Apoptotic Effect of Simvastatin on Human Dental Pulp Cells and Periodontal Ligament Fibroblasts

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Oral Health Sciences

Prince of Songkla University

2013

Thesis Title Apoptotic Effect of Simvastatin on Human Dental Pulp Cells and Periodontal Ligament Fibroblasts

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ชื่อวิทยานิพนธ์ ผลของซิมวาสแททินต่อการตายแบบอะพอปโทซิสในเซลล์เนื้อเยื่อในของฟันและเซลล์เอ็นยึดปริทันต์ในคน
ผู้เขียน นางสาวศิรินาถ แซ่หว่อง
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บทคัดย่อ

วัตถุประสงค์: มีการแนะนำซิมวาสแททินซึ่งมีฤทธิ์ยับยั้งเอ็นไซม์ทรีไฮดรอกซีทรีเมทิลกลูทาริลโคเอนไซม์เอรีดักเทสและถูกนำมาใช้เป็นยาลดระดับโคเลสเตอรอลอย่างแพร่หลาย ว่ามีผลดีต่อการสร้างกระดูกขากรรไกรและฟื้นฟูสภาพเนื้อเยื่อในของฟันและเอ็นยึดปริทันต์ ซิมวาสแททินในความเข้มข้นสูงสามารถชักนำให้เกิดการตายแบบอะพอปโทซิสได้ในเซลล์หลายชนิด แต่การศึกษาถึงผลของยานี้ที่มีต่อการตายของเซลล์ที่เกี่ยวข้องกับฟันยังมีจำนวนน้อย ดังนั้นงานวิจัยนี้จึงศึกษาผลของซิมวาสแททินว่ามีผลต่อการตายแบบอะพอปโทซิสและการเปลี่ยนแปลงลักษณะรูปร่างของเซลล์เนื้อเยื่อในของฟันและ เซลล์เอ็นยึดปริทันต์ในคนหรือไม่อย่างไร

วิธีการทดลอง: นำเซลล์เนื้อเยื่อในของฟันและเซลล์เอ็นยึดปริทันต์จากผู้ป่วย 4 คนมาเพาะเลี้ยงในสารเลี้ยงเซลล์ซึ่งมีและไม่มีซิมวาสแททินในความเข้มข้นแตกต่างกันตั้งแต่ 0.1, 1, 10 ไมโครโมลาร์เป็นเวลา 24, 48 และ 72 ชั่วโมง หลังจากนั้นวัดความมีชีวิตของเซลล์ด้วยการทดสอบวิธีเอ็มทีที และวัดปริมาณการตายแบบอะพอปโทซิสของเซลล์โดยย้อมติดสีของแอนเน็กซินไฟว์ฟิตซีและโพรพิเดียมไอโอไดด์แล้วตรวจนับจำนวนเซลล์ด้วยเครื่องโฟลไซโทเมทรี ส่วนการศึกษารูปร่างลักษณะแอกทินของเซลล์และรูปร่างนิวเคลียสทำโดยการย้อมเซลล์ด้วยไฟล์ลอยดินฟิตซีและดาพี ตามลำดับ

ผลการทดลอง: เมื่อเซลล์เนื้อเยื่อในของฟันและเซลล์เอ็นยึดปริทันต์ได้รับซิมวาสแททินพบว่าความมีชีวิตของเซลล์ลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) โดยแปรผันตามความเข้มข้นของซิมวาสแททินและเวลาที่ใช้เพาะเลี้ยงเซลล์ การตายแบบอะพอปโทซิสของเซลล์ซึ่งได้รับซิมวาสแททินความเข้มข้น 10 ไมโครโมลาร์เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ปริมาณการตายแบบอะพอปโทซิสของเซลล์ทั้งสองมีปริมาณเท่าๆกัน การย้อมสีนิวเคลียสพบลักษณะจำเพาะบ่งชี้ว่าเซลล์ทั้งสองชนิดมีการตายแบบอะพอปโทซิสกล่าวคือพบการหดตัวและชิ้นส่วนย่อยของนิวเคลียส และรูปร่างลักษณะแอกทินของเซลล์ถูกทำลายโดยแปรผันตามความเข้มข้นของซิมวาสแททินและเวลาที่ใช้เพาะเลี้ยงเซลล์

สรุปผลการทดลอง: ซิมวาสแททินลดความมีชีวิตของเซลล์เนื้อเยื่อในของฟันและเซลล์เอ็นยึดปริทันต์โดยอาจจะผ่านกลไกการชักนำการตายแบบอะพอปโทซิส

คำสำคัญ: ซิมวาสแททิน อะพอปโทซิส ไฟโบรบลาสต์ แอกทิน เนื้อเยื่อใน

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Academic Year 2012

ABSTRACT

Objective: Simvastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, and widely used as cholesterol-lowering agent, has been suggested for its beneficial effects on alveolar bone formation, regeneration of dental pulp tissue and periodontal ligament. High doses of simvastatin appear to induce apoptosis in several cell types, but little is known about its possible effect on tooth-associated cells. Therefore, the effects of simvastatin were studied on apoptosis and cell morphology of human dental pulp cells (HDPCs) and periodontal ligament fibroblasts (HPLFs).

Methods: HDPCs/HPLFs obtained from 4 patients were cultured with or without various concentrations of simvastatin (0.1, 1, and 10 μM) for 24, 48, and 72 h. The 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell viability. The levels of apoptosis of HDPCs and HPLFs were measured by flow cytometry after Annexin V/propidium iodide double staining. Phalloidin-FITC and 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining was used to examine differences in the actin cytoskeleton and nuclear morphology, respectively.

Results: The viability of HDPCs and HPLFs was significantly reduced after simvastatin treatment in a dose- and time-dependent manner ($p < 0.05$). The apoptosis of HDPCs and HPLFs was significantly increased in 10 mM simvastatin-treated cells ($p < 0.05$). The effect on apoptosis was comparable for HDPCs and HPLFs. Nuclear staining showed typical apoptotic nuclear condensation and fragmentation in simvastatin-treated HDPCs/HPLFs. A dose- and time-dependent simvastatin-induced disruption of the actin cytoskeleton was observed in both cell types.

Conclusion: Our data demonstrated that simvastatin decreased the viability of HDPCs and HPLFs, probably by inducing apoptosis.

Keywords: Simvastatin, Apoptosis, Fibroblasts, Actin, Pulp

CONTENT

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (CONTINUED)

Figure **Page**

LISTS OF ABBREVIATIONS

ANOVA	One-way analysis of variance
Apaf-1	Apoptosis activating factor-1
BSA	Bovine serum albumin
°C	Degree celsius
Caspases	cysteinyl aspartate specific proteases
CCL	Chemokine ligand
Cyr 61	Cysteine rich 61
Cyt c	Cytochrome c
DAPI	4, 6-diamidino-2-phenylindole dihydrochloride
DMSO	Dimethyl sulfomide
DMEM	Dulbecco's modified Eagle's medium
DR	Death receptors
FACS	Fluorescence-activated cell sorting
F-actin	Filamentous actin
FADD	Fas-associated protein with death domain
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FPP	Fanesyl pyrophosphate
G	Gram
GAP	GTPase-activating protein
GDI	GTP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGPP	Geranylgeranyl pyrophosphate
GGT	Geranylgeranyl transferase
GPCR	G-protein-coupled-receptor
GTP	Guanosine triphosphate
h	Hour (s)

LISTS OF ABBREVIATIONS (CONTINUED)

HCl	Hydrochloric acid
HDPCs	Human dental pulp cells
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	Hydroxymethylglutaryl CoenzymeA
HPLFs	Human periodontal ligament fibroblasts
H_2O_2	Hydrogen peroxide
IAP	Inhibitor of apoptosis
kbp	Kilobase pair
L	Litre (s)
LDL	Low density lipoprotein
M	Molarity
mg	Milligram
min	Minute (s)
ml	Milliliter
mm	Millimeter
mM	Millimolar
MMP	Matrix metalloproteinases
MTP	Mitochondrial transition pore
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NF-*k*B	Nuclear factor kappa B
ng	Nanogram
nm	Nanometer
OD	Optical density
OPG	Osteoprotegerin
PBS	Phosphate buffer saline

LISTS OF ABBREVIATIONS (CONTINUED)

PBS-A	Phosphate buffer saline- Bovine serum albumin
PBS-F	Phosphate buffer saline- Formaldehyde
PBS-T	Phosphate buffer saline- Triton X-100
PDLs	Periodontal ligament cells
PI	Propidium iodide
PM	Plasma membrane
PS	Phosphatidyl serine
PVPI	Povidone-iodine
RANKL	Receptor activator of nuclear factor kappa-B ligand
SCE	Sodium calcium exchanger
SD	Standard deviation
sec	Second
TNFR	Tumor necrosis factor receptors
TRADD	TNFR -associated protein with death domain
Trypsin-EDTA	Trypsin-ethylenediamine tetraacetic acid
α-MEM	Alpha modified Eagle's medium
α-SMA	Alpha smooth muscle
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
%	Percentage

CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONALE

Simvastatin is a member of statins family of competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. It is widely used as cholesterol-lowering drug in the treatment and prevention of cardiovascular diseases.[1] HMG-CoA reductase, which converts HMG-CoA to mevalonate, is a rate-limiting enzyme of the mevalonate cascade. Mevalonate is a precursor in cholesterol synthesis and in the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These intermediates perform as lipid attachments for isoprenylation of proteins such as small GTP-binding proteins (small GTPases) including proteins of the Ras, Rho, and Rab families.[2] Isoprenylation consists of attachment of FPP (farnesylation) or GGPP (geranylgeranylation) to the carboxyl terminus of proteins. This post-translational modification is essential for localization of small GTPases to the plasma membrane.[3] Therefore, inhibition of the mevalonate pathway by simvastatin resulting in depletion of FPP and GGPP and hence inhibition of small GTPases can affect critical cellular functions such as anti-inflammation, anticoagulation, cell cycle, apoptosis, cytoskeleton reorganization and immune activity.[4] In addition to lipid-lowering effects, inhibition of these intracellular isoprenoid-dependent proteins by simvastatin may affect several signal transduction steps relevant to these critical cellular pleiotropic effects.[1,5]

The application of simvastatin in dental treatments has been recently of interest. Periodontitis and periapical lesion are major dental problems, that relate to the inflammatory process. It is an inflammatory disease leading to alveolar bone resorption and periodontal attachment loss which adversely causes tooth loss. Administration of pharmacologic compounds which could stimulate local bone formation has been suggested in the treatment of bone defects. Simvastatin has anti-inflammatory and immunomodulatory properties.[6] Therefore, simvastatin seems to be a promising agent to achieve the goal of periodontal therapy. This is evidenced by the fact that simvastatin inhibits the progression of apical periodontitis[7] and has potential to augment alveolar bone loss.[8,9] Several studies have shown that simvastatin promotes osteoblast differentiation and

mineralization, suggesting for its therapeutic effects on regeneration of dental pulp tissue[10,11] and periodontal tissue.[12]

Apoptosis is a cell suicide program that is highly regulated and executed via activation of two main signaling pathways. The extrinsic pathway is triggered by external cues binding to death receptors, Fas and tumor necrosis factor receptors. The intrinsic pathway involves the activation of several procaspases and the mitochondrial release of apoptogenic factors such as cytochrome c and apoptotic-inducing factor into the cytoplasm. Subsequently, a cascade of events followed by the activation of proteolytic caspases results in changes of morphological, biochemical, and molecular events such as DNA fragmentation, chromatin condensation, cytoplasmic shrinkage and plasma membrane blebbing. The apoptotic bodies are then engulfed by macrophages or neighboring cells without initiating an inflammatory response.[13]

Simvastatin induces apoptosis in several cell types such as macrophage cells[14] and various cancer cell lines.[15,16] In contrast, recent studies showed that simvastatin decreases apoptosis in murine osteoblastic cells.[17,18] It has been shown that simvastatin triggers apoptosis via a variety of intracellular signaling pathways such as both caspase-dependent and -independent apoptotic pathways[14] and down-regulation of the NF-κB pathway.[19] Little is known about its possible toxic effect on tooth-associated cells. The present study examined the effects of simvastatin on apoptosis of human dental pulp cells (HDPCs) and periodontal ligament fibroblasts (HPLFs). Nuclear and cell morphology upon simvastatin treatment of these tooth- associated cell types was also analyzed.

LITERATURE REVIEW

1.1 STATINS STRUCTURE

Statins are member of statins family of competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which are the most effective class of drugs for a cholesterol-lowing agent in the treatment and prevention of atherosclerosis. They are first-line agents for patients who require drug therapy to reduce serum low-density lipoprotein cholesterol concentrations.[1]

1.1.1 Classification of statins

Statins can classified to 2 types by origin. (1) Natural statins, which are derived from metabolic fungal derivatives, include lovastatin, pravastatin, and simvastatin. (2) The newer statins (fluvastatin, atorvastatin, rosuvastatin, and pitavastatin) are synthetic compounds.[6]

1.1.2 Pharmacokinetic properties of statins

The pharmacokinetic properties of the statins are orchestrated by several factors, including their active or lactone form, their lipophilic/hydrophilic rate, and their absorption and metabolism. Lovastatin, simvastatin and pravastatin are structurally very similar. Simvastatin differs from the other two as it possesses an additional methyl group at position 2 on the butanoate lateral chain **(Fig. 1)**. While pravastatin is administered as the readily active open hydroxy-acid form, lovastatin and simvastatin are administered as inactive lactones and are hydrolyzed to its active β-hydroxyacid form, potent inhibitor of HMG-CoA reductase, after administration. Thus, lovastatin and simvastatin, are more lipophilic than their corresponding active open hydroxy-acid forms which in turn, are approximately 100 times more lipophilic than pravastatin. Lipophilic statins are considered to enter endothelial cells by passive diffusion more efficiently than hydrophilic statins, which are primarily targeted to the liver.[20] Statins are bound by serum proteins and their bioavalability are limited. The plasma concentrations of statins in patients treated for lipid disorders are in the nanomolar range and some what higher to micromolar range in situations of drug interactions **(Table 1)**.[21] Drug interactions with statins can result in markedly increased or decreased

plasma concentrations of some drugs. Concomitant use of certain drugs (fibrates, erythromycin, itraconazole, and immunosuppressive drugs such as cyclosporine) can increase blood levels of statins and, consequently, the risk for myopathy.[22] The statins have a variety of excretion pathways. Pravastatin shows significant renal excretion, while the rests are metabolised by the cytochrome P450 isoenzyme system to active hydroxy acids at the liver and excreted in the bile **(Table 2)**.[6]

1.1.3 Mechanisms of action

Acetyl-CoA is converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) via HMG-CoA synthase. The conversion of HMG-CoA to mevalonate is the rate-limiting step, and is performed by HMG-CoA reductase. Statins bind to mammalian HMG-CoA reductase at nanomolar concentrations, leading to an effective displacement of the natural substrate HMG-CoA, which bind instead at micromolar concentrations.[23] The interactions between statins and HMG-CoA reductase prevent the conversion of HMG-CoA to L-mevalonate resulting in the inhibition of the downstream cholesterol biosynthesis and numerous isoprenoid metabolites such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). Pharmacological blocking of the HMG-CoA reductase to mevalonate brings about a reduction in cholesterol synthesis which induces upregulation of LDL (low-density lipoprotein) receptors, thus it will rises the rate of elimination of LDL from plasma **(Fig. 2)**.[4]

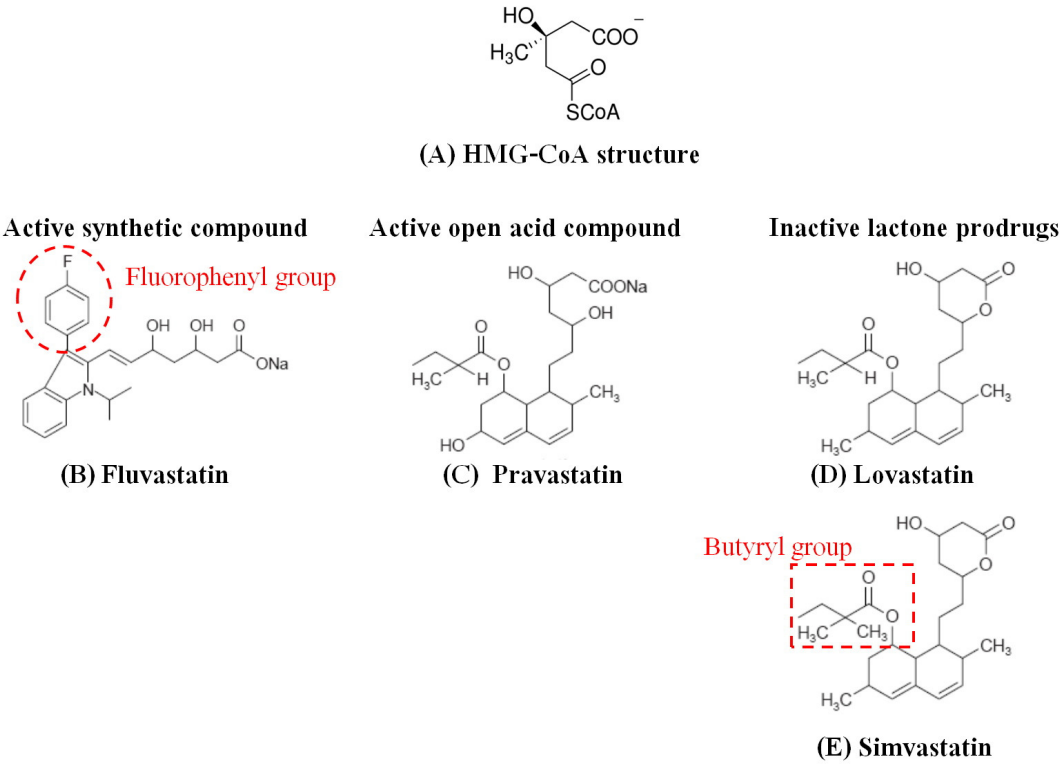

Fig. 1: Chemical structures of HMG-CoA (Hydroxymethylglutaryl CoenzymeA) reductase inhibitors: HMG-CoA structure **(A)**, Fluvastatin **(B)**, Pravastatin **(C)**, Lovastatin **(D)** and Simvastatin **(E)**.[20]

Table 1: Literature data on total and unbound plasma concentrations following administration of 40 mg doses of the different statins.[21]

Parameters	Atorvastatin acid	Simvastatin acid	Pravastatin acid	Fluvastatin acid
Average C_{max}	48-118 nM	23-78 nM	0.1 μM	1 μM
Average $C_{max(unbound)}$	1-2 nM	0.5-4 nM	55-65 nM	11 nM
Average interaction C_{max}	0.4 μM	1 μM	0.3 μM	1 μM
Average interaction $C_{max(unbound)}$	7 nM	24-60 nM	0.2 μM	11 nM
Highest observed C_{max}	1 μM	No Data	0.4 μM	2 μM
Highest observed $C_{max(unbound)}$	22 nM	No Data	0.2 μM	19 nM

Table 2: Phamacokinetics of statins.[6]

Drug	Half-life (h)	Excretion	Dose (mg)	Solubility
Atorvastatin	14	90% liver, 2% renal	10-80	Lipophilic
Fluvastatin	0.5-0.7	90% liver, 5% renal	20-80	Lipophilic
Lovastatin	3	83% liver, 10% renal	20-80	Lipophilic
Pravastatin	1.3-2.7	70% liver, 20% renal	10-40	Hydrophilic
Rosuvastatin	18-20	90% liver, 10% renal	10-40	Hydrophilic
Simvastatin	1.9	60% liver, 13% renal	10-80	Lipophilic

1.2 PLEIOTROPIC EFFECTS OF STATINS

Clinical studies suggest that the overall benefits observed with statins may not be mediated solely by their lipid-lowering properties, but possibly through cholesterol-independent or pleiotropic effects. Statins can inhibits the synthesis of mevalonate and downstream products. This has two outcomes: (a) a reduction of hepatic intracellular cholesterol content, which causes the lipid lowering effects of these compounds; (b) as mevalonate is a precursor not only of cholesterol, but also of other important intermediates, such as the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), the synthesis of these compounds is also reduced **(Fig. 2)**.[4] Isoprenoids intermediates serve as important lipid attachments for the post-translational modification of intracellular proteins such as nuclear lamins, Ras, Rho, Rac and Rap. The lipophilic isoprenyl group enables these proteins to anchor to cell membranes, which, in most situations, is an essential requirement for biological function.[3] These effects have been termed "pleiotropic effects," to indicate the ability of statins to influence multiple phenotypic traits in an organism. These pleiotroipic cholesterol-independent effects of statins on nitric oxide (NO) synthesis, oxidation, inflammation, immunomodulation, coagulation, and cell proliferation seem to be essential for atherosclerotic plaque stabilization, reduction of platelet aggregation, endothelial function improvement, reduction of hemorrhagic stress and prothrombotic state, enhancement of fibrinolytic state, and reduction of inflammatory states. These mechanisms are thought to underlie the putative therapeutic effectiveness of statins on stroke and related vascular dementia, Alzheimer's, cancer, multiple sclerosis, and rheumatoid arthritis, as summarized in Figure 3. Therefore, inhibition of the mevalonate pathway by simvastatin resulting in depletion of FPP and GGPP and hence inhibition of small GTPases can affect critical cellular functions such as anti-inflammation, anticoagulation, cell cycle, apoptosis, cytoskeleton reorganization and immune activity.[4]

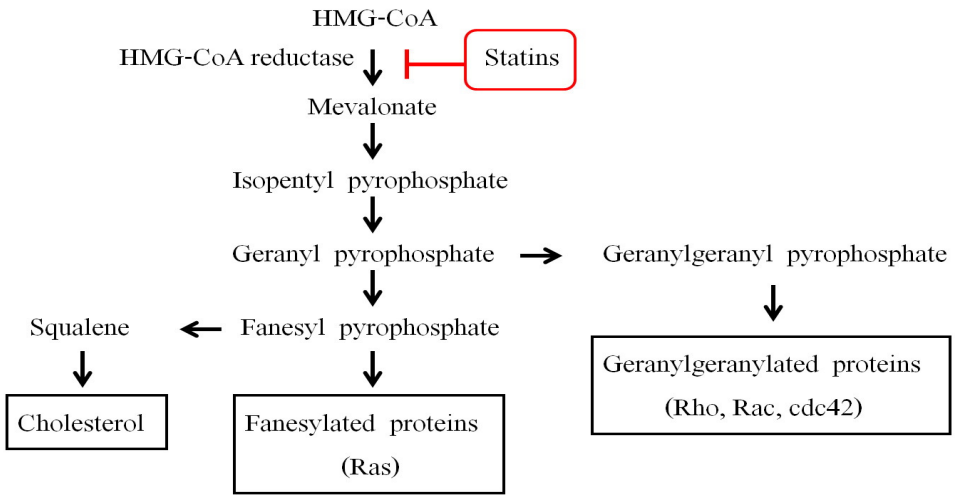

Fig. 2: Molecular mechanisms responsible for the lipid-lowering and pleiotropic effects of the statins. Statins-induced inhibition of the enzyme HMG-CoA reductase leads to a reduction in the formation of mevalonate. As cholesterol and isoprenylated protein are both products of mevalonate.[24]

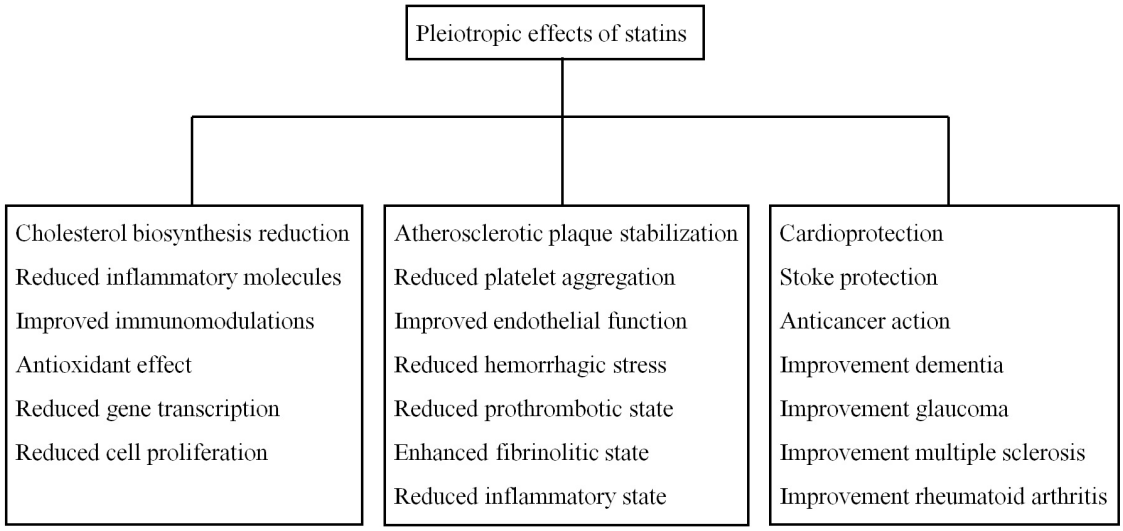

Fig. 3: The clinical benefits of statins appear to be more extensive than the expected lowering of circulating LDL-C. Statins also have direct vascular effects on endothelial cells, smooth muscle cells, monocytes, macrophages, and platelets.[4]

1.3 STATINS AND SMALL GTPases FAMILY

1.3.1 Rho GTPases in cell biology

The Rho family GTPases belong to the Ras superfamily of small GTPases. At least 10 members of the Rho subfamily are known in mammals: RhoA-E, RhoG, Rac1 and -2, Cdc42, and TC10. RhoA, -B, and -C, Rac1 and -2, and Cdc42 are the best-studied members. RhoA, -B, and -C (collectively Rho) have the same amino acid sequence in their effector domains (approximately 32–41 amino acids), appear to be regulated in a similar manner, and seem to have similar functions. The family of small GTPases is monomeric guanine nucleotide-binding proteins of approximately 20-25 kDa.The small GTPases cycle between GDP-bound (inactive) and GTPbound (active) states **(Fig. 4)**. Like other GTP-binding proteins, the Rho family GTPases exhibit both GDP/GTP-binding and GTPase activities.[25] Through these interchanging states, they serve as molecular signaling switches that regulate growth, morphogenesis, cell migration, cytokinesis and molecular trafficking. Currently, the Ras and Rho GTPase families are of special interest because they transduce extracellular stimuli to multiple intracellular signaling pathways.[26]

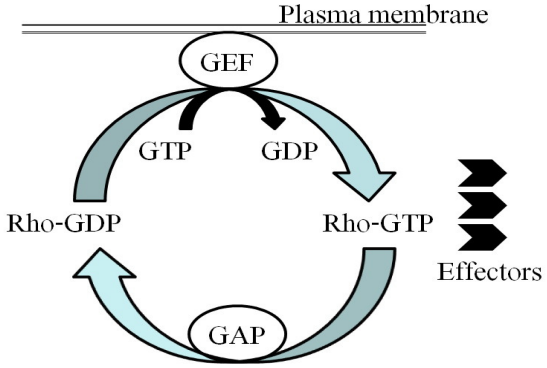

Fig. 4: The Rho GTPase cycle. They cycle between an active (GTP-bound) and an inactive (GDP- bound) conformation. The cycle is highly regulated by three classes of protein: in mammalian cells, around 60 guanine nucleotide exchange factors (GEFs) catalyse nucleotide exchange and mediate activation; more than 70 GTPase-activating protein (GAPs) stimulate GTP hydrolysis, leading to inactivation; and four guanine nucleotide exchange inhibitors (GDIs) extract the inactive GTPase from membranes.[27]

1.3.2 Regulation of the cytoskeleton by the Rho family GTPases

The ability of a eukaryotic cell to maintain or change its shape and its degree of attachment to the substratum in response to extracellular signals is largely dependent on rearrangements of the actin cytoskeleton. Cytoskeletal rearrangements play a crucial role in processes such as cell motility, cytokinesis, and phagocytosis. The actin cytoskeleton of animal cells is composed of actin filaments and many specialized actin-binding proteins.[28] Filamentous actin (F-actin) forms bundles and networks that are components of the actin cytoskeleton. Filamentous actin (F-actin) is generally organized into a number of discrete structures: **(1)** filopodia—finger-like protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. They are found primarily in motile cells and neuronal growth cones. **(2)** lamellipodia—thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many motile cells. Membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substrate and fold backward. **(3)** actin stress fibers—bundles of actin filaments that traverse the cell and are linked to the extracellular matrix (ECM) through focal adhesions. It is important, therefore, that the polymerization of cortical actin is tightly regulated. This regulation of actin polymerization, for the most part, is orchestrated by Rho GTPases.[3] Each member of the Rho GTPase family, which consists of RhoA, Rac and Cdc42, serves specific functions in terms of cell shape, motility, secretion and proliferation. This pathway is summarised in figure 5. This is supported by C3 exotoxin (a specific inhibitor of Rho) is used and is found to inhibit α-SMA (α-smooth muscle actin) expression at both the gene and protein level.[29] Rho GTPases are known principally for their pivotal role in regulating the actin cytoskeleton, but their ability to influence cell polarity, gene transcription, G1 cell cycle progression, microtubule dynamics, vesicular transport pathways and a variety of enzymatic activities ranging from an NADPH oxidase in phagocytes to a glucan synthase in yeast.[27]

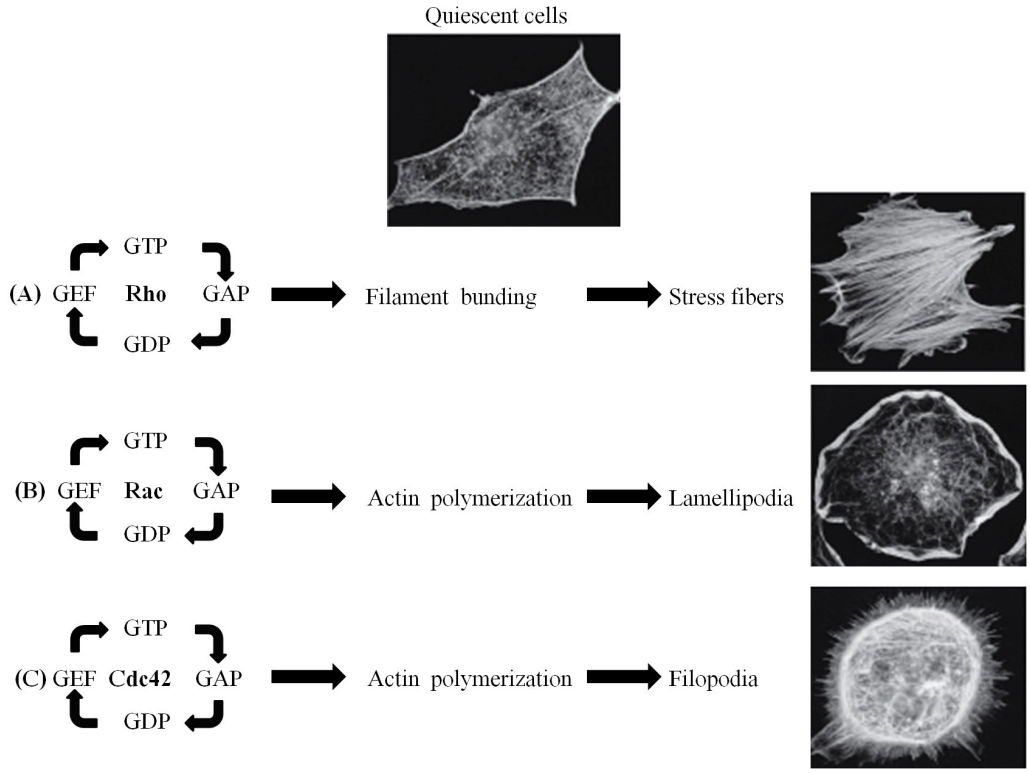

Fig. 5: The diagram illustrates proposed relationships between Rho family members and the cytoskeleton. The data are primarily obtained from studies in rhodamine phalloidin stained-Swiss 3T3 cells. The effects of Rho, Rac, or Cdc42 activation in these cells can be observed in several different ways such as with the addition of extracellular growth factors, microinjection of activated GTPases, or microinjection of guanosine diphosphate (GDP)–guanosine triphosphate (GTP) exchange factors. Addition of the growth factor lysophosphatidic acid activates Rho, which leads to stress fiber **(A)** Microinjection of constitutively active Rac induces lamellipodia **(B)** Microinjection of FGD1, an exchange factor for Cdc42, leads to formation of filopodia **(C)**.[28,30]

1.3.3 Statins and biological isoprenoid action

Protein isoprenylation permits the covalent attachment, subcellular localization and intracellular trafficking of membrane-associated proteins.[3] Members of the Ras and Rho GTPase family are the major substrates for post-translational modification by isoprenylation.[30] This process, termed isoprenylation, involves covalent attachment of farnesyl or geranylgeranyl residues to the small GTPase protein. The hydrophobic isoprenoid moiety permits anchoring of the prenylated protein to a cellular membrane and interaction with specific membrane-bound receptors. A key step in the activation of small GTP-binding proteins (e.g., Ras, Rho, Rac) is attachment of farnesiol (Ras) or geranylgeraniol (Rho and Rac), necessary for the translocation of a given inactive small GTP-binding protein from the cytosol to the membrane **(Fig. 6)**. Cell signaling is triggered when an inducer molecule binds to an enzyme-linked receptor or a membrane receptor such as a G-protein–coupled receptor (GPCR). This causes a conformational change that induces intracellular phosphorylation of the receptor or, in the case of a GPCR, G-protein activation via the release of its bound GDP and binding GTP.[27] Genarylgeneranylation is of particular interest, as it appears to be involved in numerous cell-signaling pathways. Because statins reduce the availability of GGPP, binding of prenylated proteins (e.g., Rac, Rho) to the membrane is affected, and consequently, their activity is reduced. Thus, statins that reduced protein prenylation might affect biological transduction pathways through the modulation of signaling proteins that depend on post-translational modification with isoprenoid.[4] FPP and GGPP are required for Ras farnesylation and Rho geranylgeranylation, respectively. Both Ras and Rho are necessary for a diversity of cellular events including actin cytoskeleton organization, cell adhesion and migration.[27] In endothelial cells, Ras translocation from the cytoplasm to the plasma membrane is dependent on farnesylation, whereas Rho translocation is dependent on geranylgeranylation.[31] In Swiss 3T3 fibroblasts, Rho GTPases control the assembly and organization of the actin cytoskeleton.[30] Statins inhibit both Ras and Rho isoprenylation, leading to the accumulation of inactive Ras and Rho in the cytoplasm. It is therefore not surprising to find that Rho-induced changes in the actin cytoskeleton and gene expression are related.[32] Indeed, experimental evidence suggests that inhibition of Rho isoprenylation mediates many of the cholesterol-independent effects of statins not only in vascular wall cells[31] but also in endometrial stromal cell[33] and cardiac fibroblast cells.[34] The pattern of inhibition reflects that achieve with the use of statin, therefore suggesting that inhibition of Rho signaling by statin is another pleiotropic effects of statin.

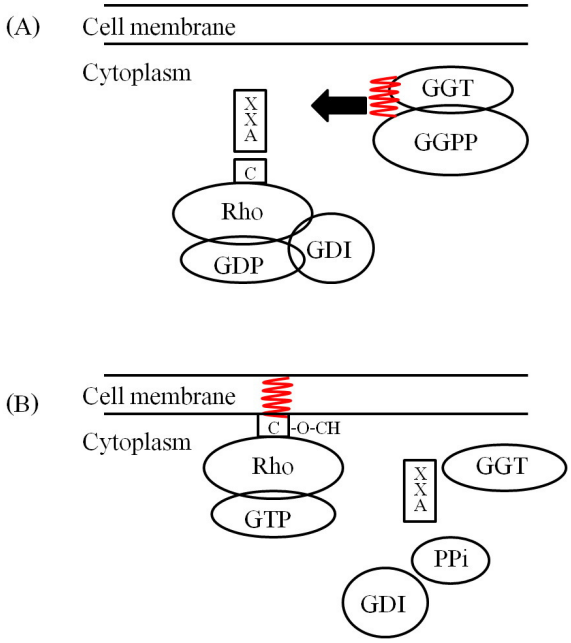

Fig. 6: Cell signaling is triggered when an inducer molecule binds to an enzyme-linked receptor or a membrane receptor such as a G-protein–coupled receptor (GPCR). As shown in the figure, geranylgeranyl transferase (GGT) transfers the geranylgeraniol group from geranylgeranyl pyrophosphate (GGPP) to, in this case, Rho **(A)**. This process permits Rho membrane attachment **(B)**. GTP dissociation inhibitor (GDI) is detached from membrane-bound Rho, which is then activated through the release of the Rho-bound GDP allowing a GTP molecule to bind in its place.[4]

1.4 APOTOTIC EFFECTS OF STATINS

1.4.1 Basic of apoptosis

Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or of distinct classes of proteases, such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics (immunogenic or non-immunogenic).[35] Apoptosis is a way of cell death in both physiological and pathological conditions. Apoptosis is accepted as an important process in different biological systems, including embryonic development, cell turnover, immune response against tumorigenesis or virus-infected cells and autoimmune disease.[13] Apoptosis morphologic features include rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), classically little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing (but maintenance of its integrity until the final stages of the process) and phagocytosis by resident phagocytes (invivo) **(Fig. 7)**.[36] Apoptosis is well suited to a role in tissue homoeostasis, since it can result in extensive deletion of cells with little tissue disruption. After fragmentary of an affected cell, the remaining fragments are rapidly disposed of by nearby intact phagocyte cells. There is no inflammation, as is elicited by coagulative necrosis, and even the lysosomal residual bodies soon disappear, possibly as a result of cell defaecation. Moreover, the process can be occurred re-utilization of cell component again.[37]

Necrosis is the death of cells through external damage, usually mediated via destruction of the plasma membrane or the biochemical supports of its integrity. The striking continuity of the morphological changes of apoptosis across cell types and species make its identification applicable to a variety of in vitro studies. Once the cell has committed to die, seemingly regardless of the mode of induction, the earliest observable ultrastructural event is condensation of chromatin to form uniformly dark, crescentic, and marginated masses adjacent to the nuclear envelope, and the nucleolar chromatin disperses forming osmiophilic aggregates in the center of the nucleus; also, the fibrillar core usually opposes the inner surface of the condensed nuclear chromatin during this time.[38]

The recognition of the significance of apoptosis in health and disease has fueled extensive research efforts aimed at elucidation of the molecular mechanisms of apoptosis and application of this knowledge for the discovery of new therapeutic regimens.

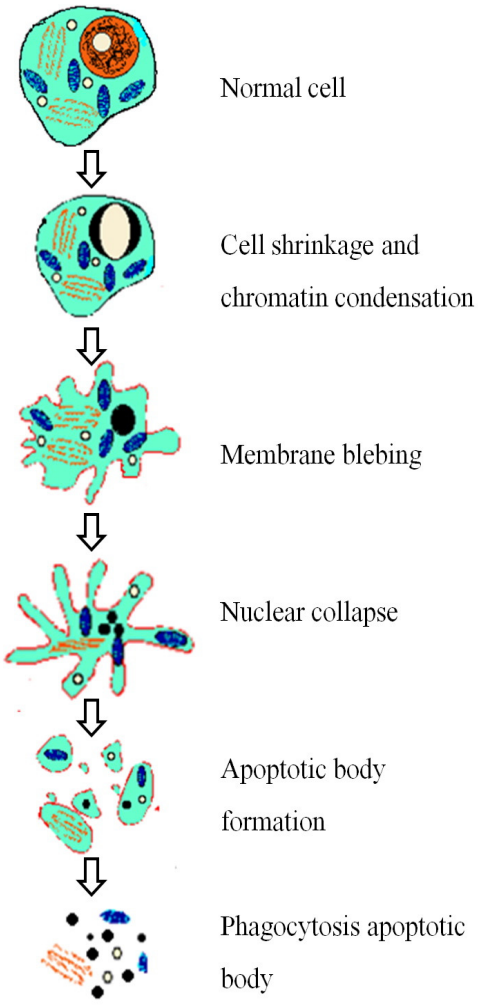

Fig. 7: The major stage of apoptosis include (1) intracellular activation (2) morphologic change (3) cell membrane change (4) phagocytosis without inflammation.[37]

Apoptosis occurs via two main signaling pathways **(Fig. 8)**.

- The extrinsic pathway or receptor-linked apoptosis involves the activation of the death receptors (DR), Fas receptors (FAS-R) and tumor necrosis factor receptors (TNFR) on the cell surface which recruits two signal transducing molecules: TNFR 1-associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD). This association leads to recruitment of adaptor molecules such as FADD or TRADD resulting in activation of initiator caspases 8 and 10, which in turn cleaves and activates executioner caspases 3, 6, and 7, culminating in apoptosis. For example in this pathway such as Cytotoxic T lymphocyte. Cytotoxic T lymphocytes are a major defense against virus infection. On antigen receptor triggering, cytotoxic T lymphocytes kill target cells by releasing perforin plus granzymes or through the action of FasL and TNF.[39]

- The intrinsic pathway or mitochondria-mediated apoptosis involves the activation of several procaspases and the mitochondrial release of apoptogenic factors such as cytochrome c. Once released, cytochrome c interacts with Apaf-1, ATP, and pro-caspase 9 to form the apoptosome. The apoptosome cleaves and activates caspase 9, which leads to caspase 3, 6, and 7 activity stimulating apoptosis and apoptosis-inducing factor into the cytoplasm.[40] Subsequently, a cascade of events are driven primarily by the activation of proteolytic caspases resulting in the processing of intracellular structural proteins and regulatory enzymes that culminates in apoptotic cell death.[41] Synthesis and/or activation of IAPs (Inhibitor of apoptosis) are under control of the transcription factor nuclear factor kappa B (NF-κB). NF-κB is present in the cytosol in an inactive form bound to a second molecule IκB. Phosphorylation of IκB leads to its enzymatic degradation. Uncoupling of IκB and NF-κB renders activation of NF-κB. NF-κB is composed of two subunits, p50 and p65, which can function independently or as a duo by translocating into the nucleus where it promotes transcription of IAPs leading to executor caspase inhibition.[42]

Caspases, a family of aspartate-specific cysteine proteases, play a crucial role in apoptosis. Caspases are at least 14 members and categorized in three groups depending on inherent substrate specificity, domain composition or the presumed role in apoptosis. They are constitutively expressed in almost all cell types as inactive proenzymes (zymogens) that become processed and activated in response to a variety of pro-apoptotic stimuli. They include initiators in apoptosis (caspase-2, 8, 9 and 10), executioners in apoptosis (caspase-3, 6 and 7) and participants in cytokine activation (caspase-1, 4, 5, 11, 12, 13 and 14).[43] The regulation of caspases is similar to other proteolytic systems. They are produced in proenzymatic form and activated by a proteolytic cleavage

(caspases can also activate each other.). While there are lots of inhibitory proteins that prevent their activity.

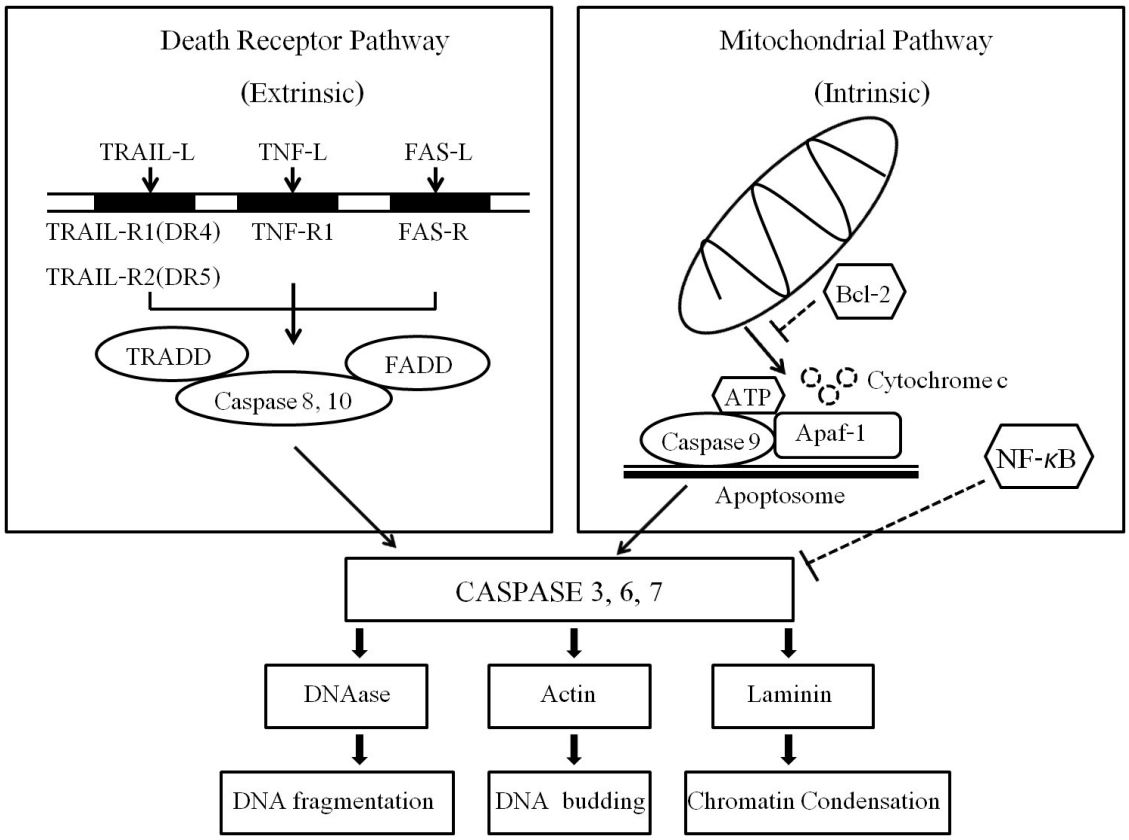

Fig. 8: Synopsis of the basic molecular mechanisms of apoptosis. The left part of the diagram portrays the extracellular (exogenous) apoptotic pathway: Specific ligands bind to death receptors on the cell membrane. The right part of the diagram depicts the intracellular (endogenous) mitochondrial apoptotic pathway, which involves mitochondrial release of cytochrome c in the cytoplasm. Both endogenous and exogenous pathways result in a chain reaction of caspase activation that culminates in apoptotic cell death.[44]

Because of the continuity in the morphological features of apoptosis, such as chromatin condensation, cell shrinkage, membrane blebbing and apoptotic body formation without the initial loss of membrane integrity, it seems likely that the core biochemical events leading to these changes should occur in all cells. The events regarded as key features of apoptotic biochemistry are represented such as[38]

- Phosphatidylserine translocation: Since apoptotic cell debris are quickly engulfed in vivo, before they rupture, the cell death program must implement plasma membrane (PM) changes in apoptotic cells which allow their recognition by phagocytes.

- DNA fragmentation: There is an initial cleavage of DNA into 300-kilobase- (kbp) pair fragments and /or 50-kbp fragments, most commonly followed by oligonucleosomalsized fragments due to double-stranded cleavage of DNA at linker regions between nucleosomes.

- Other significant changes: The dramatic morphological changes occurring in cells undergoing apoptosis implies that alterations in cytoskeletal elements and extensive cytoplasmic proteinase activities are primarily responsible for these changes. Indeed, increases in β-tubulin mRNA and cytoplasmic β-tubulin protein have been shown to occur in cells committed to die by apoptosis. Moreover, microfilament assembly is required for the cell to fragment and form apoptotic bodies.

Additionally, the caspase detection is the key proteins required for the processing, propagation and amplification of apoptotic signals that results in the destruction of cellular structures. Variable assays and experimental techniques have been developed for the study of apoptosis in cells, tissues, and organs. Several studies are available to determine whether cells are undergoing programmed cell death versus necrosis. The study of apoptosis can be compartmentalized in various ways. Table 3 summarizes features of the apoptotic process that have been exploited for detection in individual cells, in mass cell cultures, and in tissues.

Table 3: Categories of cellular changes that form the basis of apoptosis assays.[45]

Surface morphology and composition	Time-lapse surface morphology Membrane permeability: impermeable dye (PI), permeable DNA stains (DAPI, Hoechst) Phospholipid externalization (Annexin V binding)
Nuclear events and DNA cleavage	Nuclear morphology: segmentation of chromatin and nuclei DNA cleavage by gels DNA cleavage in situ
Cytoplasmic biochemical activation	Caspase activity Caspase clevage products
Mitochondrial function and intergrity	Cytochrome c release and alterations Permeability transition (vital dyes)

Knowledge of the advantages and disadvantages of each technique allows selection of the appropriate apoptotic assays or combinations thereof for accurate assessment of apoptosis in diseased cells and tissues. Implementation of these techniques in a clinical setting could facilitate the accurate diagnosis of diseases that manifest altered apoptotic rates.

1.4.2 Statins-induced apoptosis

It has been shown that statins can induce apoptosis in a variety of cell types, such as macrophage cells,[14] endometrial stromal cells[33] and various cancer cell lines.[15,16] In contrast, recent studies showed that simvastatin decreases apoptosis in murine osteoblastic cells.[17] It is thought that apoptosis of these above-mentioned cell types in diseased tissue may contribute to the pleiotropic benefits of statins. For example, apoptosis of transformed cells may prevent or slow the development of cancer[15,16] Statins are interesting options in the study for strategies to maintain plaques via macrophage-specific cell death.[46] Many of these apoptotic effects of statins are mediated by the in ability to block the synthesis of important isoprenoid intermediates, which have been shown to serve as lipid attachments for a variety of intracellular signaling molecules.[14,34] In particular, the inhibition of the small GTPase-binding proteins Rho, Cdc42, and Rac, the membrane localization of which depends on geranylgeranylation, may play an important role in mediating the direct effects of statins on cellular proliferation and survival.[34,47] Statins-induced apoptosis is likely due to the depletion of

geranylgeranylated or farnesylated proteins, which may be dependent on cell type or the state of differentiation.[24] However, the depletion of geranylgeranylated proteins is crucial role to reduce cell viability and induce apoptosis more than the depletion of farnesylated proteins.[34,47] Then, simvastatin induces apoptosis via small GTPases of the Rho family rather than Ras family proteins.[34] It has been shown that simvastatin triggers apoptosis via a variety of intracellular signaling pathways such as both caspase-dependent and -independent apoptotic pathways. The caspase-dependent apoptosis displayed typical features: nuclear DNA degradation, disruption of MMP, and caspase-3 activation. In contrast with the caspase-independent apoptosis displayed typical features: fragmented nuclei, packed DNA condensation and large-scale DNA fragmentation.[14] Additionally, simvastatin induces caspase-processing apoptotic pathways via the depletion of isoprenoid intermediates by increases cytosolic calcium concentration then activated mitochondrial-mediated apoptosis manner **(Fig. 9)**,[24] decrease of antiapoptotic proteins bcl-2[48] and down-regulation of the NF-κB pathway.[19] It is speculated that the alteration in isoprenoid synthesis with statin therapy is responsible for the tyrosine phosphorylation and stimulation of pathways causing a rise in cytosolic calcium. Sacher et al. have shown that the elevated calcium levels in response to statins activate calpain, which in turn leads to apoptosis via the translocation of Bax to the mitochondria and activation of caspase-9 and caspase-3. Inhibition of the mitochondrial transition pore, in which opening is required for cytochrome c release and thought to be regulated by Bax, prevents apoptosis and activation of caspase-9 and caspase-3.[49] Further experimentation showed that chelation of calcium completely prevented calpain, caspase-9, and caspase-3 activation. Also, coadministration of mevalonate with the statin attenuated the rise in cytosolic calcium levels, although is not completely prevent it.[49]

In conclusion, statin-induced apoptosis is largely in part due to the depletion of isoprenoids, which in turn can decrease protein geranylgeranylation and/or farnesylation, which may lead to elevated levels of cytosolic calcium and activation of calpain and the mitochondrial-mediated apoptotic signaling cascade **(Fig. 9)**.

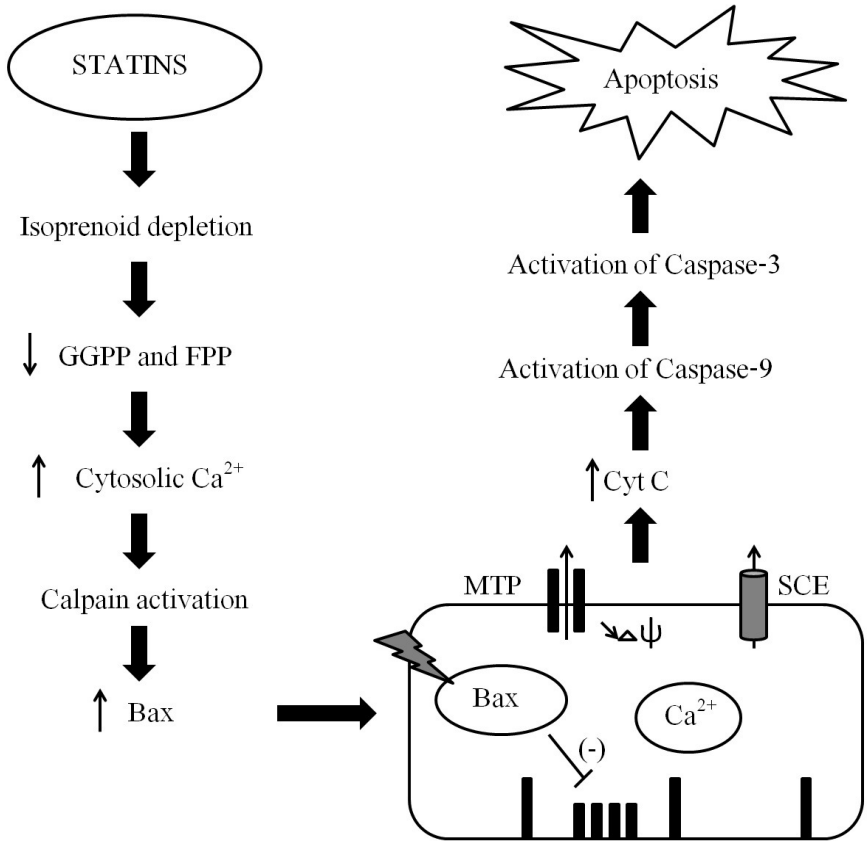

Fig. 9: Suggested effects of statin on mitochondria-mediated apoptotic signalling. HMG-CoA reductase inhibition by statins increases cytosolic calcium concentration and expression of the pro-apoptotic protein Bax. Bax in turn inhibits complex I of the mitochondrial respiratory chain (RC). This depolarises the inner membrane (ΔΨ) triggering a calcium release through the mitochondrial transition pore (MTP) and sodium calcium exchanger (SCE). Cytochrome c (Cyt C) release from the mitochondria leads to activation of procaspase-9. Active caspase-9 cleaves and activates procaspase-3, which leads to apoptosis.[50]

1.5 PLEIOTROPIC EFFECTS OF STATINS ON TOOTH-ASSOCIATED CELLS

1.5.1 The effects of simvastatin on pulp cells

Simvastatin has effect on the regeneration of injured dental pulp tissue and in the stimulation of reparative dentinogenesis because it has effect on the proliferation, induced DSPP (Dentin sialophosphoprotein) and OCN (Osteocalcin) gene expression of DPSCs (Dental pulp stem cells). This consequence can be up-regulating mineralization nodules in human pulp derived cells.[11] Consequence with another study in human pulp derived cells that, simvastatin has effect on the expression of endothelial cell adhesion molecules and angiogenic growth factors, that increase in the time-dependent and dose-dependent manner. Because angiogenesis process is important for absolute pulpal healing then simvastatin is interested in pulpal regeneration.[10]

1.5.2 The effects of simvastatin on PDL cells

An in vitro study by Yazawa et al revealed that treatment of human periodontal ligament cells (PDLs) with simvastatin results in increased levels of ALP activity and osteopontin production by these cells, indicating the potential effects of simvastatin on osteogenic differentiation of PDLs.[51] Despite these studies, the potential contribution of simvastatin to alveolar bone regeneration and the possibility of its application in periodontal therapy had further investigated. Simvastatin slightly increased the expression of osteogenic markers such as receptor activator of NF-*K*B ligand (RANKL), osteoprotegerin (OPG) in primary osteoblast cells and periodontal ligament cells, indicating its ability to influence alveolar bone formation and periodontal regeneration.[12] An increased expression of both OPG and RANKL suggests that simvastatin accelerates the turnover rate of alveolar bone. This ability of simvastatin increase alveolar bone plasticity, which is important for rapid remodeling and adaptation to mechanical load.

1.5.3 The effects of simvastatin on apical periodontitis

There are many studied that attempt to relieve bone resorption in apical periodontitis condition. Lin et al. reported that simvastatin inhibits periapical bone resorption by diminishing macrophage chemotaxis to inflammation site. This mechanism shows that simvastatin

considerably decreased TNF-α–induced Cyr61 synthesis in MG-63 cells (human osteosarcoma cells). Cysteine-rich 61 (Cyr61) and CCL2 are potential osteolytic mediators in inflammatory bone diseases, that related to reduce the apical lesion **(Fig. 10)**.[7] In additional, recent studies showed that simvastatin decreases apoptosis in murine osteoblastic cells.[17,18] Lai et al. demonstrated the alleviating effects of simvastatin on periapical lesion–induced bone destruction in animal model by suppressed apoptosis of osteoblastic cells under oxidative stress. By using a rat model of periapical lesion, simvastatin attenuates bone resorption associated with apical periodontitis, possibly through suppressing the expression of Cyr61 and apoptosis in osteoblasts.[7,18] This may be the first article highlighting the role of simvastatin in apical periodontitis-related bone destruction.

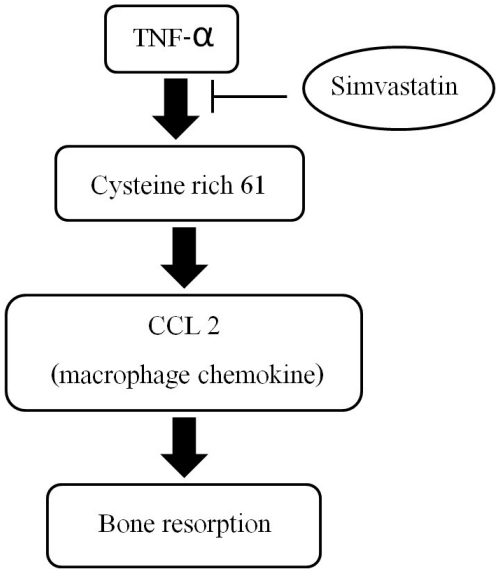

Fig. 10: Simvastatin suppresses the progression of apical periodontitis lesion. Simvastatin retards macrophage chemotaxis by acting on osteoblasts to alleviate TNF- α –stimulated synthesis of Cyr61, an inducer of CCL2. OB,Odontoblast; Cyr61,Cysteine-rich61; CCL2,Chemokine ligand-2.[7]

OBJECTIVE

It is clear the statins induce apoptosis in various cells. There are many studies to investigate pleiotropic effects of simvastin in tooth-associated cells. However, little is known about its possible toxic effects on tooth-associated cells. The objectives of this study are listed as follows:

1. The effects of simvastatin on cell viability in cultured human dental pulp cells (HDPCs) and periodontal ligament fibroblasts (HPLFs) cells.

   If the outcome of the experiments of cell viability and morphology indicate that simvastatin may induce cell toxicity, then we will further investigate the mechanism of cell death.
2. The effects of simvastatin on apoptosis in cultured human dental pulp cells (HDPCs) and periodontal ligament fibroblasts (HPLFs) cells.
3. The effects of simvastatin on cell morphology in cultured human dental pulp cells (HDPCs) and periodontal ligament fibroblasts (HPLFs) cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

2.1.1.1 Reagents for primary cell culture

Reagent name	Source
Alpha-minimum essential medium (α-MEM)	GIBCO^{R}, Invitrogen Corporation, USA
Dulbecco's modified Eagle's medium (DMEM)	GIBCO^{R}, Invitrogen Corporation, USA
Fetal bovine serum	Biochrom AG, Germany
Penicillin- Streptomycin	GIBCO^{R}, Invitrogen Corporation, USA
Fungizone	GIBCO^{R}, Invitrogen Corporation, USA
L-Glutamine	GIBCO^{R}, Invitrogen Corporation, USA
Ascorbic acid	Sigma-aldrich, USA
Sodiumbicarbonate	Merck Inc Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Sigma-aldrich, USA
0.5%Trypsin-EDTA	GIBCO^{R}, Invitrogen Corporation, USA

2.1.1.2 Reagents for apoptosis treatment

Reagent name	Source
Hydrogen peroxide	Analar^{R}, VWR International, UK
Simvastatin	Calbiochem, Merck4Biosciences, Germany

2.1.1.3 Reagents for analysis

Reagent name	Source
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT assay)	Sigma-aldrich, USA
Annexin-V FITC Apoptosis detection kit	Calbiochem, Merck4Biosciences, Germany
Fluorescein phalloidin	Molecular Probes™, Invitrogen Corporation,USA
DAPI Nucleic Acid Stain	Molecular Probes™, Invitrogen Corporation,USA

2.1.2 Instruments and special equipments

2.1.2.1 Instruments

Instrument	Company
Microflow advance biosafety cabinet-class II	Astec air BHA 48, England
CO_2 incubator	HERA cell 240, Heraeus, Germany
Centrifuge	Savant, Speed fuge HSC 15 R, USA
Centrifuge (Micro)	Hettich Mikro 220 Centrifuge, UK
Light microscope	Nikon TMS, Japan
Fluorescence microscope	Olympus corporation DP71, Japan
FC500 Flow cytometer	Becton-Dickinson, USA
Microplate Reader	Titertek Multiskan® PLUS, US
Autoclave	Tomy SS-325, US
Hemocytometer	Laboroptik. Germany
Cell counter	Need, Fuji Nishi ind, Japan
Auto pipette	Accu-jet® pro, BrandTech Scientific, Germany
Water bath	Memmert, Technical science and service, Germany

2.1.2.2 Special equipments

Special equipments	Company
6 well cell culture plates	Nunclon™, Nunc, Denmark
24 well cell culture plates	Nunclon™, Nunc, Denmark
96 well cell culture plates	Nunclon™, Nunc, Denmark
25 cm^2 cell culture flasks	Nunclon™, Nunc, Denmark
75 cm^2 cell culture flasks	Nunclon™, Nunc, Denmark
50 ml centrifuge tube	Corning Incorporation, USA
5 ml polystyrene round button tube	BD Falcon™, BD Biosciences
Microcentrifuge tube 1.5 ml (Eppendorf)	Bioline, UK
Millipore filter 0.45 µm.	Millipore corporation, USA
Pipette tip 20, 200, 1000 µl.	Bioline, UK
Microscope glass slide	Sail Brand, China
Round coverslip	Sail Brand, China

2.2 METHODS

2.2.1 Sample collection and cell culture

Human dental pulp cells (HDPCs) and Periodontal ligament fibroblasts (HPLFs) were cultured from normal human premolar or third molar teeth. The subjects aged 18-30 years were underwent tooth extraction for orthodontic reasons at the Dental Hospital, Faculty of Dentistry, Prince of Songkla University, with the approval of the Research Ethics Committee, Faculty of Dentistry, Prince of Songkla University (No. 0521.1.03/969). Informed consent was obtained from the subjects who agreed to participate voluntarily in this study. Teeth were placed in cold culture medium and transferred to the cell culture laboratory.

2.2.1.1 Primary culture of HDPCs

Isolation and culture of HDPCs were performed using an enzymatic method as described by Gronthos et al., 2000.[52] Culture media and supplements were products of Gibco (Invitrogen Corporation, NY, USA) unless indicated elsewhere. Tooth was rinsed with povidone-iodine (PVPI) topical antiseptics and washed with culture medium. Tooth surface was separated in longitudinal axis by using sterilized dental fissure burs, then split by periosteal elevator to reveal the pulp chamber. Pulp tissue was gently separated from the pulp chamber. Pulp tissue was minced with a disposable scalpel into small pieces and digested in mixed solution of 3 mg/ml of collagenase Type I and 4 mg/ml of dispase 45 min in humidified atmosphere containing 5% CO_2 at 37 °C. After centrifugation, cells were cultured in 25-cm^2 tissue culture flask (Nunc, Naperville, IL, USA) with alpha modified Eagle's medium (α-MEM), supplemented with 20% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 100 μM L-ascorbic acid (Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamate antibiotics (200 units/ml penicillin, and 200 μg/ml streptomycin), and 2.5 μg/ml fungizone and incubated in humidified atmosphere containing 5% CO_2 at 37 °C. After cells were grown to confluence, they were detached with 0.05% trypsin-ethylenediamine tetraacetic acid (Trypsin-EDTA), then transferred to 75-cm^2 tissue culture flask and passaged at a ratio of 1:3 in 10% FBS culture medium and were designated "first passage cells (P_1)". HDPCs were obtained from 4subjects. In all experiment, pulp cell passages between three and ten were used for this study.

Stocks of these cells were prepared and stored in liquid nitrogen for use in the following experiments.

2.2.1.2 Primary culture of HPLFs

Isolation and culture of HPLFs were performed using a tissue explant method as described by Somerman et al., 1988.[53] Culture media and supplements were products of Gibco (Invitrogen Corporation, NY, USA) unless indicated elsewhere. Tooth was rinsed twice with culture medium. Periodontal ligament tissue was gently removed from the middle one-third of the root surface to exclude the contamination of gingival and apical tissue using a disposable scalpel. Periodontal ligament tissue was rinsed in culture medium, cut into small piece, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), antibiotics (200 units/ml penicillin, and 200 µg/ml streptomycin) and 2.5 µg/ml fungizone in 35 mm^2 culture dish (Nunc, Naperville, IL, USA) and incubated in humidified atmosphere containing 5% CO_2 at 37 °C. After approximately 7-10 days, HPLFs could migrate out from the margin of the tissue. When cells surrounding tissue explants were confluent, they were detached with 0.05% Trypsin-EDTA and transferred to 25-cm^2 tissue culture flask and are designated "first passage cells (P_1)". Cells were continuously passed at a 1:3 ratio when 80% confluent in 75-cm^2 tissue culture flask. HPLFs were obtained from four subjects. In all experiment, HPLFs passages between three and ten were used for this study. Stocks of these cells were prepared and stored in liquid nitrogen for use in the following experiments.

2.2.2 Preparation of simvastatin

The active form of simvastatin (Calbiochem, Merck Biosciences, Darmstadt, Germany) was prepared according to the manufacturer's instructions. Briefly, 20 mg simvastatin was dissolved in 400 µl ethanol (95-100%) followed by the addition of 0.325 ml of 1 N NaOH. The solution was neutralized with 1 N HCl to pH 7.2 and brought to a 1-ml volume by deionized water. The final concentration of simvastatin was 20 mg/ml or 47.8 mM.

2.2.3 MTT assay for cell viability

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT colorimetric assay was used to measure the cellular growth and survival. MTT is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. HDPCs/HPLFs at a cell density of 2.5 X 10^3 cells/well were plated in 96-well tissue culture plates, cultured with 10% FBS for 24 h in a humidified 5 % CO_2 incubator at 37°C. Then, cells were starved in serum free medium for another 24 h. Afterwards, cells were cultured with or without simvastatin 0.1, 1, and 10 µM of simvastatin in 10% FBS culture medium for 24, 48, and 72 h. Following culture, the medium was gently removed. The 200 µl of fresh medium containing 10 mM HEPES (pH 7.4) and 50 µl MTT solution (5 mg/ml in PBS, Sigma-Aldrich) were added to each well and incubated in the dark for 4 h at 37°C. The medium and MTT were carefully removed. The formazan crystals were dissolved in 200 µl of dimethyl sulfoxide (DMSO) and 25 µl of Sorensen's glycine buffer (0.1 M glycine plus 0.1 M NaCl equilibrated to pH 10.5 with 0.1 M NaOH). The optical density (OD) of formazan production was measured at a wavelength 570 nm using a microplate reader. The mean values obtained from 5 wells under each condition were computed and statistically analyzed. The OD of formazan products was calculated for percentage of cell viability as follows:

$$\text{Percentage of cell viability} = \frac{[\,OD_{570}\ \text{treated cells}]}{[\text{avg.}OD_{570}\text{control cells}]} \times 100$$

2.2.4 Flow cytometry for apoptosis

The cellular apoptosis was measured by membrane redistribution of phosphatidyl serine (PS), based on the binding properties of Annexin V to PS and on the DNA-interacting capabilities of propidium iodide (PI). The percentage of apoptotic cells was determined by flow cytometry, using Annexin-V FITC and propidium iodide staining kit (Calbiochem, Merck Biosciences, Darmstadt, Germany). HDPCs and HPLFs were seeded at 7.5 X 10^4 cells per 2.5 ml of 10% FBS culture medium in each well within 6-well culture plates at a humidified atmosphere of 5 % CO_2 at 37°C. Cells were grown for 24 h before the culture medium was replaced with fresh serum free medium for a further 24 h. After that, cells were treated with 0, 0.1, 1, and 10 µM of simvastatin in 10% FBS culture medium, incubated for 24, 48, and 72 h. Positive control cells in this experiment contained 1 mM H_2O_2 which is used as an apoptosis inducer as described by Min et al., 2008.[54] After

incubation time, cells were harvested by centrifugation at 1,000 x g for 5 min. Cell pellets were washed with PBS and centrifugation at 1,000 x g for 5 min. Cells were gently resuspended in cold binding buffer 200 µl. After that, cell suspension was transferred to an eppendorf. Annexin V binding reagent 10 µl and Annexin V-FITC 1.25 µl were added, then incubated for 15 min at room temperature (18-24°C) in the dark. After that, cell suspension was centrifuged at 1,000 x g for 5 min at room temperature. Cell pellets were gently resuspended in cold binding buffer 200 µl. Propidium iodide 10 µl was added. The samples were analyzed by a FC 500 flow cytometer (Becton-Dickinson,BD Biosciences, San Jose, CA, USA). Fluorescence parameters were gated using unstained and single-stained untreated control cells, and 10,000 cells were counted for each treatment. Representative FACS scatter plots showed the viable cells (Annexin V -ve; PI -ve) in lower left hand quadrant, the early apoptotic cells (Annexin V +ve; PI -ve) in lower right hand quadrant, and the late apoptotic cells (Annexin V +ve; PI +ve) in upper right hand quadrant. Data are expressed as percentages of Annexin V positive cells plus Annexin V and PI double positive cells for quantitation of total apoptosis. The mean values were obtained from four cell populations in duplicate under each condition in each cell types were computed and statistically analyzed.

2.2.5 Actin and nuclear staining

Actin fibers were stained with Phalloidin-FITC (Sigma-Aldrich) and nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen), and thereafter they were observed under a fluorescence microscope. HDPCs or HPLFs were seeded at 1.2 X 10^4 cells per 1 ml on sterile glass coverslips onto 24-well culture plates at a humidified atmosphere of 5 % CO_2 at 37°C for 24 h. Cells were serum-deprived for another 24 h, and then cultured with or without 0.1, 1, and 10 µM simvastatin for 24, 48, and 72 h. After incubation time, cells were washed twice with 37°C PBS then fixed with 3.7 % paraformaldehyde/PBS for 15 min. Cells were washed twice with 37°C PBS 2 times for each 5 min and permeablized in 0.1% Triton X-100/PBS for 5 min. The cells were washed twice with 37°C PBS 2 times and blocked nonspecific binding with 0.5% BSA /PBS for 5 min. The actin filament of cells were stained with Phalloidin-FITC that diluted 40x in PBS for 30 min. Then the nuclei of cells were stained with 1 µg/ml diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min. The coverslips were mounted on slides by drop on an aqueous permanent mounting media. The cytoskeketon and nuclei were observed under a fluorescence microscope.

2.2.6 Statistical Analysis

Data are mean ± standard deviation (SD) of, at least, 4 independent experiments. Comparisons of the data between groups were performed by one-way analysis of variance (ANOVA) followed by a multiple-comparison Tukey's post hoc test. A p value less than or equal to 0.05 was considered significant.

CHAPTER 3

RESULTS

3.1 CELL VIABILITY

HDPCs and HPLFs were exposed to various concentrations of simvastatin (control, 0.1, 1, 10 μM) for 24, 48, and 72 h, then the cell viability was analyzed by MTT assay **(Fig. 11, 12)**.

HDPCs: The result showed that the viability of HDPCs was not affected when simvastatin was lower than 0.1 μM, whereas higher concentrations of simvastatin (1, 10 μM) significantly decreased the cell viability in a dose and time-dependent manner ($p < 0.05$) **(Fig. 11)**. There was significant difference the percentage of cell viability between low (0.1 μM) and high (1, 10 μM) concentration of simvastatin since 24 h incubated period ($p < 0.05$) **(Fig. 11B)**. Simvastatin (1 μM) in 48 h incubation resulted in initial significantly viability decreased 50% compared with the control group ($p < 0.05$) **(Fig. 11B)**.

HPLFs: There was significant difference the percentage of cell viability between low (0.1 μM) and high (1, 10 μM) concentration of simvastatin since 48 h incubated period **(Fig. 12B)**. The low concentration of simvastatin (0.1, 1 μM) had no significant effect on cell viability whereas higher concentrations of 10 μM had a significant inhibitory effect **(Fig. 12A)**. When HPLFs were treated with 10 μM simvastatin for 72 h, the level of cell viability decreased 50% compared with the control group ($p < 0.05$) **(Fig. 12B)**.

HDPCs and HPLFs: Similar results were found with HDPCs and HPLFs cultures. However, the sensitivity to the drug appeared to be different between the two cell types. Cell viability was decreased to 50% for simvastatin-treated HDPCs at 1 μM, after a culture period of 48 h, whereas a similar decrease in viability for HPLFs was found with a higher concentration of simvastatin, 10 μM, and at a later time point, being 72 h.

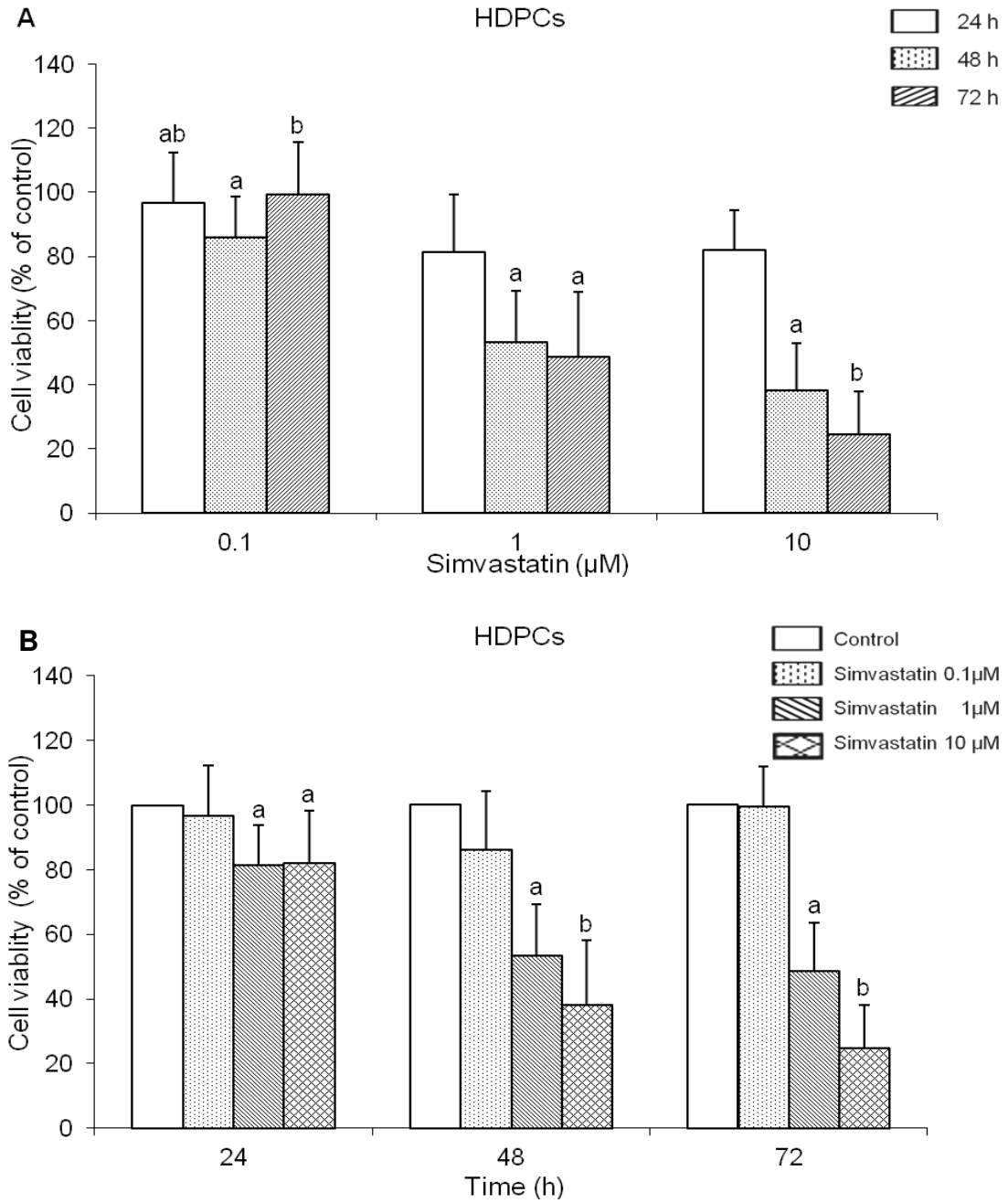

Fig. 11: Effect of simvastatin on cell viability of human dental pulp cells (HDPCs); a dose-course analysis/HDPCs **(Fig. 11A)**, a time-course analysis/HDPCs **(Fig. 11B)**. HDPCs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cell viability was determined by MTT assay. Data were represented as means ± standard deviation (SD) of four cell populations and each analyzed five times. Value with different alphabet denotes significant difference ($p < 0.05$).

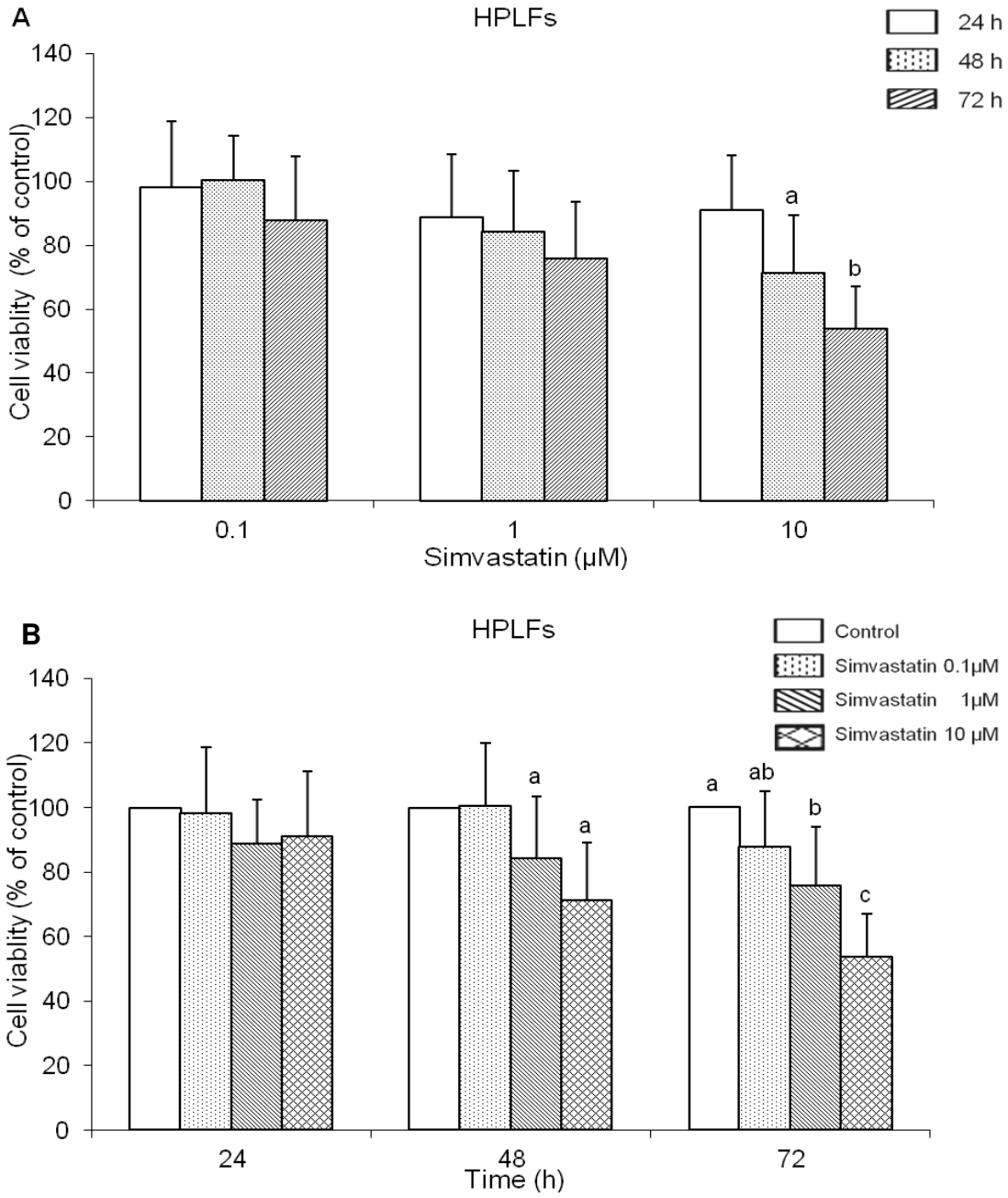

Fig. 12: Effect of simvastatin on cell viability of human periodontal ligament fibroblasts (HPLFs); a dose-course analysis/HPLFs **(Fig. 12A)**, a time-course analysis/HPLFs **(Fig. 12B)**. HPLFs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cell viability was determined by MTT assay. Data were represented as means ± standard deviation (SD) of four cell populations and each analyzed five times. Value with different alphabet denotes significant difference ($p < 0.05$).

3.2 CELL MORPHOLOGY

Morphological changes of HDPCs and HPLFs following exposure to simvastatin were investigated by the inverted microscope. **Fig 13** and **14** showed morphological images of HDPCs at 10X and 20X respectively. **Fig 15** and **16** showed morphological images of HPLFs at 10X and 20X, respectively.

HDPCs: As shown in **Fig. 13** and **14**, untreated HDPCs were generally spindle-shaped in appearance with flattened and extended cellular processes that attached to the cellular culture plate. No marked morphological changes of HDPCs were observed after exposure to 0.1 μM simvastatin. Exposure of HDPCs to 1 and 10 μM simvastatin revealed differential morphological changes. After exposure to 1 μM simvastatin for 72 h or 10 μM simvastatin for 24 h, HDPCs retracted with residual cytoskeleton and an increase in intercellular space were observed. Most HDPCs became rounded in appearance and decreased in cell density after 48 h exposure to 10 μM simvastatin.

HPLFs: Cultured HPLFs in control group were elongated, spindle in shape and cells were more confluent. The structures of HPLFs exposed to 0.1 μM simvastatin were nearly the same as those of the control, whereas after exposure to 1 μM simvastatin for 72 h or 10 μM simvastatin for 48 h, resulted in cell retraction and a decrease in cell proliferation and density. After 72 h exposure to 10 μM simvastatin, the rounding and the detachment were observed **(Fig. 15, 16)**.

HDPCs and HPLFs: Upon incubation with simvastatin, the cell size decreased of both HDPCs and HPLFs. In addition, we found shorter and thinner filamentous extensions with a reduction in cell number in a dose- and time-dependent manner **(Fig. 13-16)**.

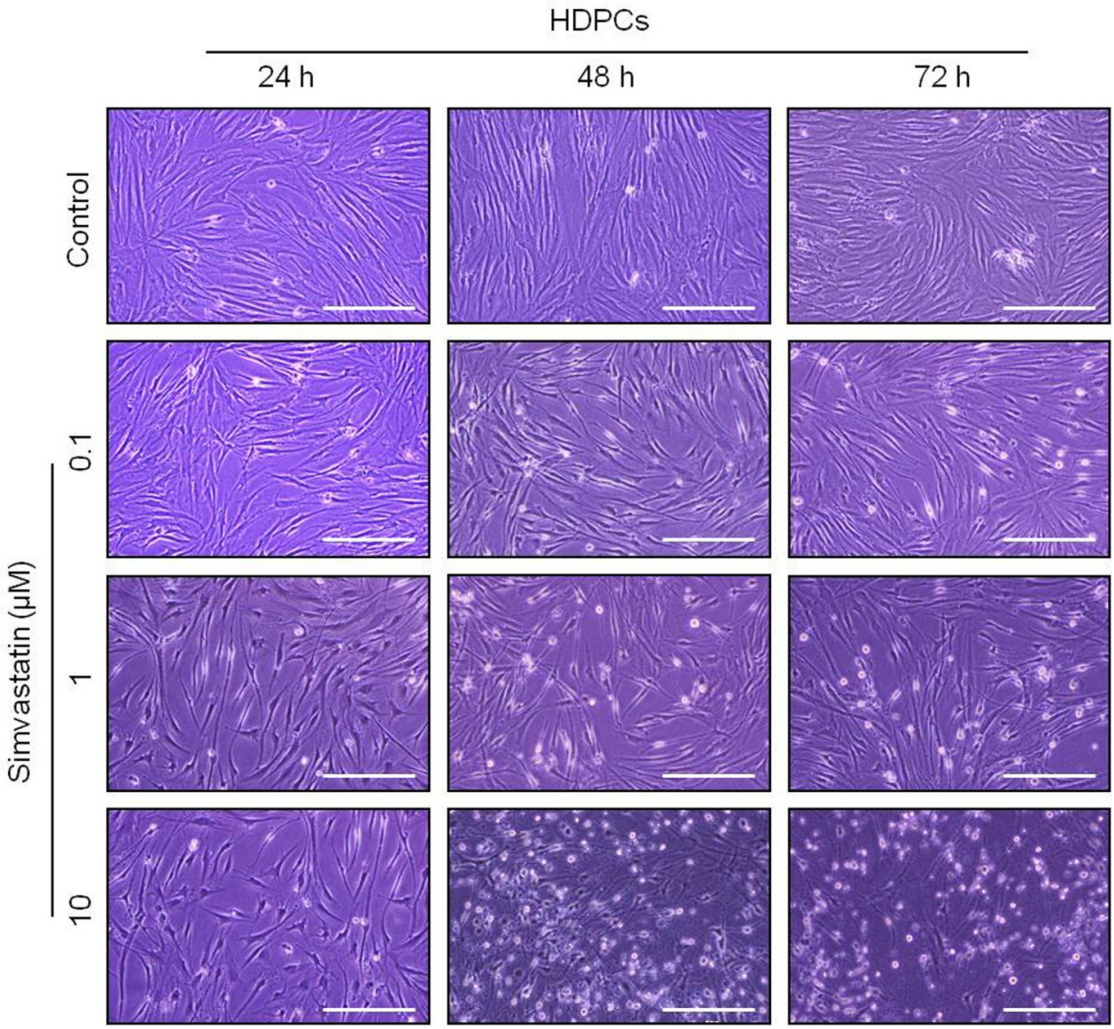

Fig. 13: Morphologic appearance of human dental pulp cells (HDPCs). Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cell morphology was examined under the inverted microscope (magnification 10X). Decreased both cell size and number of cells in simvastatin-treated cultures were observed in a concentration- and time-dependent manner. Representative images were obtained from one of four subjects. Scale bars represent 500 μm.

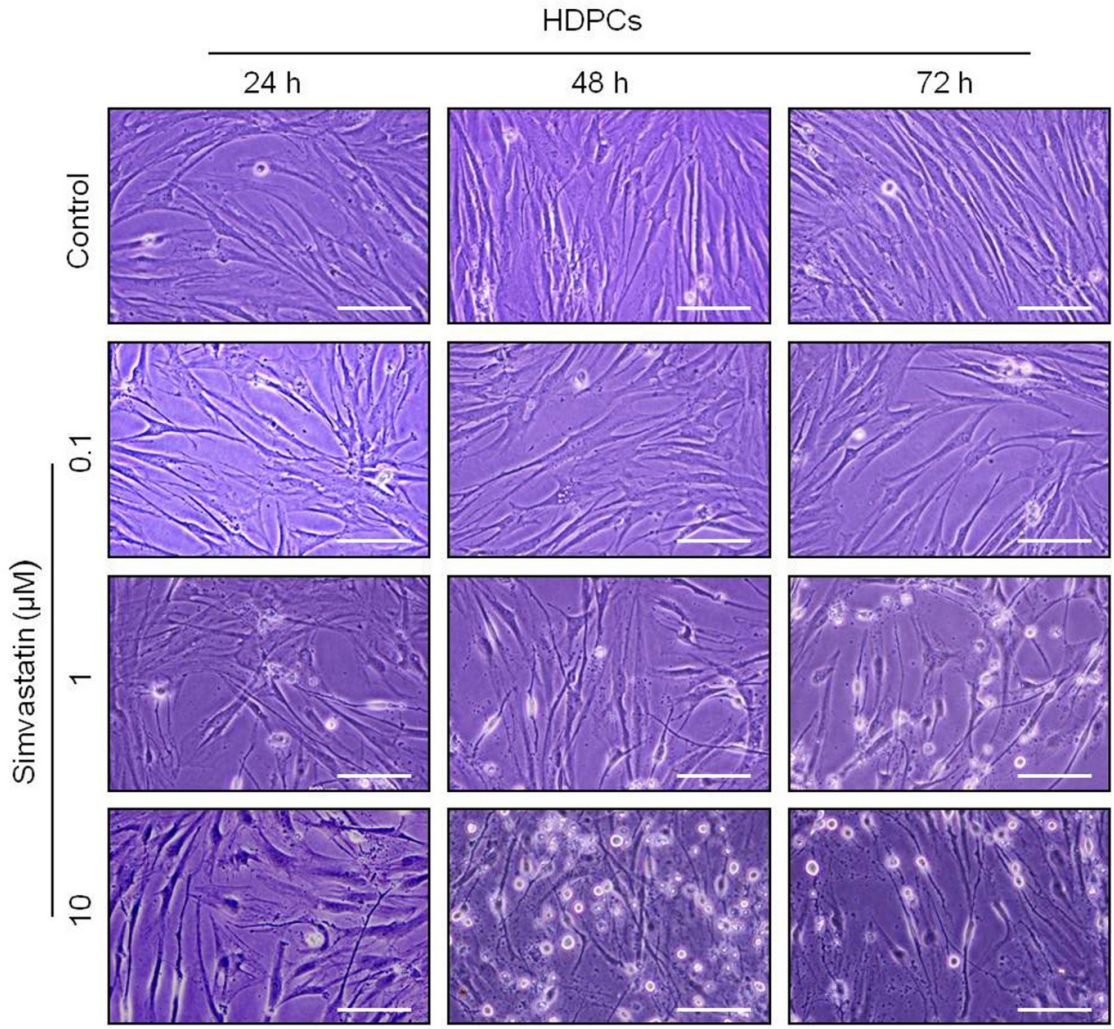

Fig. 14: Morphologic appearance of human dental pulp cells (HDPCs). Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cell morphology was examined under the inverted microscope (magnification 20X). Decreased both cell size and number of cells in simvastatin-treated cultures were observed in a concentration- and time-dependent manner. Representative images were obtained from one of four subjects. Scale bars represent 200 μm.

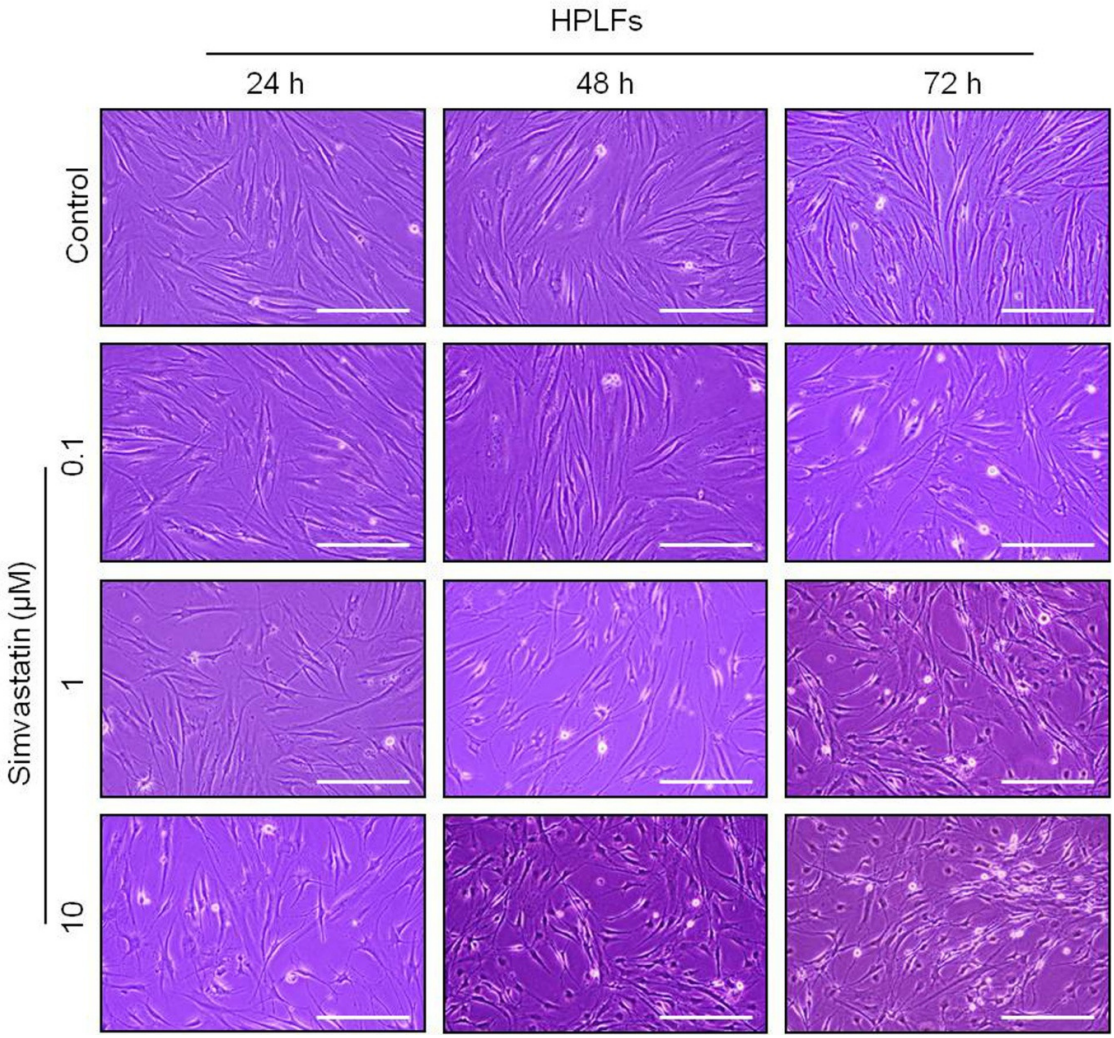

Fig. 15: Morphologic appearance of human periodontal ligament fibroblasts (HPLFs). Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cell morphology was examined under the inverted microscope (magnification 10X). Decreased both cell size and number of cells in simvastatin-treated cultures were observed in a concentration- and time-dependent manner. Representative images were obtained from one of four subjects. Scale bars represent 500 μm.

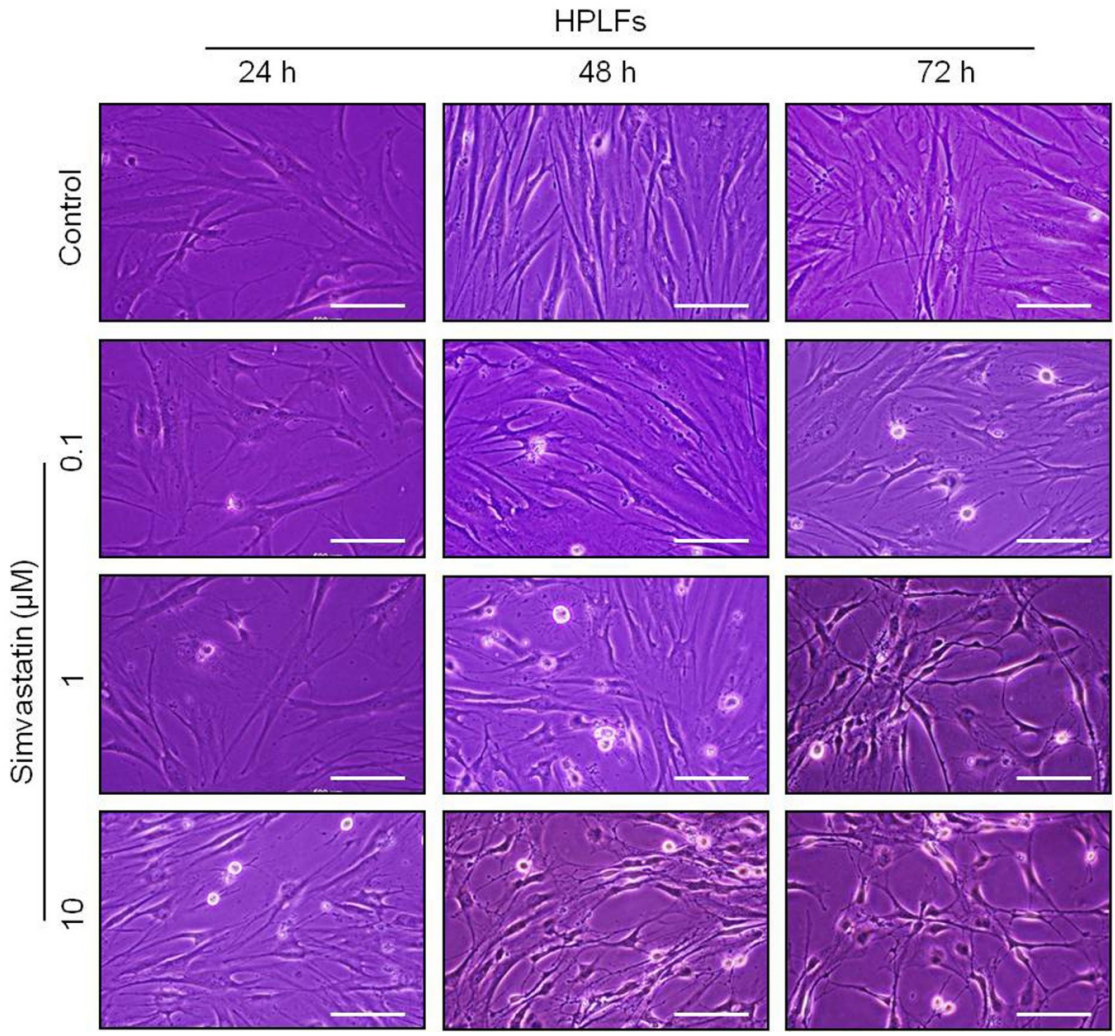

Fig. 16: Morphologic appearance of human periodontal ligament fibroblasts (HPLFs). Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cell morphology was examined under the inverted microscope (magnification 20X). Decreased both cell size and number of cells in simvastatin-treated cultures were observed in a concentration- and time-dependent manner. Representative images were obtained from one of four subjects. Scale bars represent 200 μm.

3.3 APOPTOSIS

3.3.1 FACS SCATTER PLOT OF FLOW CYTOMETRY

To examine the mechanism of cell death in simvastatin-treated cells. We used the apoptosis and necrosis-specific markers annexin V-FITC and propiodium iodide (PI) in Fluorescence-activated cell sorting (FACS) analyses. Representative FACS scatter plots showed the percentage of differently staining cells. The representative dual-parameter fluorescence density dot plot were derived from untreated cells or cells culture exposed to simvastatin (0.1, 1, and 10 μM) for 24, 48, and 72 h. Representative FACS scatter plots showed the viable cells (Annexin V -ve; PI -ve) in lower left hand quadrant, the early apoptotic cells (Annexin V +ve; PI -ve) in lower right hand quadrant, and the late apoptotic cells (Annexin V +ve; PI +ve) in upper right hand quadrant. Based on the pilot study, we chose 1 mM H_2O_2 as an apoptotic inducer for the study of apoptotic potency in HDPCs and HPLFs.

As shown in **Fig. 17 and 18**, a number of annexin V-FITC positive cells were observed after exposure to simvastatin in HDPCs and HPLFs, respectively. In accordance with the apoptotic cell data, 0.1 μM simvastatin treatment produced the lowest percentages of apoptosis both in HDPCs and HPLFs. In contrast, 1 and 10 μM simvastatin treatment induced significantly more apoptosis than control group.

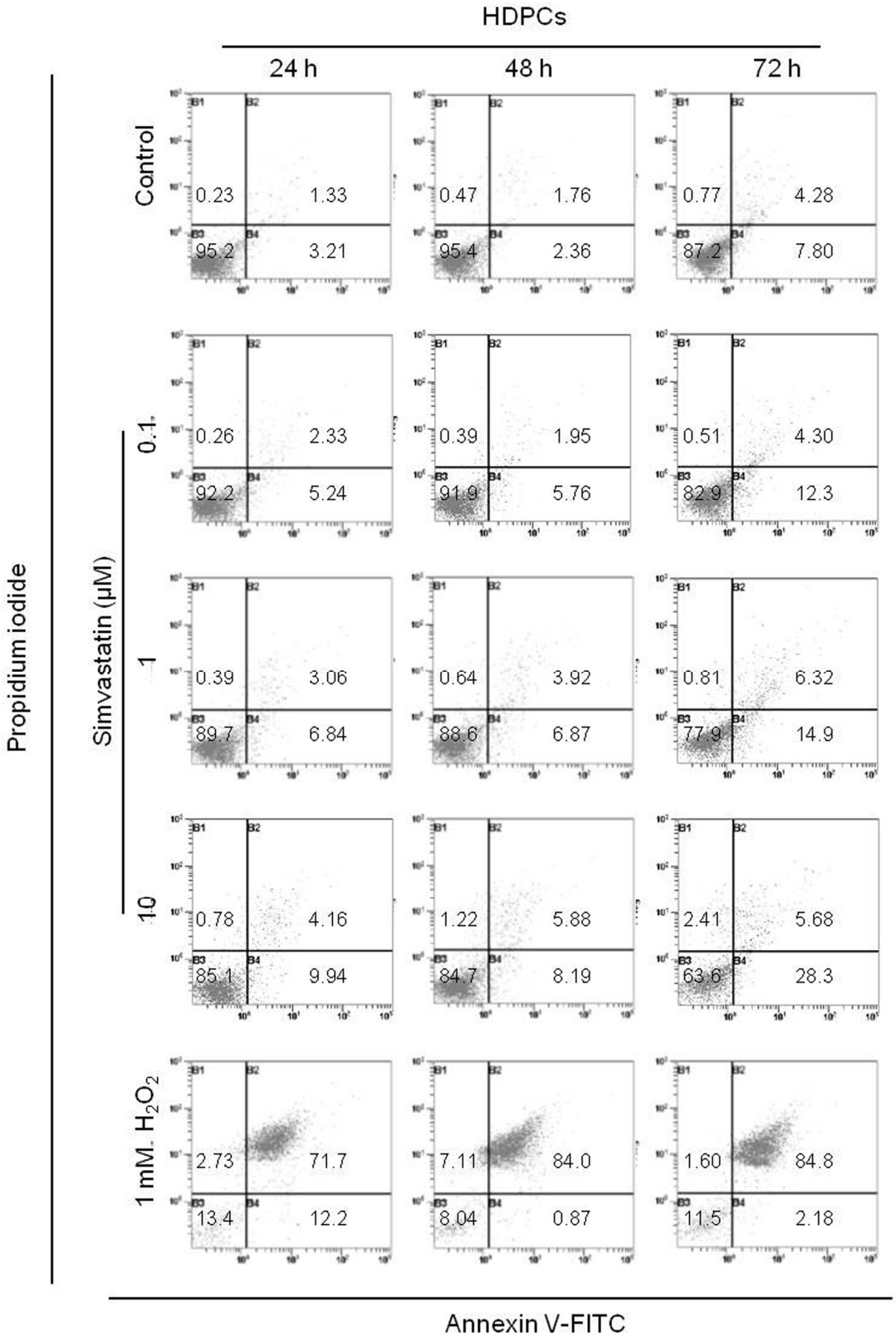
HDPCs
24 h
48 h
72 h
Control
0.1
1
10
1 mM H_2O_2
Simvastatin (μM)
Propidium iodide
Annexin V-FITC
0.23
1.33
95.2
3.21
0.47
1.76
95.4
2.36
0.77
4.28
87.2
7.80
0.26
2.33
92.2
5.24
0.39
1.95
91.9
5.76
0.51
4.30
82.9
12.3
0.39
3.06
89.7
6.84
0.64
3.92
88.6
6.87
0.81
6.32
77.9
14.9
0.78
4.16
85.1
9.94
1.22
5.88
84.7
8.19
2.41
5.68
63.6
28.3
2.73
71.7
13.4
12.2
7.11
84.0
8.04
0.87
1.60
84.8
11.5
2.18

Fig. 17: Flow cytometric analyses of simvastatin-induced cell death of human dental pulp cells (HDPCs). HDPCs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cells were stained with Annexin V-FITC and PI and subjected to fluorescence-activated cell sorting (FACS) analysis to determine the percentage of apoptotic and/or necrotic cells. Representative FACS scatter plots of HDPCs obtained from one of four subjects showed the viable cells (Annexin V -ve; PI -ve) in lower left hand quadrant, the early apoptotic cells (Annexin V +ve; PI -ve) in lower right hand quadrant, and the late apoptotic cells (Annexin V +ve; PI +ve) in upper right hand quadrant.

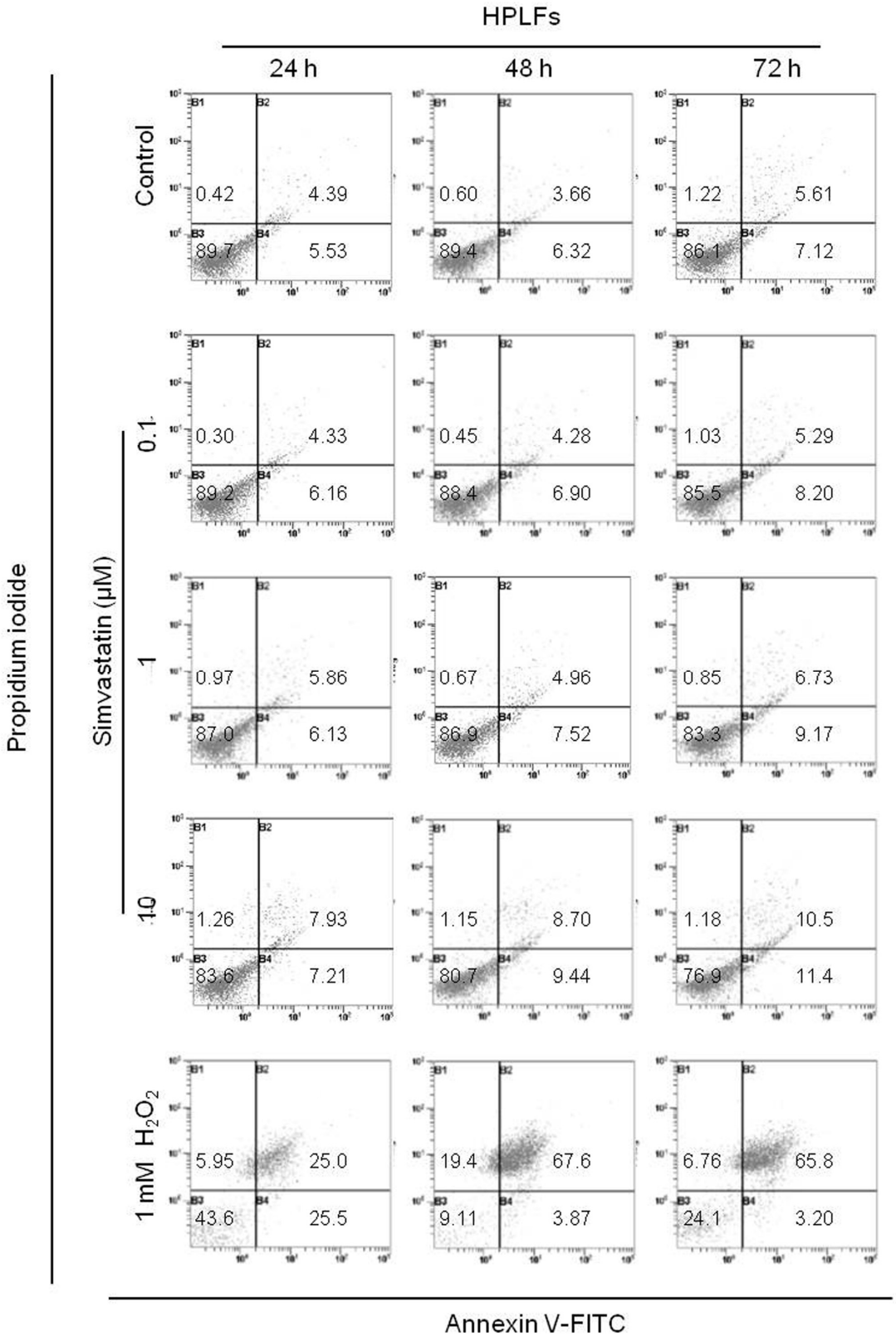
HPLFs
24 h
48 h
72 h
Control
0.1
1
10
1 mM H_2O_2
Simvastatin (μM)
Propidium iodide
Annexin V-FITC
0.42
4.39
89.7
5.53
0.60
3.66
89.4
6.32
1.22
5.61
86.1
7.12
0.30
4.33
89.2
6.16
0.45
4.28
88.4
6.90
1.03
5.29
85.5
8.20
0.97
5.86
87.0
6.13
0.67
4.96
86.9
7.52
0.85
6.73
83.3
9.17
1.26
7.93
83.6
7.21
1.15
8.70
80.7
9.44
1.18
10.5
76.9
11.4
5.95
25.0
43.6
25.5
19.4
67.6
9.11
3.87
6.76
65.8
24.1
3.20

Fig. 18: Flow cytometric analyses of simvastatin-induced cell death of human periodontal ligament fibroblasts (HPLFs). HPLFs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cells were stained with Annexin V-FITC and PI and subjected to fluorescence-activated cell sorting (FACS) analysis to determine the percentage of apoptotic and/or necrotic cells. Representative FACS scatter plots of HPLFs obtained from one of four subjects showed the viable cells (Annexin V -ve; PI -ve) in lower left hand quadrant, the early apoptotic cells (Annexin V +ve; PI -ve) in lower right hand quadrant, and the late apoptotic cells (Annexin V +ve; PI +ve) in upper right hand quadrant.

3.3.2 LEVELS OF APOPTOSIS

In this study, annexin V-FITC and PI double staining was measured by flow cytometry as a marker for apoptosis detection of HDPCs and HPLFs treated with various concentration of simvastatin (control, 0.1, 1, 10 μM) for 24, 48, and 72 h **(Fig. 19, 20)**. The number of cells that underwent apoptosis and/or necrosis was measured by annexin V-FTIC binding to phosphatidylserine expressed at the membrane (early apoptosis) and by staining the nuclei with impermeable fluorescence propidium iodide indicating lost plasma membrane integrity (late apoptotic and/or necrotic cells). The total apoptosis were determined from annexin V-FITC positive (Q4) plus annexin V- FITC/PI double positive cells (Q2).

HDPCs: All concentrations of simvastatin significantly increased apoptotic of HDPCs at 72 h ($p < 0.05$) **(Fig. 19A)**. There was significant difference the percentage of apoptotic cells between low (0.1 μM) and high (10 μM) concentration of simvastatin since 24 h incubated period **(Fig. 19B)**. When HDPCs were treated with 10 μM simvastatin for 24, 48, and 72 h, the level of apoptosis was measured to be 12.66, 15.86, and 29.73% respectively.

HPLFs: In this experiment, we observed that 10 μM simvastatin significantly increased the percentage of apoptotic cells in HPLFs, and this effect was presented in a time-dependent manner ($p < 0.05$) **(Fig. 20A)**. No effect of simvastatin at any concentration was observed at 24 h, but a dose-dependent stimulation of apoptosis was found at 48 and 72 h, compared with control group ($p < 0.05$) **(Fig. 20B)**. When HPLFs were treated with 10 μM simvastatin for 24, 48, and 72 h, the level of apoptosis was measured to be 15.27, 19.66, and 28.31% respectively.

HDPCs and HPLFs: A significant increased of apoptosis in HDPCs and HPLFs was found in the simvastatin-treated cultures at a concentration of 10 μM in a time-dependent manner ($p < 0.05$) **(Fig. 19A, 20A)**. The effect was similar for both cell populations.

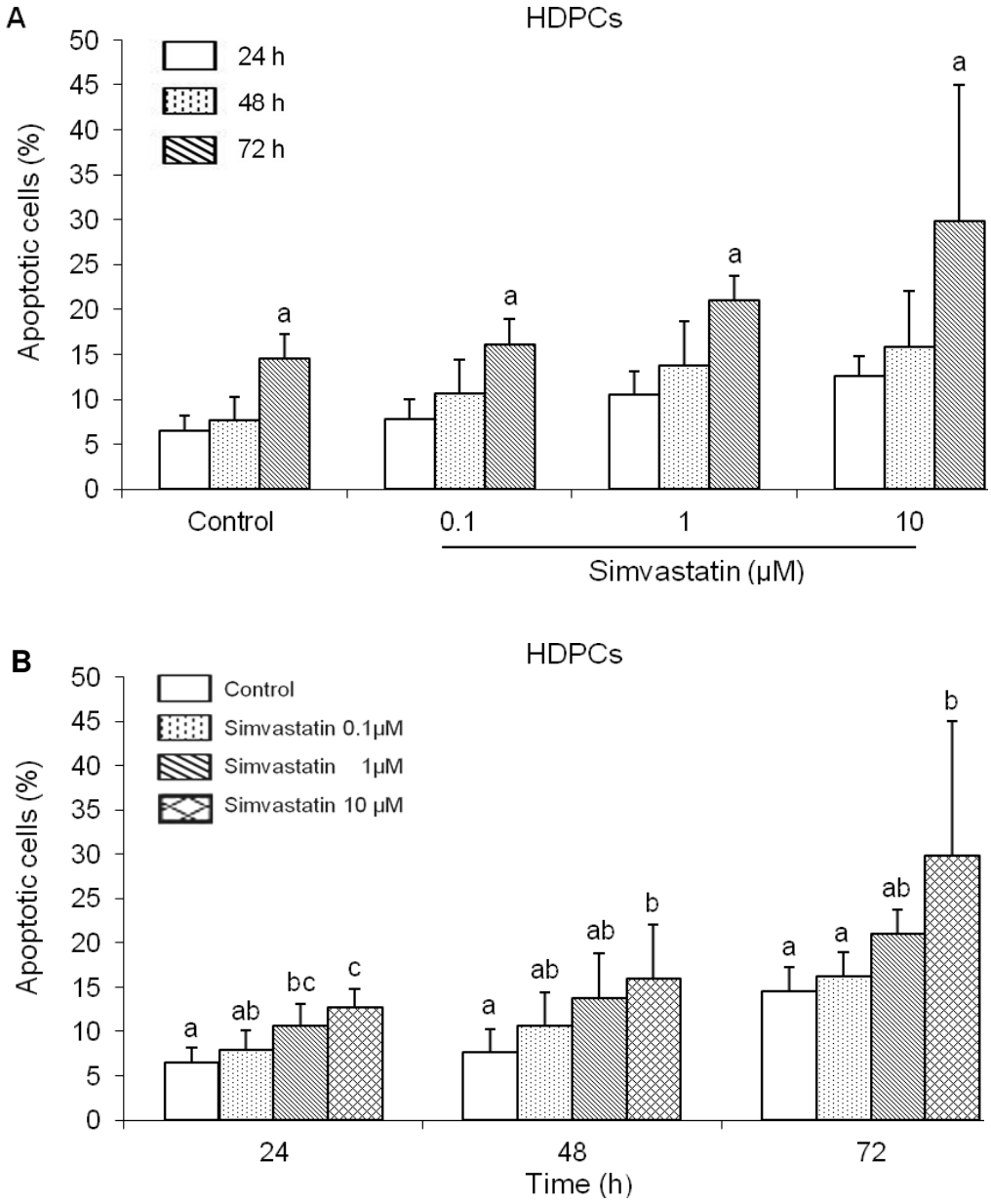

Fig. 19: The percentage of Annexin V positive cells plus Annexin V and PI double positive cells were summed for total apoptosis. A dose- **(Fig. 19A)** and time-course analysis **(Fig. 19B)** of the percentage of apoptosis of HDPCs. HDPCs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. HDPCs were stained with Annexin V-FITC and PI double staining, then analyzed by flow cytometry. Data were represented as means ± standard deviation (SD) of four cell populations and each analyzed in duplicate. Value with different alphabet denotes significant difference ($p < 0.05$).

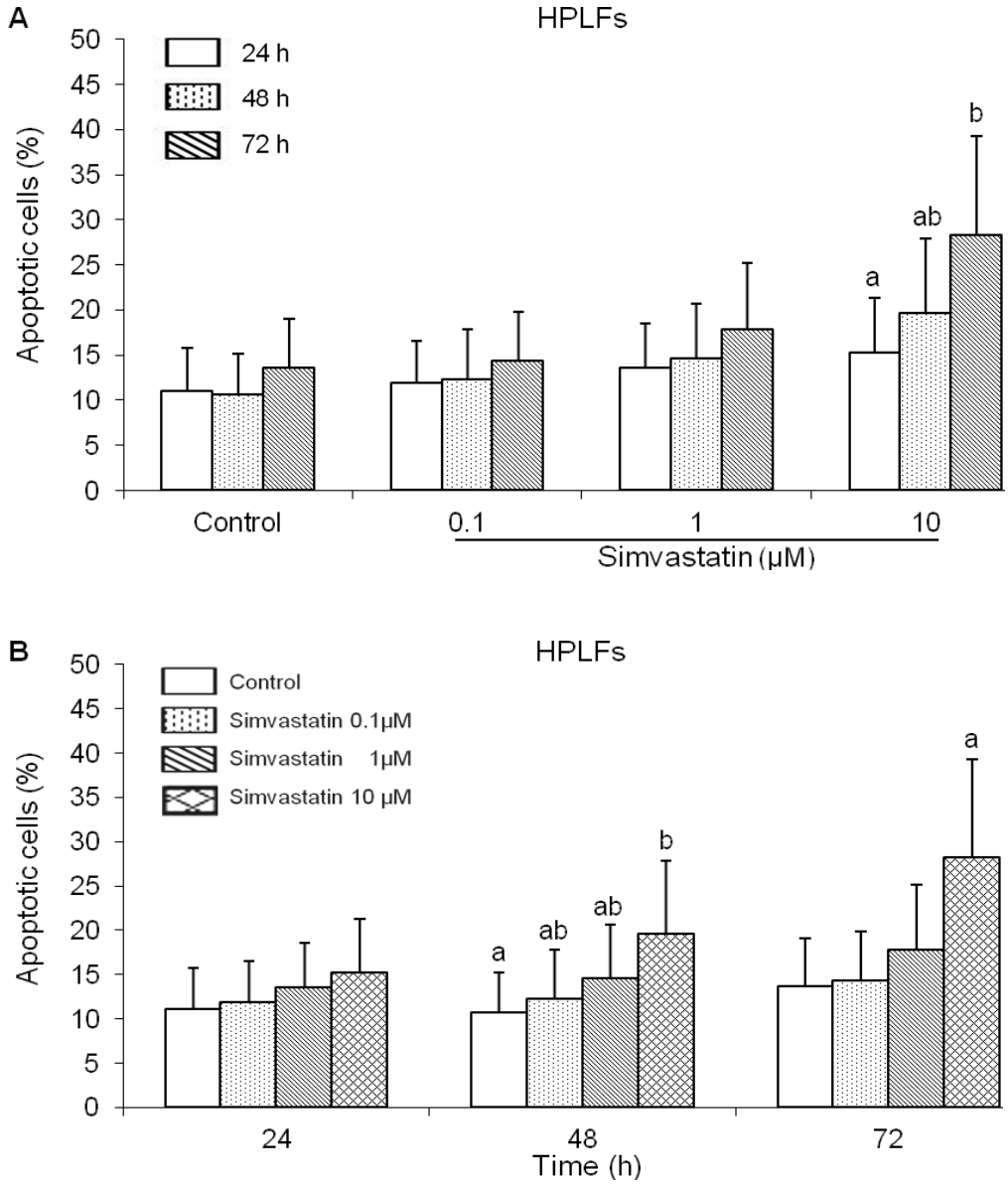

Fig. 20: The percentage of Annexin V positive cells plus Annexin V and PI double positive cells were summed for total apoptosis. A dose- **(Fig. 20A)** and time-course analysis **(Fig. 20B)** of the percentage of apoptosis of HPLFs. HPLFs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. HPLFs were stained with Annexin V-FITC and PI double staining, then analyzed by flow cytometry. Data were represented as means ± standard deviation (SD) of four cell populations and each analyzed in duplicate. Value with different alphabet denotes significant difference ($p < 0.05$).

3.3.3 STAGES OF APOPTOSIS AND NECROSIS

The percentage of early stage apoptosis, late stage apoptosis and necrotic cells of various concentrations of simvastatin treated on human dental pulp cells (HDPCs) and human periodontal ligament fibroblasts (HPLFs) for 24, 48, and 72 h after which they were stained with annexin V-FITC and PI double staining, then analyzed by flow cytometry **(Fig. 21-23)**.

The frequency of early and late stage apoptosis were increased in simvastatin treated group compared with control group. There was dose and time dependent manner in both cell types. Significant early and late stage apoptosis were observed when HDPCs were treated with 10 μM simvastatin ($p < 0.05$). The average percentage of necrosis of HDPCs and HPLFs cultured with simvastatin at the concentration of 0.1-1 μM during 24 -72 h was found to be less than 1%. At a higher dose of 10 μM simvastatin, about 1-3 % cells were identified as necrotic cells. Interestingly, we did not observed noticeable differences in the early, late stage apoptosis and necrosis response to simvastatin between HDPCs and HPLFs.

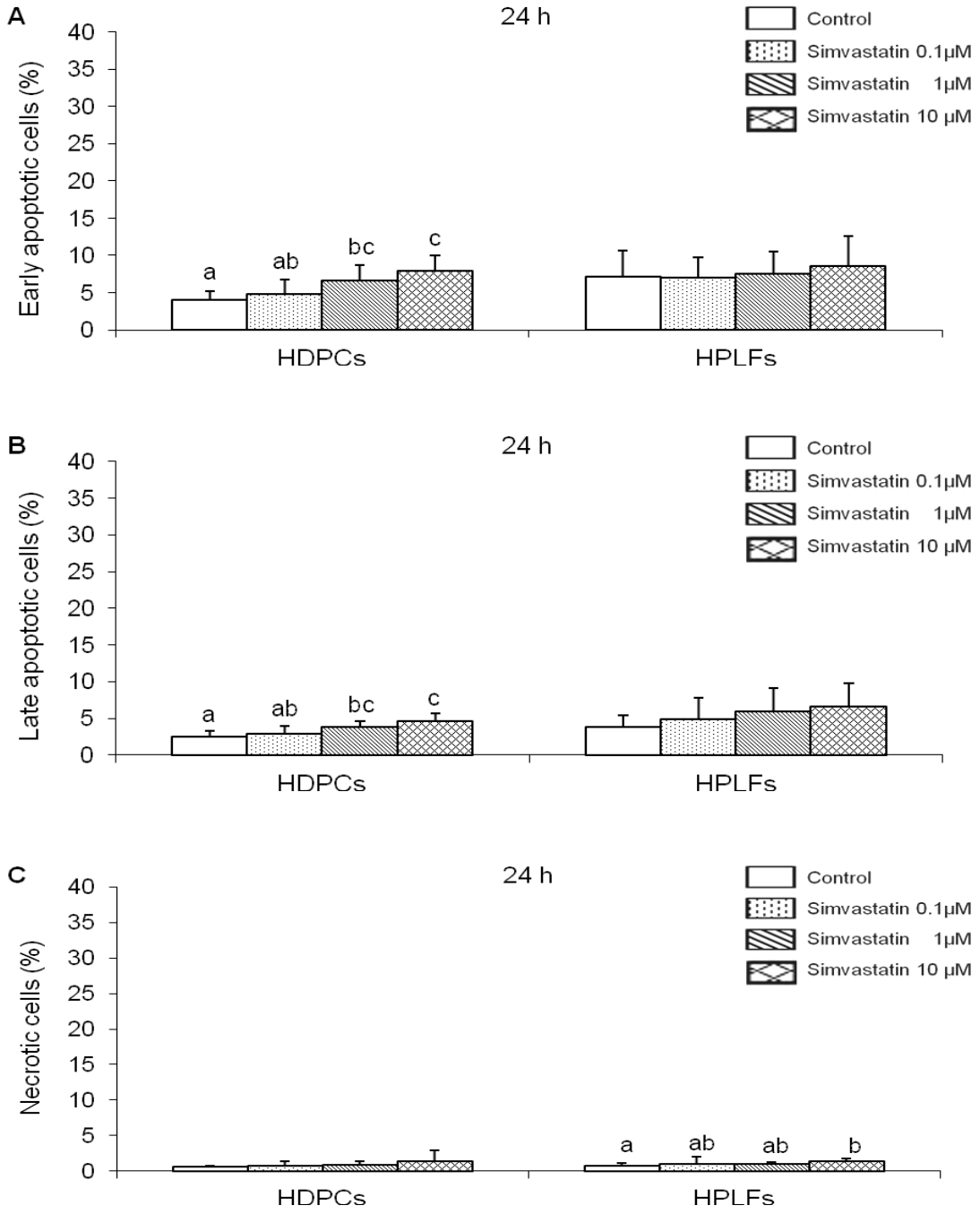

Fig. 21: The percentage of apoptotic and necrotic cells of simvastatin treated on human dental pulp cells (HDPCs) and human periodontal ligament fibroblasts (HPLFs) for 24 h after which they were stained with annexin V-FITC and PI double staining, then analyzed by flow cytometry. Early stage apoptosis **(Fig. 21A)** Late stage apoptosis **(Fig. 21B)** Necrotic cells of simvastatin treated-HDPCs and HPLFs **(Fig. 21C)**. Value with different alphabet denotes significant difference ($p < 0.05$).

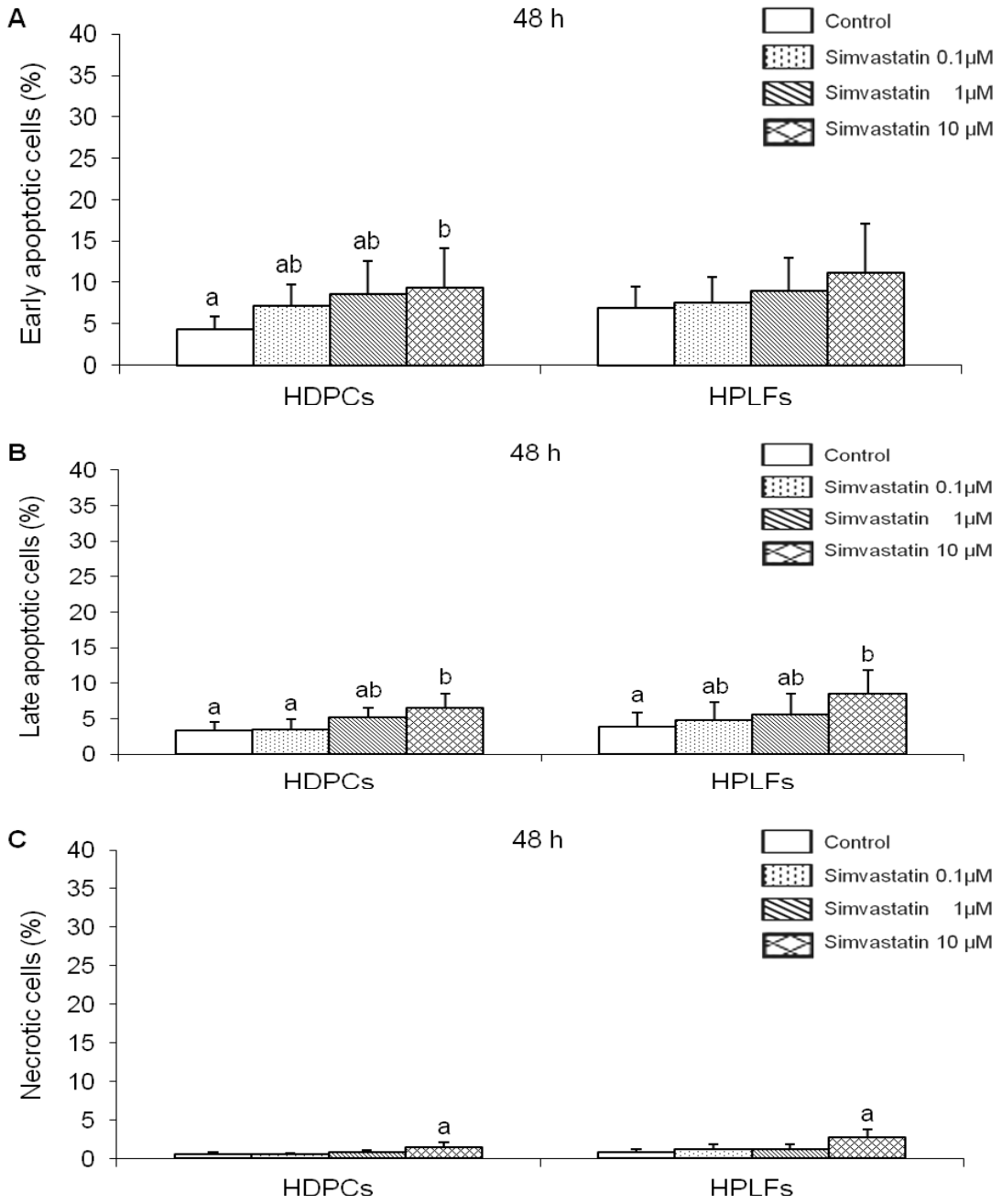

Fig. 22: The percentage of apoptotic and necrotic cells of simvastatin treated on human dental pulp cells (HDPCs) and human periodontal ligament fibroblasts (HPLFs) for 48 h after which they were stained with annexin V-FITC and PI double staining, then analyzed by flow cytometry. Early stage apoptosis **(Fig. 22A)** Late stage apoptosis **(Fig. 22B)** Necrotic cells of simvastatin treated-HDPCs and HPLFs **(Fig. 22C)**. Value with different alphabet denotes significant difference ($p < 0.05$).

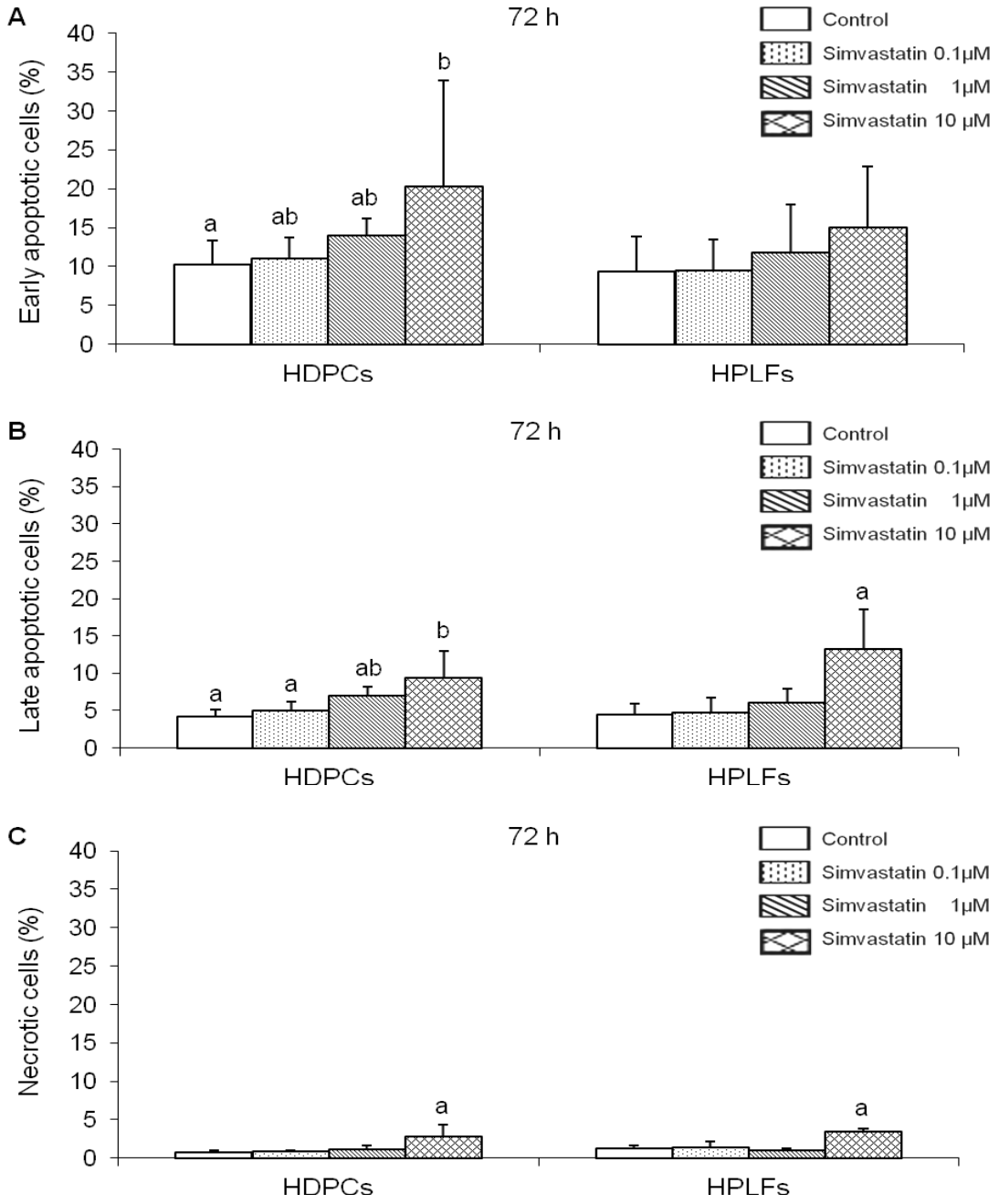

Fig. 23: The percentage of apoptotic and necrotic cells of simvastatin treated on human dental pulp cells (HDPCs) and human periodontal ligament fibroblasts (HPLFs) for 72 h after which they were stained with annexin V-FITC and PI double staining, then analyzed by flow cytometry. Early stage apoptosis **(Fig. 23A)** Late stage apoptosis **(Fig. 23B)** Necrotic cells of simvastatin treated-HDPCs and HPLFs **(Fig. 23C)**. Value with different alphabet denotes significant difference ($p < 0.05$).

3.4 NUCLEAR STAINING

Simvastatin induced-HDPCs and HPLFs resulted increase apoptosis compared with control cells. The effects of exposure to 0.1, 1, 10 μM simvastatin on altered nuclei of HDPCs and HPLFs were shown **(Fig. 24, 25)**.

HDPCs and HPLFs: Fluorescent microscopic analysis revealed that the nuclei of control group had normal structures that exhibited rounded, sharply defined nuclei and non-condensed chromatin. Under the influence of simvastatin, similar nuclear changes were noted in both cell types, being the occurrence of lobulated, condensed chromatin localized at the nuclear membrane (crescent), and the fragmentation of the nuclei accompanied by a reduction in nuclear size. These nuclear changes were found with a dose of 1 μM simvastatin or higher. However, between the two cell types a time-dependent difference was noted. In time the nuclei changed after co-incubated with 10 μM simvastatin as follows: In the 24 h-cultures, cell nuclei appeared mostly lobulated-shaped while at later periods of 48 and 72 h-cultures crescent and fragmented shapes were frequently seen. Changes in HDPCs were already noted after 24 h, whereas in HPLFs it took 48 h for the changes to occur. The nuclear morphological changes of HDPCs and HPLFs cultured with 10 μM simvastatin for 48 h were the occurrence of lobulated, chromatin condensing at the nuclear edge (crescent) and fragmented nuclei. The two different tooth-associated cell types showed similar nuclear changes **(Fig. 24, 25)**. In the 24 h-cultures, cell nuclei appeared mostly lobulated-shaped while at later periods of 48 and 72 h-cultures crescent and fragment shapes were frequently seen.

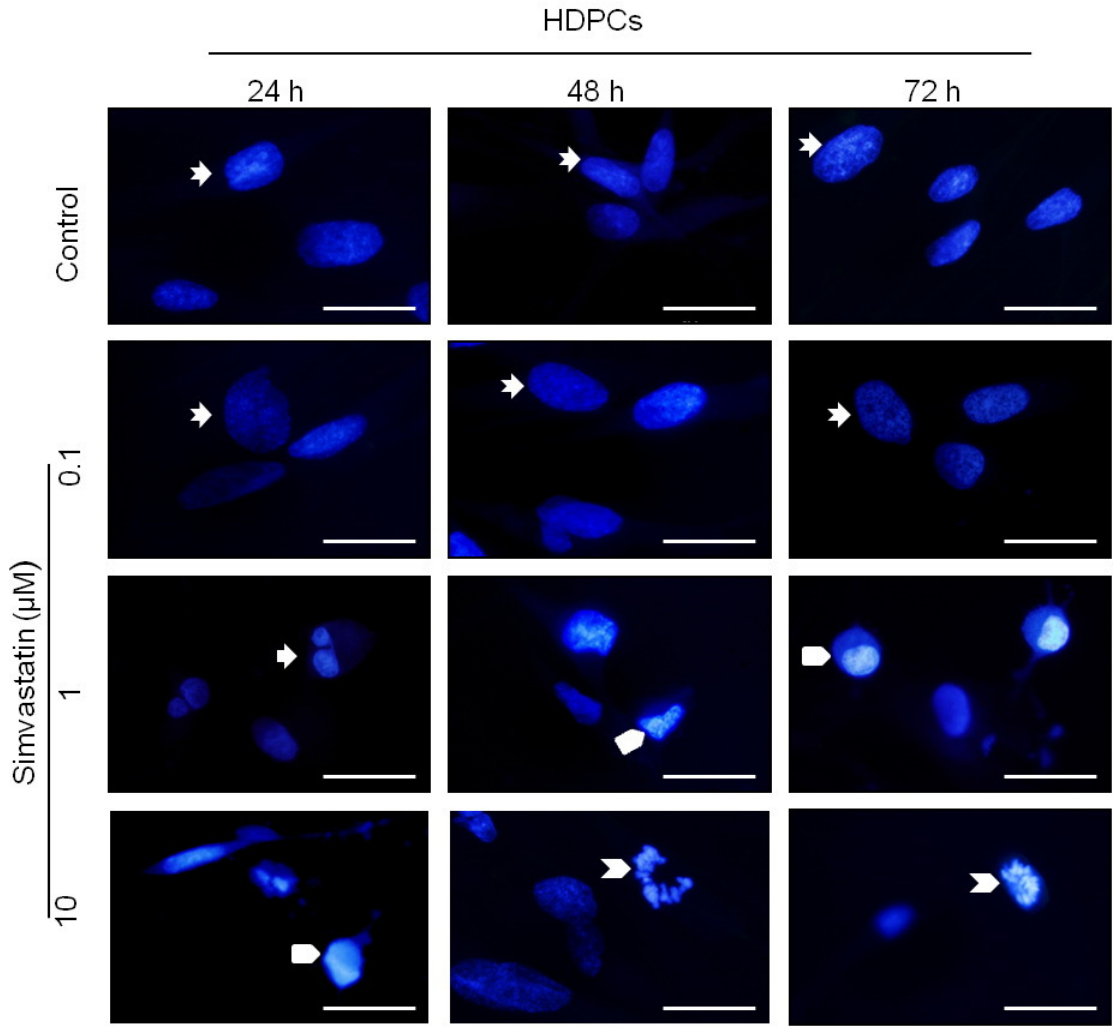

Fig. 24: Nuclear images of human dental pulp cells (HDPCs). HDPCs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h., then fixed, permeabilized, and nuclei were stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). Control nuclei showed rounded and non-condensed chromatin (notch arrow). In simvastatin-treated cultures, nuclei exhibited defined (notch arrow), lobulated (arrow), crescent (pentagon arrow), and fragmented DAPI-stained chromatin (chevron arrow). Representative fluorescence microscopy images were obtained from one of four subjects. Scale bars represent 50 μm.

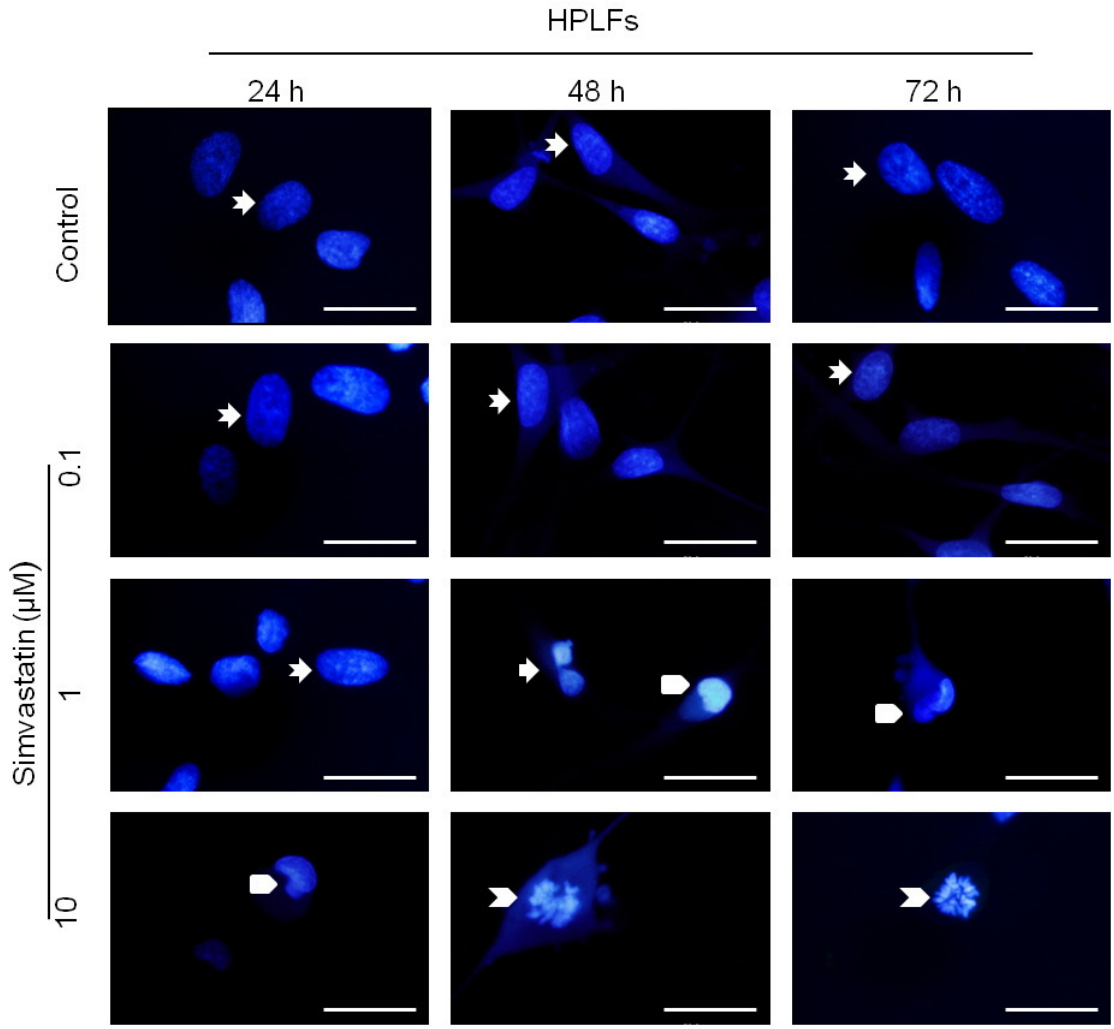

Fig. 25: Nuclear images of human periodontal ligament fibroblasts (HPLFs). HPLFs were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. then fixed, permeabilized, and nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Control nuclei showed rounded and non-condensed chromatin (notch arrow). In simvastatin-treated cultures, nuclei exhibited defined (notch arrow), lobulated (arrow), crescent (pentagon arrow), and fragmented DAPI-stained chromatin (chevron arrow). Representative fluorescence microscopy images were obtained from one of four subjects. Scale bars represent 50 μm.

3.5 CYTOSKELETAL MORPHOLOGY

We attempted to investigate cytoskeletal and nucleus morphology by Phalloidin-FITC staining. Actin filaments were visualized by staining with Phalloidin-FTIC in cultures of HDPCs and HPLFs treated with or without 0.1, 1, 10 μM simvastatin for 24, 48, and 72 h **(Fig. 26 - 29)**.

HDPCs: In the absence of simvastatin, actin fibers are organized in parallel bundles. After a 24-h exposure to 10 μM simvastatin and a 48-h exposure to 1 μM simvastatin, the actin fibers are disorganized, and their network loosened and disassembled. The shape of the cells appears indistinct. After a 72-h exposure to 1 μM simvastatin and a 48-h and 72-h exposure to 10 μM simvastatin, the cells are shrunken, with irregular outlines and the absence of identifiable fiber. Furthermore, simvastatin treated HDPCs were mostly rounded up and displayed a spindle-like appearance with actin condensed around nucleus. At three time-points, simvastatin markedly revealed apoptosis changes in HDPCs **(Fig. 26, 27)**.

HPLFs: In control group, the highly organized dense meshwork of actin stress filaments of HPLFs stained very intensely and appeared to be organized in a transverse plane throughout the cell body. Upon treatment with simvastatin, the dense net of fibers loosened and gradually disappeared. At 24 h of 10 μM simvastatin and a 72-h exposure to 1 μM simvastatin treatment, the cell spanning stress fibers began to disassemble. At 48 and 72 h of 10 μM simvastatin treatment led to a complete disintegration of the cytosolic fibers, with the cortical actin fibers still intacted **(Fig. 28, 29)**.

HDPCs and HPLFs: Disruption and loss of filamentous actin thus poor organized and less dense filaments were observed after simvastatin incubation both in HDPCs and HPLFs. This morphological alteration was detrimentally progressive in time-dependent manner. Therefore, at a final end of 72 h-cultures, a complete disintegration of filamentous actin was seen in simvastatin-treated cells.

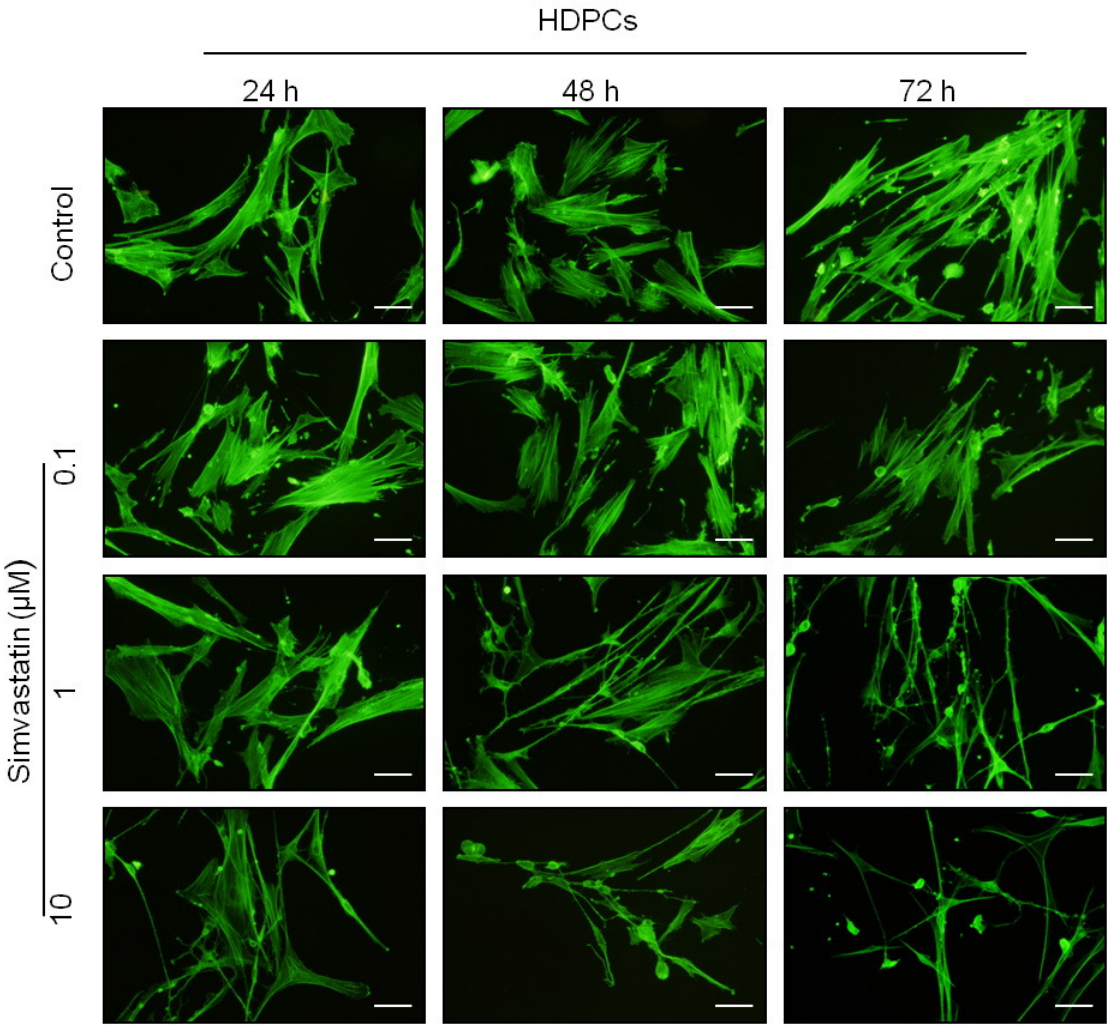

Fig. 26: Morphological appearance of human dental pulp cells (HDPCs) following staining of actin filaments. Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cells were fixed, permeabilized, and incubated with Phalloidin-FITC, then investigated by the fluorescence microscopy (magnification 20X). Representative images were obtained from one of four subjects. Scale bar represents 100 μm.

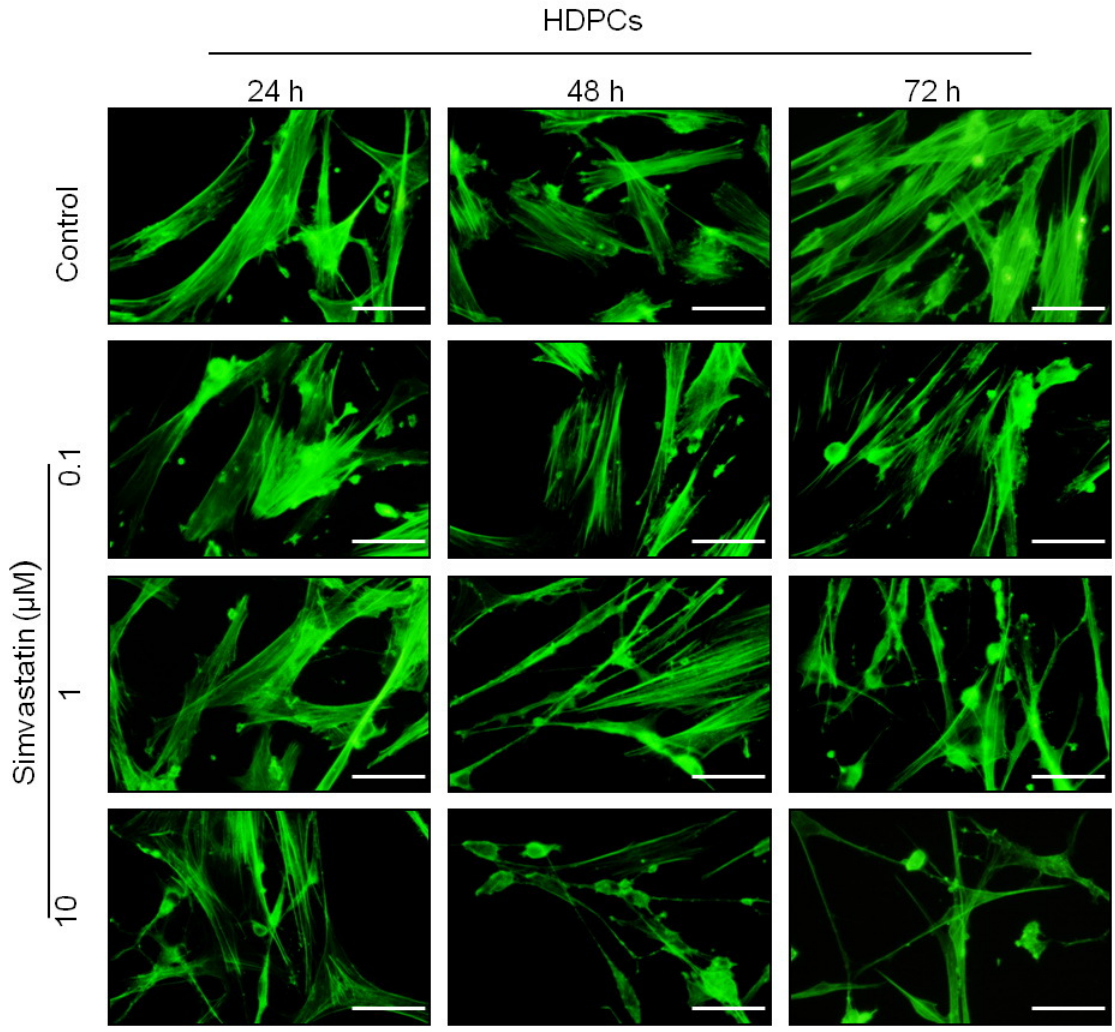

Fig. 27: Morphological appearance of human dental pulp cells (HDPCs) following staining of actin filaments. Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cells were fixed, permeabilized, and incubated with Phalloidin-FITC, then investigated by the fluorescence microscopy (magnification 40X). Representative images were obtained from one of four subjects. Scale bar represents 100 μm.

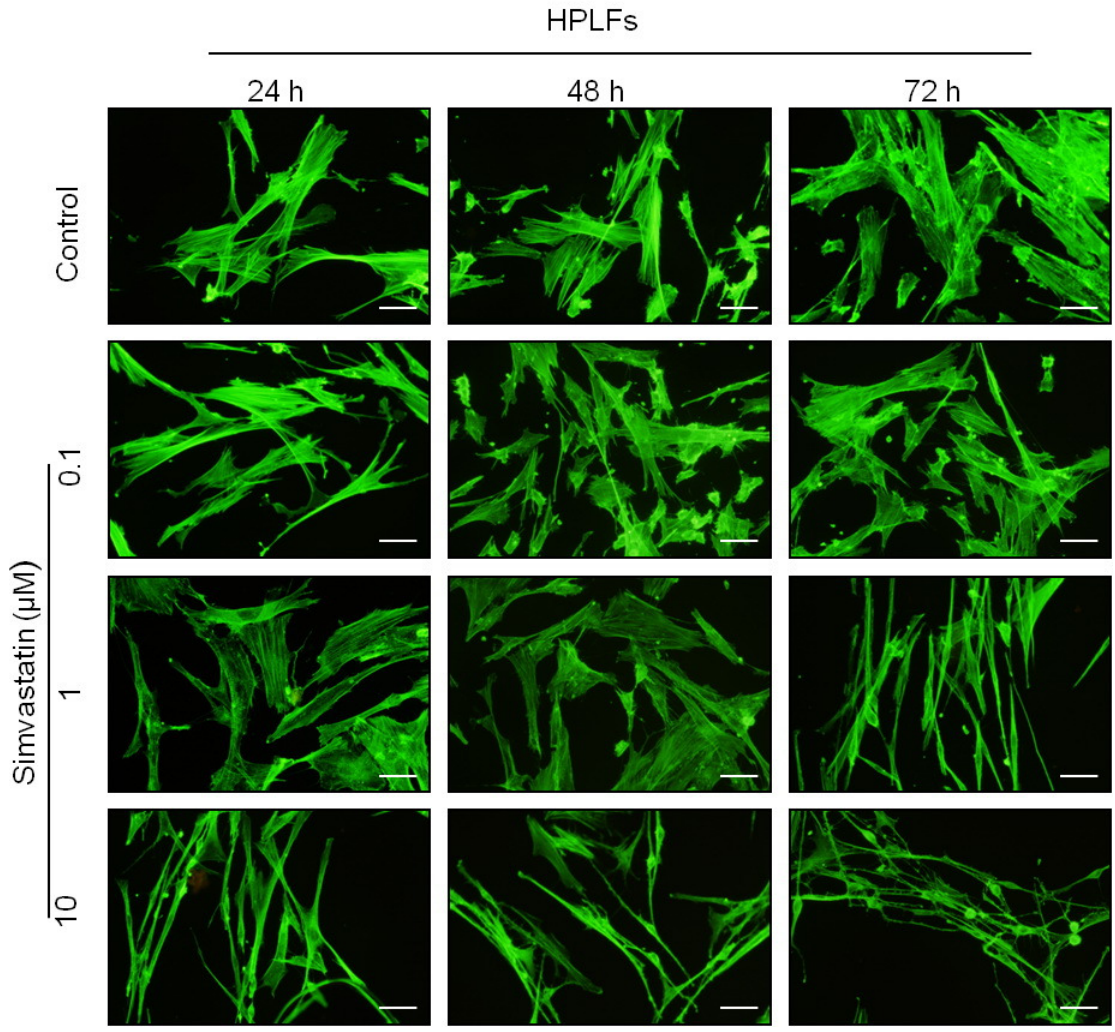

Fig. 28: Morphological appearance of human periodontal ligament fibroblasts (HPLFs) following staining of actin filaments. Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cells were fixed, permeabilized, and incubated with Phalloidin-FITC, then investigated by the fluorescence microscopy (magnification 20X). Representative images were obtained from one of four subjects. Scale bar represents 100 μm.

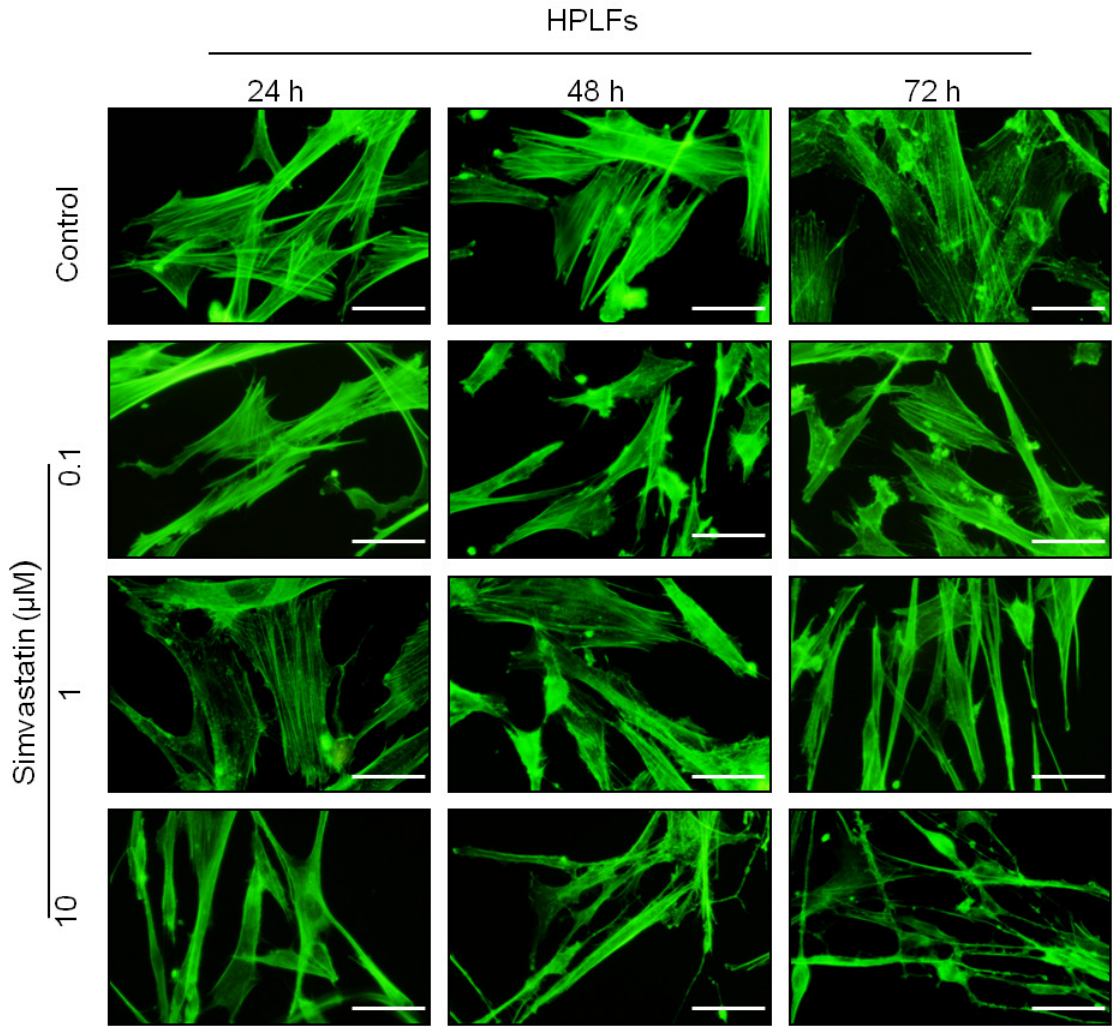

Fig. 29: Morphological appearance of human periodontal ligament fibroblasts (HPLFs) following staining of actin filaments. Cells were cultured with or without simvastatin in the concentrations of 0.1, 1 and 10 μM for 24, 48, and 72 h. Cells were fixed, permeabilized, and incubated with Phalloidin-FITC, then investigated by the fluorescence microscopy (magnification 40X). Representative images were obtained from one of four subjects. Scale bar represents 100 μm.

CHAPTER 4

DISCUSSION

4.1 CELL VIABILITY

This study was designed to evaluate the effects of simvastatin on tooth-derived cell viability and apoptosis. In the present study, we demonstrated that simvastatin significantly decreased cell viability, as measured by the activity of mitochondrial dehydrogenase, of the tooth-associated cell types HDPCs and HPLFs in a dose- and time-dependent manner. A range of statins concentrations was used similarly to that used by others.[7,11,29] In this experiment, we observed that high concentration of simvastatin seems to cause an increase in cell detachment and rounding of both cell types from the culture surface when compared to the untreated control. We found that a low concentration of 0.1 μM simvastatin had no significant effect on cell viability of HDPCs and HPLFs when compared with the control group. This is in agreement with recent studies showing no effect of simvastatin at the concentrations of 0.001, 0.01, and 0.1 μM on cell viability of alveolar osteoblasts and periodontal ligament cells after a culture period of 72 h.[12] While in cultures with higher concentrations (1-10 μM simvastatin) a significant inhibitory effect on the viability of the cells was apparent. Previous studies on rat myofibroblasts,[47] B-cells,[55] and cancer cell lines[15,16] showed similar effects. In contrast, it has been shown that no inhibitory effect of simvastatin on the viability of cells was found until high doses of simvastatin were used. This was found with gingival fibroblasts (50 μM)[56] and with osteoblastic cells (100-1000 μM).[17] Taken together, the data appear to indicate that, an effect of simvastatin on cell viability has been shown to vary according to the cell type, type of statins, and their concentration.

By comparing HDPCs and HPLFs our data showed that the effect of simvastatin on cell viability was more potent in HDPCs in terms of its minimum concentration resulting in 50% cell death. It is of note that the effect of simvastatin on cell viability is controversial and this is probably mainly due to the difference in cell type, the state of differentiation, the origin of the cells under study. Recent work showed that the high sensitivity of J774A.1 macrophages to statin-induced cell death is associated with the strong degree of HMG-CoA reductase activity in these cells.[46] Thus, the differences in sensitivity to statin-induced cell death are related to the level of HMG-CoA reductase activity in different cell types. Such a cell type related level of enzyme activity may explain the

differences between different cell types in sensitivity to simvastatin. The mechanism that may relate to simvastatin reduced-cell viability has been proposed. HMG-CoA reductase, which converts HMG-CoA to mevalonate, is a rate-limiting enzyme of the mevalonate cascade. Mevalonic acid is a precursor not only in cholesterol synthesis but also in the synthesis of isoprenoid intermediates such as FPP and GGPP. Geranylgeranylation and farnesylation are required by small G proteins such as Ras and Rho, which play crucial roles in cell growth and differentiation.[32] Statins-reduced cell viability may occur from the depletion of isoprenoid intermediates especially in GGPP. In particular, the dysfunction of RhoA is associated with statins-reduced cell viability.[47] Rho/Rho kinase signaling pathway that is target of RhoA is involved in cell survival.[30] Consequently, simvastatin may decrease cell viability, independently of cholesterol synthesis via a mechanism involving inhibition of RhoA geranylgeranylation.

4.2 APOPTOSIS

The cell viability and morphology experiments led us to hypothesize that simvastatin may induce apoptosis. We further investigated the mechanism of cell death induced by simvastatin. The present findings showed that HDPCs as well as HPLFs cultured with simvastatin underwent apoptosis. This is in agreement with several other findings using B-cell chronic lymphocytic leukemia (B-CLL),[55] skeletal muscle cells (L6 fibroblasts),[47] and endometrial stromal cells.[33] In simvastatin-treated cultures of HDPCs and HPLFs, there were insignificant numbers of necrotic cells as measured by PI staining alone. The data suggest that a primary cause of cell death of HDPCs and HPLFs treated with simvastatin is probably not due to necrosis. Consequently, this further supports that simvastatin induces apoptosis in HDPCs and HPLFs. To date several mechanisms of statin-mediated apoptosis have been proposed. The mevalonate-derived isoprenoid compounds, FPP and GGPP are transferred onto target proteins of the Ras superfamily of GTPases such as Ras and Rho by the specific enzymes farnesyl and geranylgeranyl transferases, respectively. These isoprenylations can be inhibited by statins, leading to loss of cell membrane anchorage and decreased activity of these small G proteins.[4] Simvastatin can induce apoptosis via caspase-dependent pathway. Caspases, a family of aspartate-specific cysteine proteases, play a crucial role in apoptosis.[13] Previous studies of B-cells showed that simvastatin induces apoptosis by initiation of mitochondrial caspase-9, which activates caspase-3 and 8 (executioner caspases).[55] Other studies showed that simvastatin-induced apoptosis is mediated through inactivation of RhoA[15,34] and in turn

to mitochondrial cytochrome c-induced caspase-9 which subsequently activates caspase-3.[50] Furthermore, Rho A inhibition, but not the inhibition of Cdc42 and Rac-1, induce apoptosis characterized caspase processing and a decrease of antiapoptotic proteins Bcl-2.[48] Taken together, Sacher et al. speculates that the alteration in isoprenoid synthesis with statin therapy is responsible for the tyrosine phosphorylation and stimulation of pathways causing a rise in cytosolic calcium. The elevated calcium levels in response to statins activate calpain, which in turn leads to apoptosis via the translocation of Bax to the mitochondria and activation of caspase-9 and caspase-3.[49] Not only caspase-dependent pathways, simvastatin can induce apoptosis by caspase-independent pathway such as endonuclease G, translocate to nucleus then induce tightly packed DNA and large-scale DNA fragmentation.[14] It is not known, however, which mechanism is involved in the tooth-related cell types used in the present study.

4.3 NUCLEAR STAINING

Simvastatin-triggered apoptotic cell death of HDPCs and HPLFs was confirmed by the occurrence of areas of brightly fluorescent, condensed chromatin and partial loss of the nuclear membrane. In cells at later stages of apoptosis, the nuclear membrane was lost and the chromatin dispersed into several small aggregates. DAPI is a blue nucleic acid fluorescence dyeing agent and attaches to the DNA strand spiral gutter. When a cell dies, chromosome agglutination and DNA fragmentation occur.[57] Nuclear morphology including lobulated, condensed, and pyknotic fragmented nuclei are characteristic for apoptotic nuclei.[58] In the present study this pattern of DAPI-chromatin staining was found in HDPCs as well as HPLFs after incubation with simvastatin. The data suggest that cell death caused by simvastatin is accompanied by the classical features of apoptotic nucleus. Our data of apoptotic nuclear morphology are consistent with studies performed by others.[14,47] During late stage apoptosis, fragmented nuclei morphology occur that relate to caspase-3 activation and DNA fragmentation. Caspase-3-dependent DNA cleavage enzymes, such as caspase-activated DNAse may be activate in fragmented nuclei morphology.[59] Different patterns of nuclear staining were found for different periods of culture. The present data showed that lobulated nuclei exhibited the early morphological changes of apoptosis followed by crescent and fragmentation at later periods of cultures. This nuclear pattern is possibly due to endonuclease action at a late stage in the apoptotic cascade creates small fragments of DNA.

4.4 CYTOSKELETAL STAINING

F-actin organization is essential for maintaining and modulating cellular morphology and structural integrity.[60] Several studies being in line with our data have shown that simvastatin disrupts actin filaments resulting in loss of cell structure. This was seen with endometrial stromal cells,[33] human dental pulp stem cells,[11] cardiac fibroblasts,[61] and gingival fibroblasts.[56] We found that the disruption and loss of filamentous actin in simvastatin-treated HDPCs and HPLFs was apparent after the onset of apoptosis. The data may indicate that simvastatin induces a direct change of the cellular morphology. Previous studies have shown a decrease of active RhoA protein (geranylgeranylated form of RhoA) in simvastatin-treated mesangial cells.[62] In addition, recent studies have confirmed that the effect of simvastatin on cytoskeletal organization is mediated via the pathway of geranylgeranyl-PP (GGPP) as evidenced that GGPP markedly reversed the effects of simvastatin and restored F-actin cytoskeletal rearrangement.[33] In this regard, Porter et al. demonstrated that simvastatin inhibits geranylgeranylation of RhoA, preventing its translocation to the plasma membrane and subsequent activation of downstream activators, including ROCK, and through of this mechanism disrupts the cytoskeleton and inhibits human cardiac myofibroblast.[63] Rho proteins are required for maintenance of actin filaments and cytoskeletal structure.[64] Thus, this study suggests that simvastatin inhibits Rho protein activation and signaling and consequently disrupts the actin cytoskeleton.

4.5 CLINICAL APPLICATION

There are several studies of the benefits of simvastatin in tooth-associated cells. Okamoto et al suggested that simvastatin might be an ideal active ingredient which could accelerate the differentiation of dental pulp stem cells.[11] In addition, Min et al confirmed that simvastatin has effects on the regeneration of injured dental pulp tissues and the stimulation of reparative dentinogenesis.[10] Although simvastatin benefits to these regenerative situations, the present in vitro data indicate that relatively high concentrations of simvastatin are cytotoxic to the cells. Apoptosis in skeletal muscle cells may trigger different forms of myotoxicity such as myopathy and rhabdomyolysis.[47] As discussed before, there are many factors that may be related to the different results from the previous positive effects of simvastatin. First, apparent cell type and stage of cell differentiation-dependent effects of simvastatin may give different outcomes in their activity.

Second, the conditions of experiment such as cell density, percentage of cell confluence and exposure time may be related to the response of cells. Third, the concentrations used in cell culture and animal experiments, appear to be differences in uptake and activation that may explain the variability in tissue-specific side effects. Finally, the toxicity of simvastatin is additionally complicated by the various phamacodynamics and pharmacokinetics of the drugs.

Whenever an in vitro toxicity study is conducted, the explicit or implicit objective is to draw some inference regarding the drug's behaviour in vivo. This in vivo behaviour is represented by the drug concentration-time profile in humans. Therefore, predicting a plasma concentration to target cells from these results should be considered. To understand the molecular mechanisms of simvastatin–drug toxicity, it is important to mention that simvastatin is very selective inhibitors of HMG-CoA reductase and usually does not show any relevant affinity toward other enzymes or receptor systems. However, at the pharmacokinetic level (ie, absorption, distribution, metabolism, and excretion of a given drug), the available statins have important differences, including half-life, systemic exposure, maximum plasma concentration (Cmax), bioavailability, protein binding, lipophilicity, metabolism, presence of active metabolites, and excretion routes.[65] Thus, it is difficult to conclude that simvastatin has negative effects on tooth-associated cells.

The present in vitro data emphasise that relatively high concentrations of simvastatin are cytotoxic to cells. In clinical prescription, simvastatin is orally administered in 5, 10, 20, 40, and 80 mg tablets. The highest concentration of simvastatin found in the plasma of patients having a treatment of 40 mg/day was 7.2 nM.[66] This concentration can be cytotoxic as shown in our study. Furthermore, the maximum tolerated dose of simvastatin was reported to be 15 mg/kg/day. Thus, it is of note that much higher concentrations of simvastatin could be prescribed. It is not known whether the plasma simvastatin concentration correlates to the final concentration reaching the target cells.[51] Consequently, it is practically difficult to predict the adverse outcomes when high doses are used. Further in vivo investigations are required.

CHAPTER 5

CONCLUSION

In conclusion, the present findings showed that simvastatin adversely affects cell viability of HDPCs and HPLFs probably by inducing apoptosis, characterized by classic microscopic morphology and the presence of an annexin V/ PI positive double staining detected by flow cytometry. Additionally, simvastatin disrupted the F-actin cytoskeleton in both cell types. Furthermore, simvastatin-triggered apoptotic cell death of HDPCs and HPLFs was confirmed by the occurrence of nuclear DNA degradation into nucleosome fragments. Based on these evidences, these detrimental adverse effects should be taken into consideration when implementing simvastatin in clinical use.

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APPENDICES

APPENDIX 1

MATERIALS

1. CELL CULTURE LABORATORY

1.1 Stock of α-MEM (Alpha-minimum essential medium) pH 7.2

α-MEM	13.9 g
$NaHCO_3$	2.2 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.2 with HCl. Add distilled water to 1000 ml. Sterilize the DMEM by filter and store at 4 ^{o}C.

1.2 Stock of DMEM (Dulbeeco's Modified Eagle's medium) pH 7.2

DMEM	13.9 g
$NaHCO_3$	3.7 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.2 with HCl. Add distilled water to 1000 ml. Sterilize the DMEM by filter and store at 4 ^{o}C.

1.3 Collagenases (30 mg/ml)

Collagenase	30 mg

Dissolve in 1 ml of distilled water and sterilized by filter with 0.2 μm membrane.

1.4 Dispase (40 mg/ml)

Dispase	40 mg

Dissolve in 1 ml of distilled water and sterilized by filter with 0.2 μm membrane.

1.5 L-ascorbic acid 100 mM (MW= 176.12)

L-ascorbic acid	100 mg

Dissolve in 5.68 ml of distilled water and sterilized by filter with 0.2 μm membrane.

1.6 L-Glutamate stock 200 mM

1.7 Penicillin- streptomycin stock 10^4 unit/ml

1.8 Fungizone stock 250 μg/ml

1.9 Phosphate-buffered saline (PBS) pH 7.4

NaCl	8 g
KCl	0.2 g

Na_2HPO_4 1.44 g

KH_2PO_4 0.24 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to 1000 ml. Sterilize the buffer by autoclaving and store at room temperature.

1.10 Prepare of 0.05 % Trypsin-EDTA

0.5 % Trypsin-EDTA

Dissolve 0.5 % Trypsin-EDTA in PBS pH 7.4 in ratio 1:10.

2. MTT ASSAY

2.1 MTT solution

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide 50 mg

Dissolve in 10 ml of sterilized phosphate buffer saline (PBS) and sterilize by filter with 0.2 μm membrane.

2.2 HEPES (pH 7.4) 10mM

HEPES 238 mg

Adjust the volume to 80 ml with distilled water before adjust pH to 7.4. After that, adjust volume to 100 ml with distilled water and then sterilize by filter with 0.2 μm membrane.

2.3 Sorensen' glycine buffer

Glycine 750 mg

NaCl 585 mg

Adjust the volume to 80 ml with distilled water before adjust pH to 10.5. After that, adjust the volume to 100 ml with distilled water and then sterilize by filter with 0.2 μm membrane.

3. SIMVASTATIN PREPARATION

Briefly, 20 mg simvastatin is dissolved in 400 μl ethanol (95-100%) followed by the addition of 0.325 ml of 1 N NaOH. The solution is neutralized with 1 N HCl to pH 7.2 and brought to a 1-ml volume by deionized water. The final concentration of simvastatin is 20 mg/ml or 47.8 mM.

4. H_2O_2 PREPARATION 100 mM

Density H_2O_2 30% = 1.11 g/ml.

Molecular weight = 34.01 g/mol

Dissolve H_2O_2 (9.79 M) 51 µl in deionized water 5 ml.

5. CELL STAINING

5.1 PBS-F 3.7% 20 ml

Dissolve formaldehyde 37% 2 ml in PBS 18 ml.

5.2 PBS-T 0.1% 20 ml

Dissolve Triton X-100 20 µl in PBS 20 ml.

5.3 PBS-A 0.5% 20 ml

Dissolve Bovine serum albumin 100 mg in PBS 20 ml.

APPENDIX 2

DATA

Table 4: The data of optical density (MTT assay) in human dental pulp cells (HDPCs)

Optical density (MTT assay) in human dental pulp cells (HDPCs)																				
Gr.	**control**				**simvastatin 0.1µM**				**simvastatin 1 µM**				**simvastatin 10 µM**				**H_2O_2 0.5 mM**			
Time	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**
24 hr	0.105	0.187	0.264	0.116	0.148	0.195	0.279	0.154	0.111	0.160	0.261	0.105	0.111	0.185	0.207	0.115	0.019	0.075	0.031	0.000
	0.103	0.232	0.273	0.148	0.133	0.202	0.265	0.176	0.112	0.190	0.234	0.142	0.117	0.163	0.272	0.093	0.008	0.071	0.043	0.003
	0.131	0.246	0.270	0.143	0.146	0.188	0.214	0.140	0.094	0.165	0.226	0.101	0.103	0.214	0.248	0.116	0.014	0.050	0.077	0.012
	0.131	0.244	0.243	0.168	0.112	0.212	0.256	0.131	0.107	0.185	0.277	0.095	0.108	0.140	0.301	0.093	0.018	0.066	0.046	0.003
	0.142	0.209	0.256	0.133	0.120	0.138	0.237	0.106	0.088	0.149	0.176	0.096	0.081	0.184	0.228	0.064	0.009	0.066	0.037	0.003
mean	**0.122**	**0.224**	**0.261**	**0.142**	**0.132**	**0.187**	**0.250**	**0.141**	**0.102**	**0.170**	**0.235**	**0.108**	**0.104**	**0.177**	**0.251**	**0.096**	**0.014**	**0.066**	**0.047**	**0.004**
SD	**0.017**	**0.025**	**0.012**	**0.019**	**0.016**	**0.029**	**0.025**	**0.026**	**0.011**	**0.017**	**0.039**	**0.020**	**0.014**	**0.028**	**0.037**	**0.021**	**0.005**	**0.010**	**0.018**	**0.005**
48 hr	0.118	0.191	0.216	0.100	0.099	0.161	0.209	0.141	0.077	0.117	0.079	0.096	0.050	0.090	0.017	0.088	0.001	0.052	0.008	0.002
	0.106	0.195	0.249	0.116	0.092	0.241	0.155	0.089	0.052	0.187	0.078	0.097	0.069	0.113	0.016	0.073	0.003	0.071	0.016	0.002
	0.102	0.236	0.237	0.120	0.142	0.228	0.241	0.076	0.076	0.124	0.137	0.062	0.061	0.092	0.028	0.048	0.003	0.071	0.020	0.002
	0.150	0.233	0.258	0.123	0.082	0.240	0.214	0.106	0.050	0.139	0.113	0.059	0.063	0.113	0.016	0.043	0.008	0.073	0.012	0.000
	0.155	0.264	0.227	0.131	0.107	0.194	0.130	0.101	0.072	0.097	0.065	0.052	0.066	0.109	0.017	0.042	0.010	0.052	0.009	0.000
mean	**0.126**	**0.224**	**0.237**	**0.118**	**0.104**	**0.213**	**0.190**	**0.103**	**0.065**	**0.133**	**0.094**	**0.073**	**0.062**	**0.103**	**0.019**	**0.059**	**0.005**	**0.064**	**0.013**	**0.001**
SD	**0.025**	**0.031**	**0.017**	**0.011**	**0.023**	**0.035**	**0.046**	**0.024**	**0.013**	**0.034**	**0.030**	**0.022**	**0.007**	**0.011**	**0.005**	**0.021**	**0.004**	**0.011**	**0.005**	**0.001**
72 hr	0.173	0.166	0.102	0.152	0.167	0.186	0.118	0.124	0.050	0.050	0.074	0.068	0.024	0.017	0.047	0.050	0.008	0.062	0.018	0.003
	0.214	0.181	0.117	0.186	0.174	0.241	0.145	0.134	0.048	0.112	0.074	0.089	0.028	0.019	0.042	0.046	0.018	0.062	0.014	0.001
	0.172	0.194	0.170	0.145	0.204	0.222	0.133	0.135	0.066	0.092	0.081	0.089	0.028	0.013	0.061	0.046	0.009	0.068	0.023	0.006
	0.164	0.210	0.149	0.113	0.184	0.193	0.119	0.144	0.084	0.055	0.093	0.078	0.051	0.027	0.054	0.028	0.031	0.031	0.020	0.000
	0.113	0.223	0.154	0.111	0.178	0.188	0.127	0.101	0.073	0.054	0.106	0.080	0.038	0.010	0.071	0.044	0.031	0.069	0.005	0.000
mean	**0.167**	**0.195**	**0.138**	**0.141**	**0.181**	**0.206**	**0.128**	**0.128**	**0.064**	**0.073**	**0.086**	**0.081**	**0.034**	**0.017**	**0.055**	**0.043**	**0.019**	**0.058**	**0.016**	**0.002**
SD	**0.036**	**0.023**	**0.028**	**0.031**	**0.014**	**0.024**	**0.011**	**0.016**	**0.015**	**0.028**	**0.014**	**0.009**	**0.011**	**0.006**	**0.011**	**0.009**	**0.011**	**0.016**	**0.007**	**0.003**

Table 5: The data of percentage of viable cells (MTT assay) in human dental pulp cells (HDPCs)

Percentage of viable cells (MTT assay) in human dental pulp cells (HDPCs)																				
Gr.	**control**				**simvastatin 0.1 µM**				**simvastatin 1 µM**				**simvastatin 10 µM**				**H_2O_2 0.5 mM**			
Time	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**
24 hr	86.07	83.48	101.15	81.69	121.31	87.05	106.90	108.45	90.98	71.43	100.00	73.94	90.98	82.59	79.31	80.99	15.57	33.48	11.88	0.00
	84.43	103.57	104.60	104.23	109.02	90.18	101.53	123.94	91.80	84.82	89.66	100.00	95.90	72.77	104.21	65.49	6.56	31.70	16.48	2.11
	107.38	109.82	103.45	100.70	119.67	83.93	81.99	98.59	77.05	73.66	86.59	71.13	84.43	95.54	95.02	81.69	11.48	22.32	29.50	8.45
	107.38	108.93	93.10	118.31	91.80	94.64	98.08	92.25	87.70	82.59	106.13	66.90	88.52	62.50	115.33	65.49	14.75	29.46	17.62	2.11
	116.39	93.30	98.08	93.66	98.36	61.61	90.80	74.65	72.13	66.52	67.43	67.61	66.39	82.14	87.36	45.07	7.38	29.46	14.18	2.11
mean	**100.33**	**99.82**	**100.08**	**99.72**	**108.03**	**83.48**	**95.86**	**99.58**	**83.93**	**75.80**	**89.96**	**75.92**	**85.25**	**79.11**	**96.25**	**67.75**	**11.15**	**29.29**	**17.93**	**2.96**
SD	**14.26**	**11.25**	**4.62**	**13.50**	**12.94**	**12.85**	**9.71**	**18.36**	**8.84**	**7.70**	**14.84**	**13.76**	**11.33**	**12.32**	**14.10**	**14.95**	**4.12**	**4.24**	**6.83**	**3.20**
48 hr	93.65	85.27	91.14	84.75	78.57	71.88	88.19	119.49	61.11	52.23	33.33	81.36	39.68	40.18	7.17	74.58	0.79	23.21	3.38	1.69
	84.13	87.05	105.06	98.31	73.02	107.59	65.40	75.42	41.27	83.48	32.91	82.20	54.76	50.45	6.75	61.86	2.38	31.70	6.75	1.69
	80.95	105.36	100.00	101.69	112.70	101.79	101.69	64.41	60.32	55.36	57.81	52.54	48.41	41.07	11.81	40.68	2.38	31.70	8.44	1.69
	119.05	104.02	108.86	104.24	65.08	107.14	90.30	89.83	39.68	62.05	47.68	50.00	50.00	50.45	6.75	36.44	6.35	32.59	5.06	0.00
	123.02	117.86	95.78	111.02	84.92	86.61	54.85	85.59	57.14	43.30	27.43	44.07	52.38	48.66	7.17	35.59	7.94	23.21	3.80	0.00
mean	**100.16**	**99.91**	**100.17**	**100.00**	**82.86**	**95.00**	**80.08**	**86.95**	**51.90**	**59.29**	**39.83**	**62.03**	**49.05**	**46.16**	**7.93**	**49.83**	**3.97**	**28.48**	**5.49**	**1.02**
SD	**19.67**	**13.68**	**7.08**	**9.72**	**18.21**	**15.47**	**19.29**	**20.68**	**10.55**	**15.11**	**12.54**	**18.29**	**5.76**	**5.12**	**2.18**	**17.49**	**3.02**	**4.82**	**2.11**	**0.93**
72 hr	103.59	85.13	73.91	107.80	100.00	95.38	85.51	87.94	29.94	25.64	53.62	48.23	14.37	8.72	34.06	35.46	4.79	31.79	13.04	2.13
	128.14	92.82	84.78	131.91	104.19	123.59	105.07	95.04	28.74	57.44	53.62	63.12	16.77	9.74	30.43	32.62	10.78	31.79	10.14	0.71
	102.99	99.49	123.19	102.84	122.16	113.85	96.38	95.74	39.52	47.18	58.70	63.12	16.77	6.67	44.20	32.62	5.39	34.87	16.67	4.26
	98.20	107.69	107.97	80.14	110.18	98.97	86.23	102.13	50.30	28.21	67.39	55.32	30.54	13.85	39.13	19.86	18.56	15.90	14.49	0.00
	67.66	114.36	111.59	78.72	106.59	96.41	92.03	71.63	43.71	27.69	76.81	56.74	22.75	5.13	51.45	31.21	18.56	35.38	3.62	0.00
mean	**100.12**	**99.90**	**100.29**	**100.28**	**108.62**	**105.64**	**93.04**	**90.50**	**38.44**	**37.23**	**62.03**	**57.30**	**20.24**	**8.82**	**39.86**	**30.35**	**11.62**	**29.95**	**11.59**	**1.42**
SD	**21.58**	**11.60**	**20.29**	**21.99**	**8.42**	**12.49**	**8.06**	**11.68**	**9.16**	**14.27**	**10.00**	**6.21**	**6.54**	**3.33**	**8.31**	**6.07**	**6.76**	**8.03**	**5.05**	**1.81**

Table 6: The data of optical density (MTT assay) in human periodontal fibroblasts (HPLFs)

Optical density (MTT assay) in human periodontal fibroblasts (HPLFs)																				
Gr.	**control**				**simvastatin 0.1 µM**				**simvastatin 1 µM**				**simvastatin 10 µM**				**H_2O_2 0.3 mM**			
Time	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**
24 hr	0.198	0.243	0.259	0.088	0.254	0.187	0.250	0.176	0.211	0.263	0.201	0.151	0.203	0.193	0.194	0.156	0.009	0.017	0.007	0.017
	0.219	0.215	0.263	0.145	0.282	0.241	0.260	0.118	0.204	0.216	0.172	0.143	0.191	0.158	0.252	0.185	0.003	0.016	0.006	0.009
	0.217	0.299	0.224	0.185	0.219	0.273	0.321	0.143	0.212	0.267	0.194	0.100	0.246	0.200	0.201	0.136	0.002	0.017	0.008	0.010
	0.205	0.275	0.275	0.141	0.266	0.206	0.261	0.103	0.202	0.251	0.208	0.136	0.250	0.293	0.210	0.118	0.008	0.019	0.012	0.008
	0.237	0.236	0.231	0.125	0.163	0.195	0.163	0.112	0.178	0.213	0.163	0.091	0.201	0.168	0.158	0.100	0.012	0.009	0.004	0.004
mean	**0.215**	**0.254**	**0.250**	**0.137**	**0.237**	**0.220**	**0.251**	**0.130**	**0.201**	**0.242**	**0.188**	**0.124**	**0.218**	**0.202**	**0.203**	**0.139**	**0.007**	**0.016**	**0.007**	**0.010**
SD	**0.015**	**0.033**	**0.022**	**0.035**	**0.047**	**0.036**	**0.057**	**0.030**	**0.014**	**0.026**	**0.019**	**0.027**	**0.028**	**0.054**	**0.034**	**0.033**	**0.004**	**0.004**	**0.003**	**0.005**
48 hr	0.192	0.187	0.251	0.146	0.262	0.164	0.213	0.212	0.241	0.165	0.189	0.119	0.156	0.142	0.256	0.104	0.007	0.012	0.010	0.002
	0.232	0.213	0.273	0.193	0.281	0.184	0.232	0.240	0.255	0.182	0.188	0.087	0.206	0.136	0.215	0.102	0.005	0.008	0.017	0.019
	0.206	0.229	0.259	0.179	0.283	0.188	0.260	0.195	0.244	0.168	0.203	0.164	0.127	0.166	0.174	0.086	0.008	0.009	0.025	0.004
	0.236	0.164	0.217	0.232	0.222	0.175	0.263	0.121	0.198	0.160	0.201	0.127	0.202	0.133	0.179	0.094	0.000	0.005	0.015	0.028
	0.224	0.098	0.234	0.178	0.231	0.137	0.183	0.118	0.201	0.153	0.151	0.096	0.166	0.133	0.129	0.072	0.008	0.012	0.007	0.007
mean	**0.218**	**0.178**	**0.247**	**0.186**	**0.256**	**0.170**	**0.230**	**0.177**	**0.228**	**0.166**	**0.186**	**0.119**	**0.171**	**0.142**	**0.191**	**0.092**	**0.006**	**0.009**	**0.015**	**0.012**
SD	**0.019**	**0.051**	**0.022**	**0.031**	**0.028**	**0.020**	**0.034**	**0.055**	**0.026**	**0.011**	**0.021**	**0.030**	**0.033**	**0.014**	**0.048**	**0.013**	**0.003**	**0.003**	**0.007**	**0.011**
72 hr	0.191	0.132	0.262	0.117	0.275	0.113	0.227	0.091	0.231	0.113	0.235	0.087	0.142	0.070	0.139	0.033	0.012	0.015	0.012	0.006
	0.280	0.168	0.263	0.162	0.317	0.157	0.251	0.127	0.315	0.116	0.185	0.112	0.157	0.079	0.180	0.046	0.012	0.018	0.017	0.006
	0.285	0.142	0.259	0.125	0.290	0.148	0.179	0.101	0.271	0.129	0.194	0.115	0.174	0.089	0.146	0.104	0.018	0.014	0.028	0.008
	0.319	0.157	0.284	0.152	0.346	0.108	0.204	0.129	0.240	0.087	0.194	0.119	0.195	0.089	0.170	0.058	0.009	0.009	0.013	0.003
	0.303	0.116	0.244	0.160	0.210	0.108	0.183	0.114	0.215	0.062	0.136	0.054	0.124	0.049	0.162	0.072	0.008	0.008	0.014	0.009
mean	**0.276**	**0.143**	**0.262**	**0.143**	**0.288**	**0.127**	**0.209**	**0.112**	**0.254**	**0.101**	**0.189**	**0.097**	**0.158**	**0.075**	**0.159**	**0.063**	**0.012**	**0.013**	**0.017**	**0.006**
SD	**0.050**	**0.020**	**0.014**	**0.021**	**0.051**	**0.024**	**0.030**	**0.016**	**0.040**	**0.027**	**0.035**	**0.027**	**0.028**	**0.017**	**0.017**	**0.027**	**0.004**	**0.004**	**0.007**	**0.002**

Table 7: The data of percentage of viable cells (MTT assay) in human periodontal fibroblasts (HPLFs)

Percentage of viable cells (MTT assay) in human periodontal fibroblasts (HPLFs)																				
Gr.	**control**				**simvastatin 0.1 μM**				**simvastatin 1 μM**				**simvastatin 10 μM**				**H_2O_2 0.3 mM**			
Time	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**	**Pt1**	**Pt2**	**Pt3**	**Pt4**
24 hr	92.09	95.67	103.60	64.23	118.14	73.62	100.00	128.47	98.14	103.54	80.40	110.22	94.42	75.98	77.60	113.87	4.19	6.69	2.80	12.41
	101.86	84.65	105.20	105.84	131.16	94.88	104.00	86.13	94.88	85.04	68.80	104.38	88.84	62.20	100.80	135.04	1.40	6.30	2.40	6.57
	100.93	117.72	89.60	135.04	101.86	107.48	128.40	104.38	98.60	105.12	77.60	72.99	114.42	78.74	80.40	99.27	0.93	6.69	3.20	7.30
	95.35	108.27	110.00	102.92	123.72	81.10	104.40	75.18	93.95	98.82	83.20	99.27	116.28	115.35	84.00	86.13	3.72	7.48	4.80	5.84
	110.23	92.91	92.40	91.24	75.81	76.77	65.20	81.75	82.79	83.86	65.20	66.42	93.49	66.14	63.20	72.99	5.58	3.54	1.60	2.92
mean	**100.09**	**99.84**	**100.16**	**99.85**	**110.14**	**86.77**	**100.40**	**95.18**	**93.67**	**95.28**	**75.04**	**90.66**	**101.49**	**79.69**	**81.20**	**101.46**	**3.16**	**6.14**	**2.96**	**7.01**
SD	**6.95**	**13.10**	**8.74**	**25.62**	**22.01**	**14.14**	**22.65**	**21.53**	**6.41**	**10.16**	**7.71**	**19.65**	**12.85**	**21.07**	**13.51**	**24.14**	**1.96**	**1.51**	**1.19**	**3.45**
48 hr	88.07	105.06	101.62	78.49	120.18	92.13	86.23	113.98	110.55	92.70	76.52	63.98	71.56	79.78	103.64	55.91	3.21	6.74	4.05	1.08
	106.42	119.66	110.53	103.76	128.90	103.37	93.93	129.03	116.97	102.25	76.11	46.77	94.50	76.40	87.04	54.84	2.29	4.49	6.88	10.22
	94.50	128.65	104.86	96.24	129.82	105.62	105.26	104.84	111.93	94.38	82.19	88.17	58.26	93.26	70.45	46.24	3.67	5.06	10.12	2.15
	108.26	92.13	87.85	124.73	101.83	98.31	106.48	65.05	90.83	89.89	81.38	68.28	92.66	74.72	72.47	50.54	0.00	2.81	6.07	15.05
	102.75	55.06	94.74	95.70	105.96	76.97	74.09	63.44	92.20	85.96	61.13	51.61	76.15	74.72	52.23	38.71	3.67	6.74	2.83	3.76
mean	**100.00**	**100.11**	**99.92**	**99.78**	**117.34**	**95.28**	**93.20**	**95.27**	**104.50**	**93.03**	**75.47**	**63.76**	**78.62**	**79.78**	**77.17**	**49.25**	**2.57**	**5.17**	**5.99**	**6.45**
SD	**8.51**	**28.79**	**8.83**	**16.74**	**12.92**	**11.48**	**13.57**	**29.61**	**12.10**	**6.06**	**8.47**	**16.22**	**15.16**	**7.81**	**19.29**	**7.03**	**1.54**	**1.66**	**2.81**	**5.97**
72 hr	69.20	92.31	100.00	81.82	99.64	79.02	86.64	63.64	83.70	79.02	89.69	60.84	51.45	48.95	53.05	23.08	4.35	10.49	4.58	4.20
	101.45	117.48	100.38	113.29	114.86	109.79	95.80	88.81	114.13	81.12	70.61	78.32	56.88	55.24	68.70	32.17	4.35	12.59	6.49	4.20
	103.26	99.30	98.85	87.41	105.07	103.50	68.32	70.63	98.19	90.21	74.05	80.42	63.04	62.24	55.73	72.73	6.52	9.79	10.69	5.59
	115.58	109.79	108.40	106.29	125.36	75.52	77.86	90.21	86.96	60.84	74.05	83.22	70.65	62.24	64.89	40.56	3.26	6.29	4.96	2.10
	109.78	81.12	93.13	111.89	76.09	75.52	69.85	79.72	77.90	43.36	51.91	37.76	44.93	34.27	61.83	50.35	2.90	5.59	5.34	6.29
mean	**99.86**	**100.00**	**100.15**	**100.14**	**104.20**	**88.67**	**79.69**	**78.60**	**92.17**	**70.91**	**72.06**	**68.11**	**57.39**	**52.59**	**60.84**	**43.78**	**4.28**	**8.95**	**6.41**	**4.48**
SD	**18.02**	**14.30**	**5.46**	**14.55**	**18.53**	**16.62**	**11.59**	**11.49**	**14.33**	**18.73**	**13.48**	**19.09**	**9.98**	**11.64**	**6.44**	**19.07**	**1.41**	**2.94**	**2.49**	**1.61**

Table 8: The data of apoptotic cells (Flow cytometry) in human dental pulp cells (HDPCs)

Group		control				simvastatin 0.1 μM				simvastatin 1 μM				simvastatin 10 μM				H_2O_2 1 mM			
Time	Percent	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4
24 h	Early apoptosis	2.59	4.27	3.36	3.62	3.50	7.19	3.08	5.09	6.84	7.77	3.74	6.01	9.94	9.95	6.41	5.17	1.97	5.09	46.49	2.77
	Q4 (%)	3.21	5.43	5.61	4.61	5.24	7.39	2.11	5.25	10.46	6.89	5.42	6.78	10.07	9.06	7.12	6.02	12.15	23.10	40.93	3.08
	Late apoptosis	1.17	2.31	3.60	3.09	1.47	3.17	4.59	3.30	3.06	3.36	3.58	3.31	4.16	4.02	6.13	4.14	81.29	55.73	16.24	61.41
	Q2 (%)	1.33	2.77	3.06	2.44	2.33	3.55	2.50	2.86	5.45	4.25	3.71	3.91	5.86	3.69	5.29	4.25	71.72	53.53	18.52	60.20
	Total apoptosis	3.76	6.58	6.96	6.71	4.97	10.36	7.67	8.39	9.90	11.13	7.32	9.32	14.10	13.97	12.54	9.31	83.26	60.82	62.73	64.18
	Q2+Q4 (%)	4.54	8.20	8.67	7.05	7.57	10.94	4.61	8.11	15.91	11.14	9.13	10.69	15.93	12.75	12.41	10.27	83.87	76.63	59.45	63.28
	Necrosis	0.29	0.71	0.85	0.76	0.28	0.71	1.20	0.47	0.39	0.82	1.73	0.28	0.78	0.45	4.97	0.57	1.97	5.09	46.49	2.77
		0.23	0.67	0.70	0.37	0.26	0.61	1.53	0.39	0.53	1.16	1.43	0.42	0.98	0.51	2.20	0.49	12.15	23.10	40.93	3.08
48 h	Early apoptosis	2.07	4.03	4.75	5.81	3.47	11.75	6.16	6.48	6.87	12.86	7.27	5.63	8.19	10.31	8.04	8.46	0.87	3.66	1.33	1.69
	Q4 (%)	2.36	6.02	4.96	5.02	5.76	10.33	6.57	5.96	5.33	16.55	8.05	5.68	4.38	20.50	7.74	7.08	4.44	1.53	35.78	2.01
	Late apoptosis	1.10	4.28	3.61	4.48	1.30	4.67	2.85	4.95	3.92	5.99	4.26	5.63	5.88	9.06	4.90	7.90	83.98	73.89	74.26	76.83
	Q2 (%)	1.76	3.93	3.22	3.79	1.95	4.90	3.03	4.59	3.49	7.36	4.49	6.34	3.90	8.69	4.48	7.33	78.44	87.19	27.34	73.53
	Total apoptosis	3.17	8.31	8.36	10.29	4.77	16.42	9.01	11.43	10.79	18.85	11.53	11.26	14.07	19.37	12.94	16.36	84.85	77.55	75.59	78.52
	Q2+Q4 (%)	4.12	9.95	8.18	8.81	7.71	15.23	9.60	10.55	8.82	23.91	12.54	12.02	8.28	29.19	12.22	14.41	82.88	88.72	63.12	75.54
	Necrosis	0.48	0.98	0.60	0.47	0.49	0.57	0.62	0.46	0.64	1.41	0.76	0.55	1.22	2.58	1.13	1.03	0.87	3.66	1.33	1.69
		0.47	0.69	0.70	0.45	0.39	0.71	0.67	0.47	0.73	1.21	0.77	0.57	2.25	1.34	1.00	1.57	4.44	1.53	35.78	2.01
72 h	Early apoptosis	7.80	6.72	13.58	10.09	12.34	14.16	8.61	8.89	14.95	13.63	10.76	13.43	28.29	43.45	15.84	10.09	2.18	12.29	39.37	2.72
	Q4 (%)	7.60	11.27	15.29	10.05	11.79	15.31	8.95	8.30	13.62	18.14	14.53	13.31	11.05	35.61	10.24	8.42	18.37	2.56	20.33	20.66
	Late apoptosis	4.28	4.21	3.56	5.66	4.30	4.73	3.81	6.98	6.32	6.92	8.20	6.67	5.68	9.24	7.34	9.07	84.77	68.13	19.83	86.20
	Q2 (%)	3.87	4.05	2.88	5.51	4.17	5.88	4.21	6.42	5.18	9.00	7.31	6.07	8.64	17.57	7.44	9.90	57.89	87.11	45.99	59.82
	Total apoptosis	12.08	10.93	17.14	15.75	16.64	18.89	12.42	15.87	21.27	20.55	18.96	20.1	33.97	52.69	23.18	19.16	86.95	80.42	59.20	88.92
	Q2+Q4 (%)	11.47	15.32	18.17	15.56	15.96	21.19	13.16	14.72	18.80	27.14	21.84	19.38	19.69	53.18	17.68	18.32	76.26	89.67	66.32	80.48
	Necrosis	0.77	0.86	0.67	0.80	0.51	0.88	0.66	0.90	0.81	1.84	1.00	0.56	2.41	2.42	2.06	1.87	2.18	12.29	39.37	2.72
		0.87	0.75	1.19	0.50	0.62	1.00	1.17	0.83	0.81	2.02	1.04	0.45	3.42	5.09	2.69	2.16	18.37	2.56	20.33	20.66

Table 9: The data of apoptotic cells (Flow cytometry) in human periodontal ligament fibroblasts (HPLFs)

Group		control				simvastatin 0.1μM				simvastatin 1μM				simvastatin 10μM				H_2O_2 1 mM			
Time	Percent	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4	Pt1	Pt2	Pt3	Pt4
24 hr	Early apoptosis	9.43	2.91	5.53	11.75	8.44	4.27	6.16	10.58	8.64	4.31	6.06	10.87	8.24	4.74	7.21	15.05	1.31	3.78	11.23	7.37
	Q4 (%)	7.87	2.90	5.79	11.31	6.56	3.70	5.80	10.78	8.01	4.52	5.27	12.51	10.67	3.52	6.13	13.09	16.49	20.72	25.47	11.56
	Late apoptosis	6.06	1.72	4.39	4.23	10.83	2.29	4.33	4.00	11.59	3.07	4.85	5.40	10.33	3.30	7.93	6.36	81.34	75.23	49.64	69.81
	Q2 (%)	4.25	1.69	4.16	4.49	7.21	2.39	3.98	4.42	9.92	3.15	5.35	4.76	11.61	2.59	5.86	5.53	44.44	48.15	25.03	65.33
	Total apoptosis	15.49	4.63	9.92	15.98	19.27	6.56	10.49	14.58	20.23	7.38	10.91	16.27	18.57	8.04	15.14	21.41	82.65	79.01	60.87	77.18
	Q2+Q4 (%)	12.12	4.59	9.95	15.80	13.77	6.09	9.78	15.20	17.93	7.67	10.62	17.27	22.28	6.11	11.99	18.62	60.93	68.87	50.50	76.89
	Necrosis	1.10	0.56	0.42	0.75	1.38	0.85	0.30	0.93	1.01	0.92	0.52	1.34	1.25	1.48	1.26	1.45	12.61	16.35	13.93	3.33
		1.20	0.70	0.52	1.03	1.33	0.95	0.39	1.81	1.42	1.16	0.82	0.93	1.03	2.04	0.97	1.97	6.38	10.06	5.95	3.54
48 hr	Early apoptosis	6.53	3.63	6.32	8.51	7.93	3.34	6.90	12.41	8.51	4.12	7.52	14.47	10.95	4.68	9.44	19.70	1.45	3.78	6.36	24.50
	Q4 (%)	9.08	3.57	6.03	11.15	7.48	3.71	6.63	11.22	10.10	4.72	7.00	15.09	10.87	4.92	8.70	20.14	1.57	1.13	3.87	18.92
	Late apoptosis	4.46	1.38	3.66	3.78	6.48	1.51	4.28	7.30	9.76	2.16	4.96	5.54	12.57	4.27	8.70	9.90	86.85	78.97	70.51	41.17
	Q2 (%)	7.67	1.16	3.36	5.16	5.96	1.59	4.09	7.69	9.58	2.29	4.54	6.41	12.50	3.91	6.83	9.23	78.63	49.04	67.62	53.94
	Total apoptosis	10.99	5.01	9.98	12.29	14.41	4.85	11.18	19.71	18.27	6.28	12.48	20.01	23.52	8.95	18.14	29.60	88.30	82.75	76.87	65.67
	Q2+Q4 (%)	16.75	4.73	9.39	16.31	13.44	5.30	10.72	18.91	19.68	7.01	11.54	21.50	23.37	8.83	15.53	29.37	80.20	50.17	71.49	72.86
	Necrosis	1.08	0.55	0.60	0.95	1.39	0.58	0.45	1.91	2.76	0.95	0.67	1.25	2.80	4.61	1.15	2.61	4.70	11.72	12.13	1.08
		0.82	0.52	0.65	1.68	1.63	0.56	0.58	2.32	0.95	0.72	0.78	1.22	2.73	3.89	1.58	2.29	11.89	42.28	19.40	0.36
72 hr	Early apoptosis	7.77	6.02	7.12	14.40	8.11	6.43	7.97	15.95	8.64	6.72	9.08	20.95	15.93	7.77	11.43	23.84	1.97	4.88	3.20	12.24
	Q4 (%)	7.96	5.99	6.73	18.31	8.07	6.06	8.20	15.51	10.67	7.46	9.17	22.26	14.61	6.22	11.13	29.08	2.05	4.48	23.20	6.53
	Late apoptosis	5.23	2.08	5.61	5.62	5.58	2.07	5.75	6.20	7.52	2.92	6.59	6.74	20.26	9.87	10.50	14.05	85.55	82.62	65.75	61.84
	Q2 (%)	4.30	1.89	5.58	4.92	4.73	1.93	5.29	7.07	7.04	3.21	6.73	7.45	21.74	7.55	9.32	13.20	87.67	81.92	30.99	77.73
	Total apoptosis	13.00	8.10	12.73	20.02	13.69	8.50	13.72	22.15	16.16	9.64	15.67	27.69	36.19	17.64	21.93	37.89	87.52	87.50	68.95	74.08
	Q2+Q4 (%)	12.26	7.88	12.31	23.23	12.80	7.99	13.49	22.58	17.71	10.67	15.90	29.71	36.35	13.77	20.45	42.28	89.72	86.40	54.19	84.26
	Necrosis	1.20	0.69	1.22	1.84	1.78	0.55	0.92	2.18	1.21	1.58	0.62	0.90	6.12	5.26	1.18	2.75	6.70	8.49	6.97	1.59
		1.02	0.55	1.42	1.66	1.35	0.59	1.03	2.71	0.60	1.20	0.85	0.88	4.39	5.16	1.08	1.64	5.55	9.36	4.18	1.12

Table 10: Mean percentage (SD) of apoptotic cells in human dental pulp cells and human periodontal ligament fibroblasts

Concentration of simvastatin (μM)	Stage of apoptosis	Treatment time (h)					
		24		48		72	
		HDPCS	HPLFs	HDPCS	HPLFs	HDPCS	HPLFs
Control	Early apoptosis	4.09(1.08)	7.19(3.48)	4.38(1.47)	6.85(2.63)	10.30(3.00)	9.29(4.54)
	Late apoptosis	2.47(0.85)	3.87 (1.47)	3.27(1.21)	3.83(2.08)	4.25(0.93)	4.40(1.56)
	Total apoptosis	6.56(1.67)	11.06(4.68)	7.65(2.60)	10.68(4.49)	14.55(2.71)	13.69(5.36)
	Necrosis	0.57(0.24)	0.79(0.29)	0.61(0.18)	0.86(0.39)	0.80(0.20)	1.20(0.44)
0.1	Early apoptosis	4.86(1.88)	7.04(2.67)	7.06(2.67)	7.45(3.18)	11.04(2.74)	9.54(3.91)
	Late apoptosis	2.97(0.93)	4.93(2.82)	3.53(1.44)	4.86(2.41)	5.06(1.19)	4.83(1.87)
	Total apoptosis	7.83(2.24)	11.97(4.55)	10.59(3.81)	12.31(5.51)	16.10(2.88)	14.37(5.44)
	Necrosis	0.68(0.46)	0.99(0.51)	0.55(0.11)	1.18(0.73)	0.82(0.22)	1.39(0.78)
1	Early apoptosis	6.74(1.93)	7.52(3.03)	8.53(4.04)	8.94(4.08)	14.05(2.07)	11.87(6.13)
	Late apoptosis	3.83(0.75)	6.01(3.09)	5.19(1.35)	5.66(2.88)	6.96(1.21)	6.03(1.86)
	Total apoptosis	10.57(2.50)	13.53(4.97)	13.72(5.04)	14.60(6.06)	21.01(2.70)	17.90(7.25)
	Necrosis	0.85(0.54)	1.02(0.29)	0.83(0.31)	1.16(0.68)	1.07(0.57)	0.98(0.33)
10	Early apoptosis	7.97(2.01)	8.58(4.05)	9.34(4.80)	11.18(5.90)	20.37(13.55)	15.00(7.88)
	Late apoptosis	4.69(0.93)	6.69(3.15)	6.52(1.99)	8.49(3.31)	9.36(3.58)	13.31(5.19)
	Total apoptosis	12.66(2.12)	15.27(6.05)	15.86(6.27)	19.67(8.22)	29.73(15.24)	28.31(10.96)
	Necrosis	1.37(1.57)	1.43(0.40)	1.52(0.59)	2.71(1.13)	2.77(1.05)	3.45(2.03)

APPENDIX 3

METHODOLOGY PROCEDURES

1. MTT colorimetric assay

Concept: At present colorimetric assays using the tetrazolium salt thiazolyl blue, also termed MTT, after methyl-thiazolyl-tetrazolium are widely used for assessment of cytotoxicity, cell viability, and proliferation studies in cell biology.[67] MTT gives a yellowish aqueous solution which, on reduction by dehydrogenases and reducing agents present in metabolically active cells, yields a water insoluble violet-blue formazan. The lipid soluble formazan product may be extracted with organic solvents and estimated by spectrophotometry. Consequently it has been assumed that the sites of reduction, and of formation of the formazan precipitate, were the mitochondria.

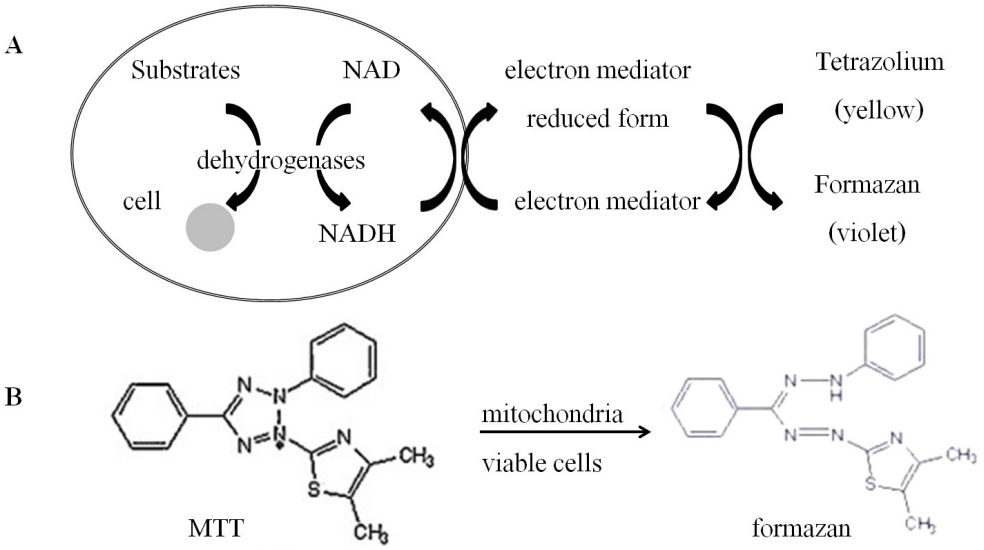

Fig. 30: MTT assay principle **(A)**. Chemical structures of MTT and Formazan **(B)**. The tetrazolium salt, MTT, is reduced through a reaction with the reduced form of 1-methoxy PMS. This mediator resides at the cell membrane and reacts directly with NADH (nicotineamido adenine dinucleotide reduced form) or NADPH (nicotineamido adenine dinucleotide phosphate reduced form). NADH and NADPH are generated from NAD+ or NADP+ by the reaction of dehydrogenase enzymes and their substrates. Therefore, the tetrazolium salt is utilized for the determination of the dehydrogenase activity or a substrate of the dehydrogenase.[68]

2. Annexin-V FITC Apoptosis and Propiodium iodide staining (Calbiochem®):

Concept: Externalization of phosphatidylserine (PS) and phosphatidylethanolamine is an important variation in the cell surface during apoptosis, that will be attached by annexin V.[69] This method is explained by extrinsically used hapten (i.e., FITC or biotin) marked annexin-V to investigate apoptosis. Annexin-V binding is able to be applied as a marker of PS externalization, using either microscopy or flow cytometry with fluorescently labeled annexin-V. Extensive molecular weight DNA binding dyes, such as propidium iodide (PI), cannot access to intact cells, without permeabilization treatments, do not label apoptotic cells until the final lysis stage. In early apoptotic cell, it can be detected by positive Annexin-V and negative propidium iodide staining. In contrast with late stage apotosis can be detected by positive Annexin-V and propidium iodide staining.[45]

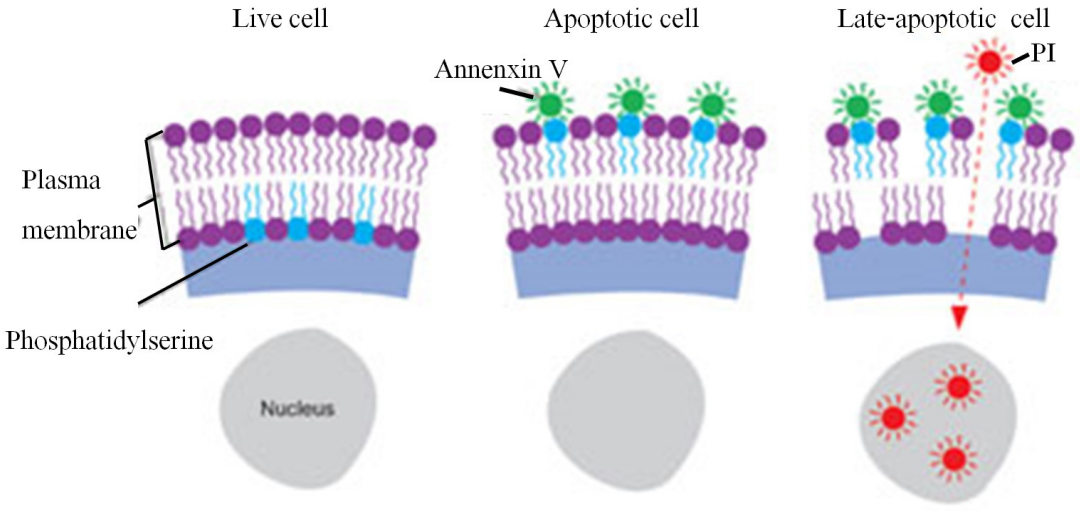

Fig. 31: The principle of apoptosis detection method based on annexin V and PI. With the aid of Ca^{2+} ions, this protein interacts with high affinity with PS heads exposed on the membrane surface. Annexin V can be labeled with fluorescent dye that allows visualization of cell with exposed PS. PI can penetrate to nucleus in late-stage apoptosis (Adapted from Biomol datasheet, Annexin V apoptosis detection kit datasheet).

2. Phalloidin cytoskeletal staining

Concept: Phallotoxins are a group of bicyclic heptapeptides from poisonous mushrooms. The major representative of this group, phalloidin, binds to actin filaments much more tightly than to actin monomers and shifts the equilibrium between filaments and monomers toward filaments, lowering the critical concentration for polymerization by 10- to 30-fold under various conditions.[70] Phallotoxins, such as phalloidin and phallacidin derivatives, bind competitively to F-actin. Fluorescent and biotinylated phallotoxins allow labeling of F-actin in fixed cells and can be used interchangeably in most applications. They stain F-actin at nanomolar concentrations and are water soluble, providing convenient probes for labeling, identifying, and quantitating F-actin.[71]

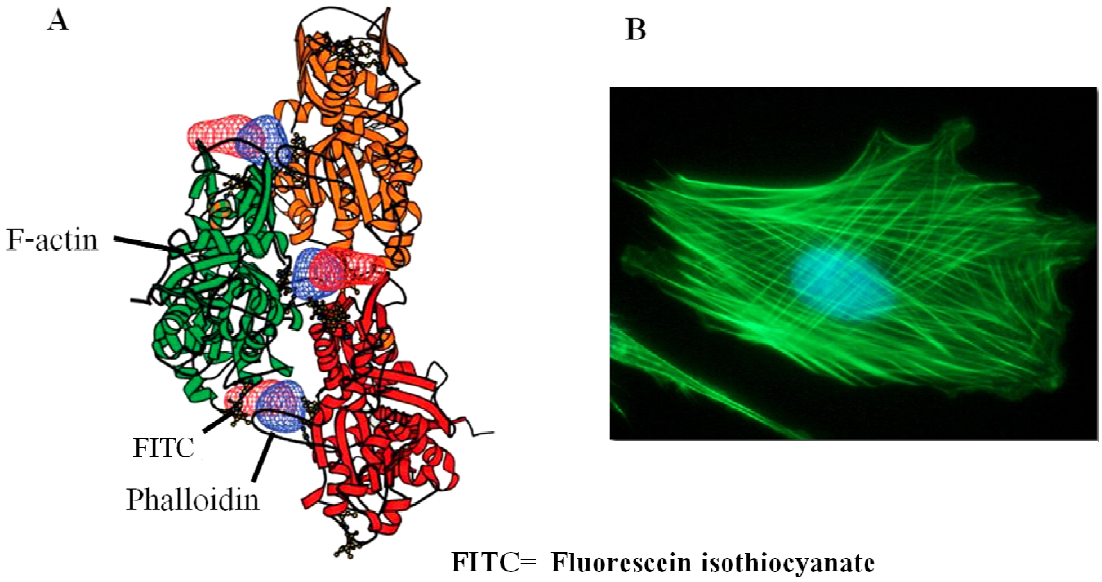

Fig. 32: The effect of bound phalloidin on contact architecture in actin filament simulations. Native conformation of F-actin allow phalloidins to bind in the groove between actin subunits. (actin subunit in orange, green, and red ribbons) Phalloidin is represented as the blue space fill matrix and the conjugated FITC as green space fill matrix **(A)**. Visualization of microtubules in fixed, permeabilized cells using FITC-phalloidin conjugates **(B)**.[71]

3. DAPI nuleus staining

Concept: DAPI (4',6-diamidino-2-phenylindole) is a DNA-specific probe which forms a fluorescent complex by attaching in the minor grove of A-T rich sequences of DNA. It also forms nonfluorescent intercalative complexes with double-stranded nucleic acids. It is used extensively in fluorescence microscopy. DAPI can pass through an intact cell membrane therefore it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower.[57]

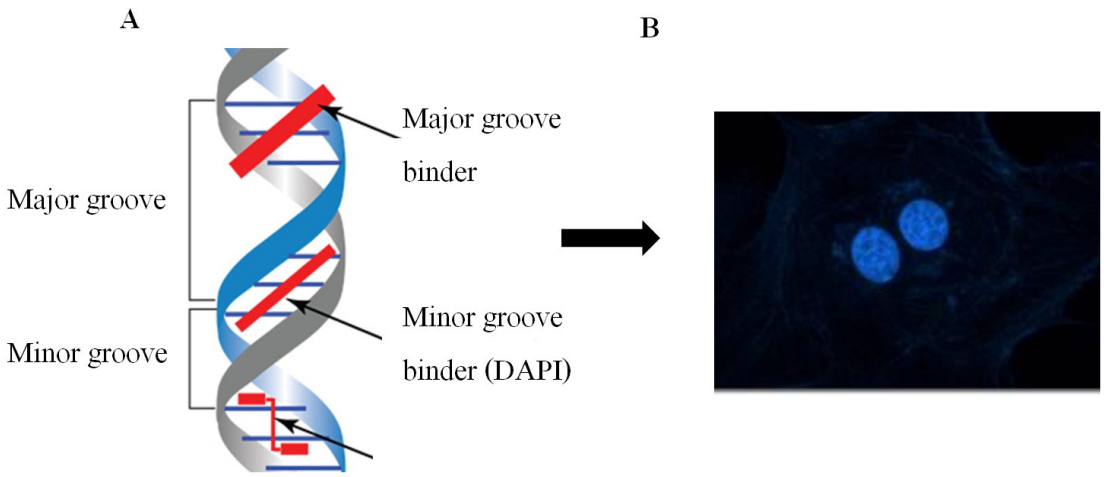

Fig. 33: The effect schematic diagram showing the binding modes of dyes to DNA, DAPI is minor groove binder type **(A)**. Representative fluorescence microscopy images of DAPI stained-cells **(B)** (Adapted from Invitrogen DNA staing kit datasheet).

APPENDIX 4

แบบเสนอโครงการวิจัย
เพื่อการพิจารณารับรองจากคณะกรรมการจริยธรรมในการวิจัย
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

1. ชื่อโครงการวิจัย

(ภาษาไทย)…**ผลของซิมวาสแตตินต่อการเกิดอะพอพโทซิสในเซลล์จากเนื้อเยื่อในโพรงประสาทฟันและเซลล์จากเอ็นยึดปริทันต์ของมนุษย์ซึ่งถูกกระตุ้นด้วยไลโพโพลีแซกคาไรด์**

(ภาษาอังกฤษ).. **Apoptotic effect of simvastatin on lipopolysaccharide-treated human pulp-derived cells and periodontal ligament cells**

1.1 ประเภทของโครงการวิจัย

☐ Intervention ☑ Non Intervention

1.2 จำนวนสถานพยาบาลที่ร่วมวิจัย

☐ Multicenters (ในประเทศ) ☐ Multicenters (ร่วมกับต่างประเทศ)

☑ Single center

2. ชื่อหัวหน้าโครงการวิจัยและที่อยู่ที่สามารถติดต่อได้สะดวก

ชื่อหัวหน้าโครงการ (ภาษาไทย)……**นางสาวศิรินาถ แซ่หว่อง**……..…

(ภาษาอังกฤษ)……**Miss Sirinart Saewong**……..

คุณวุฒิ…**ทันตแพทยศาสตรบัณฑิต** ……ตำแหน่งทางวิชาการ……..…….

ภาควิชา……**ทันตกรรมอนุรักษ์**…….โทรศัพท์…**074-429877**……

3. แหล่งทุนสนับสนุนการวิจัย ….**บัณฑิตวิทยาลัย มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่**…….

4. วัตถุประสงค์ของการวิจัย

4.1 เพื่อทดสอบความสามารถของซิมวาสแตตินต่อการเกิดอะพอพโทซิสในเซลล์จากเนื้อเยื่อโพรงประสาทฟันและเอ็นยึดปริทันต์มนุษย์

4.2 เพื่อทดสอบความเข้มข้นและเวลาของซิมวาสแตตินที่มีผลต่อความมีชีวิตและการเกิดอะพอพโทซิสในเซลล์จากเนื้อเยื่อโพรงประสาทฟันและเอ็นยึดปริทันต์มนุษย์

5. การดำเนินการวิจัย

5.1 ประชากรที่เข้ารับการศึกษา เกณฑ์การคัดเลือก (Inclusion Criteria) ประชากร และการคัดออก (Exclusion Criteria) ประชากร จำนวนประชากรทุกกลุ่ม

ประชากรที่มีสุขภาพแข็งแรง ในกลุ่มอายุ 15-30 ปี ที่มารับบริการถอนฟัน หรือผ่าตัดฟันออกเนื่องจากฟันคุด หรือถอนเพื่อการจัดฟัน ในคลินิกศัลยศาสตร์ช่องปาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ โดยที่ฟันเป็นฟันที่ไม่ผุ จากนั้นนำเอาเนื้อเยื่อในโพรงประสาทฟันและเอ็นยึดปริทันต์ไปทำการศึกษาต่อ เกณฑ์จำนวน 15 ซี่.

5.2 สถานที่ทำการวิจัย

ภาควิชาชีววิทยาช่องปาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

5.3 สิ่งที่ผู้วิจัยต้องการศึกษา (Intervention) และจะให้ (Administer)กับประชากรที่เข้าร่วมการศึกษาต้องระบุรายละเอียดดังนี้ **....ไม่มี....**

5.3.1 Intervention ที่จะศึกษาคืออะไร หาก Intervention ที่จะศึกษาเป็นยาต้องระบุชื่อสามัญ ชื่อทางเคมี และชื่อการค้าของยาที่จะนำมาใช้ในการวิจัย และต้องระบุสถานภาพของการขึ้นทะเบียนยาดังกล่าวดังต่อไปนี้

ก. ยาที่จะศึกษายังไม่ได้รับการขึ้นทะเบียนที่ประเทศใด ๆ

ข. ยาที่จะศึกษาได้รับการขึ้นทะเบียนแล้วที่ประเทศอื่น คือ (ระบุ)……..……

ค. ยาที่จะศึกษาได้รับการขึ้นทะเบียนแล้วโดยคณะกรรมการอาหารและยา กระทรวงสาธารณสุข ติดต่อสอบถามได้ที่ กองควบคุมยา สำนักงานคณะกรรมการอาหารและยา โทร. 590-7196 และ 590-7204

หากไม่ระบุสถานภาพของการขึ้นทะเบียนยาตามที่ระบุไว้ข้างต้น คณะกรรมการฯ จะยังไม่พิจารณาเอกสารทั้งหมด

5.3.2 รายละเอียด Intervention นั้นที่จะให้ (Administer) กับประชากรที่เข้าร่วมการศึกษาอย่างไร เช่น Intervention ที่เป็นยาต้องระบุขนาดยา วิธีการบริหารยา ความถี่ของการบริการยา ระยะเวลาของการให้ยา และการปรับเปลี่ยนขนาดของยา

5.4 สิ่งที่ประชากรที่ศึกษาจะได้รับหรือจะต้องปฏิบัตินอกเหนือไปจากข้อ 5.3 เช่น จำนวนครั้งที่เจาะเลือด ปริมาณเลือดที่เจาะแต่ละครั้ง จำนวนครั้งที่ผู้ป่วยต้องมาพบผู้วิจัยเป็นต้น **....ไม่มี....**

5.5 ระยะเวลาการวิจัย…..**15 เดือน**……

6. ข้อพิจารณาด้านจริยธรรม

6.1 เหตุผลและความจำเป็นที่ต้องวิจัยในคน

การพัฒนาวัสดุ pulp capping ที่ใช้รักษาเนื้อเยื่อโพรงประสาทฟันที่มีชีวิต (vital pulp therapy) วัตถุประสงค์เพื่อใช้ในฟันมนุษย์จึงมีความจำเป็นต้องทดสอบการตอบสนองของความมีชีวิตและการเกิดอะพอพโทซิสกับเนื้อเยื่อโพรงประสาทฟันและเอ็นยึดปริทันต์มนุษย์……

6.2 ประโยชน์ที่จะได้รับจากการวิจัยนี้ รวมทั้งประโยชน์ต่อประชากรที่เข้าร่วมในโครงการวิจัย **เพื่อศึกษาการตอบสนองและพัฒนาวัสดุที่จะใช้ในงานรักษาเนื้อเยื่อโพรงประสาทฟันที่มีชีวิต (vital pulp therapy)**…..

6.3 ความเสี่ยงที่ประชากรที่เข้าร่วมการศึกษาจะได้รับ ต้องระบุทั้งโอกาสที่จะเกิด รายละเอียดของ ความเสี่ยงที่อาจจะเกิด และมาตรการป้องกันและรักษารวมทั้งการชดเชยที่ผู้วิจัยได้เตรียมไว้

การศึกษานี้เป็นการศึกษานอกร่างกายโดยนำเนื้อเยื่อในโพรงประสาทฟันและเอ็นยึดปริทันต์จากฟันที่จะต้องทิ้งในภายหลังอยู่แล้ว ดังนั้นจึงไม่มีความเสี่ยงต่อประชากรที่เข้าร่วมศึกษา…….

6.4 หลักฐานหรือข้อมูล (เอกสารอ้างอิง) ที่แสดงให้เห็นว่าการวิจัยนี้น่าจะมีความปลอดภัยต่อประชากรที่เข้าร่วมการศึกษา

6.5 แบบหนังสือยินยอมเข้าร่วมการศึกษาของประชากรที่เข้าร่วมในการวิจัยที่ระบุข้อมูลต่าง ๆ ตามแบบที่คณะกรรมการฯ กำหนด

ตามเอกสารแนบท้าย…….

รายชื่อ ที่อยู่และคุณวุฒิของผู้ร่วมวิจัยทุกคน

อาจารย์ที่ปรึกษา ผศ.ดร.ทพญ. นัทธมน วัฒนอรุณวงศ์ ,D.D.S, M.Dent.Sci, Ph.D.

ผศ.ดร.ทพญ. เกวลิน ธรรมสิทธิ์บูรณ์ ,D.D.S, D.M.Sc. (Endodontics)

นักศึกษาผู้ทำวิจัย ทพญ. ศิรินาถ แซ่หว่อง, D.D.S.

7. ให้เติมข้อความต่อไปนี้ พร้อมลงลายมือชื่อหัวหน้าโครงการวิจัยและผู้ร่วมวิจัยทุกคน

ผู้เสนอโครงการวิจัยสัญญาว่าคณะผู้วิจัยจะดำเนินการวิจัยตามรายละเอียดที่ระบุไว้ในข้อเสนอโครงการวิจัยอย่างเคร่งครัด หากมีการแก้ไขข้อเสนอโครงการวิจัย ผู้เสนอโครงการจะแจ้งให้คณะกรรมการฯ ทราบโดยเร็ว เพื่อการพิจารณาอนุมัติ นอกจากนี้หากประชากรที่รับไว้ในโครงการวิจัยนี้เกิดผลข้างเคียงหรืออันตรายจากการวิจัย หรือหากมีข้อมูลองค์ความรู้ใหม่เกี่ยวกับประโยชน์หรือโทษจากแหล่งอื่นในระหว่างทำการศึกษา หัวหน้าโครงการวิจัยจะรายงานให้คณะกรรมการจริยธรรมในการวิจัย คณะทันตแพทยศาสตร์ ทราบโดยเร็ว และจะส่งรายงานการวิจัยจำนวน 1 ชุด ให้คณะกรรมการฯ ภายใน 6 เดือน เมื่อการวิจัยสิ้นสุดลงหรือเมื่อการวิจัยถูกยกเลิก

8. ลงลายมือชื่อของหัวหน้าหน่วยงานของหัวหน้าโครงการวิจัยที่อนุมัติให้ดำเนินการวิจัยได้ กรณีที่เป็นโครงการวิจัยที่เป็นวิทยานิพนธ์ จะต้องมีชื่อ พร้อมลายมือชื่อของผู้ควบคุมวิทยานิพนธ์ด้วย

ลงชื่อ ……..……...……..นักศึกษาผู้วิจัย
(ทพญ. ศิรินาถ แซ่หว่อง)

ลงชื่อ ……..……...……..…..อาจารย์ที่ปรึกษาโครงการ
(ผศ.ดร.ทพญ. นัทธมน วัฒนอรุณวงศ์)

ลงชื่อ ……..……...……..…..อาจารย์ที่ปรึกษาโครงการ
(ผศ.ดร.ทพญ. เกวลิน ธรรมสิทธิ์บูรณ์)

ลงชื่อ ……..……...……..หัวหน้าภาควิชาทันตกรรมอนุรักษ์
(ผศ .ทพ.ปรินทร หริรักษาพิทักษ์)

ใบเชิญชวน

ขอเชิญเข้าร่วมโครงการวิจัยเรื่อง……**ผลของซิมวาสแตตินต่อการเกิดอะพอพโทซิสในเซลล์จากเนื้อเยื่อในโพรงประสาทฟันและเซลล์จากเอ็นยึดปริทันต์ของมนุษย์ซึ่งถูกกระตุ้นด้วยไลโพโพลีแซกคาไรด์**……

เรียน ท่านผู้อ่านที่นับถือ

ข้าพเจ้า…**ทพญ. ศิรินาถ แซ่หว่อง**…ใคร่ขอเล่าถึงโครงการวิจัยที่กำลังทำอยู่และขอเชิญชวนท่านเข้าร่วมโครงการนี้ กล่าวคือ โครงการนี้เป็นโครงที่ศึกษาการตอบสนองของเนื้อเยื่อโพรงประสาทฟันและเอ็นยึดปริทันต์ โดยทำการศึกษานอกร่างกาย คือ นำเนื้อเยื่อโพรงประสาทฟันและเอ็นยึดปริทันต์มาทดสอบการตอบสนองต่อยาซิมวาสแตติน และไลโพโพลีแซคคาไรด์ของแบคทีเรีย….

ในโครงการวิจัยนี้ ผู้วิจัยจะขอใช้**<u>เนื้อเยื่อโพรงประสาทฟันและเอ็นยึดปริทันต์</u>**ของฟันที่ถูกถอนแล้วของผู้ป่วยที่มารับการถอนฟัน เนื่องจากฟันคุดหรือเพื่อการจัดฟันในคลินิกศัลยศาสตร์ช่องปาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ โดยไม่ได้กระทำการใดๆกับร่างกายผู้ป่วย จึงไม่ได้เพิ่มความเสี่ยงต่อผู้ป่วยแต่อย่างใด

หากท่านมีข้อสงสัยประการใดเกี่ยวกับโครงการวิจัยนี้ กรุณาติดต่อข้าพเจ้า **ทพญ. ศิรินาถ แซ่หว่อง** สาขาวิชาวิทยาเอ็นโดดอนต์ ภาควิชาทันตกรรมอนุรักษ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ โทรศัพท์ 074-429877, 081-8980310

ไม่ว่าท่านจะเข้าร่วมในโครงการวิจัยนี้หรือไม่ ท่านจะยังคงได้รับการรักษาที่ดีเช่นเดียวกับผู้ป่วยคนอื่น ๆ และถ้าท่านต้องการที่จะถอนตัวออกจากการศึกษานี้เมื่อใด ท่านก็สามารถกระทำได้อย่างอิสระ

หากท่านมีคำถามใดๆก่อนที่จะตัดสินใจเข้าร่วมในโครงการนี้ โปรดซักถามคณะวิจัยได้อย่างเต็มที่

ขอขอบคุณเป็นอย่างสูง

ลงชื่อ ……..……..……..นักศึกษาผู้วิจัย

(ทพญ. ศิรินาถ แซ่หว่อง)

ลงชื่อ ……..……..……..…..อาจารย์ที่ปรึกษาโครงการ

(ผศ.ดร.ทพญ. นัทธมน วัฒนอรุณวงศ์)

หมายเหตุ :- กรุณาอ่านข้อความให้เข้าใจก่อนเซ็นชื่อยินยอมเข้าร่วมโครงการ

แบบยินยอมเข้าร่วมการศึกษา

โครงการวิจัยเรื่อง...**ผลของซิมวาสแตตินต่อการเกิดอะพอพโทซิสในเซลล์จากเนื้อเยื่อในโพรงประสาทฟันและเซลล์จากเอ็นยึดปริทันต์ของมนุษย์ซึ่งถูกกระตุ้นด้วยไลโพโพลีแซกคาไรด์**.....

วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้า.....อายุ.....ปี อาศัยอยู่บ้านเลขที่.....ถนน.....

ตำบล.....อำเภอ.....จังหวัด.....

ได้อ่าน/ได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจากการวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว โดยผู้รับผิดชอบโครงการคือ

ทพญ. ศิรินาถ แซ่หว่อง สถานที่ติดต่อ สาขาวิชาวิทยาเอ็นโดดอนต์ ภาควิชาทันตกรรมอนุรักษ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตหาดใหญ่ โทรศัพท์ 074-429877 , 081-8980310

หรือเมื่อมีปัญหาใด ๆ เกิดขึ้นเนื่องจากการทำวิจัยในเรื่องนี้ข้าพเจ้าสามารถร้องเรียนไปที่คณบดีคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ.หาดใหญ่ จ.สงขลา 90112 โทรศัพท์ 074-28-7510

หากผู้วิจัยมีข้อมูลเพิ่มเติมทั้งด้านประโยชน์และโทษที่เกี่ยวข้องกับการวิจัยนี้ ผู้วิจัยจะแจ้งให้ข้าพเจ้าทราบอย่างรวดเร็ว โดยไม่ปิดบัง

ข้าพเจ้ามีสิทธิที่จะของดการเข้าร่วมโครงการวิจัยโดยมิต้องแจ้งให้ทราบล่วงหน้าโดยการงดการเข้าร่วมการวิจัยนี้ จะไม่มีผลกระทบต่อการได้รับบริการหรือการรักษาที่ข้าพเจ้าจะได้รับแต่ประการใด

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะที่เกี่ยวกับตัวข้าพเจ้าเป็นความลับ จะไม่เปิดเผยข้อมูลหรือผลการวิจัยของข้าพเจ้าเป็นรายบุคคลต่อสาธารณชน จะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย หรือการเปิดเผยข้อมูลต่อผู้มีหน้าที่ที่เกี่ยวข้องกับการสนับสนุนและกำกับดูแลการวิจัย

ข้าพเจ้าได้อ่าน/ได้รับการอธิบายข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ จึงได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจโดยนักวิจัยได้ให้สำเนาแบบยินยอมที่ลงนามแล้วกับข้าพเจ้าเพื่อเก็บไว้เป็นหลักฐาน จำนวน 1 ชุด

ลงชื่อ……...……ผู้ยินยอม

ลงชื่อ ……..……...……..นักศึกษาผู้วิจัย
(ทพญ. ศิรินาถ แซ่หว่อง)

ลงชื่อ ……...……...……...…..อาจารย์ที่ปรึกษาโครงการ
(ผศ.ดร.ทพญ. นัทธมน วัฒนอรุณวงศ์)

ลงชื่อ..……...……พยาน
ลงชื่อ..……...…พยาน

<u>หรือ</u>ในกรณีผู้ถูกทดลองยังไม่บรรลุนิติภาวะ จะต้องได้รับการยินยอมจาก<u>ผู้ใช้อำนาจปกครอง (บิดา/มารดา/ผู้ปกครอง)</u> **<u>ให้ผู้เกี่ยวข้องเซ็นชื่อ ดังนี้</u>**

ลงชื่อ..……...……ผู้ยินยอม
ลงชื่อ..……..บิดา/ผู้ใช้อำนาจปกครอง
ลงชื่อ……มารดา
ลงชื่อ……...……หัวหน้าโครงการ
ลงชื่อ……พยาน
ลงชื่อ……พยาน

ที่ ศธ 0521.1.03/ 969

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
ตู้ไปรษณีย์เลขที่ 17
ที่ทำการไปรษณีย์โทรเลขคอหงส์
อ.หาดใหญ่ จ.สงขลา 90112

หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง "ผลของซิมวาสแตตินต่อการเกิดอะพอพโทซิสในเซลล์จากเนื้อเยื่อในโพรงประสาทฟันและเซลล์จากเอ็นยึดปริทันต์ของมนุษย์ซึ่งถูกกระตุ้นด้วยไลโพโพลีแซกคาไรด์"

หัวหน้าโครงการ ทันตแพทย์หญิงศิรินาถ แซ่ห่วง

สังกัดหน่วยงาน นักศึกษาหลังปริญญา ภาควิชาทันตกรรมอนุรักษ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ แล้ว ในคราวประชุมครั้งที่ 7/2554 เมื่อวันที่ 10 สิงหาคม 2554

ให้ไว้ ณ วันที่ 10 ต.ค. 2554

(รองศาสตราจารย์ ดร.รวี เถียรไพศาล)
รองคณบดีฝ่ายวิจัย
ประธานกรรมการ

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ทพ.นพ.สุรพงษ์ วงศ์วัชรานนท์)

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