EFFECT OF P85, F68 AND F127 PLURONIC BLOCK COPOLYMERS ON OSTEOGENIC, CHONDROGENIC AND ADIPOGENIC DIFFERENTIATION OF HUMAN TOOTH GERM STEM CELLS (HTGSCs)

by

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this thesis is dedicated to my family

and my teachers up to this time…

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ABSTRACT

EFFECT OF P85, F68 AND F127 PLURONIC BLOCK COPOLYMERS ON OSTEOGENIC, CHONDROGENIC AND ADIPOGENIC DIFFERENTIATION OF HUMAN TOOTH GERM STEM CELLS (HTGSCs)

The use of stem cells for therapeutic applications of disorders such as Parkinson, Alzheimer, diabetes, spinal injuries and cancer have been shown to be a promising approach for the regenerative medicine. Human tooth germ stem cells which have mesenchymal stem cell characteristics have been proven to be a significant adult stem cell type with high proliferation and differentiation capacity. Biomaterials are indispensible tools in tissue engineering and among them, pluronics have gained a great interest in recent years. Pluronics, also known as "poloxamers", which consist of hydrophilic poly (ethylene oxide) and hydrophobic poly (propylene oxide) chains, are one of the most attractive polymers used as vehicles for therapeutic agents such as drugs and growth factors. In this study, we tested the effects of three pluronics P85, F68 and F127 on osteogenic, chondrogenic and adipogenic differentiation of the mesenchymal stem cells (MSCs) from human impacted 3rd molar tooth germs (HTGs). The results showed that none of the pluronics used in this study were toxic for the cells. On the other hand, during differentiation while F68 increased differentiation efficiency of MSCs into osteogenic, chondrogenic and adipogenic cell types remarkably, P85 exerted cytotoxic effect. It was found that F127 has not significantly altered the differentiation of MSCs. These data suggest for the first time that F68 has great potential to increase osteogenic, chondrogenic and adipogenic differentiation of MSCs, which might be used for the development of new tissue engineering strategies in the regenerative medicine.

ÖZET

P85, F68 ve F127 PLURONİC POLİMERLERİN İNSAN DİŞ GERM KÖK HÜCRELERİNİN KEMİK, KIKIRDAK VE YAĞ HÜCRELERİNE FARKLILAŞMASI ÜZERİNE OLAN ETKİSİ

Parkinson, alzaymır, diyabet, omurilik yaralanmaları ve kanser gibi hastalıkların terapötik uygulamalarında kök hücrelerin kullanılmasının yenileyici tıp için umut verici bir yaklaşım olduğu gösterilmiştir. Mezenkimal kök hücre özelliklerine sahip olan insan diş germ kök hücreleri üstün çoğalma ve farklılaşma kapasiteleriyle önemli bir yetişkin kök hücre tipi olarak kanıtlanmıştır. Biyomalzemeler doku mühendisliğinin vazgeçilmez araçlarıdır ve içlerinden pluronicler son yıllarda büyük ilgi görmüştür. Hidrofilik polietilenoksit ve hidrofobik polipropilenoksit zincirlerinden oluşan poloxomer olarak da bilinen pluronic polimerleri ilaç ve büyüme faktörleri gibi terapötik ajanların taşınmasında kullanılan en ilgi çekici polimerlerdendir. Bu çalışma kapsamında P85, F68 ve F127 pluronic polimerinin insan yirmi yaş dişlerinden elde edilen mezenkimal kök hücrelerin kemik, kıkırdak ve yağ hücrelerine farklılaşması üzerine olan etkileri test edilmiştir. Elde edilen sonuçlar çalışmada kullanılan hiçbir pluronic polimerin hücreler için toksik olmadığını gösterdi. Diğer taraftan farklılaşma sürecinde F68 mezenkimal hücrelerin kemik, kıkırdak ve yağ hücre tiplerine farklılaşma yeteneklerini önemli ölçüde artırırken P85 toksik etki göstermiştir. Çalışmada F127'nin mezenkimal kök hücre farklılaşmasını önemli ölçüde değiştirmediği bulundu. Sonuçlar ilk kez F68'in mezenkimal kök hücrelerin kemik kıkırdak ve yağ farklılaşması için önemli bir potansiyele sahip olduğunu göstermiştir, bu da yenileyici tıp için yeni doku mühendisliği stratejileri geliştirilmesine olanak sağlayacaktır.

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LIST OF SYMBOLS / ABBREVIATIONS

G	Gram
h	Hour
mM	Milimolar
M	Molar
min	Minute
ml	Mililiter
N	Normal
nm	Nanometer
v	Volume
w	Weight
µL	Mikroliter
ACAN	Aggrecan
ALP	Alkaline phosphatase
ASCs	Adipose derived Stem Cells
ATP	Adenosine Tri Phosphate
ATPase	Adenosine Tri Phosphatase
BCRP	Breast Cancer Resistance Protein
BGLAP	Bone gamma-carboxyglutamic acid-containing protein
BLAST	Basic Local Alignment Search Tool
BBMEC	Bovine Brain Microvessel Endothelial Cell
BMMSCs	Bone Marrow Mesenchymal Stem Cells
BMP-2	Bone Morphogenetic Protein 2
CD	Cluster of Differentiation
CFU-F	Colony Forming Unit Fibroblast
CHO	Chinese Hamster Ovary
CMC	Critical micelle concentration
Col I	Collagen Type I
Col II	Collagen Type II

DAPI	4',6-diamidino-2-phenylindole
DFPCs	Dental Follicle Precursor Cells
DMEM	Dulbecco's modified eagle medium
DNA	Deoksiribonükleik asit
DPSCs	Dental Pulp Stem Cells
DSCs	Dental Stem Cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FA	Fatty Acid
FABP4 (AP2)	Fatty Acid Binding Protein 4 (adipocyte protein)
FACS	Fluorescence Activated Cell Sorter
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT1	Glucose transporter
GSH	Glutathione
HA	Hyaluronic Acid
HCL	Hydrochloric Acid
HepG2	Human hepatocellular liver carcinoma cell line
HLB	Hydrophilic-lipophilic balance
HSC	Hematopoietic Stem Cell
HTGSCs	HumanTooth Germ Stem Cells
IDPSCs	Immature dental pulp stem cells
MCT1	Monocarboxylate Transporter 1
MDR	Multi Drug Resistant
MRP	Multidrug Resistance Proteins
MSCs	Mesenchymal Stem Cells
MTBE	Methl-Tert Butyl Ether
MTS	Methyl Tetrazolium Salt
NaOH	Sodium hydroxide
NCBI	The National Center for Biotechnology Information
NPMS	Nonporous microspheres

Oct4	Octamer-binding transcription factor 4
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDLSCs	Periodontal Ligament Stem Cells
PE	Phycoeritrin
PEO	Polyethylene oxide
Pgp	P- glycoprotein
PMS	Porous microspheres
PPO	Polypropylene oxide
PSA	Penicilin Streptomycin Amphicilin
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SCAP	Stem Cells from Apical Papilla
SHED	Stem cells from human exfoliated deciduous teeth
SSEA	Stagespecific Embryonic Antigens
TGF-β1	Transforming Growth Factor-Beta 1
TRA	Tumor Recognition Antigens

1. INTRODUCTION

1.1. GENERAL OVERVIEW OF STEM CELLS

Stem cells are undifferentiated cells with proliferation, self renewal and multilineage differentiation capacity [1]. Each stem cell divides and gives two cells; one of them remains as a stem cell and the other one is a specialized cell with different functions [2]. Stem cells are uncommitted cells till they receive a signal from the surrounding environment to turn into a special cell type. Stem cells are able to differentiate into several specialized cell types leading to the repair of damaged tissues in the body.

It has been reported that embryonic stem cells obtained from embryo are pluripotent and able to differentiate into all cell types in the body. In contrast, adult stem cells are restricted in their differentiation capacity. Stem cells play role in homeostasis as well as maintaining blood, skin turnover [1]. Based on their differentiation capacity they can be classified into three groups. Totipotent stem cells are derived from early embryo (morula stage until 16 cell) and they are able to give rise to a complete organism. Pluripotent stem cells reside in the inner cell mass of the blastocyst and can develop to almost 200 different cell types in the body. Finally multipotent stem cells which are derived from adult tissues are able to differentiate into a limited number of cell types [3].

Depending on their sources stem cells can be divided into five groups including embryonic stem cells, embryonic germ cells, fetal stem cells, umbilical cord blood stem cells and adult stem cells shown in Figure 1.1. Embryonic stem cells can be obtained from zygote or 2-cell, 4-cell, 8-cell and morula stages of embryo. In addition to this, inner cell mass of the 5 to 6 day of blastocyst is another source for embryonic stem cells [4]. Human embryonic germ cells which are originated from the 5 to 9 week old fetuses are pluripotent stem cells and are able to give all cell types derived from three germ layers [5]. Fetal stem cells are obtained from fetus and expanded in culture easily [6].

Umblical cord blood stem cells are much more primitive than bone marrow derived stem cells and do not cause a severe immune response. Moreover, they can be cryopreserved for later use. The matrix of umblical cord blood also termed as Wharton's jelly has been shown to be a source for mesenchymal stem cells such as Placenta [7]. Finally adult stem cells, which are found in adult body after embryonic development and disperse into the different places of the human body, are promising candidates for stem cell based therapy. Progenitor or precursor cells in adult tissues are moderately differentiated cells with a limited differentiation potential [8]. Adult stem cells are not found in all organs and they are rare. The main job of adult stem cells is to provide homeostasis by replacing cells in injury or disease [9-10].

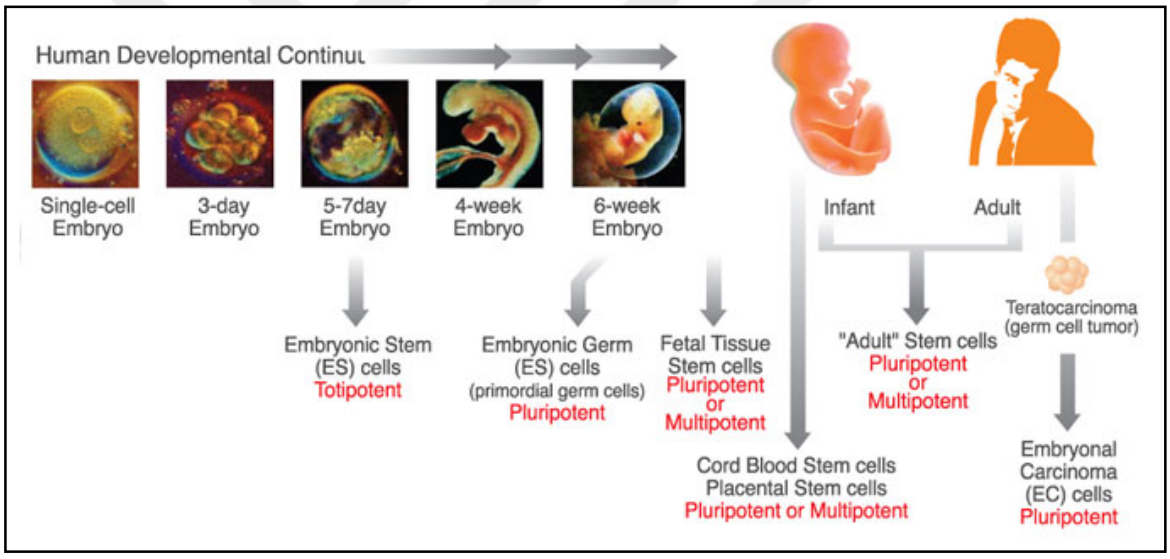


Figure 1.1. Sources of stem cells [11]

Adult stem cells are mostly clonogenic and have self renewal potential. They should give identical cells that can differentiate into cell types of the tissue to which they belong. Hematopoietic stem cells and mesenchymal stem cells are two classes of adult stem cells. Hematopoietic stem cells derived from bone marrow are naturally able to differentiate into blood cells and they were reported to differentiate into osteogenic and neurogenic cells [12].

Mesenchymal stem cells are generally found in nonhematopoietic bone marrow but bone marrow is not the only source of mesenchymal stem cells. Mesenchymal stem cells (MSCs) can be isolated from different sources including bone barrow, adipose tissue, synovial membrane, skeletal muscle, dermis, pericytes, trabecular bone, human umbilical cord, lung, dental pulp, amniotic fluid, fetal liver, and even peripheral blood, suggesting that MSCs are widely dispersed in the body [13]. It has been considered for a long time that adult stem cells are restricted about producing specific cell types rather than three cell types: osteogenic, chondrogenic and adipogenic cells which are originated from mesodermal layers [14]. In the following part we will discuss MSCs in detail.

1.2. BIOLOGY OF MESENCHYMAL STEM CELLS

MSCs were first identified in guinea pig bone marrow by Friedenstein in 1970s [15]. MSCs have self-renewal and differerentiation capacity which makes them popular for tissue engineering applications [16]. Cultured mesenchymal stem cells can easily grow and adhere to the culture dishes and are characterized by using different methods. Obtaining the antigenic profile of MSC surface by fluorescence activated cell sorting is an essential and reliable method for characterization of stem cells. Mesenchymal stem cells are positive for mesenchymal stem cell markers such as CD29, CD73, CD90, CD105 and CD 166 and negative for hematopoietic antigens such as CD45, CD34 and CD14. Colony forming unit fibroblast assay (CFU-F) is also used to identify MSCs [15].

Mesenchymal stem cells have multipotent differentiation capacity which means that mesenchymal stem cells can differentiate into mesodermal originated cell lines including osteoblasts, chondroblasts, and adipocytes under appropriate *in vitro* and *in vivo* conditions. However, a lot of evidence suggests that under appropriate *in vitro* conditions MSCs have the ability to undergo unorthodox differentiation (meaning that bone marrow stromal cells can differentiate into unrelated tissues), giving rise to cells with characteristics of visceral mesoderm, neuroectoderm and endoderm [17]. Mesenchymal stem cells from different species such as human, canine, rabbit, rat, and mouse were demonstrated to differentiate into some types of tissues including bone [18-19], cartilage

[20], tendon [21-22], muscle [23-24], adipose tissue [25-26] and hematopoietic supporting stroma [26].

Different strategies have been developed to increase differentiaton potential of mesenchymal stem cells including use of specific growth factors and culturing MSCs in the presence of biomaterials with the aim of providing the natural niches during stem cell differentiation. Molecular mechanisms that control MSCs differentiation have not been completely understood yet but Baksh *et al.* in 2004 suggested a model combining two continuous yet distinct compartments shown in Figure1.2. Transcriptional modification occurs in the first compartment that givinig rise to precursor cells with no obvious changes in phenotype and self renewal capacity. Precursor cells divide symetrically and give rise to tripotent and bipotent precursor cells which are similar to multipotent MSCs morphologically but quiete different in terms of their gene transcription profile. Precursor cells continue to divide and give rise to unipotent precursor cells followed by entering to the commitment compartment and acquiring lineage specific properties [27]. Transcription factors, cytokines, growth factors, and different extracellular matrix molecules take place in the commitment and differentiation of MSCs to mature cell types [28-29].

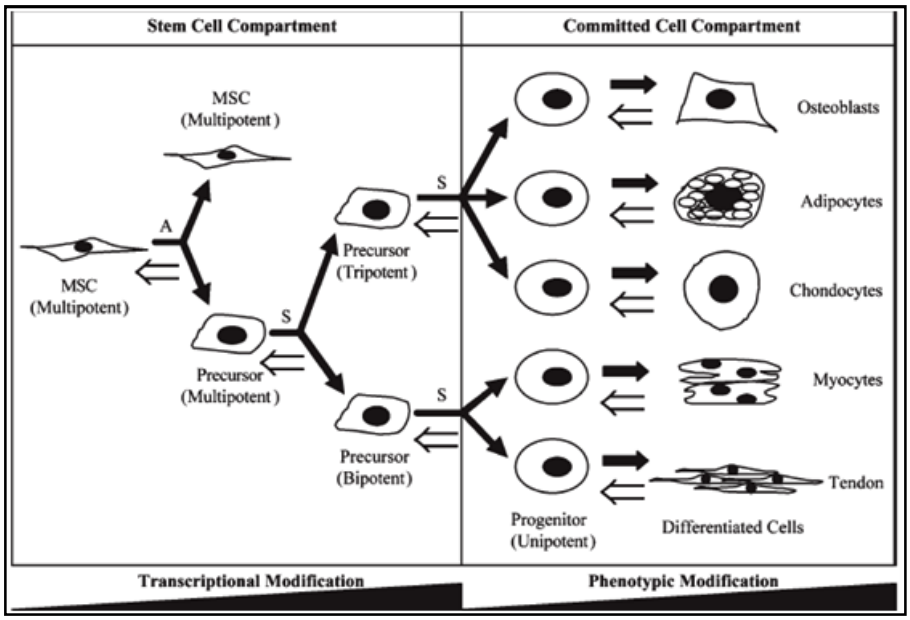


Figure 1.2. Model of adult stem cell differentiation [27]

Plasticity is a physiological need for tissue homeostasis and plasticity of bone marrrow stem cells gives them the multidifferentiation potential [30]. Although chondrocytes, osteoblasts, adipocytes and reticular cells belong to different tissues, they come from the same precursor cell carrying a osteogenic commitment marker. This plasticity is called as orthodox. On the opposite, the unorthodox plasticity means that bone marrow stromal cells can differentiate into unrelated tissues. In the past years, it was shown that bone marrow cells could generate neural cells[31] and hepatocytes [32].

In our study, we used dental derived MSCs. Although bone marrow is the primary source for mesenchymal stem cells, there are some disadvantages in using these cells in clinical trials. Isolation procedure of bone marrow MSCs is invasive and painful, on the other hand, dental derived mesenchymal stem cells are easy to obtain from waste dental tissues and expanded in culture plates with almost no ethical problems. In the following part, we will give a brief overview of the dental stem cells.

1.3. DENTAL STEM CELLS

Dental stem cells have gained a big attention in recent years as a source of adult stem cells. Dental tissues can be isolated from different sources; deciduous teeth, third molar teeth or other teeth derived as a result of different dental treatments. [33]. Dental mesenchyme is named as ectomesenchyme because of its interaction with neural crest. Thus, stem cells that are isolated from these dental tissues have mesenchymal stem cell characteristics and can differentiate into several other cell types including neurogenic cells.

Dental pulp is a soft connective tissue in the center of the tooth that contains nerves and blood vessels and surrounded by the dentine. Dental pulp is the combination of both ectodermic and mesenchymal components, including multipotent neural crest cells [34]. From the outer layer to the inner layer the pulp is divided into four parts: The first part (external layer) contains odontoblasts that produces dentin, the second part, named “cell free zone”, rich about extracellular matrix content, the third part, also named as “cell rich zone”, rich in progenitor cells that have plasticity and pluripotential properties, the inner part, that includes the vascular area and nervous plexus [35]. Post-natal dental pulp contains stem cells which take place in dentin formation. Adult pulp stem cells were first

identified by Gronthos and collages in 2000 [36]. Dental pulp and bone marrow stem cell express the same stem cell surface markers, including CD44, CD106, CD146 and Stro-1 [37]. It has been suggested that STRO-1 (cell surface protein) in dental pulp identifies cells with both odontogenic and multilineage potential [38].

Both dental pulp stem cells and bone marrow stem cells produce matrix proteins related with mineral tissue formation incluiding alcaline phosphatase and osteocalcin. Instead of their dentinogenic differentiation potential dental pulp stem cells are able to differentiate into adipogenic and neurogenic cells [39]. It was shown recently that Dental pulp stem cells could also differentiate into osteogenic, chondrogenic and myogenic cells [40]. Dental pulp includes stem cells with high odontogenic differentiation capacity [41]. The dental stem cells obtained from human exfoliated deciduous teeth also known as SHED and are shown to be highly proliferative and also named as immature dental pulp stem cells (IDPSCs) expressing embryonic stem cell (ESC) markers such as Oct4, Nanog, stagespecific embryonic antigens (SSEA-3, SSEA-4), and tumor recognition antigens (TRA-1-60 and TRA-1-81) [34]. Devoloping teeth contain a soft tissue named as apical papilla and this part of the tooth includes a cell rich zone where stem cells can be isolated [42]. Dental follicle cells are derived from dental follicles originating from an ectomesenchymal tissue containing progenitor cells which are able to generate the periodontium (as an axample cementum, periodontal ligament and alveolar bone) [43]. Dental follicle contains the developing tooth and odontogenic organ and it can differentiate into periodontal ligament. The dental follicle is an ectomesenchymal tissue surrounding the enamel organ and dental papilla of the tooth germ includes stem cells and progenitor cells for cementoblasts, periodontal ligament cells and osteoblasts. Dental follicle coordinates the tooth eruption [44]. Periodontal ligament stem cells are isolated from periodontal ligament of the tooth that contains cementum-forming cells (cementoblasts) and bone-forming cells (osteoblasts). Periodontal ligament stem cells take place in tissue homeostasis and the regeneration of peridontal tissue [45-46]. Periodontal ligament is a connective tissue that fix the attachment of the teeth whose fibers are reached to the cementum and the alveolar bone and embedded in. The differentiation of dental follicle cells are controlled by growth factors and cytokines. Cells in different parts of the dental follicle differentiate into different tissues such as, some cells differentiate into cementoblasts which produce cementum and some of them differentiate into osteoblasts

which secrete the bone matrix. Stem cells in the periodontal ligament and dental pulp from human third molars and deciduous teeth were recently identified. These cells are colony forming cells and look like bone derived human mesenchymal stem cells [33-36].

1.3.1. Human Tooth Germ Stem Cells (HTGSCs)

Human tooth germ stem cells are isolated from the third molar human tooth germs of young adults at the ages between 10 and 16. They have been demonstrated to have mesenchymal stem cell characteristics. The tooth germ is an aggregation of the progenitor cells that forms a tooth. It consists of the dental papilla, the dental follicle, and the enamel organ. HTGSCs are primarily derived from the immature dental pulp and dental follicle tissues shown in Figure 1.3 [47]. Third molar tooth germs were reported to develope after the age of six but remain undifferentiated before this time. This actually lays behind the fact that MSCs derived from third molar tooth germs are highly proliferative and mutipotent [34].

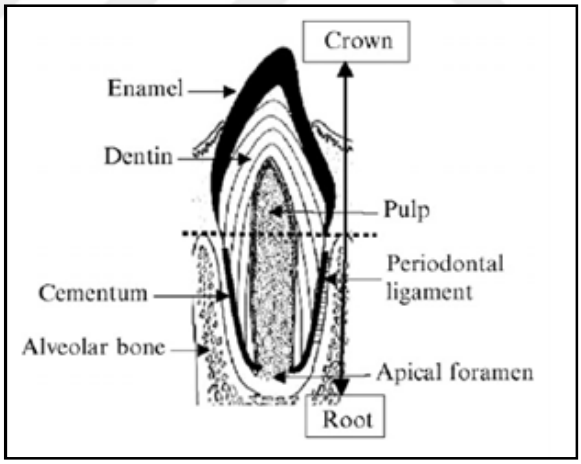


Figure 1.3. Structure of tooth [47]

It was also reported that stem cells that reside in tooth germs have the ability to differentiate into several cell lines that are originated from 3 germ layers: ecto-, meso- and endo-derm [48].

1.4. TISSUE ENGINEERING OF BONE, CARTILAGE AND ADIPOSE TISSUE

Osteogenesis is an important process that takes place in fetal development and through the life. Various pathological conditions such as trauma, inflammation and surgical treatment for neoplasia cause the loss of the bone tissue. In all these situations, it is crucial to find out methods to replace the damaged bone tissue or to help the body repair its damage by increasing the osteogenesis [49-50]. Tissue engineering and regenerative medicine approach generally combine the suitable progenitor cells with the appropriate biomaterial scaffolds to repair tissue damages. Different types of stem cells such as bone marrow stem cells, adipose derived stem cells or even induced pluripotent stem cells might be used to produce osteogenic tissues [51]. In addition these biomaterials, which serve as vehicles or scaffolds, are successful in the tissue engineering. Appropriate three dimensional structure of biomaterials and their interaction with the cellular and microenvironmental components affects the incorporation of the engineered tissues into the endogenous tissues. Scaffolds to be used in bone regeneration must have properties such as osteocunduction capacity (the ability to provide cell attachment, migration and proliferation), osteoinduction (the ability to provide environmental osteogenic stimula), biocompatibility and biodegradability [51].

Cartilage formation is an important process for the body during the fetal development and postnatal lifetime. In orthopaedic surgery articular cartilage damage is one of the essential issue for therapy. Cartilage defects which are caused by congenital abnormalities, diseases and traumas have limited the intrinsic healing potentials since the cartilage tissue does not have enough blood supply and wound healing response. This naturally results in chondrocytes that can not proliferate and regenerate the damaged cartilage tissue effectively [52]. Although hyaline cartilage can repair its small losses, the fibrous tissue and fibrocartilage which are different from normal hyaline cartilage in terms of their biochemical and biomechanical properties are necessary for larger defects to be repaired [53]. There are different methods for the cartilage repair but the most promising one is the mesenchmal stem cell based cartilage tissue engineering. In bone marrow stimulation method it is expected that bone marrow-derived chondroprogenitor cells heals the cartilage lesion. The technique is favoured by orthopaedic surgeons because it is cheap compared with the others. In these method, the defects can only be repaired by the help of fibrous

tissue or fibrocartilage tissue, because, the induced chondroprogenitor cell number is too small to provide a full cartilage repair [54]. Mosaicplasty is another method which uses the autologous osteochondral transplantation to the cartilage defect lession. The method is good for providing the hyaline cartilage but it is not easy to obtain autologous osteochondral plugs which limits the utility of this method [55].

In autologous chondrocyte implantation method chondrocytes are collected from biopsy and are expanded *in vitro* and at the end the cells were injected into the cartilage defect. Autologous chondrocyte implantation has succesful results for clinical applications but it has some disadvantages such as the loss of transplanted cells, hypertrophy of periosteum [56-57], loss of chondrogenic phenotype for cells in monolayer culture and the invasiveness of surgical method [58]. In cartilage engineering, multipotent mesenchymal stem cells can be isolated from different sources. However, it is important to take signals from microenvironment to induce mesenchymal stem cell differentiation into cartilage and as well as protecting their phenotype. Chondrogenic differentiation can be managed by specific factors *in vitro* giving rise to chondrogenic cells that can be used in tissue engineering approach. On the other hand productivity of matrices is of great importance for tissue repair. Tissue engineering can overcome these problems by using *ex vivo* techniques and compatible biomaterials. Therefore, design of new generation biomaterials are crucial for chondrocytes to differentiate, grow and maintain their phenotypes [59].

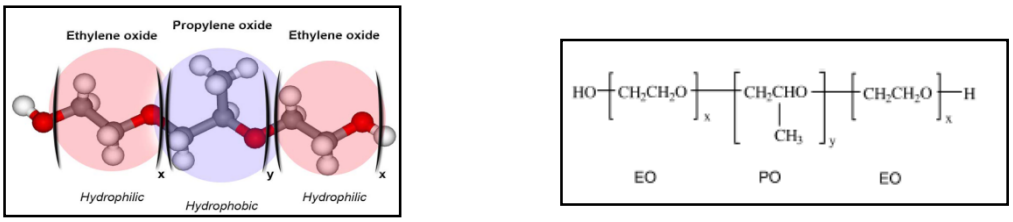
Adipogenesis starts during embryonic development and continues thorough the life in a controlled way forming soft tissues in the body. Soft tissue repair is higly crucial for clinical approach. Adipose tissue defects, as usually seen after traumas, abnormalities, deep burns, tumor resections, mastectomies [60], cosmetic facial problems of cheek, chin, and jaw [61-62], and lipodystrophies related with type II diabetes [63] need to be repaired with the adipose tissue engineering. Although autologous and allogenic adipose tissues have a limited proliferation capacity and replacing potential of adipocyte damage, they can be used as therapeutic materials for the adipose tissue engineering [64]. It has been recently shown that cell based adipose tissue engineering which combines adipogenic progenitor cells with biomaterials, may be a promising method for the soft tissue engineering. [65-66]. It was also shown that mesenchmal stem cells can be an unlimited source of adipogenic cells in the presence of specific factors. However, it is limited to

create and protect a long term stable, viable and functional adipose tissue by exogenously-delivered stem cell populations because of biomaterials with insufficient biocompatibility and rapid degradation rates [65-67]. Therefore, appropriate biocompatible biomaterials combined with injectable adipogenic precursor cells need to be developed for the successfull regenerative therapy applications.

Variety of biopolymers have been reported to be very successful in tissue repair and replacements therapies. Among them pluronics are good candidates for tissue engineering because of their properties such as biocompatibility, biodegredability and low cost [68]. In following part some detailed information about these functional biopolymers will be provided with a specific focus on three biopolymers used in this study.

1.5. PLURONIC TRIBLOCK COPOLYMERS

Polymer based technology is one of the most attractive approach for pharmaceutical research and applications. Pluronics, also known as “poloxamers” which consist of hydrophilic poly(ethylene oxide) and hydrophobic poly (propylene oxide) chains are one of the most attractive polymers used as vehicles for therapeutic agents, drugs or genes. Polyethylene oxide (PEO) and polypropylene oxide (PPO) blocks are formed in A-B-A tri-block structure: PEO-PPO-PEO [69]. Pluronic molecule and molecular formula of pluronic was shown in Figure 1.4 [70-71].



a b

Figure 1.4.a. Pluronic molecule and 1.4.b. molecular formula of pluronic [70-71]

Different types of pluronics can be found as commercial products and these pluronics differ depending on their numbers of PPO and PEO blocks. These copolymers are named with the letter "P" (for poloxamer) generally followed by two or three digits. For three

digits; the first two digits x 100 give the roughly molecular mass of the polyoxypropylene chain, and the last digit x 10 gives the percentage of polyoxyethylene units. For two digits; the first digit x 300 gives the approximate molecular mass of the polyoxypropylene chain, and the last digit x 10 gives the percentage of polyoxyethylene units. Pluronics are coded with letters according to their physical form at room temperature (L = liquid, P = paste, F = flake (solid)) [70-72].

Different numbers of ethylene oxide and propylene oxide units can alter the hydrophilic-lipophilic balance (HLB) of block copolymers. Thanks to their amphiphilic character which is the result of tri-block structure, these polymers exhibit surfactant properties such as interaction the with biological membranes. Pluronic block copolymers are synthesized by the consecutive polymerization of poly ethylene oxide and poly propylene oxide units with an alkaline catalyst [73]. Concentrations higher than critical micelle concentration (CMC) in aqueous solutions cause self-assembley of copolymers forming micelles. The diameter of pluronic micelles changes between 10 nm and 100 nm [69]. Hydrophobic PPO blocks generate the core of the micelles having the capacity absorbing different therapeutic reagents or drugs. Figure 1.5 shows pluronic micelle.

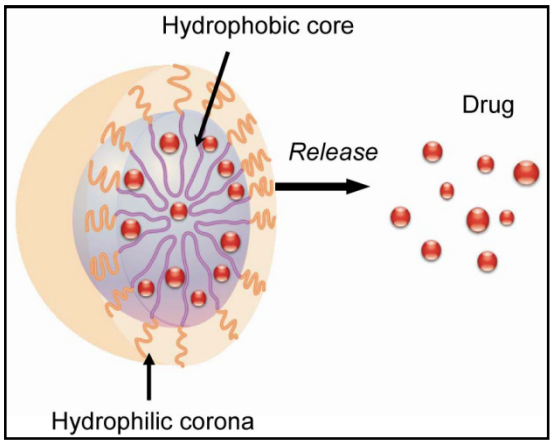


Figure 1.5. Pluronic micelle with a solubilized drug [70]

Unimers are single block copolymer molecules formed at concentrations below the critical micellization concentration. Unimers have the ability of incorporation and translocation through the cellular membranes. The hydrophobic PPO chains of Pluronic triblock copolymers interact with hydrophobic areas of the membrane which causes change

of the membrane structure, and reduces membrane microviscosity ("membrane fluidization"). However, at high concentrations pluronic block copolymers produce micel forms in which PPO chains are hidden in the micellar core preventing pluronics from interacting with the cellular membranes [74].

Pluronic triblock copolymers can be used for different applications and have different effects on cell metabolism which was shown in Figure 1.6 [70]. When they are used as drug delivery agents they are inert and prevent drug from degradation and enhance the uptake of drug by tissues [75]. Interaction of pluronics with the plasma membrane causes inhibition of Pgp (P-glycoprotein) or MRP (Multidrug Resistant Protein) ATPase activity [70]. On the other hand, micellar concentrations cause repair of Pgp ATPase activity. There is a complex mechanisms about pluronic effects on MDR (Multidrug Resistant) cells [70]. It was shown that pluronic block copolymers:

- Interact with membranes by altering microviscosity [70]
- Inhibit Pgp [76-77-78], multidrug resistance proteins (MRPs) [79] and breast cancer resistance protein (BCRP) [80-81]
- Increase secretion of cytochrome C and reactive oxygen species (ROS) levels in the cytoplasm [70]
- Trigger proapoptotic pathway and prevent anti-apoptotic mechanism in MDR cells [82]
- Inhibit the glutathione/glutathione S-transferase detoxification system [74]
- Inhibit drug sequestration within cytoplasmic vesicles [83]
- Control shear stress in bioreactors increasing cell survival under stress conditions [84].

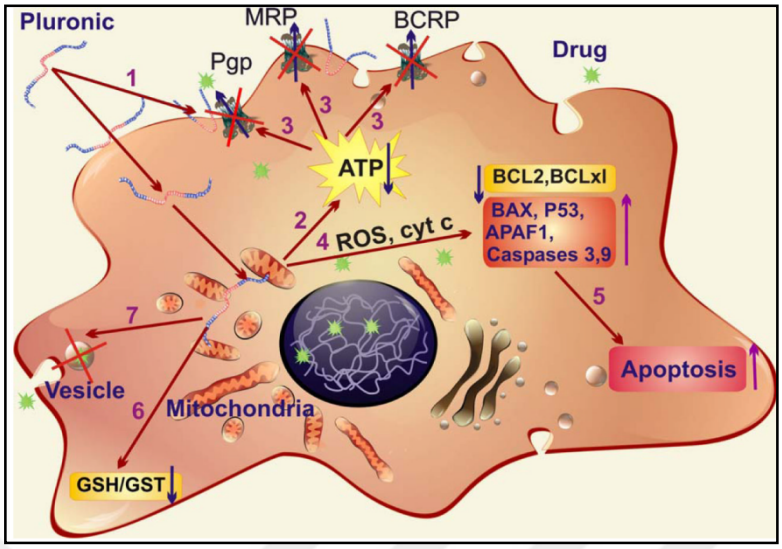


Figure 1.6. Effects of pluronic block copolymers on MDR cells [70]

Inhibition of ATP production in MDR cells are one of the most important effect of pluronics on cell metabolism resulted in the sensitization of MDR cancer cells to chemotherapeutic agents [85]. It was demonstrated *in vitro* [86] and *in vivo* [77] that transition of therapeutic agents across the brain microvessel endothelial cells and intestinal barrier cells is enhanced with the presence of pluronics. Normal cells are sensitive for glucose and utilize glucose as energy source, while MDR cells are sensitive for fatty acids and use fatty acids and glucose [87]. Interaction of hydrophobic chains of pluronics with fatty acids causes the immobilization of fatty molecules inside the micelle cores of pluronics affecting the respiration mechanism as a concequence of altering the activity of membrane bound enzymes.

Pluronic transition across plasma membrane into intracellular compartments is done by caveole- mediated endocytosis [88] directing these triblock copolymers to the endoplasmic reticulum [89] and to the mitochondria [90-91]. Interaction of pluronics with mitochondrial membrane might have important effects on cellular metabolism. In addition to these effects, one of the most important roles of pluronics is triggering the apoptotic pathway. Interaction of pluronic molecules with mitochondria membranes leads to change of the membrane structure resulting in the excessive release of cytochrome C and enhances ROS (Reactive Oxygen Species) levels in the cytoplasm. Accordingly all these events may

also increase the mitochondria dependent apoptosis [82]. Different kinds of pluronics incluiding the ones used in this study were reported to severely induce the expression of proapoptotic genes and inhibit that of anti-apoptotic genes when they are combined with apoptotic agents. Glutathione (GSH) detoxicification system is an important mechanism in the cell to hinder the oxidative stress dependent cell death. GSH is a tripeptide protecting cells against ROS. In MDR cells some pluronics such as P85 cause a reduction in GSH levels leading to apoptosis [92].

Pluronic block copolymers can also be useful for gene therapy applications by increasing the transfection efficiency. It was previously demonstrated that Pluronic block copolymers induce plasmid DNA transfection and expression in the mice antigen presenting cells and efficiently enhanced the plasmid DNA expression in the skeletal muscle, spleen, and lymph nodes [93-94]. It was shown that pluronic block copolymers can be suitable agents for nonviral gene therapy by increasing the gene tranfer and inducing the transcription of the genes.

Another important field for pluronic research is tissue engineering. Pluronics can be used in tissue engineering directly or in combination with other biomaterials and appropriate growth factors. It was shown that some pluronics act as cell encapsulation agents and exert positive effects on the tissue repair [95]. In this study, we focused on three pluronics, P85, F68 and F127, which have been reported to have different pharmaceutical properities. In the following part, detailed information is given about these three pluronics.

1.5.1. Pluronic P85

P85 pluronic block copolymer is a symmetric tri-block copolymer with the $EO_{25}PO_{40}EO_{25}$ formula that is consisted of a central poly(propylene oxide) block with poly(ethylene oxide) blocks on the each end. Figure 1.7 shows structure of P85. It is in paste form and its solubility in water is higher than 10% [96].

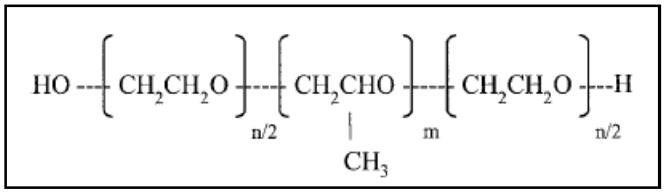


Figure 1.7. Structure of P85, n:52, m:40

In solutions at the critical micelle concentration (CMC) unimers self assemble to produce micelles. P85 have 67 mM CMC value and 4600 dalton molecular weight. These polymers inhibit P-gp drug efflux system by increasing the uptake of P-glycoprotein-dependent drugs (Pgp) to the cells. However, P85 has the ability of permeabilization at concentrations below the CMC [97], especially when combined with a substrate of an ATP-dependent drug efflux mechanism [86-74]. P85 is widely used to enhance of drug transport to the brain because it can easily transport from the blood brain barrier. P85 is generally used in bovine brain microvascular endothelial cells (BBMECs) to inhibit drug transporters [98] or against cancer cells along with anticancer agents [99].

1.5.2. Pluronic F68

Pluronic F-68, also named as Pluronic PE 6800, is a, non-ionic and low foaming surfactant composed of a central polypropylene oxide and two polyethylene oxide groups. Figure 1.8 shows F68 structure [100-101].

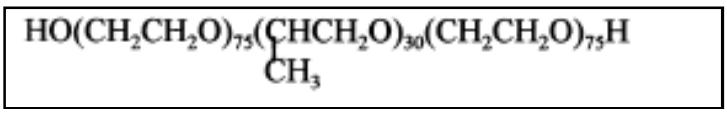


Figure 1.8. Structure of F68

Pluronic F-68 is a white powder with a 8350 Daltons molecular mass. It is soluble in water and has a neutral pH. The percentage of polypropylene oxide (% hydrophobicity) is about 20. It disperses calcium and magnesium salts [101]. The critical micelle concentration (CMC) is 1.1 mM [102]. Pluronic F-68 does not create micelles but produces two to three layers of the block polymers which attach to the membrane surface and prevent aggregation [101]. Up to this time, the general use of Pluronic F-68 is to prevent

cells from the effects of shear forces in culture situations (shaker and spinner cultures). It does this action by monitoring the cell bubble interactions [103].

1.5.3. Pluronic F127

Pluronic F127 is a polyoxyethylene-polyoxypropylene triblock copolymer with the $EO_{106}PO_{70}EO_{106}$ formula. F127 is a white powder with an average molar mass of 13,000 Daltons [104-105]. Hydrophilicity of the F127 is due to the 70% ethylene oxide content [106-107]. Figure 1.9 shows structure of F127.

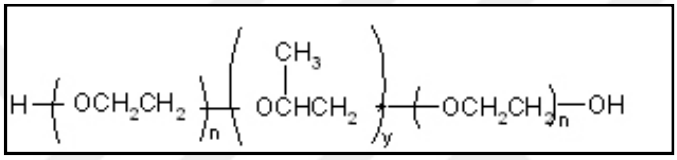


Figure 1.9. Structure of F127, n:100, y:65 [108]

Because of effective solvation and hydrogen bonding at lower temperatures F127 can dissolve more in cold water than in hot water [109]. F127 is a perfect carrier for drugs and has many applications for different drug formulations. It is also used in scaffold systems and has a usage in the field of tissue engineering [110-111].

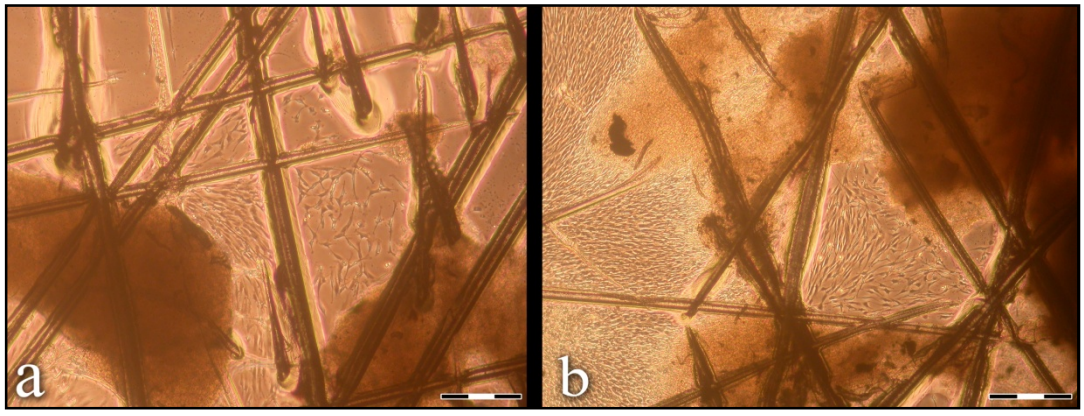
1.6. AIM OF THE STUDY

In this study, it was aimed to demonstrate the effects of three pluronics, P85, F68 and F127 on osteogenic, chondrogenic and adipogenic differentiation of HTGSCs with MSCs charecteristics. By testing these functional polymers on MSCs it is expected to develop a new strategy for increasing the effectiveness of MSC based tissue engineering applications in bone, cartilage and adipose tissue regeneration. This study, for the first time, demonstrates pluronics` (P85, F68 and F127) potential roles in the differentiation of MSCs in a comparative way.

2. MATERIALS AND METHODS

2.1. ISOLATION OF HTGSCS

Human tooth germs were taken from the wisdom teeth of 13 year old patient. Isolated tooth germs were harvested into small pieces and plated in six well plates with Dulbecco's modified essential medium (Invitrogen, Gibco, UK, cat # 31885) supplemented with 10 % fetal bovine serum (Invitrogen, Gibco, UK, cat # 10270-106) and 1 % PSA (Invitrogen, Gibco, UK, cat # 15240-062). Cells started to expand on six well plate surface in three to four days and reached 80% confluency after eight days. Figure 2.1 shows expanded cells. After this time cells were removed from plates with 0.25% (w/v) tyrpsin-EDTA (Invitrogen, Gibco, UK, cat # 25200). Medium was added to unattached cells to block trypsin negative effects on cells. After centrifugation at 1500 rpm for 5 minutes cell pellet was suspended in fresh medium and transfered to T-75 (Zelkultur Flaschen, Switzerland, cat # 90075) flask. These cells were cultivated in an incubator (Thermo, US, model no: 3131) at 37 °C with 5% CO2 and 95% humidity. Medium was changed every day and cells were passaged on alternate days [112].



Figüre 2.1.a. Isolated Human Tooth Germ Stem Cells at day 3 and 2.1.b. day 8, Scale bar: 400µm

2.2. CHARACTERIZATION OF HTGSCs

Characterization of cells was done according to the procedure used by our group [112]. Cells were removed from flask with tyrpsin-EDTA (Invitrogen, Gibco, UK, cat # 25200) and incubated with primary antibodies prepeared in PBS (Invitrogen, Gibco, UK, cat # 10010, pH 7,4) for one hour. In the study, primary anti-bodies against CD29 (cat #BD556049), CD34 (cat # SC-51540), CD45 (cat # SC-70686), CD90 (cat # SC-53456), CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat # SC-53551) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA, 100 testes in 2ml) and CD73 (cat # BD 550256) (Zymed, San Francisco, CA, USA) were used with 1:100 dilution. Cells were washed with PBS (Invitrogen, Gibco, UK, cat # 10010, pH 7,4) to discard excess primary antibodies. After that cells were incubated with fluorescein-iso-thio-cyanate (FITC)-conjugated chicken antimouse secondary antibodies (cat # SC-2989, 200µg/0.5ml) at 4°C for one hour, except for CD29 against which phyco-erythrin (PE) – red light-harvesting protein containing chromophore – conjugated monoclonal antibody was used. The flow cytometry analysis of the cells were completed using Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA, USA, model no: 342975) flow cytometry system. 5000 cells were counted for each sample.

2.3. PREPARATION OF PLURONICS

Pluronics were prepared according to the protocols described by Exner *et al.* in 2005 [113]. P85 (BASF, USA, cat # 30085877), F68 (BASF, USA, cat # 52389638) and F127 (BASF, USA, cat # 55401892) pluronic block copolymers were dissolved in PBS at 10% (w/v) concentration by vortexing and incubated on ice to disperse the polymer. Because pluronics exhibit thermoreversible gelation which means that they can easily dissolve at low temperatures and form gels at room temperature for high concentrations. 10% (w/v) concentrations were diluted to 1% (w/v) concentration in medium with no serum (DMEM) and sterilized with 0.2µm filter (Sartorius, minisart, Germany, cat # 16534). Solutions were kept at 4ºC until use.

2.4. TOXICITY ASSAY FOR DIFFERENT CONCENTRATIONS OF PLURONICS

Four concentrations (0.01%, 0.02%, 0.05%, 0.1% w/v) of three pluronics were prepared in DMEM with 10% (v/v) fetal bovine serum and 1% (v/v) PSA from 1% stock concentrations. HTGSCs at passage 2 were seeded on 96 well plates (BIOFIL, TCP, Switzerland, cat # 011096) at a concentration of 5000cells/well followed by addition of four concentrations of the three pluronics. The cell viability was measured by the MTS-assay (CellTiter96 Aqueous One Solution, Promega, UK, cat # 2587530) according to the manufacturer's instructions. MTS (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2- (4-sulfo-phenyl)-2H-tetrazolium) is a tetrazolium-salt based colori-metric assay for detecting the activity of enzymes (mostly in the mitochondria) that reduce MTS to formazan, giving a purple color [112]. Briefly after incubating the cells with pluronics for 24, 48 and 72 hours, 10µl MTS reagent with 100µl growth medium was added to each well incubated for two to three hours followed by reading absorbance at 490 nm with an ELISA (Biotek, model no: EL800) plate reader. The cell viability was also measured during differentiation on day 1, day 3, day 5 and day 7 in order to see the effect of pluronic P85 to differentiating cells.

2.5. DIFFERENTIATION PROCESS

In this study, HTGSCs were induced to differentiate into osteogenic, chondrogenic and adipogenic cells. For osteogenic and adipogenic differentiation, HTGSCs at passage 2 were counted and seeded in 24 well plates (BIOFIL, TCP, Switzerland, cat # 011024) at a concentration of 15.000cells/well followed by addition of pre-made differentiation media (STEMPRO, Gibco, UK, cat # A10069-10066, A10410-10065) to the cells with and without pluronics, P85, F68 and F127 at concentration of 0.05% (w/v). The cells were incubated in a humidified (95%) incubator at 37^{o}C with 5% CO2 for 7-10 days changing the differentiation media with and without pluronics every other day. For chondrogenic differentiation micromass culture system was applied as previously described [114]. Shortly, 12,5 µl medium droplets containing 250 000 cells placed in center of each well of 24 well plate. The plates were put in incubator for two hour to provide cell attachment followed by addition of 500µl pre-made chondrogenic medium (STEMPRO, Gibco, UK, cat # A10069-10064) and changing the medium every other day for 15 days.

2.6. ALP ACTIVITY ASSAY

ALP enzyme activity assay was done to prove osteogenic differentiation. After osteogenic differentiation the cells were trypsinized and centrifuged at 1000 rpm for 5 minutes. The pellets were resuspended in 500µl cell lysis buffer containing 0.2% (v/v) triton-X-100 diluted in PBS incubating for 30 minutes at room temperature shaking at 850 rpm. After lysis of cells, 25µl of protein lysate and 75 µl of Randox reagent (ALP ligand) (Randox ALP detection kit-RANDOX, UK, cat # AP542) were mixed in 96-well plate and incubated for 15 mins followed by measuring absorbance at 405nm by using an ELISA (BIOTEK, model no: EL800) plate reader in order to detect enzyme activity.

2.7. VON KOSSA STAINING

Von kossa staining was done to show calcium deposition as a parameter of osteogenic differentiation. After 10 days of incubation with osteogenic medium the cells in 24 well plate were fixed with 2% (w/v) Paraformaldehyde (Sigma, US cat # 158127) at 4ºC for 30 mins. Cells were rinsed with distilled water after fixation and stained with Von Kossa kıt (Bio optica, Italy, cat # 04-170801) according to the protocol described by manufacturer. Briefly, 10 drop Reagent A (Lithium carbonate saturated solution) was put on the cells and left to act for 10 mins. Then the cell were rinsed with distilled water and 10 drops of reagent B (Silver nitrate solution) was put on the cells and left to act in dark for 1 hour. After incubation the cells were rinsed with distilled water and then 10 drop of reagents C (Reducing Solution) and 5 drop of distilled water were placed on the cells for 5 mins (until silver salts become black). The cells were rinsed in distelled water and ten drop of regent D (Sodium sulphate solution) was placed on samples and left to act for 5 mins. Then the samples were rinsed in distelled water and ten drop of reagent E (Mayer's Carmalum) was put on the cells and left to act for 5 mins. Finally, the cells were rinsed with distilled water and dehydrated by applying 99% alcohol followed by observation under light microscope.

2.8. ALCIAN BLUE STAINING

Alcian blue staining was done to prove chondrogenic differentiation. Alcian blue staining solution was preapered by dissolving 1g alcian blue dye (Sigma, USA, cat #

335630) in 100 ml 3% (v/v) acetic acid. The cells were first fixed with 2% (w/v) paraformaldehde for 30 min and stained with Alcian blue staining solution by incubating for 30 mins. After incubation staining solution was removed and the cells were washed with PBS for three times. Samples were observed under light microscope [115].

2.9. OIL RED STAINING

Oil red staining was done to show lipid vesicles as a parameter of adipogenic differentiation. Oil red staining solution was prepeared by dissolving 0.5 g oil red (Sigma, USA, cat # O0625) in 100ml isopropanol. The cells were fixed with 2% (w/v) paraformaldehyde for 30 min. After that they were rinsed with PBS, stained with oil red diluted (6:4) in PBS for one hour. Then the cells were washed with PBS and observed under light microscope [116].

2.10. IMMUNOCYTOCHEMISTRY ANALYSIS

HTGSCs were fixed in 2% (w/v) paraformaldehyde by incubating for 30 mins at 4ºC. The cells were washed three times for 5 minutes with PBS by gentle shaking on the plate shaker. Permeabilization of cells was done by incubating the cells with 0.1% (v/v) Triton-X 100 diluted in PBS for 5 minutes at room temperature. The cells washed again with PBS three times for 5 minutes. Then they were incubated with 2% goat serum (Sigma, US cat # G9023) diluted in PBS for 20 min at 4ºC in order to block non-specific binding of antibodies followed by washing with PBS three times for 5 minutes. The cells were incubated with primary antibodies with 1:100 dilutions (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4ºC. For osteogenic differentiation collagen type I (Santa-Cruz 59772) and osteocalcin (Santa-Cruz 30044, 200µg/ml), for collagenic differentiation collagen type II (Santa-Cruz 28887, 200µg/ml) and for adipogenic differentiation FABP4 (Santa-Cruz 136150, 50µg/0.5ml) primary antibodies were used. After incubation with antibodies the cells were washed with PBS three times for 5 mins to remove unbound primary antibodies. Goat anti rabbit IgG Alea Fluor 488 (Invitrogen, USA, cat # A11008), Goat anti mouse IgG Alea Fluor 488 (Invitrogen, USA, cat # A11001) secondary antibodies were added to the samples and waited for 1 hour at 4ºC. The cells were rinsed with PBS three times for 5 minutes. The nuclei of the cells were stained with DAPI (4',6-diamidino-2-phenylindole) (Applichem, Germany, cat #

A40990010) by adding DAPI (100:0.1 dilution) on the cells and incubating for 20 min at 4ºC followed by rinsing with PBS for three times. The stained samples were observed under fluorescence microscope (Nicon Eclipse TE200, Germany, Model no: CCD1300B).

2.11. PRIMER DESIGNING and RT-PCR ANALYSIS

Primers for collagen type II, aggrecan and FABP4 genes were designed by using Primer BLAST online software of The National Center for Biotechnology (NCBI) and ordered from Invitrogen company to be synthesized at 50 nmoles. The other primers sequenced were used as previously described in the literature. Table 2.1 shows primers that were used in this study.

Table 2.1. Primers that were used in this study.

Primer	Sense (5'-3')	Antisense(5'-3')	Base pair
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA	123 [117]
Osteonectin	ATGAGGGCCTGGATCTTCTT	CTGCTTCTCAGTCAGAAGGT	576 [118]
Col II	GTGTGGAAGCCGGAGCCCTG	GGTCCTGGTTGCCCACTGGC	170
Aggrecan	ACTGCTGCAGACCAGGAGGT	TCCTCGGGGGTGACGATGCT	152
FABP4	GGGTCACAGCACCCTCCTGA	TGGTGGCAAAGCCCACTCCTAC	162

Total RNAs from differentiated samples were isolated using High Pure RNA isolation kit (ROCHE, USA, cat # 11828665001) according to the manufacturer's instructions. cDNA was made by using cDNA High Fidelity cDNA synthesis kit (ROCHE, USA, cat # 05081955001). SYBRgreen real time PCR method was used to detect the gene levels. cDNAs from cells incubated with and without pluronics during differentiation and that from control cells were mixed with primers and SYBR Premix Ex Taq (TAKARA, Japan, cat# RR041) in a final volume of 20µL. Table 2.2 shows amount of reagents. GAPDH (glycer-aldehyde-3-phosphate-de-hydrogenase) gene was used as the house-keeping gene for normalization of the data. All RT-PCR experiments were done

using iCycler RT-PCR (Bio-Rad, Hercules, CA, USA, icycler iQ Optical Module) detection system. PCR conditions were shown in Table 2.3.

Table 2.2. Regents in PCR

Reagents	Volume
SYBRGreen	10µl
Primer Forward (10µM)	0.4µl
Primer Reverse (10µM)	0.4µl
Distilled water	4.2 µl
Template (100ng/ml)	5 µl

Table 2.3. PCR conditions

cycle	repeats	step	dwell time	Set point
1	1	1	3 min	93°C
2	40	1	30 sec	93°C
-	-	2	40 sec	61°C
-	-	3	45 sec	72°C
3	1	1	10 min	72°C
4	110	1	12 sec	40°C
5	1	1	-	4°C

2.12. FATTY ACID ISOLATION

Fatty acid isolation was done to find out pluronics effect on membrane fatty acid profile. Fatty acid isolation was carried out to find out the effect of pluronics on fatty acid profile. Isolation was done according to the protocol described by our group previously [119]. HTGSCs were counted and seeded in petri dishes at a concentration of 1 million cells/dish. The next day growth media with 0.05% (w/v) Pluronic P85, F68 and F127 were added to the cells. At day three, the cells were trypsinized and cell pellets were collected by centrifugation at 1000 rpm for 5 mins. The cells pellets were transfered to the glass screw cap tubes. 1ml of 1.2 M NaOH dissolved in 50% aqueous methanol was added to

pellets and incubated in a water bath at 100 ºC for 30 mins. They were left at room temperature for 25 mins for cooling followed by addition of 2ml of 54% (w/v) 6 N HCL prepared in 46% aqueous methanol (v/v) and incubated in a water bath at 80ºC for 10 mins. After rapid cooling on ice, fatty acids were extracted in 1.25 ml solution containing 50% methl-tert butyl ether (MTBE) in hexane leading to formation of a bottom and top phases in the tube. The bottom phase was removed with a pasteur pipette. The top phase was mixed with 3ml of 0.3M NaOH and cleaned with the anhydrous sodium sulfate followed the transfer into a 1ml clean glass vials for analysis in MIDI Fatty Acid Analysis System (Agilent Technologies, USA, model no: 6890-N).

2.13. STATISTICAL ANALYSIS

Graphics were drawn using Microsoft Office Excel and GraphPad Prism5 softwares and Standard errors and t-test values were calculated using GraphPad Prism5 software. For statistical analysis student t test was used by the GraphPad prism5 programme p value less than 0.05 was considered statistically significant.

3. RESULTS

3.1. CHARACTERIZATION OF HUMAN TOOTH GERM STEM CELLS

HTGSCs were characterized for their surface antigens CD29, CD34, CD45, CD90, CD105, CD133, CD166 and CD73 by using Flow cytometry. Cells were shown to be positive for CD29, CD105, CD90, CD73, CD166 and negative for CD34, CD45, CD133. Results were shown in figure 3.1. This data prove that HTGSCs are positive for MSCs markers and negative for HSC markers.

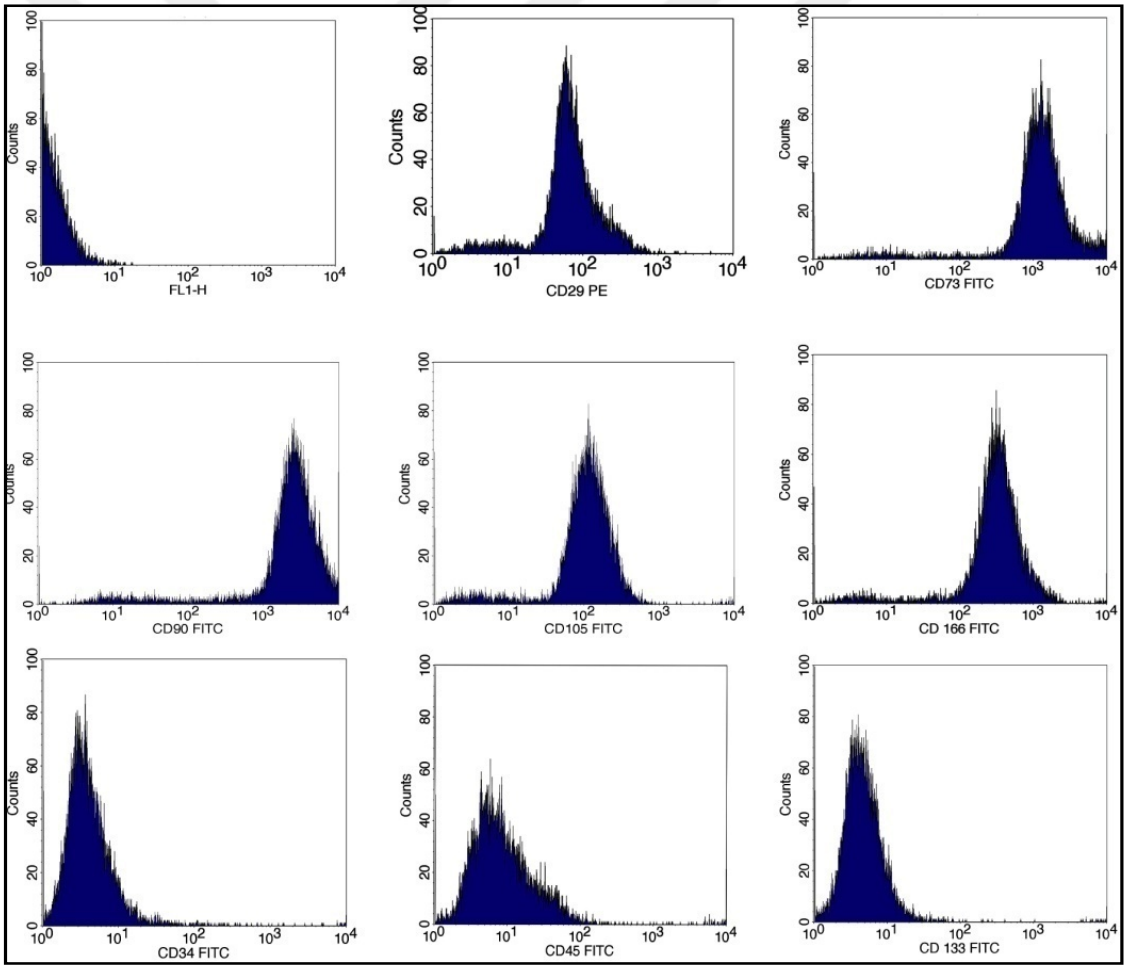


Figure 3.1 Flow cytometry analysis of HTGSCs

3.2. TOXICITY OF PLURONIC BLOCK COPOLYMERS

Depending on the litrature, toxicity assay was performed at four concentrations of three pluronics for three days. The results showed that none of the pluronics were toxic for the cells. It was shown in figure 3.2.a, b, c.

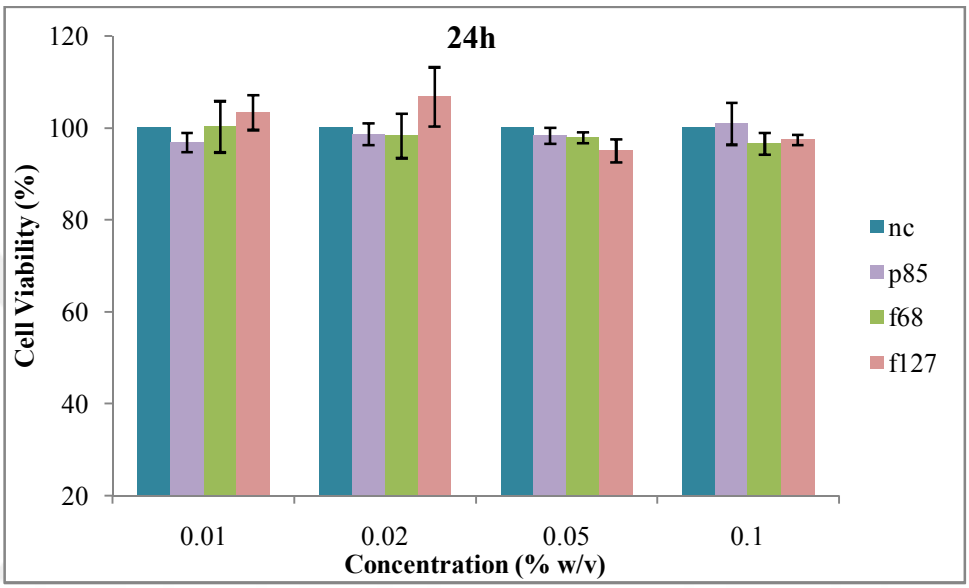


Figure 3.2.a. Toxicity results of four concentrations of three pluronics for 24h

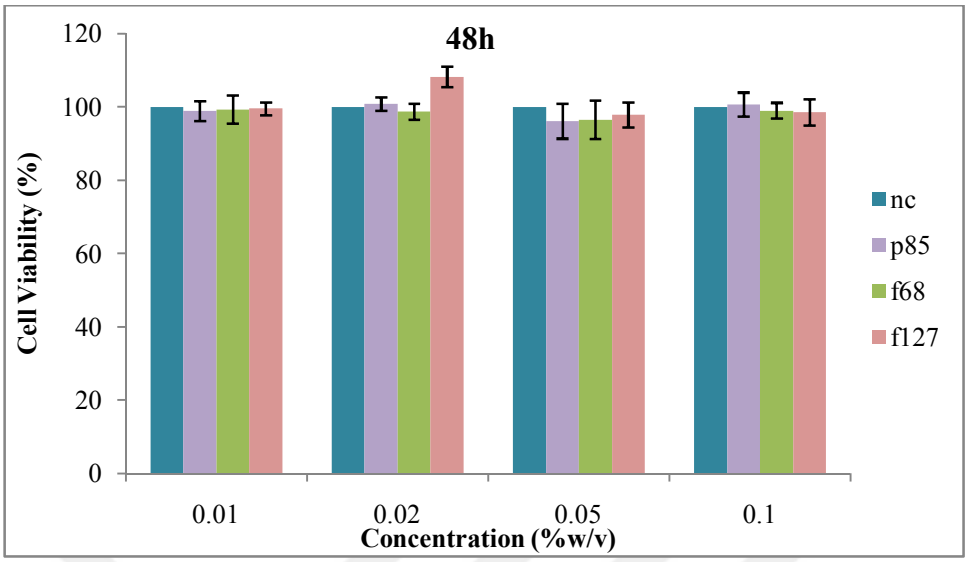


Figure 3.2.b. Toxicity results of four concentrations of three pluronics for 48h

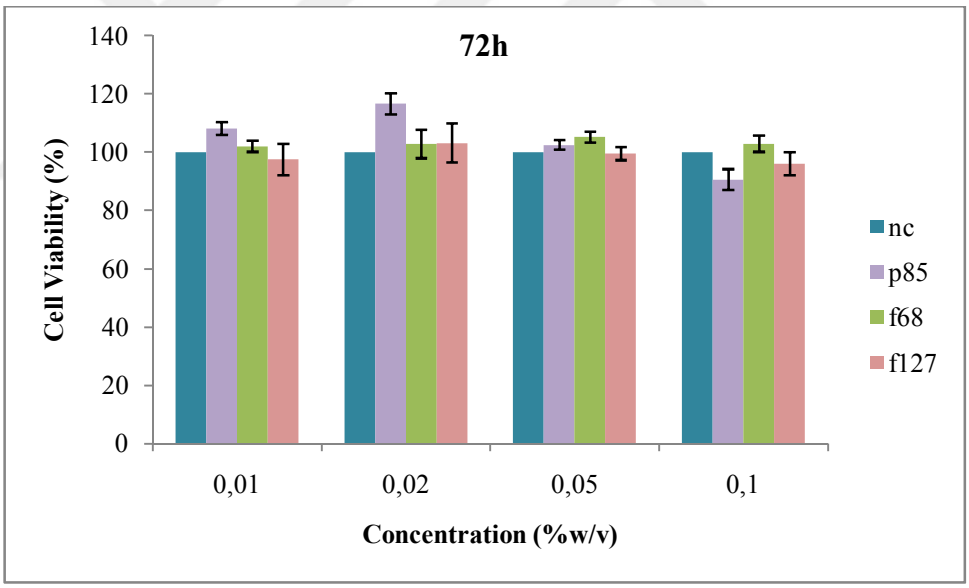


Figure 3.2.c. Toxicity results of four concentrations of three pluronics for 72h

3.3. P85 TOXICITY DURING DIFFERENTIATION

During the study we realized that P85 exerted toxic effects starting on the fifth day of differentiation process. Upon this we checked the cytotoxicity of P85 during osteogenic, chondrogenic and adipogenic differentiation on 1st, 3rd, 5th and 7th days. It was shown that P85 started to show toxic effects on the first day of the differentiation at 0.05% concentration and on the fifth day of the differentiation at 0.01% concentration. Figure 3.3.a, b and c show the results.

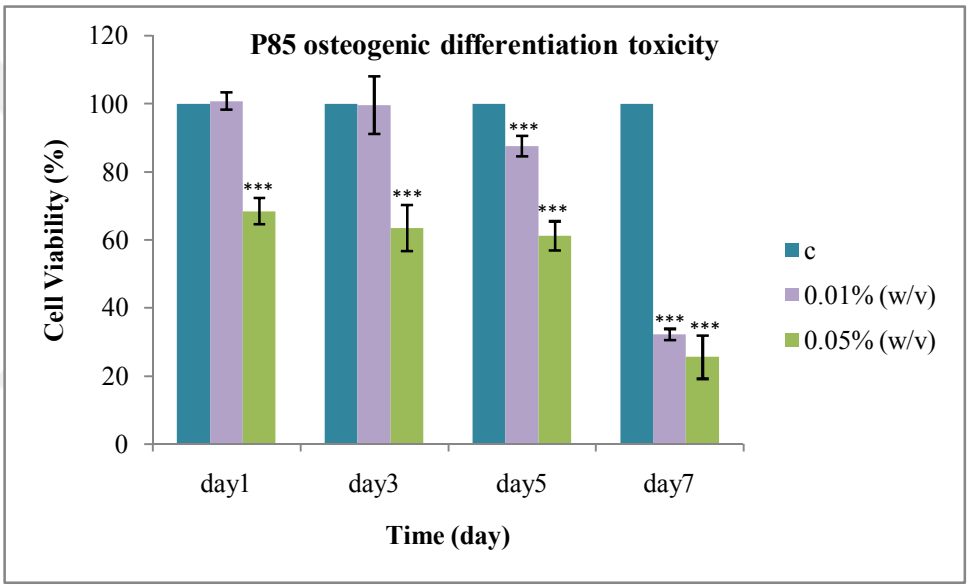


Figure 3.3.a. P85 Osteogenic differentiation toxicity (*p<0.05). 0.05% (w/v) concentration of P85 started to exert toxic effects at day1 and 0.01% (w/v) concentration of P85 started to exert toxic effects at day 5

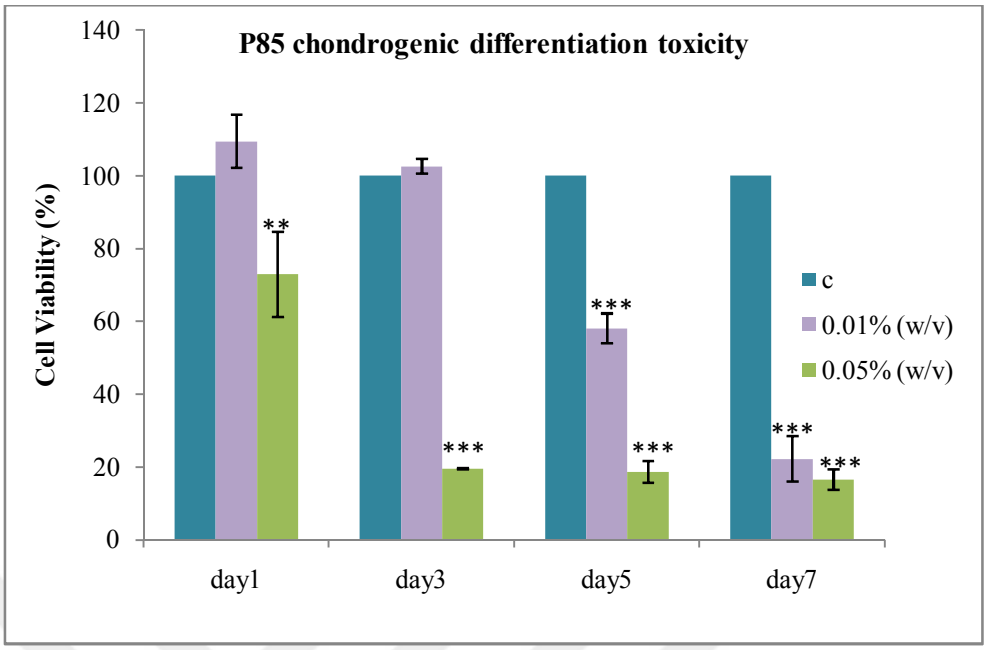


Figure 3.3.b. P85 Chondrogenic differentiation toxicity (*p<0.05). 0.05% (w/v) concentration of P85 started to exert toxic effects at day1 and 0.01% (w/v) concentration of P85 started to exert toxic effects at day 5

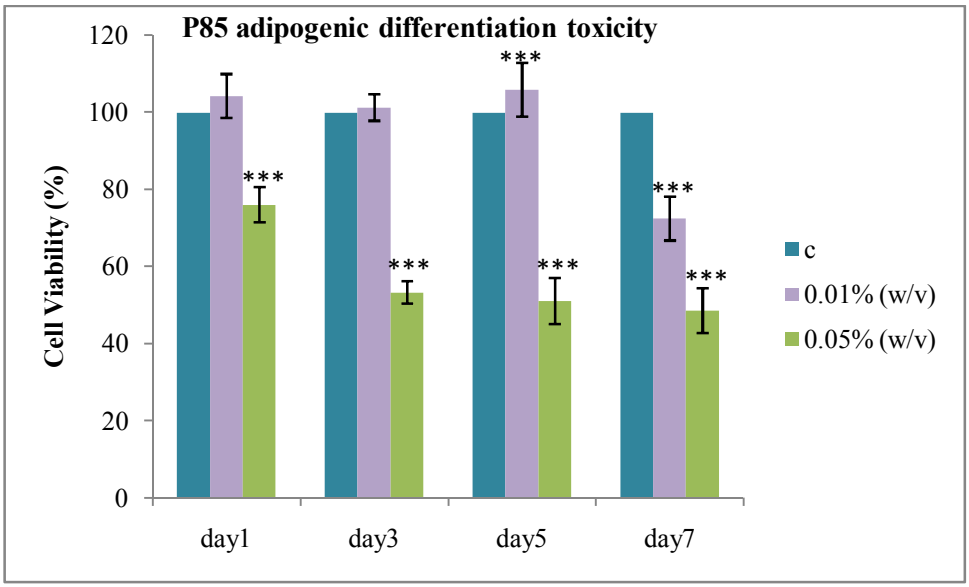


Figure 3.3.c. P85 Adipogenic differentiation toxicity (*p<0.05). 0.05% (w/v) concentration of P85 started to exert toxic effects at day1 and 0.01% (w/v) concentration of P85 started to exert toxic effects at day 7

3.4. FATTY ACID ISOLATION

FA analysis showed that P85 and F127 had a slight effect on fatty acid profile of the cells compared to the control group. Hovewer, F68 were demonstrated to alter the fatty acid profile significantly compared to control group. The results revealed that pluronic P85 caused cells to lose two fatty acid (sum 4 and sum in 4) and gain a new FA (18:2w6c). Pluronic F127 exerted similar effects with P85. The only different FA found in F127 group was 22:1w9c alcohol. Pluronic F68 changed FA profile of cells by causing cells to gain four new unsaturated fatty acids (16:1w9c (1.9%), 16:1w7c (2.2%), 18:2w6c (4.6%) and 22:1w9c alcohol). F68 also caused significant decrease in the levels of saturated FAs Figure 3.4.a, b and c show the results.

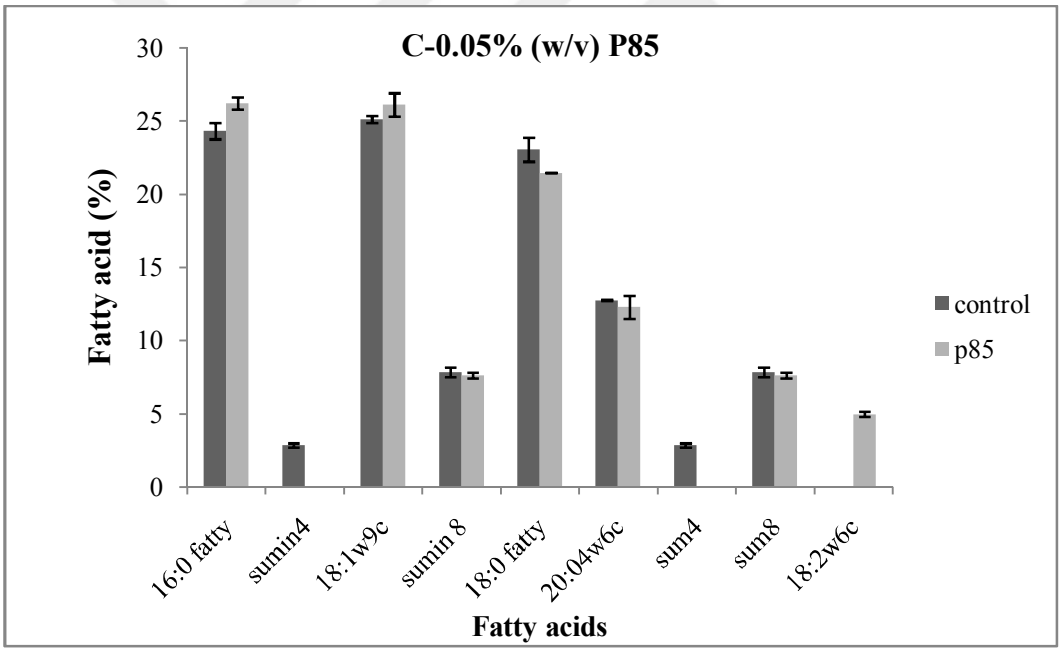


Figure 3.4.a. Fatty acid profile comparison between P85 and Control. Summed featured 4 (summed in featured 4): 15:0 iso 2-OH, 16:1ω7c and summed featured 8 (summed in featured 8): 18:1ω7c, 18:1ω6c

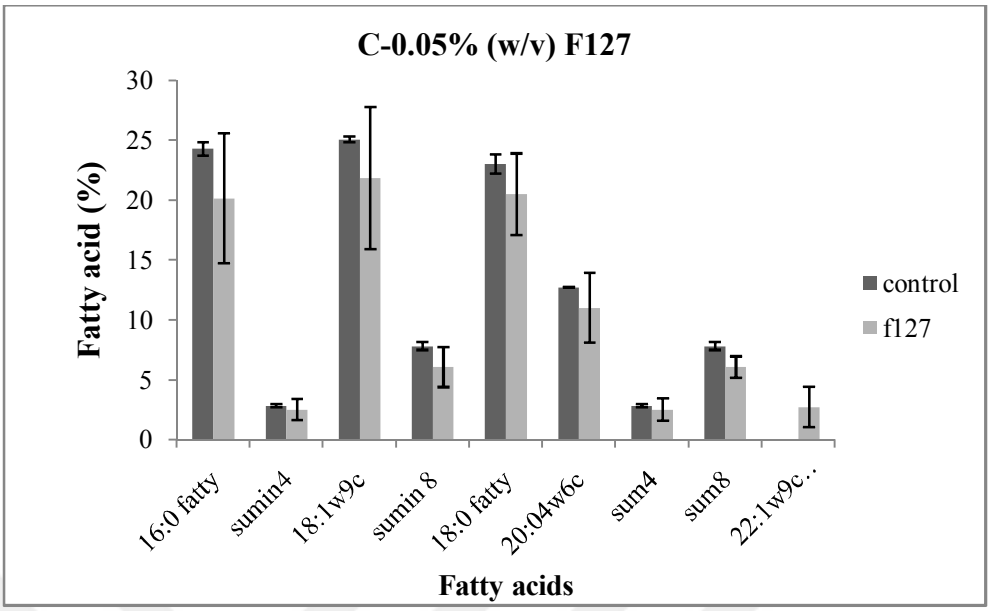


Figure 3.4.b. Fatty acid profile comparison between F127 and Control. Summed featured 4 (summed in featured 4): 15:0 iso 2-OH, 16:1ω7c and summed featured 8 (summed in featured 8): 18:1ω7c, 18:1ω6c

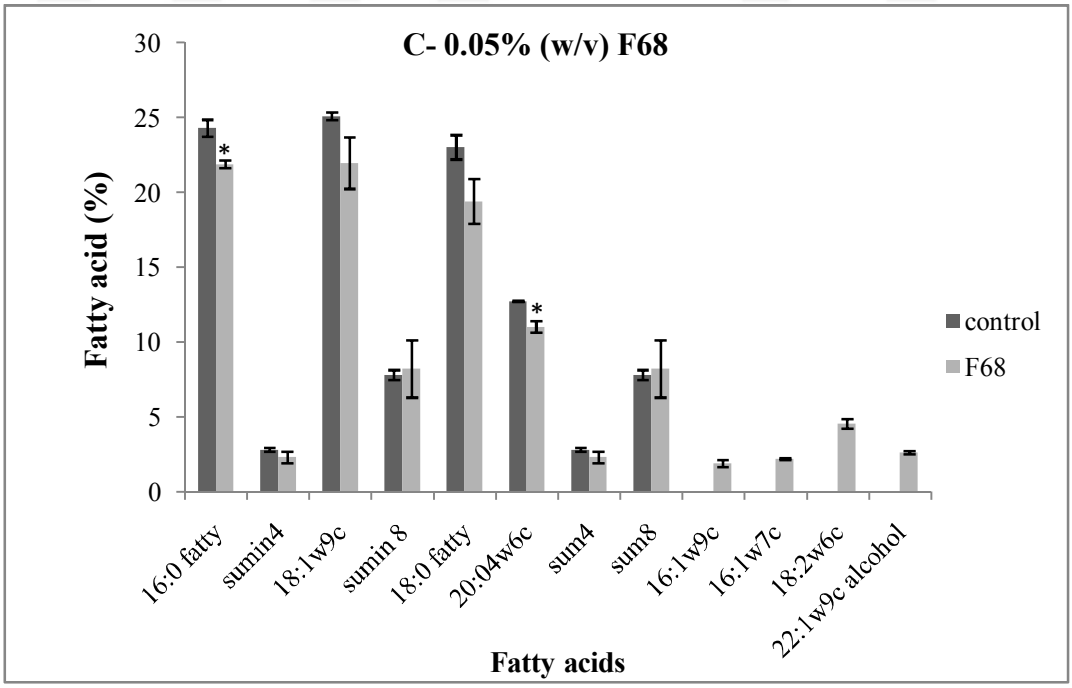


Figure 3.4.c. Fatty acid profile comparison between F68 and Control. Summed featured 4 (summed in featured 4): 15:0 iso 2-OH, 16:1ω7c and summed featured 8 (summed in featured 8): 18:1ω7c, 18:1ω6c (*$p<0.05$)

3.5. OSTEOGENIC DIFFERENTIATION

3.5.1. ALP Activity in Osteogenic Differentiation

Alkaline phosphatase is a kind of hydrolase enzyme which removes phosphate groups from different molecules, including nucleotides, proteins, and alkaloids. The enzyme is active in the alkaline environment [120]. High concentrations of alkaline phosphatase is produced in the growing bone and in bile and necessary for the deposition of minerals in the bones and teeth. In clinical applications, alkaline phosphatase levels are measured for bone diseases [121]. In the study, we determined ALP activity to demonstrate osteogenic differentiation. Figure 3.5 shows ALP activity results.

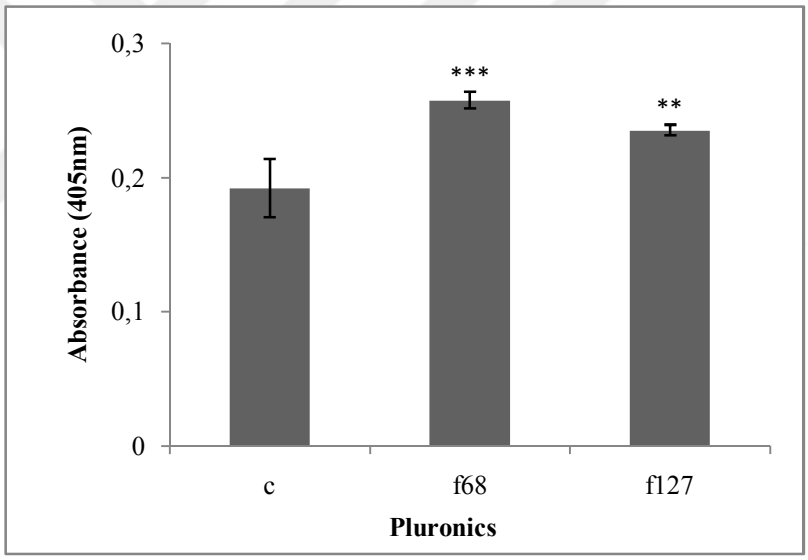


Figure 3.5. ALP activity results of control, F68, F127 (*p<0.05)

The results showed that both F68 and F127 increased the activity of ALP compared to the control group.

3.5.2. von Kossa Staining in Osteogenic Differentiation

Osteogenic differentiation was confirmed by showing the calcium mineralization with von Kossa staining which is a standart protocol for demonstrating deposits of calcium or calcium salt as a paramater of osteogenic differentiation. In all groups, calcium depositions were detected by using this protocol proving osteogenic differentiation. Results were shown in figure 3.6.

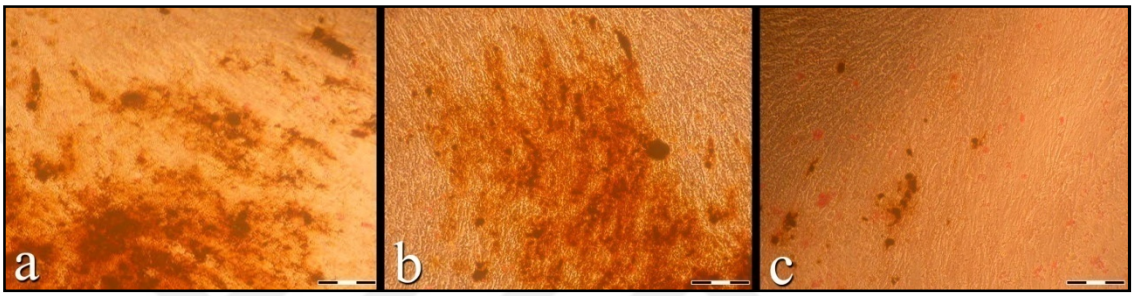


Figure 3.6.a. von Kossa staining of control, 3.6.b. F68 and 3.6.c. F127, Scale bar: 400µm

3.5.3. Immunocytochemistry Analysis of ColI and Osteocalcin

Collagen type I and osteocalcin expressions were also shown to prove osteogenic differentiation. Type I collagen is the most common collagen in vertebrates and 90% of the skeleton contains this type of collagen. It is found in the organic part of the bone. Osteocalcin, a noncollagenous protein found in bone and dentin is known as bone gamma-carboxyglutamic acid-containing protein (BGLAP) [122]. Osteocalcin is produced only by osteoblasts and have a role in metabolic regulation, bone building, bone mineralization and calcium ion homeostasis [123]. Results showed that control, F68 and F127 groups expressed collagen type I and Osteocalcin proteins. Figüre 3.7-3.8 show immunostaining results.

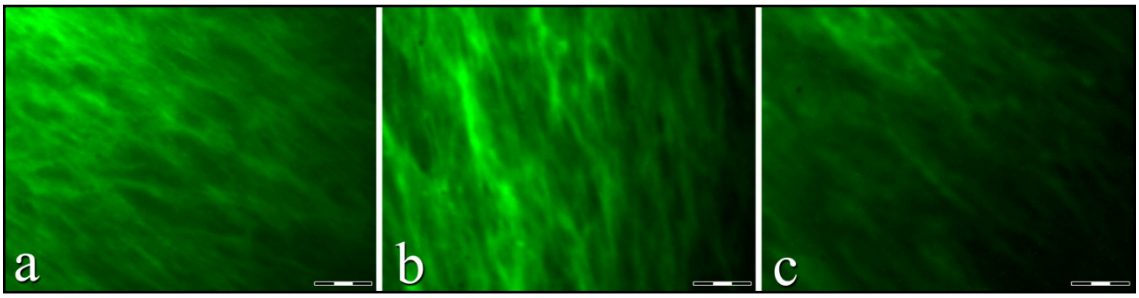


Figure 3.7.a. Immunostaining of Collagen type I control, 3.7.b. F68 and 3.7.c. F127. All groups were stained positive for collagen type I shown in fibrillary form labelled with FITC conjugated secondary antibody, Scale bar: 100µm

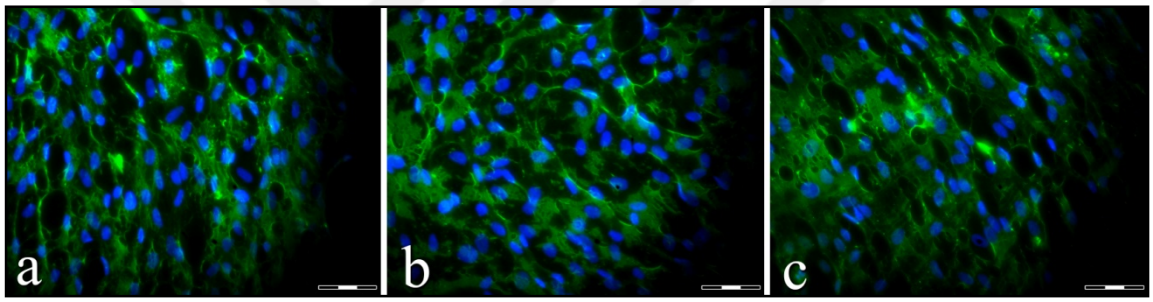


Figure 3.8. Immunostaining of Osteocalcin. All groups were stained positive for Osteocalcin labelled with FITC conjugated secondary antibody. 3.8.a. control, 3.8.b. F68 and 3.8.c. F127 Scale bar:100µm

3.5.4. Real time PCR Analysis of Osteonectin mRNA levels

Osteonectin is a glycoprotein found in the bone and binds calcium. Osteoblasts secrete this protein during bone formation and protein has role in bone mineralization, cell-matrix interactions, and collagen binding [124]. In this study, osteonectin gene levels were determined by real time PCR method. GAPDH was used as a house keeping gene to normalize results. According to the results the highest level of osteonectin was determined in F68 group. Results were shown in figure 3.9.

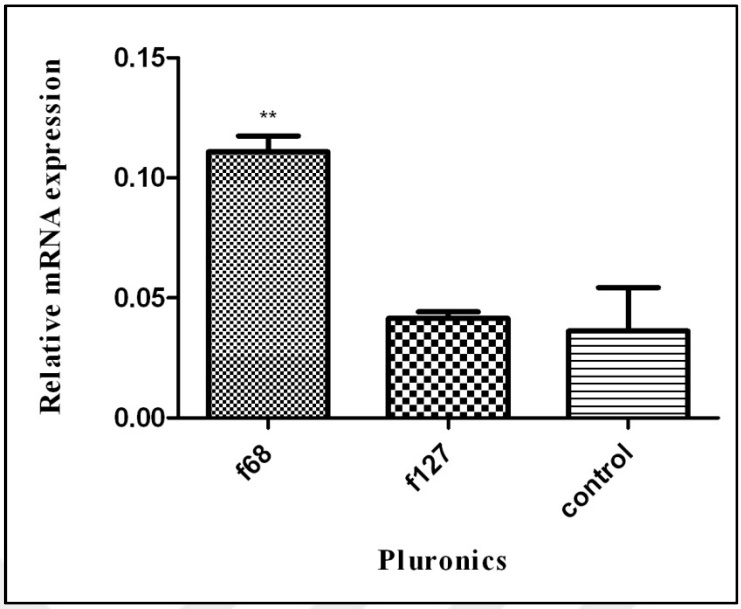


Figure 3.9. Osteonectin gene expression in control, F68 (0.05% w/v) and F127 (0.05% w/v) groups (*p<0.05)

3.6. CHONDROGENIC DIFFERENTIATION

3.6.1. Alcian Blue Staining in Chondrogenic Differentiation

Alcian blue staining was performed to show the chondrogenic differentiation. Mucopolysaccharides and glycosaminoglycans formed in the chondrogenic differentiation are stained with this dye. In this study, control, F68 and F127 were all positively stained with Alcian blue. Figure 3.10 shows alcian blue staining results.

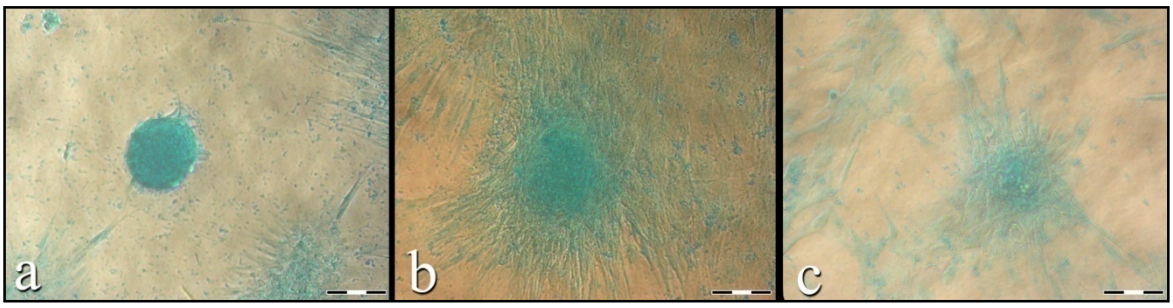


Figure 3.10.a. Alcian blue staining of control, 3.10.b. F68 and 3.10.c. F127, Scale bar: 400µm

3.6.2. Immunocytochemistry Analysis of ColII

Type-II collagen is the basic protein for articular cartilage and hyaline cartilage. It composes 50% of all protein in cartilage and 85-90% of collagen of articular cartilage. Expression of Collagen type II was checked with immunocytochemistry. Results showed that control, F68 and F127 are expressing Collagen type II. Figure 3.11 shows immunostaining results.

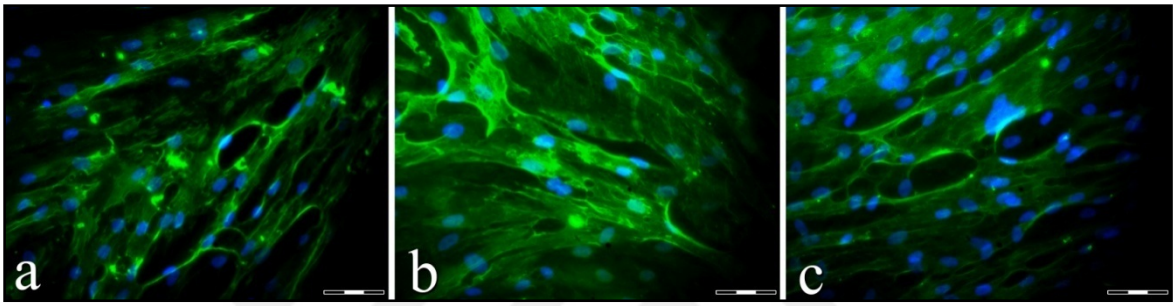


Figure 3.11. Immunostaining of Collagen type II. All groups were stained positive for Collagen type II labelled with FITC conjugated secondary antibody. 3.11.a. control, 3.11.b. F68 and 3.11.c. F127, Scale bar: 100µm

3.6.3. Real Time PCR Analysis of ACAN and Col II mRNA levels

Expression levels of Collagen type II and Aggrecan, known as large aggregating proteoglycan or chondroitin sulfate proteoglycan 1, forms a major structural component of cartilage with Type-II collagen [125], was detected by using Real time PCR. GAPDH was used as a house keeping gene to normalize the results. Collagen type II expression was highest in cartilage tissue and is checked for a marker of chondrogenic differentiation. The results showed collagen type II and agrecan gene expressions were highest in F68 treated group. Figure 3.12 and Figure 3.13 show the results.

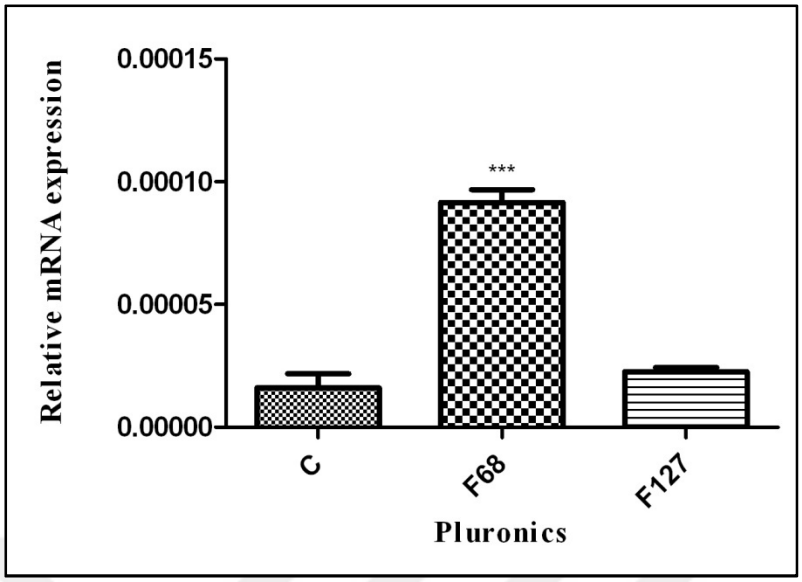


Figure 3.12. Collagen type II relative mRNA expression levels of control, F68 (0.05% w/v) and F127 (0.05% w/v) groups (*p<0.05)

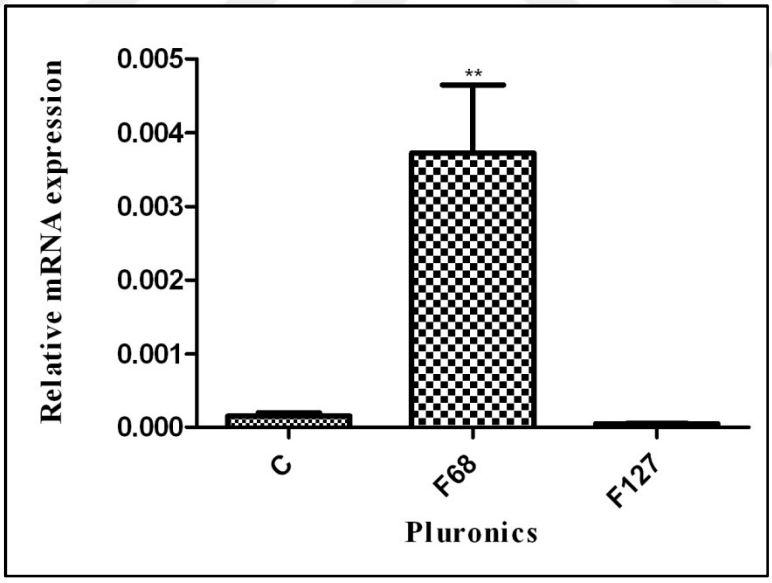


Figure 3.13. Aggrecan relative mRNA expression levels of control, F68 (0.05% w/v) and F127(0.05% w/v) groups (*p<0.05)

3.7. ADIPOGENIC DIFFERENTIATION

3.7.1. Oil Red Staining in Adipogenic Differentiation

In this study, we performed oil red staining to visualize the intra cellular lipid vesicles. Oil red is a lysochrome diazo dye that could be used to stain neutral triglycerides and lipids. Upon adipogenic differentiation we could detect lipid vesicles stained with oil red. Figure 3.14 shows oil red staining results.

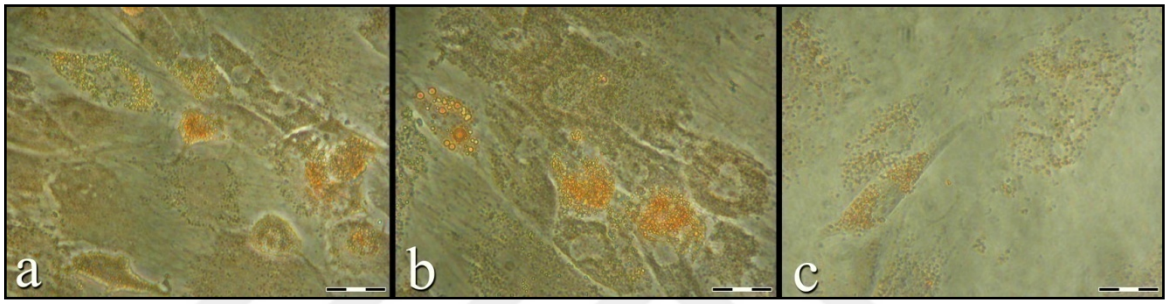


Figure 3.14.a. Oil red staining of control, 3.14.b. F68 and 3.14.c. F127, Scale bar: 400µm

3.7.2. Immunocytochemistry Analysis of FABP4

FABP4, known as fatty acid binding protein 4 (FABP4) is a carrier protein for fatty acids and it is mostly found in adipocytes and macrophages [126]. FABP4 expression was checked by using immunocytochemistry to demonstrate adipogenic differentiation. Results showed that all groups were positive for FABP4. Figure 3.15 shows results.

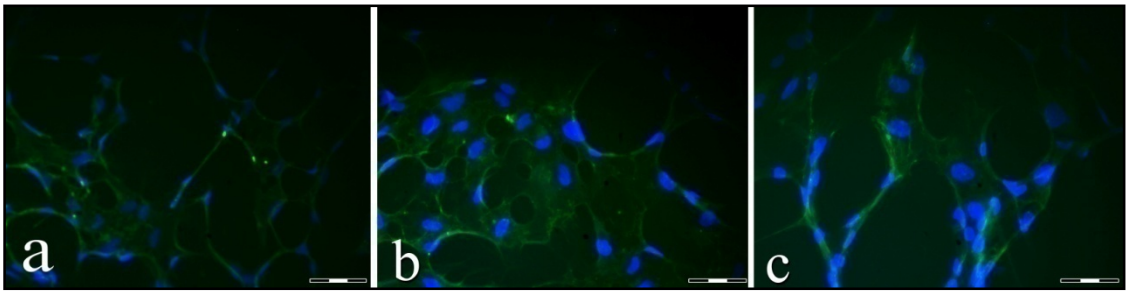


Figure 3.15. Immunostaining of FABP4. All groups were stained positive for FABP4 labelled with FITC conjugated secondary antibody. 3.15.a. control, 3.15.b. F68 and 3.15.c. F127, Scale bar: 100µm

3.7.3. Real Time PCR Analysis of FABP4 mRNA levels

FABP4 expression levels were detected by using Real Time PCR. The results showed that FABP4 expression was highest in pluronic F68 group. Figure 3.16 shows FABP4 gene expression levels.

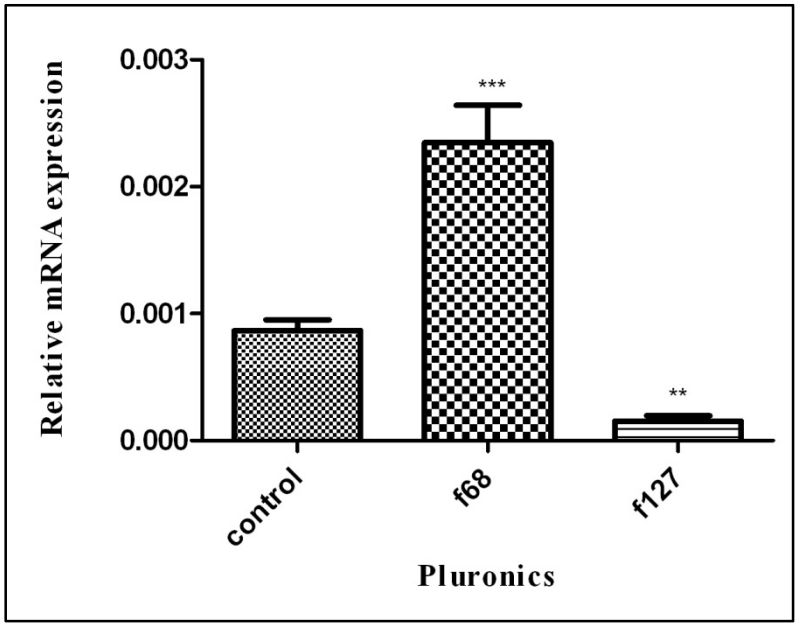


Figure 3.16. FABP4 relative mRNA expression levels of control, F68 (0.05% w/v) and F127 (0.05% w/v) groups (*p<0.05)

4. DISCUSSION

For bone, cartilage and fat tissue repair development of appropriate biomaterials which must be non-toxic, biocompatible and bioabsorble is of great importance [106]. Tissue engineering is a promising approach for regenerative therapy and biopolymers are indispensable tools for cell based tissue engineering. Bone, cartilage and fat tissue injuries or losses are very serious problems for many people. Accidents, inflammation, trauma, injuries, some disorders and burns are factors related with tissue damage and cell based tissue engineering is popular and promising for replacing the damaged tissue. In order to create a vehicle for cells, it is necessary to use appropriate biomaterials in tissue engineering applications [51]. The regenerative capacity of human tissues are varies depending on the tissue type. For instance, epithelial tissue can repair injuries and regenerate the defected area throughout life, however connective tissues including bone or cartilage have limited regenerative capacity, and finally myocardium and neural tissues have almost no regenerative capacity when compared to epithelial tissues.

Stem cells are candidate tools for regenerative therapy because of their amazing proliferation and differentiation capacity [127]. One of the main problems for stem cells in therapeutic use is to find sources containing adequate number of stem cells. The number of pluripotent stem cells is limited in the adult body but recent studies showed that there are a number of tissues such as bone marrow, adipose tissue, dental tissues where stem cells can be isolated [128]. On the other hand isolation of stem cells from some tissues such as nervous system in adult body does not seem feasible. The ideal approach in stem cell research is to obtain sufficient number of stem cells from the body without long cultivation processes with no risk for donors. Although it is known that stem cells have an excellent proliferation capacity to serve as good candidates for therapeutic applications, their interaction with biomaterials leads to the development of clinicaly applicable strategies.

In this case, it is necessary for researchers to find right stem cell source containing the stem cells with proliferation, self renewal and differentiation capacity. Due to the ethical problems concerning embryonic stem cell research adult stem cells have gained a lot of interest for stem cell based regenerative therapy. Adult stem cells are good

alternatives for regenerative therapy and there are several sources in the adult body for stem cell isolation. Since bone marrow was first identified as a rich multipotent MSC source, MSCs from other tissues have been characterized based on the properties determined for these cells [26-129-130-131-132]. Bone marrow mesenchymal stem cells (BMMSCs) can be easily isolated and expanded *in vitro*. Their ability to self renew themselves and differentiate into cells derived from mesenchymal lineage made them good candidates for regenerative medicine [133]. Isolation procedures that are caused by surgical process, contamination risk or bone marrow related diseases are problems associated with these cells [134]. BMMSC extraction procedure is traumatic and the amount of cells isolated from this tissue is limited. Therefore, new adult stem cell sources and isolation procedures are of great interest. Although in human body, bone marrow is the well-accepted source for mesenchymal stem cells, there are different tissues including adipose tissue, synovium, skeletal muscle, lung and dental tissues reported to comprise the MSCs [135]. Efforts to find the most suitbale stem cell source and to develope most convenient isolation procedures are still ongoing.

Dental tissues including follicle or pulp are promising candidates as alternative stem cell sources because they seem to bring a sort of solution to the problems related with stem cell isolation and cultivation. Dental stem cells (DSCs) are easily isolated because of simple surgical access and they are mostly the waste materials of dental applications. In addition to these advantages, DSCs do not cause any ethical controversy [136]. Isolation procedure is extremely efficient and after the operation dental tissues show very low morbidity in the operated site. Gronthos *et al*. in 2000 was first to isolate DSCs from pulp tissue and these cells were termed as postnatal dental pulp stem cells (DPSCs) [36]. After this, DSCs from different teeth and different parts of the tooth were isolated and characterized such as exfoliated deciduous teeth (SHED) [33], periodontal ligament stem cells (PDLSCs) [137], stem cells from apical papilla (SCAP) [138] and dental follicle precursor cells (DFPCs) [43]. Stem cells display a great differentiation potential. It was demonstrated that DSCs have the capacity to give rise to three cell lineages: osteo/odontogenic, adipogenic, and neurogenic. Moreover, there are studies in the literature about dental stem cells and their use in tissue-engineering studies and pre-clinical applications [139]. In this study, we used HTGSCs derived from human impacted third molars (wisdom teeth) that have MSC properties and tested the effects of three different

pluronics on differentiation of these cells. This is the first study in the literature explaining the effects of pluronic triblock copolymers on differentiation of DSCs.

Pluronics are triblock copolymers arranged in a A-B-A structure: Polyethylene oxide- Polypropylene oxide- Polyethylene oxide. There are several types of pluronic block copolymers that are commercially available in the market and are used for different applications such as drug delivery or tissue engineering [95]. In the present study, P85, F68 and F127 pluronics have been shown to posess some pharmaceutical benefits but not fully tested on stem cells.

At the critical micelle concentration P85 generally forms micelles and below the critical micelle concentration it has a great permeabilization ability [100]. One of the important actions of P85 is the inhibition of P-gp with a range of concentrations from 0.0001 to 5% [140]. At the concentrations below the critical micelle concentrations similar effects like P-gp inhibition was shown in multidrug resistant cancer cells [141]. Researches demonstrated that P85 does its inhibiton effect on P-gp by causing an energy depletion in the ATP pool [85]. Inhibition of P-gp drug efflux system by P85 is crucial for drug transport through the blood brain barrier and combination of P-gp dependent drugs with P85 causes an increase in efficiency of transport of drugs to the brain. P85 does its inhibitory effect by two mechanism: first one, it causes a cellular ATP depletion in brain microvessel endothelial cells and the second one, it decreases the affinity of drug efflux systems to ATP and drug molecules [86]. P85 has the ability to interact with cell membranes easily and incorporate to the lipid bilayer which causes an ATP depletion along with entering to the membrane. Briefly it increases membrane fluidity and inhibits ATPase activity of P-gp in drug resistant cells [74]. Elena *et al*. in 2004 showed that P85 affects two transporters, GLUT1 and MCT1, which are related with energy metabolism in the brain. After applying different concentrations of P85 to the brain microvessel endothelial cells, the group measured the inhibitor effect on GLUT1 and MCT1 transporters demonstrating that low doses of P85 activated GLUT1 and it was also shown with flux studies that P85 block MCT1 in BBMEC [98]. Ken *et al*. in 2002, examined the effect of P85 on increasing opioid peptide analgesia. In the study, they used concentrations below and above the critical micelle concentration and they proved that P85 enhanced opioid peptide analgesia with P-gp and MRP inhibition [97]. Batrakova *et al.* in 2001

tested the transport of digoxin that is a substrate for P-gp in the presence of pluronic P-85 *in vitro* and *in vivo*. The study revealed that P85 increased cellular accumulation of digoxin in porcine kidney epithelial cells exrepressing MDR1 gene. P85 was able to enhance transport of digoxin to the brain by inhibiting P-gp drug efflux system [77]. Exner *et al.* in 2005 examined the effect of capboplatin combined with P85, F127, and L61 on rat colorectal carcinoma cell line. They showed that only P85 exerted significant toxicity for this cell line [113]. P85 was also tested with doxorubicin which is a anticancer agent. It was shown that doxorubicin combined with P85 triggered pro-apoptotic pathway and prevented activation of anti-apoptotic pathway [99]. P85 were suggested to be a safe and efficient agent for non viral gene transfer and previous studies showed that P85 increases gene transfection in HepG2 cell line [142]. The fact that lay behind of this might be that P85 trafficking through the membrane is managed by the endocytic pathway and this property can be used for gene delivery applications [88]. In our study, the effect of P85 on differentiation of MSCs was evaluated. The results showed that P85 exerts toxic effect during the differentiation. The possible explanation of this might be that P85 facilitates the intake of chemicals, which are inducing differentiation, for example dexamethasone used in osteogenic differentiation, is also an anti cancer drug, and casue them to be toxic for the cells. Another reason for this result might be that exposure of cells to this pluronic on alternate days with differentiation media might trigger apoptosis in the cell.

F68 is another pluronic block copolymer which is widely used as a surfactant to protect cells from shear stress in bioreactors. In mechanically agitated gas-sparged bioreactors F68 can be used to prevent cell death caused by shear [143] and it can be used in the serum free culture systems [144]. F68 was also showed protective effects in suspension cultures for mammalian cells [145-84-146], insect cells [147] and plant cells [148]. F68 prevent cell death by slowing down bubbles velocity [149], hindering bubble break up [150] and decreasing cell-bubble interactions [151-152-153]. It was suggested that plasma membrane fluidity of hibridomas was decreased by F68 [84]. TB/C3 hybridoma cells strength was increased with 0.05% (w/v) F68 and this was measured bursting membrane tension and compressibility modulus [147] and F68 can exert its effect after removal from the medium [154].

F68 is suggested to stimulate cell growth and cell attachment to the culture dish surfaces [155-156]. F68 was shown to incorporate to the biologic membranes and interact with lipid bilayer to stabilize it [157]. Anne *et al*. in 2008 showed that F68 interacts with mebmranes of chondrocytes and CHO (Chinese Hamster Ovary) cells differently also representing that F68 enter the cells through the endocytic pathway [158]. Hellung *et al*. in 2000 tested the effect of F68 on Tetrahymena cells under different stress conditions. It was shown that F68 prolongs the survival under the conditions of hyperthermia, hypothermia, starvation and high ion concentrations. The protective effect was observed for 0.001-0.1% (w/v) concentration range [159]. In conculusion, it can be said that pluronic F68 is generally used to overcome shear stress problem in bioreactors and to protect cells against toxic conditions, and also it was demonstrated to be used for the drug delivery. Hovewer, it has not yet been used for differentiation experiments. The data in the present study indicated first time F68 increases differentiation efficiency of the MSCs.

Pluronic F127 is a commercially available triblock copolymer and non toxic, dissolves easiliy in water, and a good tool for the drug delivery. It is more soluble at low temperatures and forms gel at high temperatures [160]. F127 is a perfect drug delivery system because it has reverse thermal gelation and low toxicity [161]. It has been reported that F127 is the least toxic commercially available copolymer [109]. Pluronic F127 has many kinds of applications and one of them is using F127 gel formulations for dermal delivery of drugs. For local pain using F127 with analgesic or anti-inflammatory drugs is a promising approach due to the effective transport of drug through the skin. Miyazaki *et al.* in 1995 applied indomethacin with F127 in 20% (w/v) gel form and observed that percutaneous absorbtion improved [162]. Topical administration can also be suitable for anticancer agents and there are several studies regarding this. 5- flurouracil and adriamycin combined with F127 were used by Miyazaki *et al*. in 1984 and they showed that the higher concentration of pluronic caused the decreased release of the drug [163].

Effects of pluronics on permeability was studied with weak acids and bases which made contributions to the drug delivery by pluronics through the skin. It was shown that permeation of large molecules such as 2-n-undecylmalonic acid and doxorubicin can be facilitated by pluronic F127 [110]. Pillai *et al*. in 2003 used insulin with F127 for skin permeation experiments which might be an alternative way of taking insulin for Diabetics

[164]. Pluronic F127 is used for wound healing because it helps protein transport such as epidermal growth factor (EGF) [165-166]. Thermoreversible property of F127 is an advantage for using this polimer as a cell encapsulating material especially in tissue engineering. Sarwat *et al*. in 2005 tried to use F127 with membran stabilizing agents such as hydrocortisone, glucose, and glycerol on HepG2 cells. The results showed that F127 combined with membran stabilizing agents can be a suitable cell encapsulation material [167].

Although there has been no attempts to study the effects of P85 and F68 on differentiation of MSCs, F127 has been used in some differentiation experiments in the past. Aditya *et al*. in 2008 used F127 for adipogenic differentiation of BMMSCs. They tried to use gel formulation of F127 (20%, w/v) as scaffold for fat tissue engineering combined with the collagen type I. They concluded that pluronic F127 can be a suitable 3D environment for bone marrow derived mesencyhmal stem cells [111]. Huang *et al.* in 2006 tried to use F127 with Interpore and they managed to differentiate rabbit MSCs to osteogenic cells. They loaded MSCs into a scaffold made by Interpore and 20% (w/v) F127 with the presence of induction medium. At the end of the study they showed osteogenic differentiation with alizarin red staining, PCR and electron microscopy. Thus, they showed that rabbit BMMSCs can differentiate to osteoblasts in Interpore scaffold combined with F127 [168]. Osteoinductivity potential of recombinant human bone morphogenetic protein-2 (BMP-2) was combined with F127 by Abdala *et al*. in 2010. The group combined BMP-2 with monoolein and F127 for critical bone defects in rats. They designed control groups, monoolein groups, poloxomer groups and groups together with monoolein and poloxomer. After two weeks they took bones from rats for the histological analysis. They found that, in all groups they had similar results with control group [169]. In a recent study, F127 hydrogel microspheres combined with the hyaluronic acid (HA) enabling controlled release of dexamethasone was tested on chondrogenic differentiation of rabbit BMMSCs. Constructs were loaded with PMS (Porous Microspheres) or NPMS (Nonporous Microspheres). Hydrogel microspheres were injected subcutaneously in nude mouse. After 2 and 4 weeks constructs were taken from animals. Sox 9 and type II collagen which are cartilage specific markers were shown to increase in the PMS-loaded construct than in the NPMS-loaded one. Moreover alcian blue staining and immunohistochemistry of collagen type II showed that PMS group enabled better

production of chondrogenic tissue [170]. In a recent document Jung *et al*. in 2010 used Pluronic F127 derivatives and hyaluronic acid to induce chondrogenic differentiation of human adipose derived stem cells (ASCs). Hyaluronic acid was used in hydrogel as a physical stabilizer and a chondrogenic growth factor, transforming growth factor-beta 1 (TGF-β1) was combined with this hydrogel consisting of 20% (w/v) F127. Hydrogel was injected subcutaneously into nude mice and into the defected articular cartilage of rabbit knee. Collagen type II immunostainings and alcian blue stainings were performed to show succesfull differentiation suggesting that TGF- β1 conjugated hydrogel is a effective tool for cartilage formation from human ASCs [171]. In conclusion, it can be said that F127 is a suitable tool for the controlled release applications and it was used for differentiation experiments succesfuly. In this study, we checked the use of F127 in osteogenic, adipogenic and chondrogenic differentiation and we compared all three pluronics, F127, F68 and P85 for their potential to increase differentiation potential of the MSCs.

The fatty acid profiles of HTGSCs were analyzed after incubating them with pluronics at 0.05% (w/v) concentration in order to check the effects of the pluronics on the membrane structure. According to our results P85 led some of the fatty acids, Sum in feature 4 and sum 4, dissapear when compared with the control group. P85 treated group gained a new FA, 18:2w6c. Level of other FAs did not change significantly in this group. For F127 treated group the only difference is a monosaturated fatty acid 22:1w9c alcohol (3.4%). This FA was not found in control group after incubation. Other FAs are found unchanged both in the control group and F127 treated group. For F68 there was an interesting profile compared with the control group. F68 caused a significant decrease in 16:0 and 20:4w6c and an insignificant decrease in 18:1w9c, 18:0 and sum 4. Sum in feature 8, sum in feature 4 and sum 8 are almost same with control group. However there are four new unsaturated fatty acids; 16:1w9c (1.9%), 16:1w7c (2.22%), 18:2w6c (4.56%) and 22:1w9c alcohol (2.6%) appeared in F68 treated group. Results showed that P85 and F127 did not cause a significant difference in the fatty acid profile of the cells. However, F68 changed fatty acid profile and increased the number of unsaturated fatty acids in the membranes of the cells. Unsaturated fatty acids in lipid bilayer increases membran fluidity and provides functionality to the membrane [172]. It was shown that F68 stabilizes membrane by interacting with lipid bilayer [158]. Changes in lipid composition can alter structural properties of the membrane as well as its function [173]. It was reported that

peroxisomal oxidation of polyunsaturated fatty acids caused a decreased turnover of membrane fatty acids which leads an ineffective protection against free radical peroxidation [174]. As a conclusion it can be said that F68 affected cell membrane in a positive way and making it more flexible, dynamic and protecting membrane integrity which may facilitate the differentiation process. This also lead increase in cells` interaction with their environment preventing cell aging. In this study, F68 changed membrane unsaturated lipid content which might be one of the resasons of the increased differentation efficiency compared to the control group. It might be suggested that there is a link between FA profile and differentiation capacity of MSCs. Moreover, for the first time it was shown that pluronics have effects on FA profile of MSCs.

Pluronics F127, F68 and P85 were shown to be non toxic in the literature at small concentrations hovewer at high concentrations they can be toxic [95]. In this study, toxicity assay results supported the data in the literature concluding P85, F68 and F127 are not toxic for HTGSCs at 0.05% (w/v) concentration which is mostly prefered concentration for pluronics [141-147]. One exception was that P85 exerted toxic effect during osteogenic, chondrogenic and adipogenic differentiation at both 0.05% (w/v) and 0.01% (w/v) concentrations. This results may be due to changes in premeabilzation of cells becasue of P85. P85 has a great interaction and permeabilization ability through the membrane and by the help of this characteristic it is a suitable agent to be used as an inhibitor of drug efflux systems especially combined with drugs [100]. In our experiment, the differentiation media which contain different chemicals such as dexamethasone, is also used as a chemotherapeutic drug. This means that P85 may have facilitated transport of chemical in differentiation medium including dexamethasone to the cells excessively leading the cells death. Hovewer, F68 and F127 did not exert the same effect during the differentiation process.

In order to confirm osteogenic differentiation we showed ALP activity, von kossa staining results, osteocalcin and collagen type I immunostaining as well as osteonectin mRNA expression by real time PCR. ALP which is an early and common marker for osteogenic differentiation was chosen in this study to show osteogenic differentiation. ALP roles in mineralization of calcifying tissues [175]. Our results showed that wells treated with Pluronic F68 showed the highest ALP activity compared with control and F127

groups. The second marker for the osteogenic differentiation is calcium deposits visualized by qualitative von kossa staining method. Control, F68 and F127 groups were shown to form calcium depositions. Collagen type I and osteocalcin are two bone extracellular matrix proteins that collagen type I accounts for 90% of the bone matrix. [176]. Osteocalcin is noncollagenous protein synthesized by osteoblastic cells in the late stage of cell maturation [177] and binds to the minerals in the bone and regulating the bone growth [178]. Both of these proteins are considered to be osteogenic differentiation markers. In this study, it was demonstrated that collagen type I and osteocalcin are expressed in all groups after osteogenic differentiation which was confirmed by the immunocytochemisty analysis. Osteonectin is a bone specific protein, produced by osteoblasts and related with the bone extracellular matrix formation [179]. Real time PCR analysis showed that F68 group showed the highest expression of osteonectin compared with the control and F127 groups suggesting that F68 increases osteogenic differentiation.

Chondrogenic differentiation was confirmed by alcian blue staining, immunostaining of aggrecan and collagen type II and real time PCR analysis. Alcian blue stains glycosaminoglycans and mucopolysaccharides showing the deposition of cartilage matrix proteoglycans [180]. In this study, control, F68 and F127 groups were positively stained with Alcian blue. Collagen type II is an indispensable component of articular and hyaline cartilage. It is the basic collagen of cartilage and can be used to prove chondrogenic differentiaition. Aggrecan also known as large aggrecating proteoglycan is a kind of proteoglycan which forms the major component of cartilage along with type II collagen [181-182]. The results showed all groups expressed collagen type II and aggrecan confirming chondrogenic differentiation [183-184]. On the other hand it was found that the highest expression of collagen type II and aggrecan was detected in F68 group suggesting that F68 is accelarating chondrogenic differentiation.

Adipogenic differentiation was confirmed by oil red staining, immunocytochemistry of FABP4 as well as Real time PCR experiments. Oil red stain is used to stain neutral triglycerides and lipids. In this study, all of the groups formed lipid vesicles stained with oil red. FABP4, fatty acid binding protein 4 also known as adipocyte protein 2, is a carrier protein for fatty acids and can be used as an adipogenic differentiation marker [126]. Immunocytochemistry results revaled that FABP4 is expressed by all groups whereas real

time PCR data showed that F68 treated group show the highest expression of FABP4 among all the groups.

In conclusion, this study revealed that F68 is an appropriate pluronic block copolymer which might be used in tissue engineering of bone, cartilage and adipose tissue. So far there is no study describing the effect of P85 and F68 on differentiaiton of MSCs. Although there are some studies investigating the interaction between F127 and mesenchymal stem cell differentiaiton, it is still less known about how F127 as well as F68 and P85 changes membrane FA profile and the expression of differentiation markers. In this study, effects of these pluronics were investigated for the first time on the differentiation of HTGSCs which were proven to be MSCs showing the ability to differentiate into osteogenic, chondrogenic and adipogenic cell types [48].

The results showed that P85 is toxic during the differentiation of cells which had not been reported before our study. The data revealed that P85 is not toxic for cells in cell culture but when it is applied with the differentiaiton medium it starts to be toxic at day one or at day five based on its concentration. It might be due to accumulation of dexamethasone inside the cells since P85 facilitates the transport of dexamethasone into the cells. F127 is not toxic for differentiation but it exerted the same effects with control group and did not increase differentiation potential of HTGSCs. On the other hand, F68 was demonstrated to increase osteogenic, chondrogenic and adipogenic differentiaiton most probably by interacting with the cell membrane FAs.

Beside being non toxic and biocompatible, use of F68 might increase the success of various tissue engineering applications since it increases the survival of the cells and support them to differentiate. HTGSCs and F68 may be useful for tissue engineering, especially about dental tissue engineering. For the future it will be possible to use F68 pluronic block copolymer for different applications such as combining different growth factors with these polimers and provide suitable scaffold systems for tissue engineering. Bone, cartilage and fat tissue engineering promise the treatment of many serious disease since the knowledge about stem cells` biology and quality of biomaterials enhances day by day. Dental tissue engineering including the issues such as development of periodontal ligaments, pulp regeneration and alveolar bone engineering will benefit from the use of

stem cells with the dental tissue origin. This study suggests that the use of both a relatively new stem cell source, HTGSCs, and pluronics with pharmaceutical properties in combination might lead the development of highly productive regenerative therapies for various of diseases.

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