with cancer, and it is hypothesized that omega-3 fatty acids may be the cause (Forouhi and Wareham, 2019).

2.7 Diagnosis of DM

Diabetes diagnostic criteria were first introduced by the American Diabetes Association (ADA) in 1997, then again in 2003 and 2010. In 2003, ADA recommended using a 75-gram oral glucose tolerance test (OGTT) and fasting plasma glucose (FPG) to diagnose diabetes. The International Expert Committee recommended that **total glycosylated haemoglobin (HbA1c) 6.5%** be added to the diagnostic criteria in 2009.

The decision was endorsed by ADA, the European Diabetes Study Society, and the World Health Organization (Orçun et al., 2003). The decrease of FPG, which determines diabetes, from 140 mg/dl to 126 mg/dl was the most significant adjustment made in the WHO criteria in 1985 (Uygur and Yavuz, 2017). A diagnosis of diabetes is assured in a person with a FGP value of more than 126 mg/dl if the FPG value is more than 126 mg/dl or postprandial plasma glucose is higher than 200 mg/dl when the FPG value is re-measured, according to the given criteria. In addition, according to the criteria determined by the Japanese Diabetes Association (JDS), DM can be diagnosed with FPG ≥ 126 mg/dl when HbA1c $\geq 6.5\%$ is confirmed.

If FPG ≥ 126 mg/dL, plasma glucose measured at the second hour of 75 g OGTT is ≥ 200 mg/dL or HbA1c $\geq 6.5\%$. The glycated hemoglobin (A1C) test reveals the percentage of blood sugar linked to hemoglobin, the protein that transports oxygen from the lungs to tissues and organs in red blood cells. An A1C level between 5.7 and 6.4 percent is defined as prediabetes. HbA1c allows the determination of the average blood sugar level of the previous three months. Normal values are those that fall below 5.7 %. Diabetic mellitus can be diagnosed if the results are verified again (Association, 2021; ATMACA, 2012). In the presence of hyperglycemia symptoms, random plasma glucose of ≥ 200 mg/dL is also diagnostic. Individuals with pre-DM may be detected using the same techniques.

2.8 Treatment of DM

It is possible to manage diabetes by making lifestyle changes, such as increasing physical activity and paying attention to diet, before implementing to drug therapy (Skyler, 2004). When these approaches are insufficient, pharmacologic therapy is required. There are several types of diabetes medications available today, and each class has a different effect on glucose regulation.

There are two types of drugs known as oral anti-diabetic agents: Glinides, Sulfonylureas, Glitazones, α -Glucosidase inhibitors, Biguanides and insulin preparations (Short-Acting Preparations, Intermediate-Acting Preparations, Long-acting Preparations). The most prevalent treatment for T2DM is metformin combined with a secretagogue. Third-class oral drugs (glitazone or glucosidase inhibitor) and an intermediate or long-acting drug can be added if sufficient glycemic control is still not accomplished. Consequently, the natural progression of T2DM, people with diabetes will eventually need insulin therapy (Skyler, 2004). Acupoint Therapy is a different type of treatment for this disease that does not involve medication. Diabetes and its chronic complications, such as hyperglycemia, neuropathy, nephropathy, and hepatopathy, have all been studied using this treatment method. Electroacupuncture, manual acupuncture, point injections, and catgut implantation are the most prevalent methods of this therapy. Electroacupuncture can decrease blood glucose levels because of the relationship between the endocrine and nervous systems (Feng et al., 2018). According to a study comparing bariatric surgical intervention to lifestyle intervention therapy for diabetes treatment, the surgical intervention group has more supplies and maintains glycemic control rates (Courcoulas et al., 2020). Stress has an effect on metabolic activity as well, causing blood glucose levels to rise and insulin action to fall. According to some studies, managing stress through pharmacological or behavioral interventions may help with diabetes treatment. However, more long-term research is required (Surwit and Schneider, 1993).

2.9 FFA-induced impairment of insulin signaling

It has been hypothesized that expression of IR is immoderate in the cells of adipose, skeletal muscle and liver tissues. Except for adipose tissues, liver tissues and muscle tissues, there is no other tissue where glucose is accumulated and stored. When the blood glucose levels of normally healthy adult individuals are examined, it is observed that it is between 90-12 mg/dl and this level rises to 250-300 mg/dl in postprandial blood sugar (after the meal). In healthy individuals, the fasting insulin level can rise to 15 units per milliliter of blood, and the postprandial blood glucose can rise to 40 units per milliliter of blood. The decrease in postprandial glucose is dependent on an increase in insulin level. It takes about 2 hours to bring blood sugar levels back to normal. Hyperglycemia is removed from the bloodstream by insulin, and glucose is provided by glucose uptake and storage in myocytes, adipocytes, and hepatocytes' target cells. Because myocytes store 75% of glucose and hepatocytes and

adipocytes store the rest, myocytes are insulin's primary target cell. When insulin fails to restore the blood glucose that increased post-prandial glucose to normal levels, hyperglycemia remains in the blood circulation longer, as in T2DM. For this reason, in T2DM patients and insulin resistance, problems such as abnormal fatty acid oxidation symptoms, increased circulatory concentration and lipid accumulation in myocytes may occur frequently (Boden, 1997; McGarry, 2002). It increases insulin activity on myocytes, hepatocytes and adipocytes due to hyperlipidemia, but hyperlipidemia levels reduce insulin activity, the increase of FFA in the blood due to hyperlipidemia decreases glucose uptake into the cell stimulated by insulin (Moller, 2001).

IR is one of the places where insulin resistance occurs. This is due to insulin resistance fatty acid. Insulin resistance is created by fatty acids in different ways. The phosphorylation status of the kinase domain, changes in IR expression, activities of receptor tyrosine kinases and binding of ligands are implicated in explaining insulin resistance. There may be a functional defect that may be associated with genetically based defects, or it may affect its binding to ligands, its expression in the IR, and its tyrosine kinase activity (Pessin and Saltiel, 2000).

To investigate what causes homozygous *IR*-null mice to die shortly after nativity, the *IR*s of knockout mice were examined and it was concluded that homozygous *IR*-null mice died not long after birth due to excessive insulin resistance (Accili et al., 1996; Joshi et al., 1996). Homozygous knockout mice did not show a significant phenotype since they did not have a single allele of the *IRS-1* gene, but it was concluded that the *IRS-1* gene was homozygous disrupted in mice with mild insulin resistance (Araki et al., 1987).

Comparing double heterozygous mice (*IR* -/+, *IRS* -/+) and *IRS-1* homozygous null mice (*IRS-1* -/-), possibly due to pancreatic beta cell compensation, no significant diabetic phenotypic was observed in *IRS-1* homozygous null (*IRS-1* -/-) mice did not indicate a definite diabetic phenotypic expression, but mice with double heterozygosity (*IR* -/+, *IRS* -/+) developed both diabetes and insulin resistance (Brüning et al., 1997). It was concluded that homozygous deformation in the *IRS-2* gene is the cause of impaired insulin secretion with diabetes and peripheral insulin resistance (Withers et al., 1998).

Insulin resistance examined in *IRS-2*-impaired animals may be thought to reflect secondary events related to changes in cell function that make *IRS-2* appear unnecessary for exercise or insulin-induced glucose transport (Higaki et al., 1999). Thereby, it can be said that changes in

the insulin secretion pattern affect the findings of studies in cell-specific *IR* knockout mice that improve diabetes and peripheral insulin resistance (Kulkarni et al., 1999).

It has been demonstrated that increased IRS-1 serine phosphorylation is associated with reductions of insulin-induced FFA and IRS-1 tyrosine phosphorylation, and IRS-1-based PI3K activity. This disrupts downstream effectors and reduces RS-1 tyrosine phosphorylation (Yu et al., 2002). Downstream insulin signals can be further disrupted by FFA. The application of saturated fat causes Akt/PKB to block insulin activation because of the rise in the amount of diacylglycerol and ceramide in muscle cells exposed to the culture medium (Chavez et al., 2003). *Glucose transporter type 4 (Glut-4)* increases the speed of Glut-4 traffic across the membrane by actively sequestering it in an intracellular location by insulin (Martin et al., 2000). There is only one way for glucose to be transferred into the cell. This pathway is made possible by Glut-4 making the membrane translocation for insulin the final step in signaling. In addition, it has been proven that Glut-4 translocation defects are the cause of lipid-induced insulin resistance (Pessin et al., 1999).

2.10 Prevention of DM

It has been stated that the risk of young-onset T2DM is low in breastfed infants (Taylor et al., 2005). Obesity stands out as an important risk factor for T2DM. According to the statement made by Health Canada, it emphasized the importance of healthy nutrition and physical activity in children to prevent diabetes (Health Canada, 2013). Studies have shown that orlistat or metformin induce short-term weight loss in obese adolescents and children (Rogovik et al., 2010).

2.11 Pterostilbene

Pterostilbene (trans-3, 5-dimethoxy-4 hydro-hydroxystylbene; PTS) is known as a naturally dimethylated analog of resveratrol and its main source is grapes and blueberries (Paul et al., 2010). Pterostilbene with its phenol structure is chemically related to resveratrol. Comparing PTS and resveratrol, PTS has a higher lipophilicity effect than resveratrol because it contains two methoxyl groups, resulting in higher bioavailability, longer half-life and lower toxicity (Benlloch et al., 2016; Kapetanovic et al., 2011).

Pterostilbene is found naturally in grapes and blueberries. Pterostilbene includes one hydroxyl group and two methoxy groups whereas resveratrol has three hydroxyl groups is the main

structural difference that distinguishes Pterostilbene and resveratrol from each other. Pterostilbene is more lipophilic because it has two methoxy groups, which increases cellular uptake and oral absorption occurs with a higher potential efficiency to pterostilbene (Lin et al., 2009). When the half-lives and high oral bioavailability of PTS and resveratrol are compared, the half-life of PTS stands out as 105 minutes and high oral bioavailability of 80%, while the half-life of resveratrol is 14 minutes and high oral bioavailability of 20%, significantly lower than the values of PTS. (Asensi et al., 2002; Kapetanovic et al., 2011; Nutakul et al., 2011; Remsberg et al., 2008). In addition, PTS has representing low total body clearance followed by broad tissue distribution. (Kapetanovic et al., 2011).

PTS and resveratrol have biological functions such as anti-inflammatory, anti-oxidant, anti-diabetic, anti-cancer and anti-oxidant activities (Bhakkiyalakshmi et al., 2016). Oral administration of 40 mg/kg PTS for 6 weeks showed that 56% decrease in blood glucose and a concomitant significant increase in serum insulin in an in vivo study with a streptozotocin (STZ)-induced rat diabetes model (Pari and Satheesh, 2006).

Extensive animal studies have been conducted examining PTS for its efficacy and safety. Animal studies showed their efficacy on cardiometabolics as well as cognition mediators and cancer (Mizuno et al., 2008; Pan et al., 2008; Rimando et al., 2005). Generally Recognized as Safe (GRAS) substances are acquitted from pre-market Food and Drug Administration (FDA) inspection. In 1958, the GRAS status criteria were introduced in the Food Additives Amendment to the Federal Food, Drug and Cosmetics Act. A foodstuff has been scientifically investigated by scientists and considered safe for human consumption after 1958 to receive GRAS approval. (U.S. Department of Health and Human Services, Food and Drug Administration, 2011).

It is now accepted that insulin resistance (hyperinsulinemia) has an important contribution to the development of dyslipidemia, hypertension and glucose intolerance in patients with NIDDM (www.diabetes.org). Considering the useful of dietary polyphenols in the prevention of cardiovascular disease and control of glucose homeostasis has been investigated (Borriello et al., 2010; Nadtochiy and Redman, 2011).

2.12 Molecular pathway

It has been demonstrated that several defects in insulin signaling such as changed insulin receptor (IR) kinase activity, abnormalities in glucose transporter translocation, reduces in phosphorylation of intracellular substrates and decreases in IR number or affinity are effective in the development of peripheral insulin resistance (DeFronzo, 1997). On the other hand, the molecular mechanisms that are the actual cause of the relative dysfunction of β -cells in T2DM remain unidentified as a result of extreme complexity. Maturity-onset diabetes of the young (MODY) formation is related with primary defects in pancreatic β -cells, *hepatocyte nuclear factor (HNF) 1-alpha*, *HNF 4-alpha* (Yamagata et al., 1996), *HNF 1-beta* (Horikawa et al., 1997) or *IPF1* (Stoffers et al., 1997) and mutations in genes encoding Glucokinase (Froguel et al., 1992) has been stated. Despite intensive research, the genetic basis of the prevalent T2DM form is still not determined.

Numerous reports have been reported of excessive lipid demand due to increased circulating FFA, which conduce to the formation of T2DM (Boden, 1997; McGarry, 2002). Consequently, insulin resistance is caused by an increase in intramyocellular lipids, and an increase in intramyocellular lipids is due to the storage of excess FFA in non-fat stores (Boden et al., 2001; Kuhlmann et al., 2003). The primary manifestations of this disease are the onset of loss of insulin activity and insulin resistance. It is now well known that the main factors affecting insulin resistance are FFAs, but the underlying mechanism of how insulin resistance is affected by FFAs is still not resolved.

The FFA concluded that insulin-mediated signals cause inhibition of glucose transport. Phosphorylation of IRS-1 and activation of IRS-1-related PI3K occur through the linkage of insulin to the heterotetrameric membrane receptor (Chavez et al., 2003; Dresner et al., 1999; Yu et al., 2002). Glucose uptake into the cell occurs by the transport of the Glut-4 to the membrane; this happens by influencing Glut-4, which is activated by downstream effectors such as Akt/PKB (Hill et al., 1999). Inhibition of the Gluconeogenic key enzyme, Phosphoenolpyruvate Carboxykinase (PEPCK), and phosphorylation of its downstream targets could not be achieved by the mutant kinase. It proposes that the disruption of insulin activity is due to impairments in the insulin signaling pathway. They are insulin signaling molecules that have a role in cellular insulin resistance and also take part in metabolic and mitogenic effects (Greene et al., 2004; White, 2002; Zick, 2001). Some Protein kinase C (PKC) isoforms that

have been proposed to have a regulatory effect on insulin signaling have been shown in several recent reports. Insulin resistance has been found to be interrelated with the expression levels and activity of several PKC isoforms. Lipid infusion is effective in disrupting insulin-based glucose release in muscle in rats and humans with simultaneous activation of specific PKC isoforms (Boden and Shulman, 2002; Griffin et al., 1999; Yu et al., 2002).

2.13 Insulin receptor and signaling

When the target cell surface receptor binds, the effect of insulin initiates, which is the component of the receptor molecule that has a role in the activation of tyrosine kinase. The IR is a heterotetrameric complex of two α subunits ($\alpha_1\alpha_2$) and two β subunits ($\beta_1\beta_2$) bound by disulfide bonds. Transmission of signals across the plasma membrane occurs when insulin binds to the extracellular α subunit then activates the β subunit's tyrosine kinase C terminal domain.

The effect of a subunit on a set of intramolecular transphosphorylation reactions in which its adjacent partner phosphorylates on a certain tyrosine residue is caused by the binding of insulin to the IR (Pessin and Saltiel, 2000). Their expression in cells of muscle, fat and liver tissues is quite high, although IRs are found on the surfaces of almost all cells (BRUNETTI et al., 2001). The catalytic activity of the receptor tyrosine kinase that recruits IRS proteins is stimulated by autophosphorylation of the IR tyrosine residue (Pessin and Saltiel, 2000; Rosen, 1987). These processes provide increased effector enzyme activity (White, 1998; Yenush et al., 1998). With, IRS-1 and IRS-2 proteins phosphorylate certain phosphoinositols must be targeted by PI3 kinase for the formation of phosphatidylinositol 3,4,5 triphosphate (PIP3) from phosphatidylinositol 4,5 biphosphate (PIP2). Accordingly, ser/thr kinase, that is, phosphoinositide-dependent kinase-1 (PDK1), is activated (Le Good et al., 1998). Ser/thr kinase Akt/PKB is phosphorylated or activated by activated PDK1. Akt directly interacts with PIP3 by containing a specific PH domain (Saltiel and Kahn, 2001). Activation of Glut-4 depends on Akt. Glut-4 moves across the cell membrane to take glucose from the cell surface into the cell. With the movement of Glut-4 to the membrane, it is understood that Glut has a significant role in the transport of glucose into the cell. Glut-4 is an insulin-dependent transporter protein. Binding of Glut-4 to the insulin signaling pathway begins via Akt. Akt has a large role in the connecting of Glut-4 to the insulin signaling pathway (Kupriyanova and

Kandror, 1999; Martin et al., 2000; Pessin et al., 1999). The insulin signaling pathway that operates in the insulin target cell is briefly described by Figure 1.

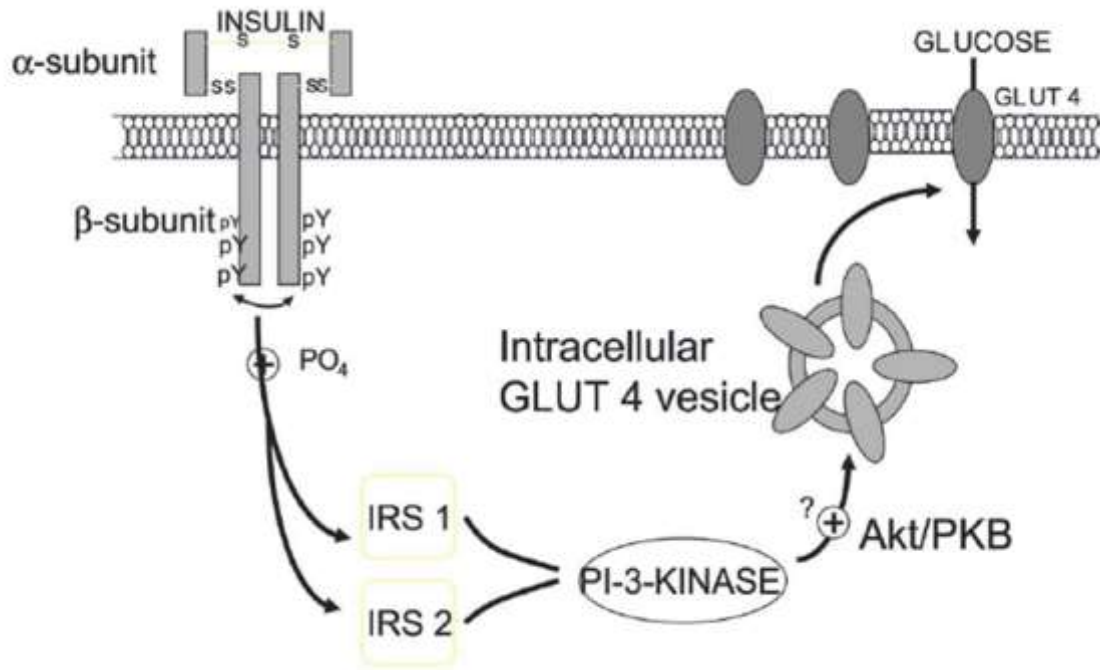


Figure 1. Insulin signaling pathway

3. MATERIALS AND METHODS

3.1 PTS preparation

In this study, the range of using PTS concentrations (1-20 μ M) were determined based on the scientific reports (Bedi et al., 2020; Hsu et al., 2012; Seo et al., 2017). PTS (HPLC purity, 99.8%) was obtained from Sabinsa. PTS was dissolved in dH₂O to 1M and 1mM stock solutions and then stored at -20 C° under shade until use.

3.2 Cell culture and proliferation

3T3-L1 (CRL-173) preadipocyte cells were taken from the American Type Culture Collection (ATCC, Manassas, VA) and seeded into T25 culture flasks at a density of 1×10^6 cells/cm² containing expansion medium. 3T3-L1 cells expansion medium was prepared in 90% DMEM medium prepared with FBS to a final concentration of 10% supplemented with 1% Penicillin/streptomycin and 1% L-Glutamine. In T25 culture flasks, incubations were carried out in a humidified environment at 37 °C, 5% CO₂ and 95% air in an incubator (İldam, ILD-CI-80, Turkey). Culture media was changed every 3 days in a biosafety cabinet (DEMAIR, MSC-II A, Turkey). In addition, images of cells were obtained using an inverted microscope (Leica, Dmii Led Fluo, Thermo Fisher, Germany).

3.3 Cells viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrasodium bromide] was utilized to investigate the effect of PTS on 3T3-L1 preadipocytes viability and to determine the appropriate dose of PTS to be used in further studies (Mosdam, 1983). 3T3-L1 preadipocytes were cultured into 96-well-plates at a density of 2×10^3 cells/well and incubated. The medium was removed when 3T3-L1 preadipocytes reached 80-90% confluency. The cells were treated with DMEM medium supplemented with various concentrations of PTS (1-2.5-5-7.5-10-12.5-15-17.5-20 μ M) and 0.1% (v/v) dimethyl sulfoxide (DMSO) except DMEM controls.

For MTT assay, the culture media was aspirated and cells were rinsed with PBS. MTT solution was prepared 0.5 g/mL in PBS and 100 μ L of the solution was applied to wells. The cells were incubated for at 37 °C for 4h. Afterwards, 100 μ L of DMSO was supplied to the wells for 30 min. The relative cell viability was measured with a spectrophotometer (Uvmini-1240, Shimadzu, Kyoto, Japan) optical density (OD) at wavelength of 570 nm. The relative cell-

viability was plotted in graphs. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = (\text{Average OD}_{570} \text{ of treated cells} / \text{Average OD}_{570} \text{ of control cells}) \times 100 \quad (1)$$

3.4 Differentiation

The 3T3-L1 preadipocytes were differentiated into mature adipocytes in adipocyte maintenance medium in the presence or absence of PTS (Green and Kehinde, 1975). The differentiation medium was made up of 90% DMEM, 10% FBS, 1% L-Glutamine, 1% Penicillin/streptomycin, 1 μ M dexamethasone (DEX), 0.5 mM isobutylmethylxanthine (IBMX) and insulin (1 μ g/mL). First of all, 3T3-L1 cells were cultivated at 1×10^6 in a 25 cm² flask filled with preadipocyte growth media. Preadipocyte cells were induced to differentiate using differentiation medium supplemented with either in the presence or absence of PTS. After 48 hours for differentiation induction. The differentiation medium was aspirated and adipocyte maintenance medium which contains DMEM with 10% FBS and insulin (1 μ g/mL) was added (Figure 1). An inverted microscope was used to examine differentiated adipocyte cells. Also, Oil Red O staining was performed for observing lipid accumulation in the cells (Hsu et al., 2012; Seo et al., 2017).

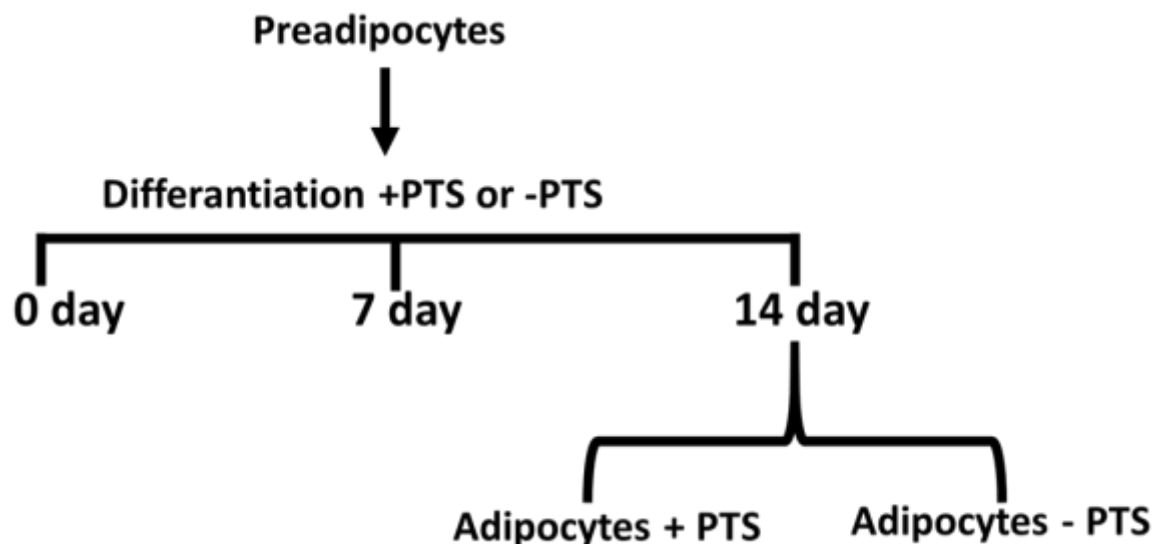


Figure 2. Differentiation protocol of the 3T3-L1 adipocytes

3.5 Oil Red O staining

Oil Red O Staining Kit was applied to examine and determine of the lipid accumulation in preadipocytes, differentiated adipocytes and the efficacy of PTS in 3T3-L1 adipocytes (Hsu et al., 2012; Hu et al., 2018; Seo et al., 2017). The 3T3-L1 preadipocytes, differentiated adipocytes and PTS loaded adipocytes were seeded on coverslips (Nunc Thermanox Coverslips, Thermo Scientific, USA) plated in 12-well plate (1×10^5 cells/well) for Oil Red staining. Coverslips were taken at day 14 and medium was aspirated. Then, cells were gently rinsed with PBS. After that, cells were fixed using 1 mL 10% (v/v) of formalin for 30 min. After fixation, cells were washed with distilled water and then added 1 mL 60% isopropanol (v/v) for 5 min. Then isopropanol was discarded. The Oil Red O working solution containing 60% Oil red O stock solution and 40% distilled water was filtered through Whatman no:1. The cells were stained directly with 1 mL Oil Red O solution for 20 min. After incubation, the Oil Red O solution was aspirated and cells were rinsed with distilled water until the stain is gone. Hematoxylin was immersed into the wells and cells were incubated for 1 min and then rinsed 2-5 times with distilled water. Briefly, images of cells were obtained using a microscope. In addition, total lipid content in cells was measured by washing with isopropanol to remove stain residues. Also, spectrophotometer was employed to measure OD at wavelength of 500 nm (Nanoprop 2000c, Thermo Scientific, USA) (Hsu et al., 2012; Hu et al., 2018; Seo et al., 2017).

3.6 Live-dead assay

In order to analyze the effect of PTS on cell viability, live-dead cell assay was used (Derici et al., 2021). Ethidium homodimer-1 (Eth-1) binds to the DNA of cells with impaired cell membrane integrity, showing dead cells with red fluorescence. Calcein-AM (C-AM), on the other hand, is a non-fluorescent acetomethoxy-derived compound, but it is broken down by esterase enzymes in the cytoplasm and turns into Calcein, which makes living cells appear green with the effect of fluorescence. For the live/dead cell assay, 3T3-L1 preadipocytes, differentiated adipocytes in presence or absence of PTS were seeded on coverslips into a 24-well plate. The assay was performed on 0, 7 and 14 days. Firstly, cells were washed twice with 250 μ L of +/+ PBS. For the continuation of the staining process, 1 μ M Eth-1 and 1 μ M C-AM were added to the wells. Following 30 min incubation which is under room temperature and darkness, added solutions were discarded from the wells, and then rinsed by 250 μ L +/+ PBS. Cells were examined at 10X to assess the distribution of cells on the coverslip with the aid of a

fluorescent microscope. After the examinations, images were taken for each coverslip. ImageJ software was used to determine the relative cell viability in each area. Calculation of cell viability (%):

$$\text{Viable Cells (\%)} = [\text{Viable cells} / (\text{Viable cells} + \text{Dead cells})] \times 100 \text{ (2)}$$

3.7 Phase contrast microscopy

The 3T3-L1 preadipocytes, 3T3-L1 adipocytes in presence or absence of PTS were followed and monitored while cultivation process continues by using phase contrast microscope (Leica, Dm1e Led Fluo, Thermo Fisher, Germany). Since the cells were visible in the plate, different staining procedures were not required. The cells were imaged at day 0, day 7, and day 14 under 10X magnification.

3.8 Immunofluorescence staining

Immunofluorescence (IF) staining was applied to examine the effect of PTS on the intracellular level of Glut-4 and Adipoectin in 3T3-L1 cells and cell morphology. The immunofluorescence staining was performed on 0. day, 7. day and 14. day. The 3T3-L1 preadipocytes, differentiated adipocytes and PTS loaded adipocytes were cultivated into a 6-well plate. 24 h later, the coverslips were washed with 1X PBS. After the washing process was finished, the coverslips in the 6-well plate were transferred to the 24-well plate, one for each well, with the help of sterile needle and tweezers. For the fixation process, 250 μ L of 4% (w/v) paraformaldehyde was applied on the coverslips and incubated at room temperature for 20 min. Then, paraformaldehyde was discarded from the wells and the coverslips washed 3 times with cold 300 μ L 1X PBS. Permeabilization is a very important process since the target protein is intracellular. 250 μ L of 0.1% Triton X-100 (thawed in 1X PBS, v/v) was applied on the coverslips and incubated for 5 min at room temperature for permeabilization. Triton X-100 is the most common detergent used to improve the penetration of the antibody. Along with this feature, it also destroys the membranes, which is not suitable for membrane-bound antigens. The triton X-100 on the cells was aspirated and the wells washed for 5 min with 250 μ L of 1X PBS for three times. After permeabilization, 250 μ L 1% (w/v) of BSA in PBST (PBS + %0.1 Tween 20) was applied to each well for 30 min at ambient temperature. Afterwards, cells were

incubated with 250 μ L 1% (w/v) anti-Beta Actin, 1:100 (v/v) of Adinopectin and anti-Glut4 in PBST overnight in a humidified room at 4 °C. Then, cells were gently washed with PBS. 250 μ L 1:1000 (v/v) of Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) was applied to wells as a secondary antibody and cells were incubated for 45 min at room temperature. After that, the cells were rinsed with PBS for three times. To perform the immunostaining procedure, 250 μ L Phalloidin-iFluor 488 Reagent 1:100 (v/v) in %1 (w/v) BSA was added into the wells for incubation.

Also, for nucleus staining, DAPI 1:1000 (v/v) in PBS was added to the wells and incubated in darkness for 30 min at ambient temperature. After the incubation, solutions were aspirated and the wells were rinsed 3 times with PBS. Finally, the coverslips, on which all staining procedures were applied, were taken on the slides with the cellular parts on top for visualization. 3 μ L of BrightMount/Plus was added to the coverslips taken on the slides. The IF staining was visualized at 10X and 20X magnifications using a fluorescence microscope and ImageJ software was used to analyses fluorescein signals (imagej.nih.gov) (Schindelin et al., 2015).

3.9 Giemsa staining

Giemsa staining was used to analyze the effect of PTS on cell morphology. The 3T3-L1 preadipocytes, differentiated adipocytes in presence or absence of PTS were seeded on coverslips into a 24-well plate (Derici et al., 2021). The coverslips were taken at day 7 and day 14 for fluorescence staining. The wells were eluted with 250 μ L PBS twice. Then, cells were fixed with 250 μ L 60% (v/v) of cold methanol in PBS for 1 h. After that, 250 μ L 100% (v/v) methanol was added to wells and incubated for 10 min at room temperature. Then, the wells were rinsed with 250 μ L of PBS twice. 0.1 g of Giemsa dye was dissolved in 10 mL of methanol, kept in hot water for 20-30 minutes and passed through a 0.22 μ m filter. 1 mL of Giemsa was added to cover the surfaces of the cells whose fixation process was completed and incubated for 20 min at room temperature. Finally, the dye solution was discarded and the wells were rinsed three times. Cell morphologies were examined at 10X under an inverted microscope. The ImageJ program (imagej.nih.gov) was employed to measure the mean cell diameter in each three areas in images for three wells.

3.10 RNA isolation and Real-time RT-PCR

To observe the effect of PTS on insulin resistance and adipogenesis, the expression of *Glut-4* and *Adiponectin* genes expression level in 3T3-L1 cells was evaluated using Real-time PCR (Li et al., 2016). The 3T3-L1 preadipocytes, 3T3-L1 adipocytes in presence or absence of PTS were cultured into a 6-well plate (3×10^5 cells/well) and harvested at 14 days after the initiation of differentiation for Real-time RT-PCR. TRIzol reagent (Life Technologies, Rockville, MD, USA) was used to isolate total RNA. The RNA was thawed with DEPC water. Concentrations RNA was stored at -80°C until it was used. Concentrations of RNA samples were determined using a spectrophotometer (Nanodrop 2000c, Thermo Scientific, USA). RNA was converted to cDNA using ProtoScript II first Strand cDNA Synthesis Kit (NEB, New England Biolabs Inc., USA) according to the manufacturer's instructions. One Taq 2X Master Mix with Standard Buffers (New England Bio labs Inc., USA) used for amplification of cDNA were made up to 25 μL in volume by thermal cycler using the primer pairs primer pairs synthesized by Sentebiolab.:

Glut-4, 5'-TAGGAGCTGGTGTGGTCAATACG-3' (forward) and 5'-
TAAAAGGGAAGGTGTCCGTCG-3' (reverse);

Adiponectin 5'-TACAACCAACAGAATCATTATGACGG-3' (forward) and 5'-
GAAAGCCAGTAAATAGAGTCGTTGA-3' (reverse);

Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh), 5'-TCAACGGCACAGTCAAGG-3'
(forward) and '5'-ACTCCACGACATACTCAGC-3' (reverse).

The GoTaq® 2-Step RT-qPCR System (Promega, ABD) was used for real-time RT-PCR by Qiagen Rotor-Geneq (Qiagen, Hilden, Germany). The PCR steps were shown in Table 1. The comparative threshold cycle CT method ($\Delta\Delta\text{CT}$) method was used for analysis of expression of target genes and normalisation of each gene expression results were measured with the *Gapdh* gene expression (Livak and Schmittgen, 2001). The Rotor Gene Q Software 1.2 program (Qiagen, Germany) was used qRT-PCR results.

Table 1. The protocol real-time RT-PCR

PCR step	Temperature (°C)	Time (min)	Cycle
Initial denaturation	94	3	1
Denaturation	94	0.5	35
Annealing	52-58	0.5	35
Extension	72	1	35
Final extension	72	5	1

3.11 Statistical analysis

Graph Pad Prism software 8.4.3 version (Graphpad Software Inc., USA) was performed for statistical analysis of the data. Data were expressed as mean $\pm$ standard deviation (SD). Moreover, each experiment was performed done in triplicate. It was examined whether the results found in experiments were normally distributed or not, and Student's t-test was used to determine differences among the groups. For multiple comparisons of results, one-way ANOVA analysis followed by Tukey's test were used.

4. RESULTS AND DISCUSSION

4.1 Effects of PTS on cell viability in 3T3-L1 preadipocytes by MTT assay

In order to assess the efficiency of PTS on the viability of 3T3-L1 cells and to determine the appropriate dose for use in future studies, the cells were treated with PTS with different concentrations between 0-20 μM for 24, 48 and 72 h, and the results were expressed as % cell viability has been shown in Figure 3. At the end of the 24 h incubation, a 10% reduction in the viability of the cells to which PTS concentrations higher than 5 μM was applied compared to the control was observed and the reduction in cell viability continued significantly at higher concentrations ($P = 0.6081$ for 1 μM ; $P < 0.0001$ for 2.5 μM ; $P = 0.0001$ for 5 μM ; $P < 0.0001$ for 7.5 μM and higher concentrations) (Figure 3A).

After 48 h of incubation, a 10% decrease in the viability of cells exposed to concentrations of PTS higher than 7.5 μM was observed compared to the control, and the reduction in cell viability continued significantly at higher concentrations ($P = 0.3262$ for 1 μM ; $P = 0.0017$ for 2.5 μM ; $P = 0.0012$ for 5 μM ; $P < 0.0001$ for 7.5 μM and higher concentrations) (Figure 3A). Similarly, after 72 h of incubation, a 10% reduction in viability of cells exposed to concentrations of PTS higher than 8.5 μM was observed compared to control, and the reduction in cell viability continued significantly at higher concentrations ($P = 0.2545$ for 1 μM ; $P = 0.0791$ for 2.5 μM , $P = 0.0005$ for 5 μM , $P = 0.0073$ for 7.5 μM , $P < 0.0001$ for 10 μM and higher concentrations) (Figure 3A). Moreover, the PTS concentration (IC_{50}) causing 50% reduction in viability of cells for 24, 48 and 72 h' incubation was observed as $23.04 \pm 1.89 \mu\text{M}$, $17.37 \pm 1.22 \mu\text{M}$ and $14.60 \pm 2.88 \mu\text{M}$, respectively (Figure 3B, 3C, 3D). Based on this data, doses of 5 and 7.5 μM of PTS were selected to apply to cells in subsequent experiments. The results showed that the addition of PTS to the medium reduced the proliferation of 3T3-L1 preadipocytes in a time- and dose-dependent manner.

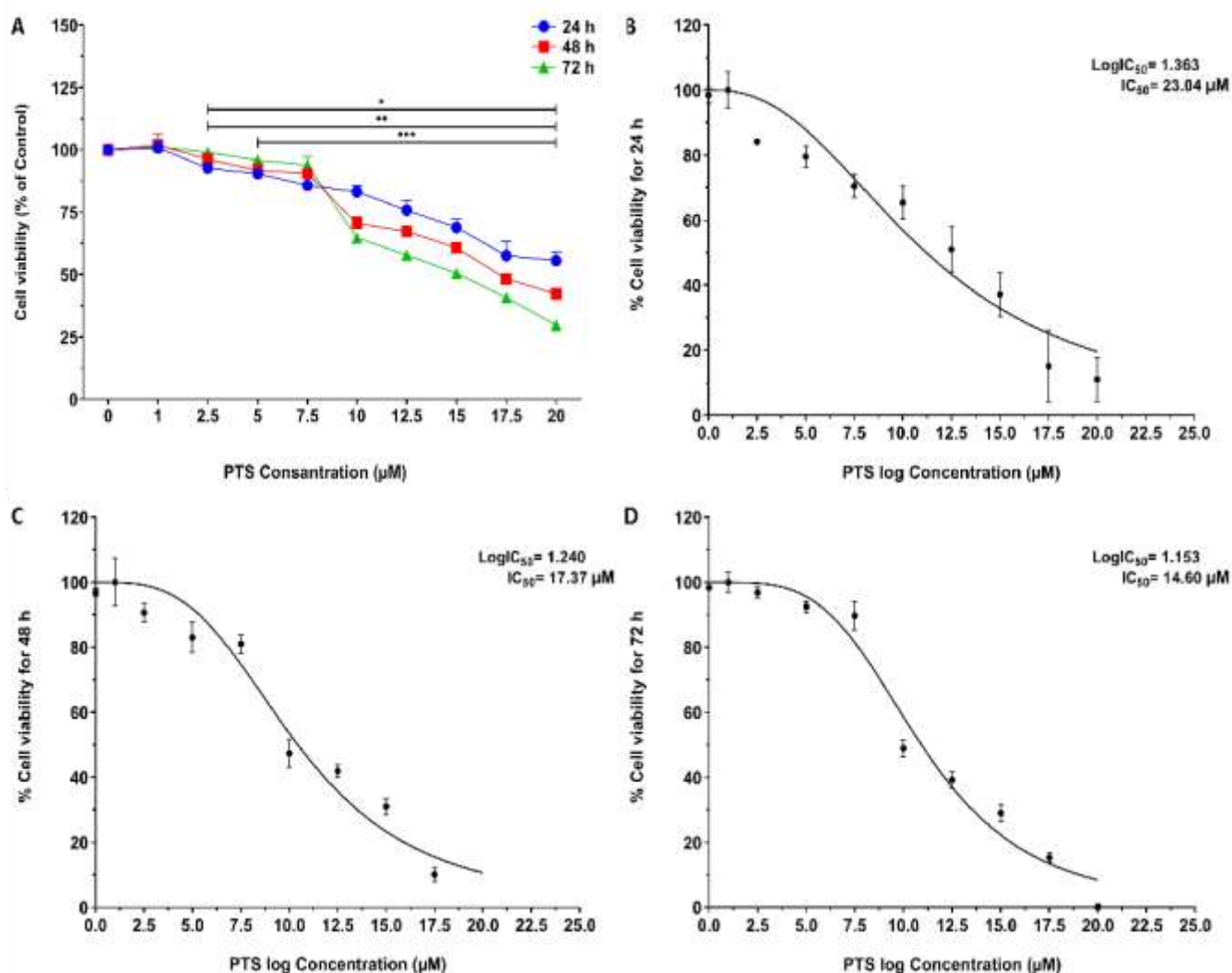


Figure 3. Effect of PTS on the viability of 3T3-L1 cells.

A. MTT assay data of PTS. 3T3-L1 preadipocytes cultured in 96-well plate were subjected to PTS treatment with increasing concentrations for 24, 48 and 72 h. Cytotoxicity was then calculated by MTT assay. In addition, the cell in the medium without PTS was used as the control and the cell in the medium without PTS but containing 0.01% DMSO was used as the vehicle control. **B. C. D.** IC₅₀ values of PTS for 24, 48 and 72 h. IC₅₀ was calculated by nonlinear regression model. Data are presented as mean $\pm$ SD from three independent experiments and expressed as fold change compared to control. One-way ANOVA test was used for statistical analysis. For 24, 48 and 72 h, respectively; * $P < 0.005$, ** $P < 0.005$, *** $P < 0.005$ significantly different from untreated 3T3-L1 preadipocyte. PTS: Pterostilbene; MTT: Methylthiazole tetrazolium; DMSO: Dimethyl sulfoxide; IC₅₀: half (50%) maximum inhibition concentration; SD: Standard deviation.

4.2 Effects of PTS on terminal differentiation of 3T3-L1 preadipocytes and adipogenesis by Oil-Red O staining

In order to confirm the adipocyte phenotype of 3T3-L1 cells and to evaluate the effect of PTS on the terminal differentiation phase of adipogenesis, 3T3-L1 preadipocytes were differentiated into mature adipocytes in adipocyte maintenance medium in the presence or absence of PTS (5 μ M and 7.5 μ M) for 14 days. Terminal adipocyte differentiation and lipid accumulation in cells were evaluated by Oil-Red staining at day 14 of adipogenesis (Figure 4). In the study, undifferentiated and non-PTS-treated preadipocytes and differentiated and non-PTS-treated adipocytes were used as controls. Comparing the intensity of Oil-Red-O staining in cells examined under an inverted microscope was considerably more intense in untreated adipocytes than in preadipocytes. However, an overall reduction in Oil-Red-O staining was observed in mature 3T3-L1 treatment group adipocytes treated with different doses of PTS compared to untreated adipocytes. Treatment of cells with a 7.5 μ M dose of PTS markedly reduced the level of Oil-Red-O staining compared to the 5 μ M dose treatment group of PTS (Figure 4A).

Furthermore, for quantification of lipid content in adipocytes, Oil-Red O dye from 3T3-L1 cells was measured by eluting with isopropanol. Lipid accumulation was increased by $85.96 \pm 2.16\%$ in untreated adipocytes compared to preadipocytes ($P < 0.0001$). Lipid accumulation was reduced by 30.64 ± 1.36 percent in 3T3-L1 adipocytes treated with a 5 μ M dose of PTS compared to untreated mature 3T3-L1 adipocytes ($P < 0.0001$). Intracellular lipid accumulation was reduced by approximately 54.93 ± 3.14 in 3T3-L1 adipocytes treated with a 7.5 μ M dose of PTS compared to untreated mature 3T3-L1 adipocytes ($P < 0.0001$). In addition, lipid accumulation was reduced by 35.02% ($P < 0.0001$) in adipocytes treated at the 7.5 μ M dose compared to the 5 μ M dose of PTS (Figure 4B). Treatment of 3T3-L1 preadipocytes with PTS significantly attenuated terminal differentiation in a dose-dependent manner, in agreement with other data. These data show that PTS treatment inhibits adipogenesis in 3T3-L1 cells by reducing lipid accumulation in adipocyte differentiation.

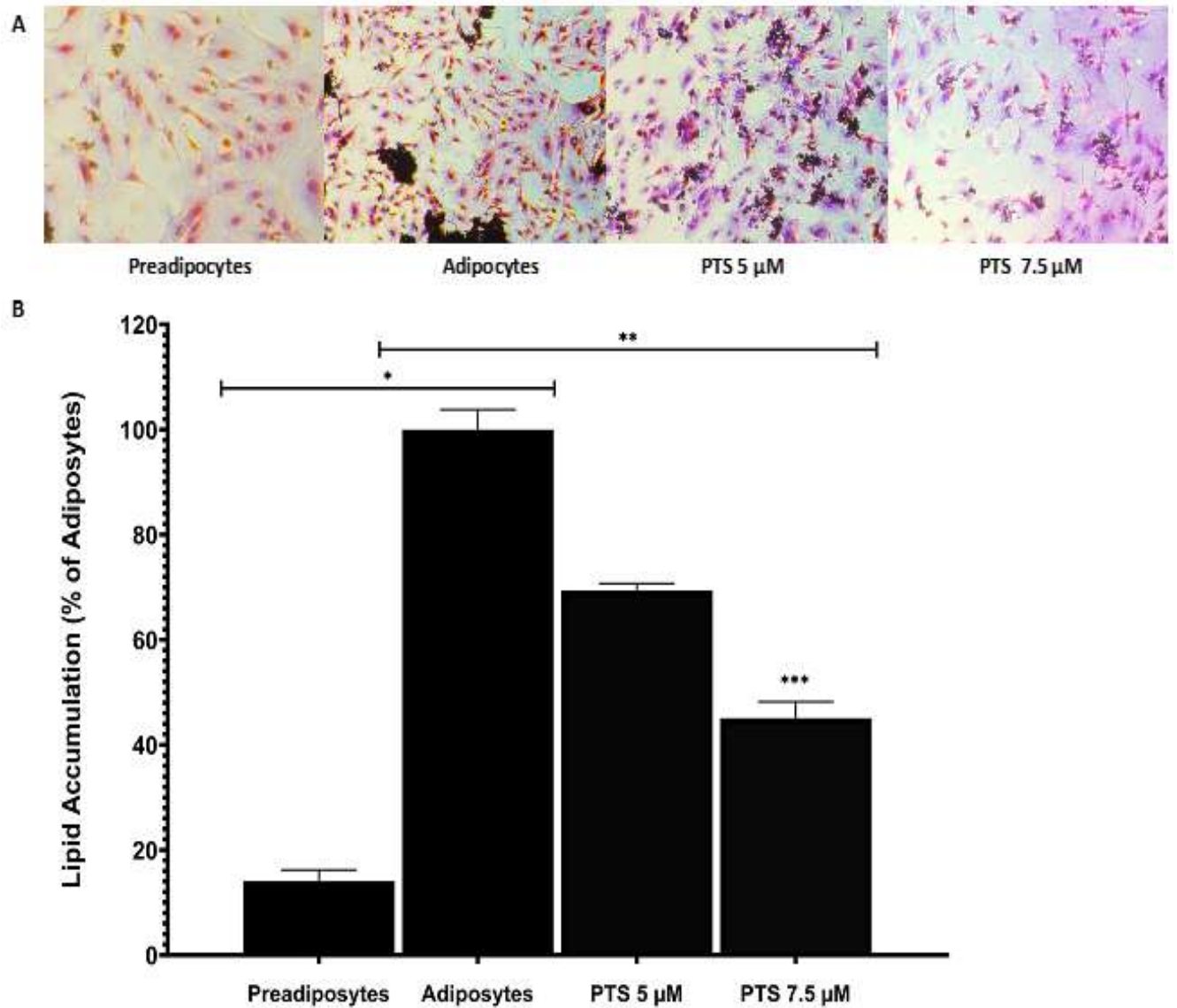


Figure 4. Effect of PTS on adipogenesis and lipid accumulation.

A. Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μ M or 7.5 μ M) of PTS in adipocyte maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. Representative microscopic images of cells stained with Oil-Red O at 10X magnification. **B.** Quantitative data of Oil-Red O staining. Data with relative change of OD500 from three independent experiments are presented as mean $\pm$ SD and expressed as fold change compared to adipocyte control. One-way ANOVA test was performed for statistical analysis. * $P < 0.0001$ and ** $P < 0.0001$ differ significantly from untreated 3T3-L1 adipocyte. *** $P < 0.0001$ is significantly different from the 5 μ M dose of PTS. PTS: Pterostilbene; OD: Optical density; SD: Standard deviation.

4.3 Effects of PTS on cell viability of 3T3-L1 in adipogenesis by live/dead assay

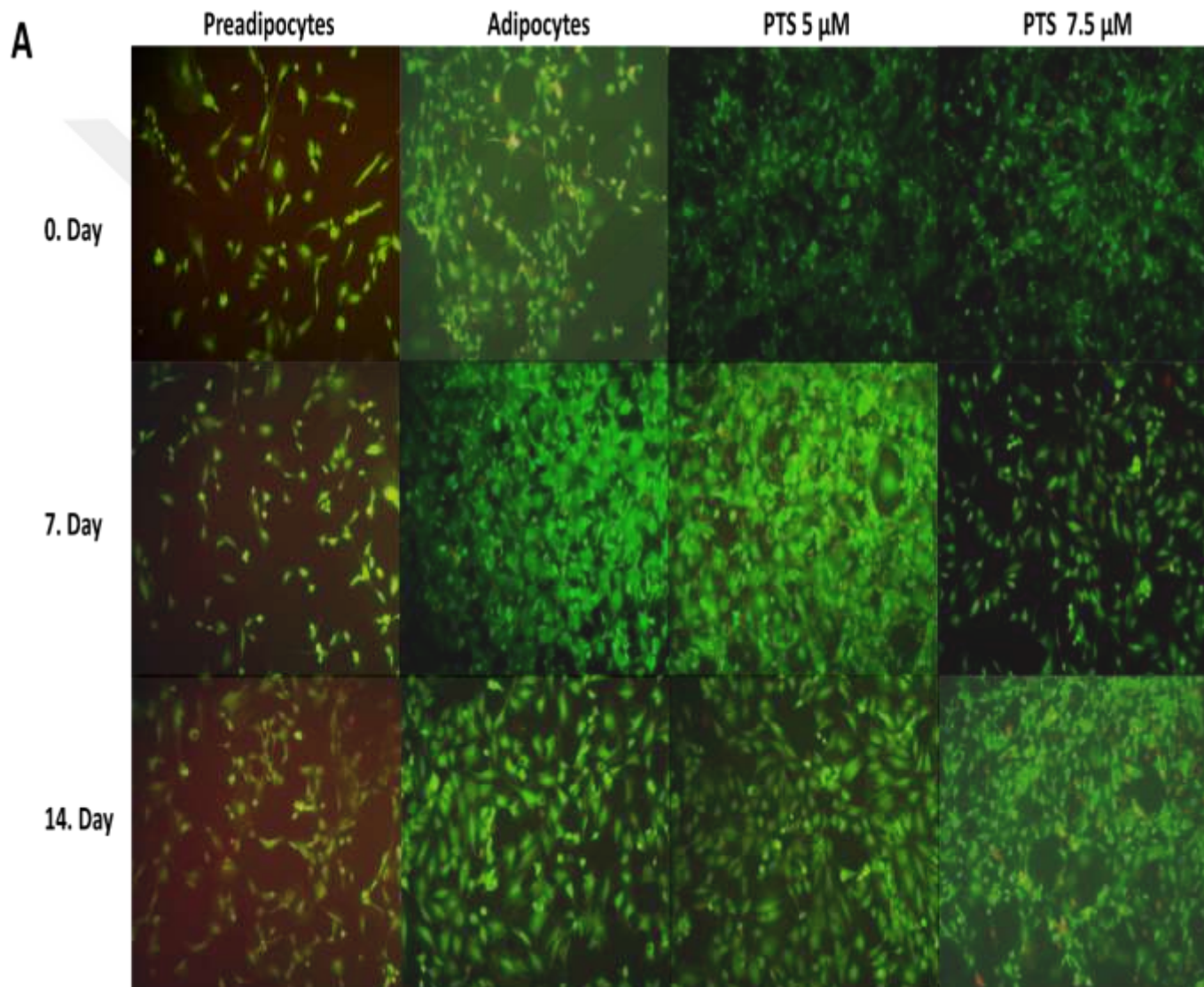
The effect of PTS on the viability of 3T3-L1 cells in the differentiation phase was evaluated using the live/dead assay. Two different media were created for 3T3-L1 cells based on the presence or absence of PTS (5 μ M and 7.5 μ M) for 14 days, and the cells were differentiated into mature adipocytes in adipocyte maintenance media. Dual staining was performed on days 0, 7 and 14 to separate live (green) and dead (red) cells using Calcein-Acetomethoxy (C-AM) and Ethidium homodimer-1 (Eth-1), respectively. Representative images of live and dead cells in PTS-loaded adipocytes obtained by preadipocytes, adipocytes and live dead assay are demonstrated in Figure 5A.

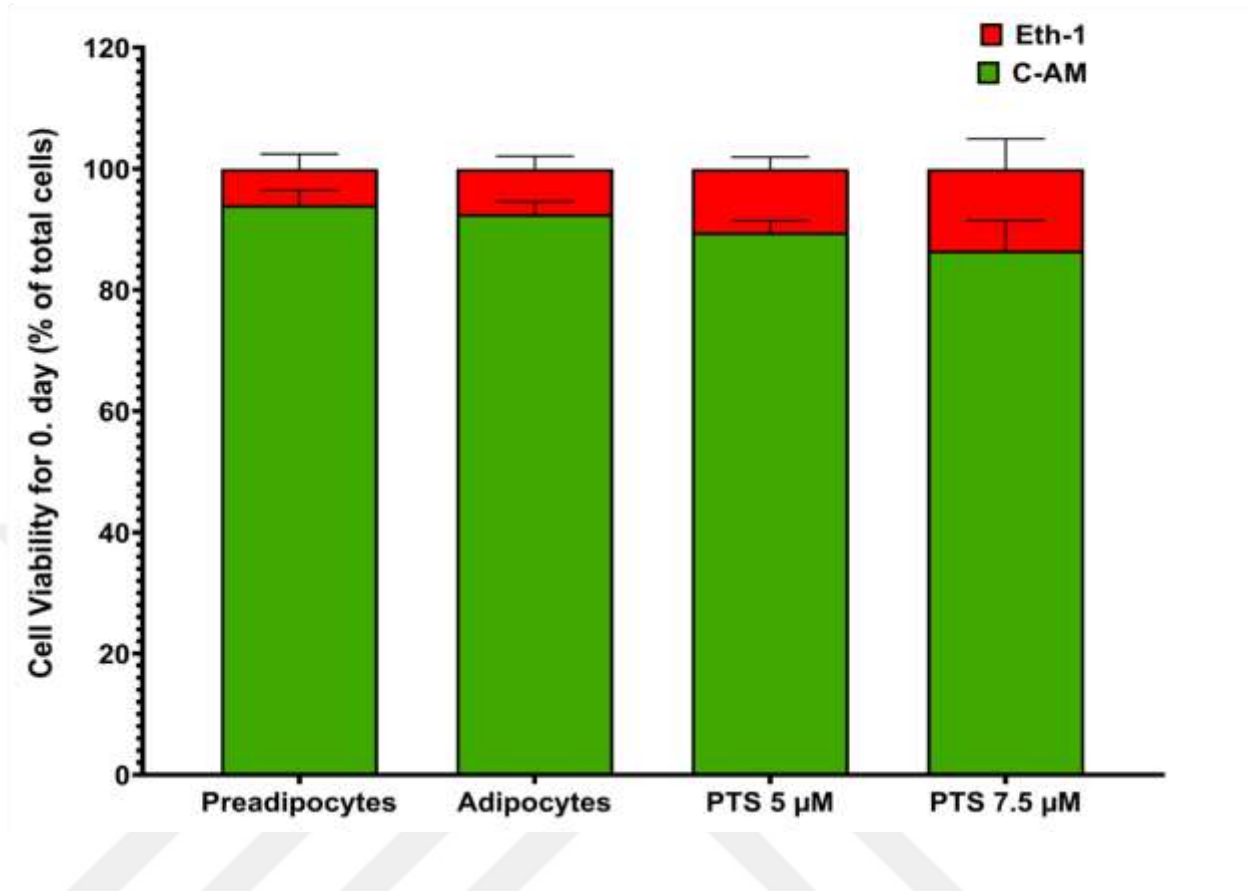
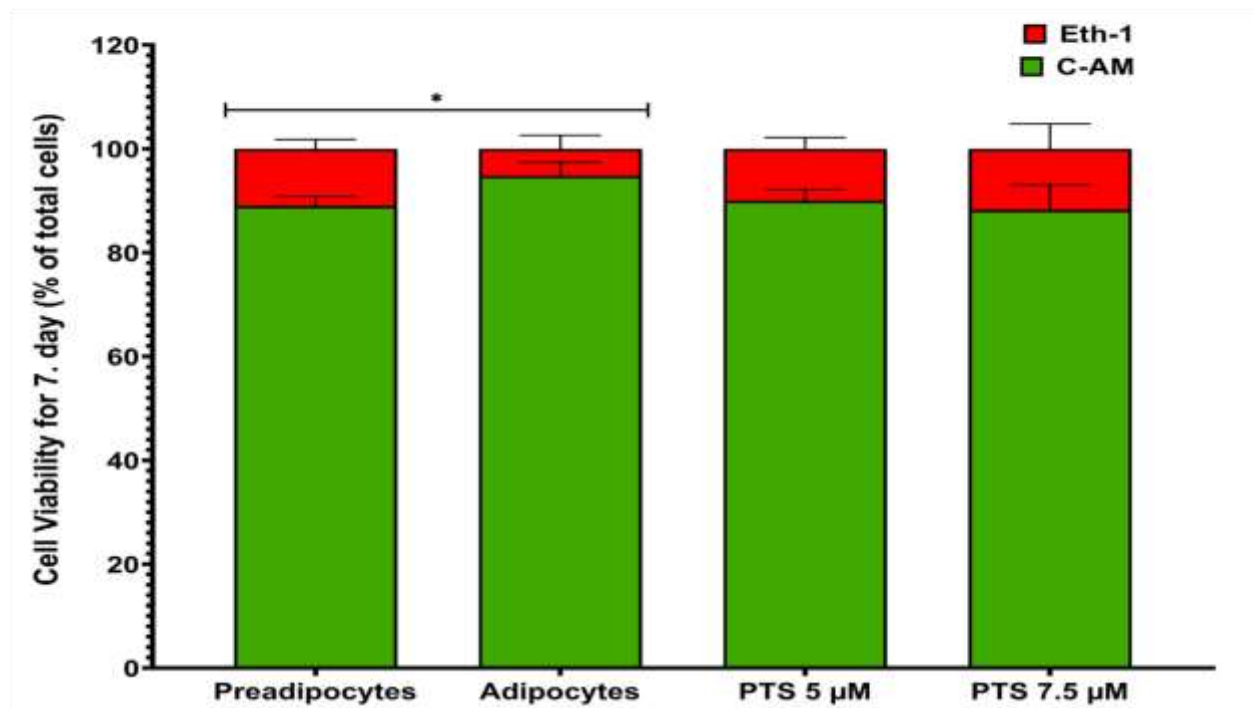
Under the inverted microscope for day 0 of differentiation, high-intensity green fluorescence and low-intensity red fluorescence were observed in all groups (Fig. 3A). The percentage of viable cells for day 0 in preadipocyte and untreated adipocytes were $94\% \pm 2.40$ and $92.50 \pm 2.08\%$, respectively ($P= 0.4860$). Also, similar to mature 3T3-L1 adipocytes, the percentage of viable cells for day 0 after 5 μ M and 7.5 μ M treatment of PTS was $89.50 \pm 1.90\%$ and $86.50 \pm 4.09\%$, respectively ($P= 0.2071$ and $P= 0.1516$). Moreover, the percentage of viable cell change was similar for the 5 μ M and 7.5 μ M treatments of PTS, with 3.24% and 6.48%, respectively ($P= 0.1540$) (Figure 5B).

On the 7th day of differentiation, under the inverted microscope, low red fluorescence intensity was observed with high green fluorescence intensity in all groups. However, green fluorescence intensity was lower in untreated adipocytes compared to preadipocytes (Figure 3A). Viability increased for day 7 in untreated adipocytes compared to preadipocytes, and the percentage of viable cells was $89.00 \pm 1.82\%$ and $94.75\% \pm 2.62$, respectively ($P= 0.0114$). Also, similar to mature 3T3-L1 adipocytes, the percentage of viable cells at day 7 after 5 μ M and 7.5 μ M treatment of PTS was $90.00 \pm 2.16\%$ and $88.25\% \pm 4.78\%$, respectively ($P= 0.1414$ and $P= 0.0536$). Furthermore, the 5 μ M and 7.5 μ M treatments of PTS, the percentage of viable cell change was similar. These values were 5.01% and 6.86%, respectively ($P= 0.6110$) (Figure 5C).

Under the inverted microscope for the 14th day of differentiation, low red fluorescence intensity was observed with high green fluorescence intensity in all groups. However, green fluorescence intensity was lower in untreated adipocytes compared to preadipocytes. Similarly, red fluorescence intensity was partially increased in treatment group adipocytes compared to untreated adipocytes (Figure 5A). Viability increased at day 14 in untreated adipocytes

compared to preadipocytes and the percentage of viable cells was $81.25\% \pm 2.62$ and $95.50 \pm 1.41\%$, respectively ($P= 0.0005$). Also, unlike mature 3T3-L1 adipocytes, the percentage of viable cells at day 14 after $5\ \mu\text{M}$ and $7.5\ \mu\text{M}$ treatment of PTS was 88.50 ± 3.10 and $79.75 \pm 1.25\%$, respectively ($P= 0.0319$ and $P= 0.0008$, respectively). Therewithal, the $5\ \mu\text{M}$ and $7.5\ \mu\text{M}$ treatments of PTS, the percentage of viable cell change was different from each other. These values were $\%6.84$ and $\%16.05$, respectively ($P= 0.6110$) (Figure 5C).



B**C**

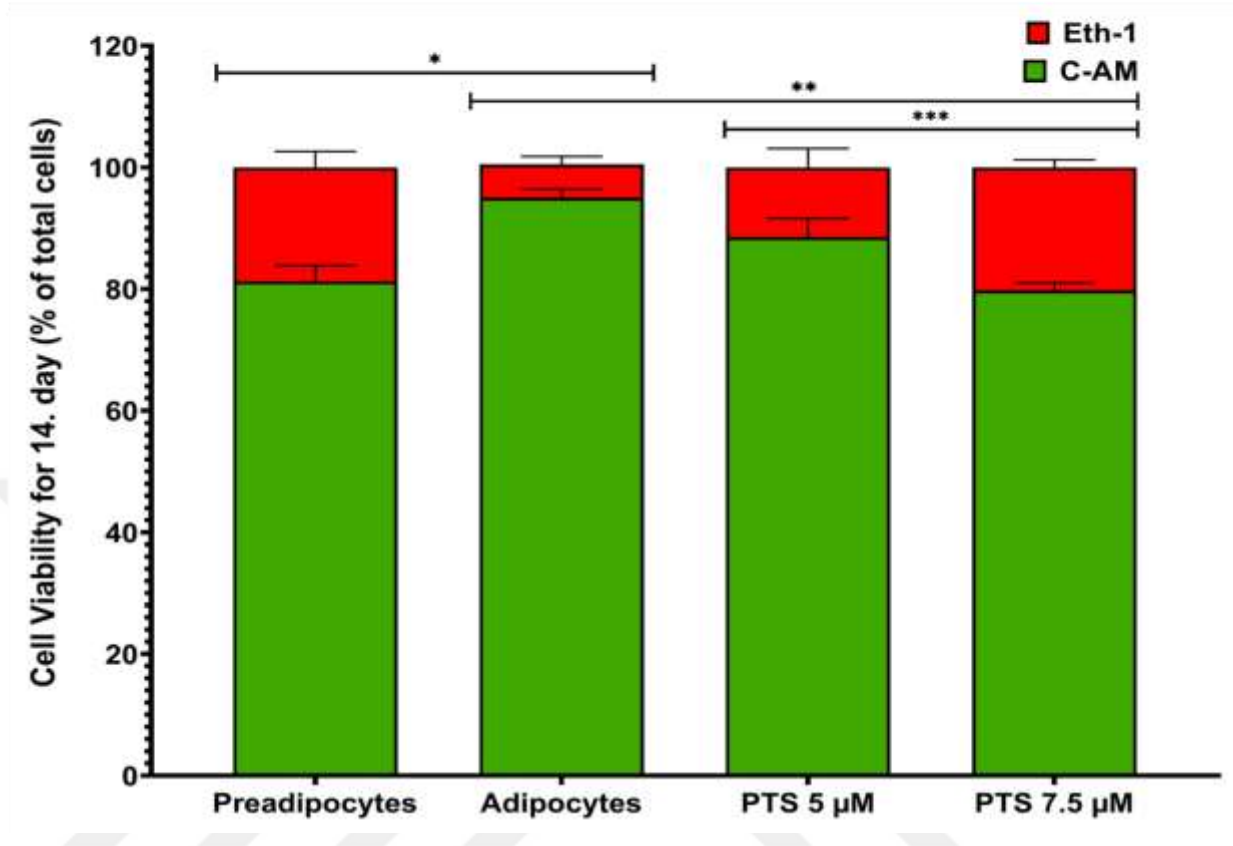
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Figure 5. Effect of PTS on the viability of 3T3-L1 cells in the differentiation phase.

A. Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μ M or 7.5 μ M) of PTS in adipocyte maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. The microscopic images at 10X magnification of cells stained with C-AM and Eth-1 at day 0, 7 and 14 of differentiation. C-AM and Eth-1 staining showed live and dead cells, respectively. **B. C. D.** Quantitative data of live-dead staining for days 0, 7 and 14 of differentiation, respectively. Three coverslips were evaluated for all cell groups. Six randomly selected areas on each coverslip were imaged microscopically and determined using ImageJ program. Data were stated as the total percentage of live and dead cells showed as mean $\pm$ SD. One-way ANOVA test was used for statistical analysis. * $P < 0.05$ and ** $P < 0.05$ differ from untreated 3T3-L1 adipocytes. *** $P < 0.05$ is significantly different from the 5 μ M dose of PTS. PTS: Pterostilbene; C-AM: Calcein-Acetomethoxy; Eth-1: Ethidium homodimer-1; SD: Standart Deviation.

4.4 Effects of PTS on cell morphology of 3T3-L1 in adipogenesis by phase-contrast microscopy, giemsa staining and immunofluorescence staining

4.4.1 Phase contrast microscopy

Adipocyte culture differentiated from 3T3-L1 preadipocytes displays a monolayer of large, spherically shaped cells that partially overlap (Green and Kehinde, 1975). Cells were followed by phase contrast microscopy to observe the effect of PTS on the morphology of 3T3-L1 cells in the differentiation phase. 3T3-L1 preadipocytes were differentiated into mature adipocytes in adipocyte maintenance medium in the presence or absence of PTS (5 μ M and 7.5 μ M) for 14 days. Phase-contrast microscopic images of cells on days 0, 7, and 14 are presented in Figure 4.

A decrease in mitosis was observed on days 0, 7 and 14 of 3T3-L1 preadipocyte cultures used as controls. In addition, although 3T3-L1 preadipocytes preserved their spindle-shaped morphology, a decrease in density was also observed. However, an increase in mitosis was observed on the 0, 7 and 14 days of 3T3-L1 adipocyte cultures, which were used as a control in adipogenesis and differentiated without treatment in adipocyte maintenance medium. Over time, the cell developed an adipogenic phenotypic and acquired a rounded body and less elongated morphology by lipid accumulation. In the group treated with 5 μ M or 7.5 μ M doses of PTS in Adipocyte maintenance medium, a dose-dependent partial adipogenic phenotypic cell clumps were observed over time. Adipocytes treated with PTS 7.5 μ M on day 14 showed less intense lipid deposition and a more spindle-like morphology compared to the other treatment group.

4.4.2 Giemsa staining

In Giemsa staining, compared to 3T3-L1 adipocytes differentiated without treatment, 3T3-L1 preadipocytes showed characteristic fibroblastic cell morphology at days 0, 7, and 14 with longer, spindle-like structure and wide spread. Adipocytes differentiated by treatment with 5 μ M or 7.5 μ M doses of PTS were partially round and small in a time- and dose-dependent manner. However, especially on day 14, adipocytes treated with PTS 7.5 μ M compared to the other treatment group grew dramatically, gaining a long, spindle-like structure as in preadipocytes (Figure 6).

4.4.3 F-actin staining

In F-Actin staining by immunofluorescence, 3T3-L1 preadipocytes exhibited a spindle morphology with multiple cell extensions at days 0, 7, and 14. In addition, a morphological change from spindle to spherical was observed over time in 3T3-L1 adipocytes differentiated without treatment, and the cells were partially overlapped. Adipocytes differentiated by treatment with 5 μ M or 7.5 μ M doses of PTS formed cell clusters with a morphology between preadipocytic and adipogenic phenotype in a time- and dose-dependent manner. However, especially on the 14th day, compared to the other treatment group, actin filaments were filamentous in adipocytes treated with PTS 7.5 μ M, with less integrity and similarly oriented filaments as in preadipocytes (Figure 8).

4.4.4 DAPI staining

Nuclear staining with DAPI showed increased chromatin condensation, a characteristic of apoptosis over time, to 3T3-L1 preadipocytes compared to 3T3-L1 adipocytes differentiated without treatment. Adipocytes differentiated by treatment with 5 μ M or 7.5 μ M doses of PTS exhibited slightly increased DNA condensation in a time- and dose-dependent manner. However, chromatin condensation was quite high in adipocytes treated with PTS 7.5 μ M, as in preadipocytes, especially on day 14 compared to the other treatment group (Figure 9).

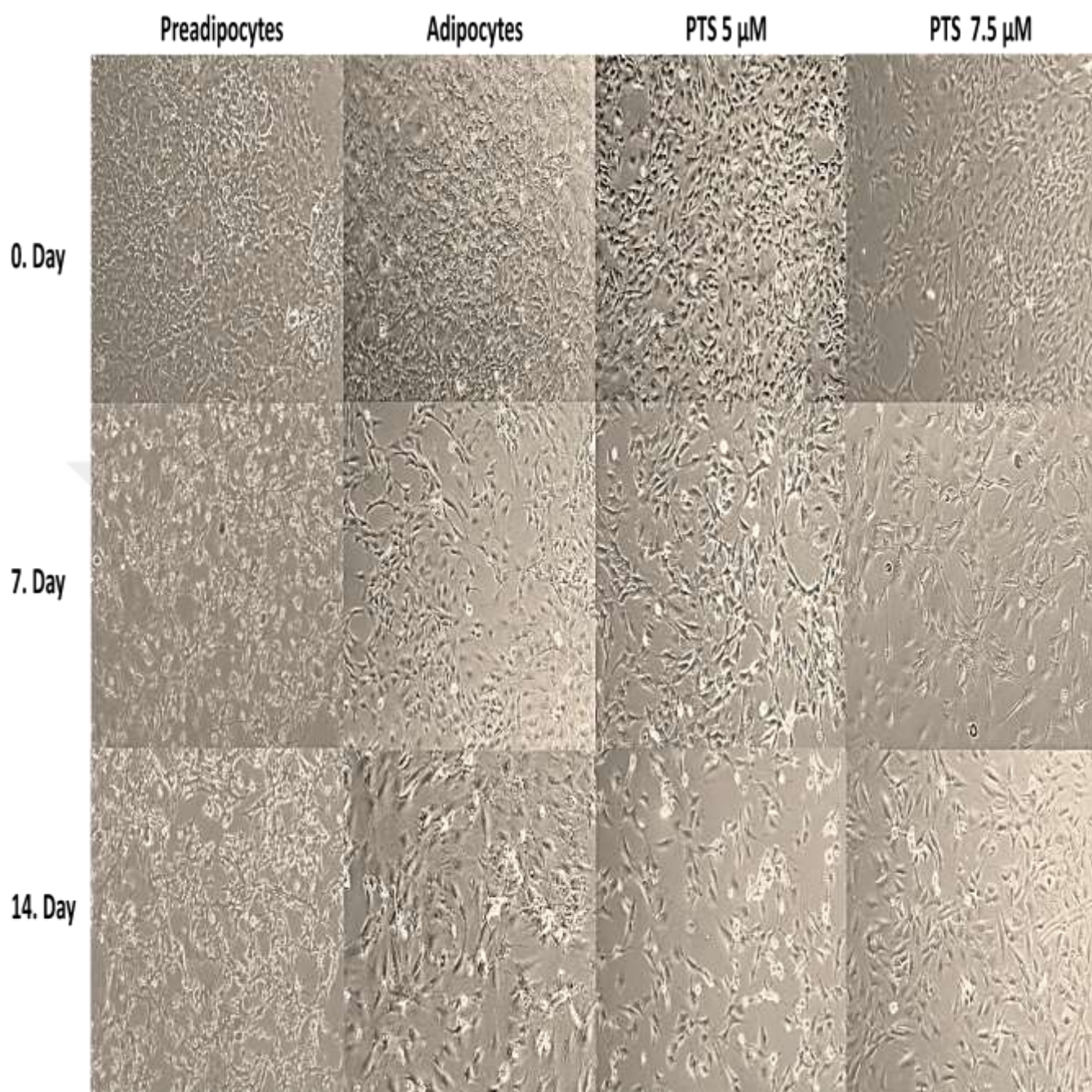


Figure 6. Phase-contrast microscope data showing the effect of PTS on the morphology of 3T3-L1 cells in the differentiation phase.

Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μM or 7.5 μM) of PTS in adipocyte maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. Representative phase-contrast microscopic images of cells at day 0, 7, and 14 of differentiation at 10X magnification.

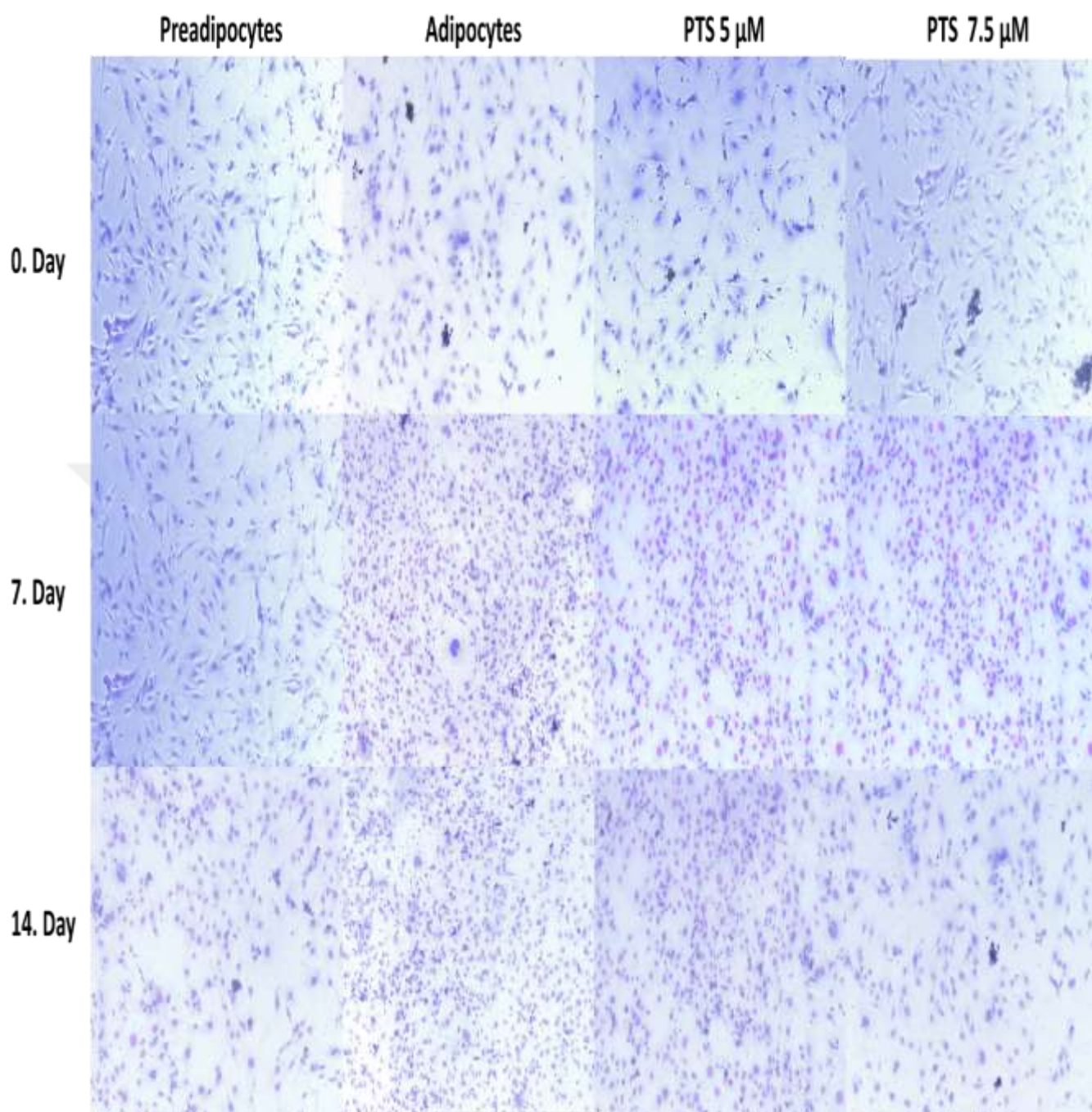


Figure 7. Giemsa staining data showing the effect of PTS on the morphology and cell size of 3T3-L1 cells in the differentiation phase.

Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μM or 7.5 μM) of PTS in adipocyte maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. Representative phase-contrast microscopic images at 10X magnification of Gimsa-stained cells at day 0, 7, and 14 of differentiation (Scale bar: 200 μm).

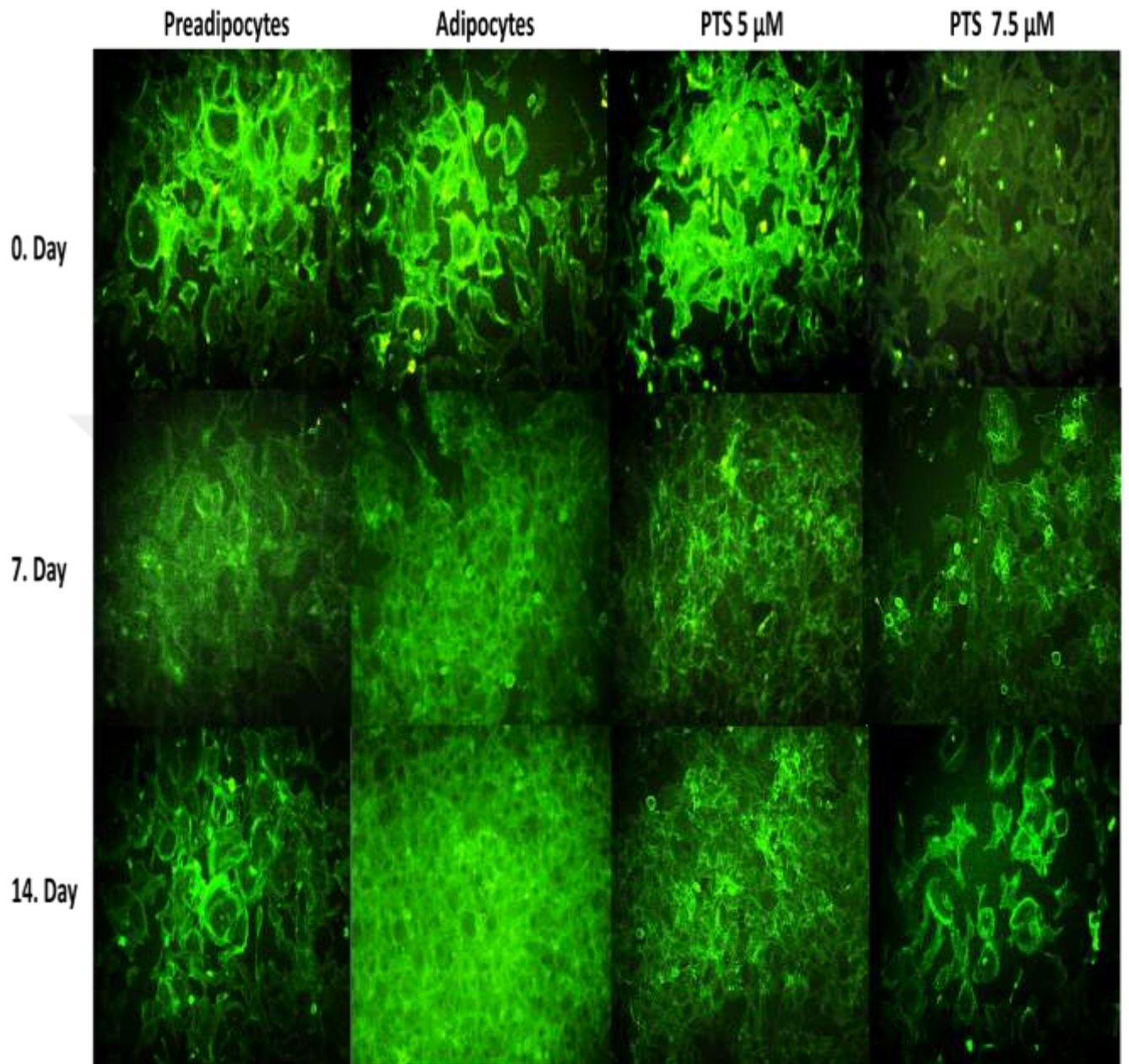


Figure 8. Immunofluorescence staining data showing the effect of PTS on cellular shape and integrity of 3T3-L1 cells in the differentiation phase.

Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μM or 7.5 μM) of PTS in adipocyte maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. Representative phase-contrast microscopic images at 10X magnification of cell cytoskeleton stained with Phalloidin (Alexa Fluor-488) at day 0, 7, and 14 of differentiation (Scale bar: 200 μm).

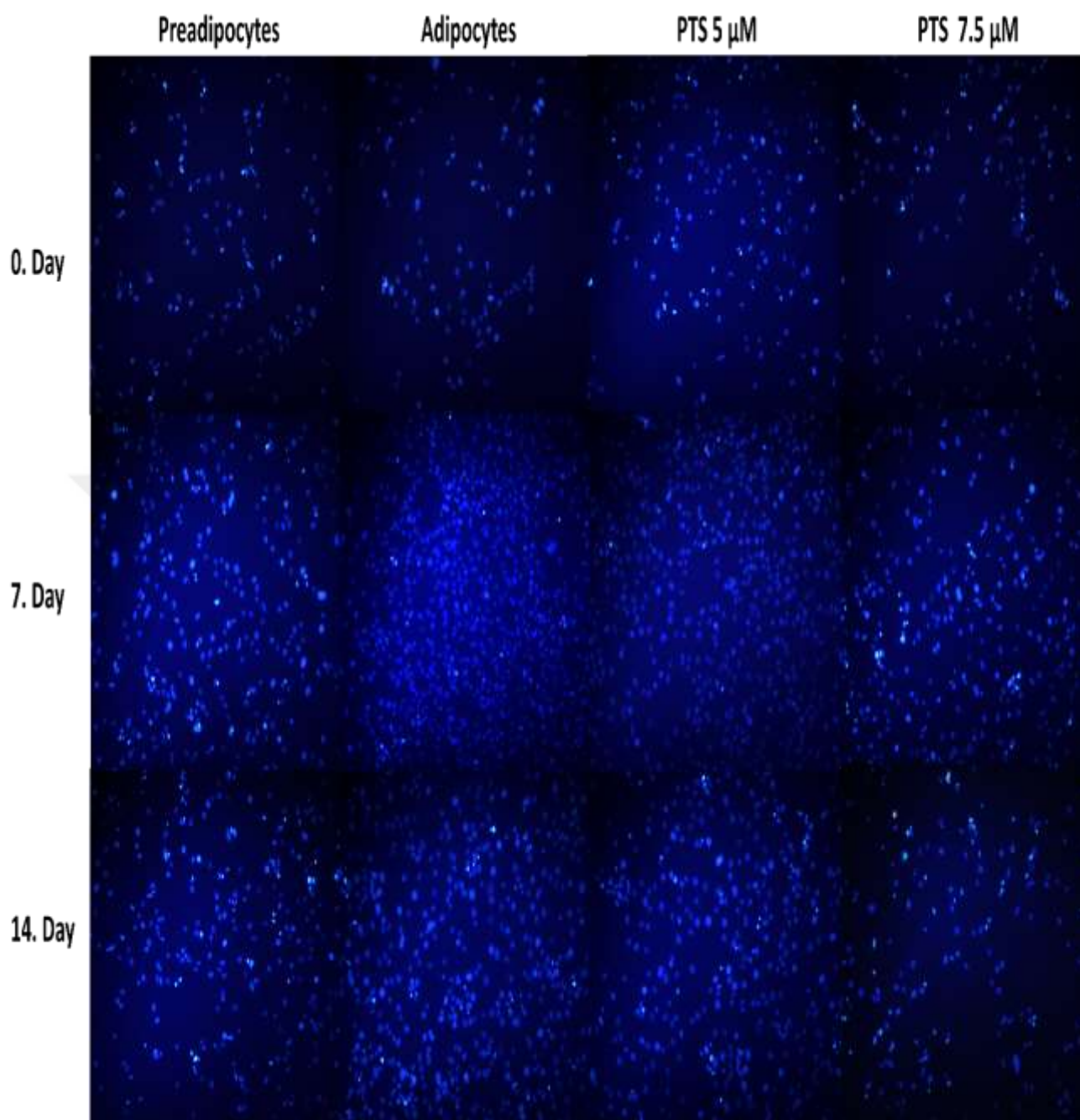


Figure 9. Immunofluorescence staining data showing the effect of PTS on DNA condensation of 3T3-L1 cells in the differentiation phase.

Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μM or 7.5 μM) of PTS in adipocyte maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. Representative phase-contrast microscopic images at 10X magnification of DAPI-stained cell nuclei at day 0, 7, and 14 of differentiation (Scale bar: 200 μm).

4.5. Effects of PTS on mRNA level expression of *Adiponectin* and *Glut-4* in 3T3-L1 by immunofluorescence staining and RT-PCR

3T3-L1 preadipocytes were differentiated into mature adipocytes in adipocyte maintenance medium in the presence or absence of PTS (5 μ M and 7.5 μ M) to investigate the effect of PTS on insulin resistance and adipogenesis, for 14 days. Glut-4 and Adiponectin expression in cells at day 14 of adipogenesis was evaluated by immunofluorescence staining and RT-PCR (Figure 10 and Figure 11). In the study, undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls.

Glut-4 and Adiponectin fluorescence was intensely observed in preadipocytes compared to adipocytes in immunofluorescent staining. However, the fluorescence intensity of both was dose-dependently increased in adipocytes treated with either 5 μ M or 7.5 μ M doses of PTS compared to untreated adipocytes. Moreover, the fluorescence intensity for Glut-4 and Adiponectin was significantly increased in adipocytes treated with PTS 7.5 μ M compared to the other treatment group (Figures 10A, 11A). Based on quantitative analysis of immunofluorescent staining, antibody fluorescence intensity for anti-Glut-4 and Adiponectin increased $331.09 \pm 11.97\%$ and $553.31\% \pm 15.10\%$ in preadipocytes compared to 3T3-L1 adipocytes, respectively ($P < 0.0001$ for all). The fluorescence intensity for Adiponectin increased to 72.81 ± 7.44 , $202.04 \pm 15.24\%$ for Glut-4 and $102.98\% \pm 12.89\%$, $348.99 \pm 17.34\%$ for Adiponectin in adipocytes treated with 5 μ M or 7.5 μ M doses of PTS compared to untreated adipocytes. In addition, fluorescence intensity for Glut-4 and Adiponectin increased by 74.78% and 121.19%, respectively ($P < 0.0001$ for all) in adipocytes treated with PTS 7.5 μ M compared to the other treatment group (Figure 10B, 11B).

Glut-4 mRNA expression was increased $298.35 \pm 4.7\%$ in untreated differentiated preadipocytes compared to adipocytes ($P = 0.0001$). *Glut-4* mRNA expression was increased $64.58\% \pm 10.00\%$ and $190.20\% \pm 3.40\%$ in adipocytes treated with 5 μ M or 7.5 μ M doses of PTS compared to untreated adipocytes ($P = 0.0796$ and $P = 0.0001$), respectively. However, *Glut-4* mRNA expression was increased by 76.32% ($P = 0.0007$) in adipocytes treated with PTS 7.5 μ M compared to the other treatment group (Figure 10C).

Adiponectin mRNA expression was increased $514.54\% \pm 10.03$ in untreated differentiated preadipocytes compared to adipocytes ($P < 0.0001$). *Adiponectin* mRNA expression was increased $88.91 \pm 10.43\%$ and $270.35\% \pm 10.99\%$ in adipocytes treated with 5 μ M or 7.5 μ M

doses of PTS, respectively, compared to untreated adipocytes ($P = 0.0046$ and $P = 0.0017$). However, *Adiponectin* mRNA expression was increased by 96.04% ($P = 0.0029$) in adipocytes treated with PTS 7.5 μM compared to the other treatment group (Figure 11C).

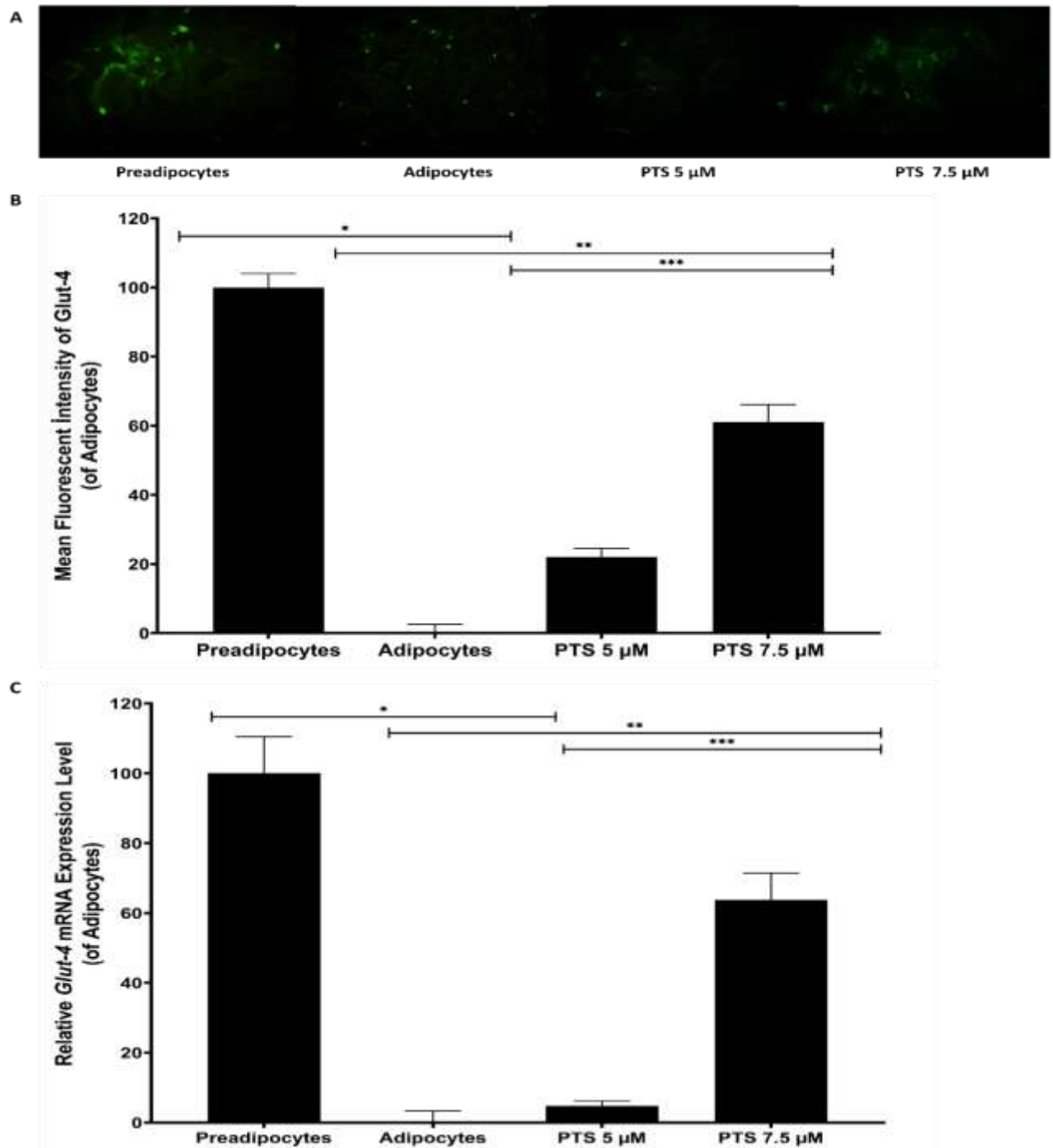


Figure 10. Immunofluorescence staining and RT-PCR data showing the effect of PTS on *Glut-4* expression.

Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μM or 7.5 μM) of PTS in adipocyte

maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. **A.** Representative phase-contrast microscopic images of cells stained with anti-Glut-4 antibody at day 14 at 20X magnification (Scale bar: 200 μ m). **B.** Quantitative data of relative intensity of anti-Glut-4 immunofluorescent staining. Coverslips were evaluated for each cell group. Six individual regions that randomly selected were imaged on each coverslip and the relative intensity of the immunofluorescent dye was measured using ImageJ software. Immunofluorescent staining analysis data were defined as relative variation of fluorescent signal intensities and mean $\pm$ SD. **C.** Expression of the *Gut-4* gene at the mRNA level was determined by qRT-PCR, normalized with *Gapdh*. Data were analyzed using the comparative Ct method ($\Delta\Delta$ Ct) from three independent experiments. Data with relative change of Ct were presented as mean $\pm$ SD and expressed as fold change compared to adipocyte control. One-way ANOVA test was used for statistical analysis. * $P < 0.05$ and ** $P < 0.05$ differ significantly from untreated 3T3-L1 adipocyte. *** $P < 0.05$ is significantly different from the 5 μ M dose of PTS. PTS: Pterostilbene; Glut-4: Glucose transporter type 4; RT-PCR: Quantitative Reverse Transcription PCR; Ct: Cycle threshold SD: Standard deviation.

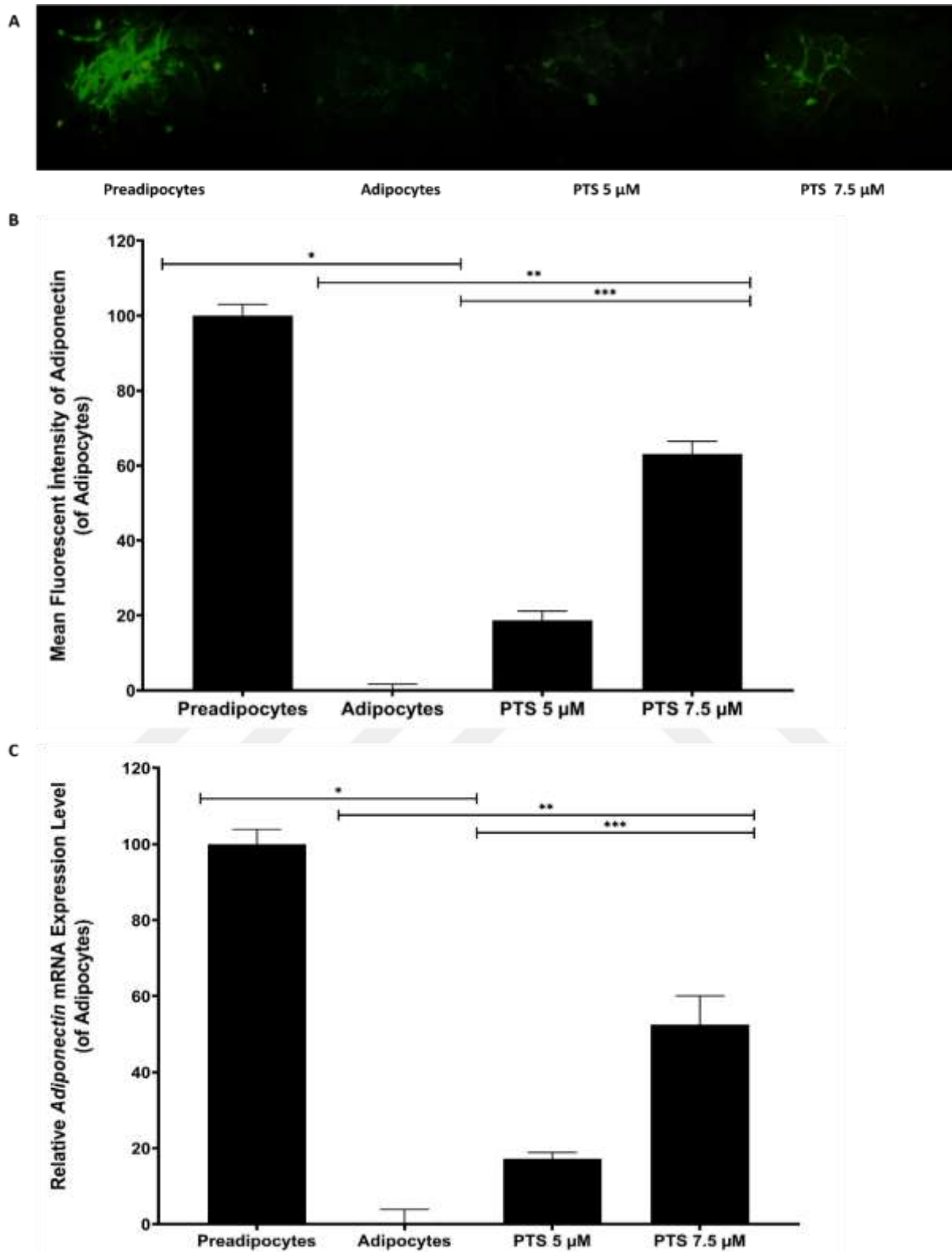


Figure 11. Immunofluorescence staining and RT-PCR data showing the effect of PTS on Adiponectin expression.

Preadipocytes were differentiated into mature adipocytes for 14 days in the absence of PTS treatment or in the presence of different doses (5 μ M or 7.5 μ M) of PTS in adipocyte

maintenance medium. Undifferentiated and untreated preadipocytes and differentiated and untreated adipocytes were used as controls. The level of mRNA and intracellular expression of the Adiponectin gene in cells was evaluated by Immunofluorescent staining and qRT-PCR. **A.** Representative phase-contrast microscopic images of cells stained with anti-Adiponectin antibody at day 14 at 20X magnification (Scale bar: 200 μ m). **B.** Quantitative data of relative intensity of anti-Adiponectin immunofluorescent staining. At least three coverslips were evaluated for each cell group. Six individual regions that randomly selected were imaged on each coverslip and the relative intensity of the immunofluorescent dye was measured using ImageJ software. Immunofluorescent staining analysis data are presented as the relative variation of fluorescent signal intensities and mean $\pm$ SD. **C.** Expression of the *Adiponectin* gene at the mRNA level was determined by qRT-PCR, normalized with *Gapdh*. Data were analyzed using the comparative Ct method ($\Delta\Delta$ Ct) from three independent experiments. Data with relative change of Ct were presented as mean $\pm$ SD and expressed as fold change compared to adipocyte control. One-way ANOVA test was used for statistical analysis. * $P < 0.05$ and ** $P < 0.05$ differ significantly from untreated 3T3-L1 adipocyte. *** $P < 0.05$ is significantly different from the 5 μ M dose of PTS. PTS: Pterostilbene; RT-PCR: Quantitative Reverse Transcription PCR; Ct: cycle threshold SD: Standard deviation.

5. DISCUSSION

DM is a metabolic disease characterized by chronic hyperglycemia, mostly accompanied by the insufficiency of insulin or its effect. DM has become a global public health problem due to its close relationship with many diseases such as obesity, cancer, cardiovascular and neurological (Cho et al. 2018). The high prevalence of DM, together with its accompanying diseases, has led to increased interest in research on improving insulin sensitivity and inhibiting adipogenesis (Saeedi et al., 2019; Al-Jaber, et al., 2021). 3T3-L1 preadipocytes are one of the cell models that allow the in-vitro study of adipogenesis (Poulos, et al., 2010.). In adipogenesis, increased insulin resistance, adipocyte hypertrophy, lipid accumulation and energy demand cause an increase in intracellular oxidative stress (Gregoire, et al., 1998). Studies have reported that inhibition of preadipocyte differentiation, adipogenesis, lipid accumulation, as well as reduction of cell density, mitosis, and insulin resistance may be important mechanisms in the fight against DM (Balcazar et al., 2021; Deepa, et al., 2015). PTS is a natural phenolic compound found predominantly in blueberries. PTS has been reported as a natural, small molecular drug candidate for many chronic diseases with its pharmacokinetic properties, antioxidant properties, anti-inflammatory activity, and anti-cancer capacity. In vivo studies have demonstrated the antidiabetic properties of PTS (Satheesh, et al., 2006). Therefore, in our study, we focused on the effect of PTS on differentiation and adipogenesis by evaluating cell proliferation, lipid accumulation, cell morphology, DNA condensation, *Glut-4* and *Adiponectin* expression levels in 3T3-L1 preadipocytes and differentiated mature adipocytes.

Our study showed that the addition of PTS to the medium decreased cell survival in 3T3-L1 preadipocytes in a time- and dose-dependent manner, consistent with the literature (Hs, et al., 2012) In addition, treatment of 3T3-L1 preadipocytes with PTS appeared to reduce their viability in a time- and dose-dependent manner, with IC₅₀ values of $23.04 \pm 1.89 \mu\text{M}$ at 24 h, $17.37 \pm 1.22 \mu\text{M}$ at 48 h, and $14.60 \pm 2.88 \mu\text{M}$ at 72 h. In contrast, at least approximately 90% of 3T3-L1 preadipocytes remained viable and had no cytotoxicity in treatment with 5 μM and 7.5 μM PTS. Therefore, these doses were applied to cells in further analyzes to evaluate the effect of PTS on differentiation and adipogenesis. Moreover, live-dead staining data similarly showed that PTS treatment significantly reduced cell viability in 3T3-L1 adipocytes compared to control, in a time- and dose-dependent manner. These results support that low doses of PTS may be an attractive cytotoxic agent. 3T3-L1 preadipocytes begin to differentiate by

adipogenesis when they are exposed to adipogenic factors. Adipogenesis is a process characterized by cell growth arrest, mitotic clonal expansion, morphological changes and lipid accumulation (Guru, et al., 2021). Studies have shown that many polyphenol compounds such as fucoxanthin, kaempferol, gallocatechin gallate, resveratrol and PTS have inhibitory effects on lipid accumulation in 3T3-L1 adipocytes (Rayalam, et al., 2012). In our study, the developed adipocytic phenotype was confirmed by staining the oil droplets. In addition, exposure of 3T3-L1 adipocytes to PTS at 5 μ M and 7.5 μ M concentrations decreased lipid accumulation by 30.64 ± 1.36 and 54.93 ± 3.14 , respectively, compared to the control. Therefore, PTS may be beneficial in preventing adipocyte hyperplasia by inhibiting adipogenesis by stimulating lipolysis in 3T3-L1 adipocytes.

Adipocyte culture differentiated from 3T3-L1 preadipocytes displays a monolayer of large, spherically shaped cells that partially overlap. Increased lipid synthesis and visible intracellular lipid vesicles in adipogenesis cause cells to spheroid (Gregoire, et al., 1998). In our study, the follow-up of Giemsa staining and F-actin staining with using phase contrast microscopy, it was observed that PTS treatment caused a decrease in mitosis, cell density, integrity and fat droplets, an increase in cell size, a similar orientation of actin filaments in filamentous structure, and a fibroblastic shape with a long spindle-shaped structure in adipocytes compared to control, in a time- and dose-dependent. PTS may contribute to the loss of spherical morphology of cells and inhibit adipocyte hyperplasia-hypertrophy by inducing lipolysis in adipocytes.

Apoptosis induction is a normal and healthy cellular process required to get rid of abnormal or expired cells without an inflammatory response. Nuclear chromatin condensation is the first morphological change observed in a cell signaling apoptosis (Elmore, 2007). In DAPI staining, it was observed that PTS induced nuclear chromatin condensation via apoptosis in 3T3-L1 adipocytes in a dose-dependent manner, especially after 7 days of treatment. Thus, PTS exerts an anti-adipogenic effect on 3T3-L1 adipocytes by inducing apoptosis and inhibiting differentiation. Preadipocytes differentiate into adipocytes upon growth arrest in adipogenesis, resulting in the formation of adipose tissue (Elmore, 2007; Gregoire et al., 1998). Therefore, induction of apoptosis in differentiated adipocytes has been proposed as an important strategy to fight against diseases such as DM and obesity (Rayalam et al., 2008). PTS treatment may prevent adipose tissue hyperplasia, as the induction of apoptosis may reduce the number of preadipocytes, causing them to differentiate into fewer adipocytes.

Adiponectin, an insulin-sensitizing adipocytokine, has been shown to decrease expression in 3T3-L1 adipocytes during adipogenesis (Fasshauer et al., 2002). Similarly, in-vivo studies have reported that fasting insulin concentrations are negatively correlated with Adiponectin plasma concentrations (Yu et al., 2002). Therefore, induction of *Adiponectin* expression is critical importance in the fight against DM and obesity (Yamauchi et al., 2001). In a study, it was observed that the mRNA and protein expression of the *Adiponectin* gene decreased in 3T3-L1 adipocytes that differentiate in adipogenesis (Li et al., 2016; Hsu et al., 2012). Consistent with the literature in this study, treatment of 3T3-L1 cells with PTS increased both mRNA and intracellular expression of *Adiponectin* in a time- and dose-dependent manner. Our results support the suggestion that PTS may increase Glut-4 production in adipocytes through increased Adiponectin AMP kinase and increased phosphorylation of acetyl-CoA carboxylase (Ceddia et al., 2005).

Insulin accelerates glucose entry into skeletal muscle and adipose tissue by stimulating the translocation of Glut-4, a glucose transporter, from the cell to the plasma membrane (Martin et al., 2000). Increased insulin resistance, weakened Glut-4 translocation, and decreased intracellular glucose uptake following insulin stimulation in DM are a major defect (Szymczak-Pajor and Śliwińska, 2019). Therefore, some drugs used in the treatment of DM target *GLUT-4* with some genes and increase intracellular glucose uptake by promoting Glut-4 translocation in adipocytes with its up-regulation (Kubota et al., 2006). It has been reported that *Glut-4* gene decreases mRNA, protein expression and thus translocation in 3T3-L1 adipocytes in adipogenesis (Li et al., 2016). In an in-vivo study, it was reported that PTS treatment significantly increased Glut-4 protein expression in adipose tissue of diabetic rats (Sun et al., 2019). In our study, PTS treatment significantly up-regulated the mRNA and intracellular expression of the *Glut-4* gene during differentiation of 3T3-L1 adipocytes. Considering the roles of Adiponectin and Glut-4 in adipogenesis and insulin resistance, as well as the antioxidant capacity of PTS, all these results suggest that PTS may be an attractive phytochemical with the potential to regulate adipocyte differentiation in the fight against DM.

6. CONCLUSION

In conclusion, our data show that PTS inhibits cell proliferation and growth, reduces lipid accumulation, preserves fibroblastic morphology, and induces apoptosis in differentiated 3T3-L1 adipocytes. In addition, it was observed that PTS inhibited insulin resistance and expression of adipogenic genes at mRNA and intracellular level in differentiated 3T3-L1 cells. These results show that PTS suppresses adipogenesis and reduces the differentiation of preadipocytes into mature adipocytes. Increased insulin resistance, increased preadipocyte proliferation and progressive adipogenesis are highly related to adipose tissue hyperplasia. Therefore, reducing adipocyte growth by suppressing the differentiation of preadipocytes and adipogenesis may be an alternative treatment strategy for DM. All our results show that PTS can play an important role in the prevention and treatment of DM by reducing the differentiation of preadipocytes with its anti-adipogenic effect.

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