EFFECT OF F68, F127 AND P85 PLURONIC BLOCK COPOLYMERS ON ODONTOGENIC DIFFERENTIATION OF HUMAN TOOTH GERM STEM CELLS (hTGSCs)

by
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ON
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HUMAN TOOTH GERM STEM CELLS (hTGSCs)

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this thesis is dedicated to my family, my colleagues
and my teachers up to this time…

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ABSTRACT

EFFECT OF F68, F127 AND P85 PLURONIC BLOCK COPOLYMERS ON ODONTOGENIC DIFFERENTIATION OF HUMAN TOOTH GERM STEM CELLS (hTGSCs)

Mesenchymal Stem Cells are non-embryonic adult stem cells, they can differentiate into a variety of tissues, including bone, tooth, cartilage, tendon, muscle and adipose in an optimum conditions. This potential of MSCs can be used in tissue engineering applications such as bone, tooth, skin and cartilage tissue repair. Stem cells isolated from 3rd molar tooth germs as hTGSCs, were recently shown to be pluripotent and these stem cells can be used in tissue engineering as well. Biomaterials are indispensible tools in tissue engineering and among them, pluronics have gained 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, chemicals and growth factors. In this study, we tested the effects of three pluronics F68, F127 and P85 on odontogenic differentiation of mesenchymal stem cells (MSCs) from human tooth germ stem cells (hTGSCs) taken from the 3rd molar tooth. The results showed that none of the pluronics used in this study were toxic for the cells except fornthe P85 at high concentration. Long term treatment of P85 can cause toxicity. F68 remarkably increased the cell viability and the differentiation efficiency of MSCs into odontogenic cell types. P85 showed toxic effect for cells during the differentiation process. It was found that F127 has not significantly altered the cell viability and differentiation of MSCs. These data suggest, for the first time, that F68 has a great potential to increase odontogenic differentiation of MSCs which might lead to the development of new tissue engineering strategies for regenerative medicine.

ÖZET

F68, F127 ve P85 PLURONİC POLİMERLERİNİM İNSAN DİŞ GERM KÖK HÜCRELERİNİN DİŞ HÜCRELERİNE FARKLILAŞMASI ÜZERİNE OLAN ETKİSİ

Mezenkimal kök hücreler embriyonik olmayan ve uygun şartlar altıda diş, kemik, yağ, kıkırdak ve kas gibi birçok dokuya farklılaşabilen hücrelerdir. Mezenkimal kök hücrelerin farklılaşabilme yetenekleri doku mühendisleri tarafından kemik, diş, kıkırdak gibi dokuları oluşturmada kullanılabilir. 3. Molar diş germünden elde edilen kök hücreler pluripotent olması ve doku mühendisliğinde kullanılabilirliği gösterilmiştir. Son yıllarda doku mühendisliğinde kullanılan biyolojik materyallerden olan pluronikler büyük ilgi çekmiştir. Poloksomer olarak ta bilinen pluronikler hidrofilik (etilen oksit) ve hidrofobik (propilen oksit) gruplarından oluşmaktadır. Bu polimerler ilaçların, kimyasalların ve büyüme faktörü gibi terapotik ajanların taşınmasında kullanılması ilgi çekici faktörlerden biridir. Bu çalışmada üç pluroniğin F68, F127 ve P85'in insane 3. Molar diş hücresinden elde edilmiş mezenkimal kök hücrelerin diş hücresine farklılaşmada ki etkisini ölçtük. Sonuçlar gösteriyor ki P85'in yüksek konsantrasyonları hariç tüm pluronikler hiç bir toksisite göstermiyor. F68 mezenkimal kök hücrelerinin hücre canlılığını ve farklılaşma kapasitesini arttırıyor. Farklılaşma sırasında uzun süre P85'e maruz kalan kök hücre canlılığı azalma gösteriyor. F127'in ne hücre canlılığına ne de farklılaşma sürecine hiç bir etkisi olmadığı görülmüştür. Bu verilerden yola çıkarak ilk defa pluronik F68'in diş hücresine farklılaşmasında olumlu olarak etkili olduğu gösterilmiştir, bu da doku mühendisliğinde yeni stratejilerin geliştirilmesine yol açabilir.

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LIST OF SYMBOLS / ABBREVIATIONS

h	Hour
kDa	Kilo Dalton
mM	Mili Molar
M	Molar
ml	Mili Liter
N	Normal
nm	Nano Meter
nM	NanoMolar
µL	Mikroliter
µM	Mikro Molar

ALP	Alkaline Phosphatase
ATP	Adenosine Triphosphate
BBMEC	Bovine Brain Micro Vessel Endothelial Cells
BMP 2	Bone Morphogenic Protein 2
BMP 7	Bone Morphagenic Protein 7
BMSCs	Bone Marrow derived Mesenchymal Stem Cells
CHO	Chinese Hamster Ovary
COL1A	Collagen Type 1
DAPI	4',6-Diamidino-2-Phenylindole
DFSCs	Dental Follicle Stem Cells
DMEM	Dulbecco's Modified Eagle Medium
DMP 1	Dentin Matrix Protein 1
DNA	Deoxyribonucleic Acid
DPSCs	Dental Pulp Stem Cells
DSP	Dentin Sialo Protein
DSPP	Dentin Sialophospho Protein
DXM	Dexamethasone
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay

ESCs	Embryonic Stem Cells
EtBr	Etidium Bromide
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
hES	human Embryonic Stem Cells
hTGSCs	human Tooth Germ Stem Cells
MDR	Multidrug Resistance
MLRs	Mixed Lymphocyte Reactions
MS	Multiple Sclerosis
MSCs	Mesenchymal Stem Cells
MTS	Metil Tetrozolium Salt
NCBI	National Center for Biotechnology Information
NSCs	Neural Stem Cells
P gp	Permeability Glycoprotein
PBS	Phosphate Buffered Saline
PDLSCs	Periodontal Ligament Stem Cells
PEG	Polyethylene Glycol
PEO	Polyoxyethylene Oxide
PPO	Polyoxypropylene Oxide
PSA	Penicilin Streptomycin Amphicilin
RNA	Ribonucleic Acid
SCAPs	Stem Cells from the Apical part of the Papilla
SHEDs	Stem cells from human exfoliated deciduous teeth
TAE	Tris-Asetic Acid-EDTA
UV	Ultraviole

1. INTRODUCTION

1.1. GENERAL OVERVIEW OF STEM CELLS

Stem cells have noticeable potential to turn into many different cell types in the body during the early development and growth. In other words they are the genre of undifferentiated cells that are able to differentiate into many cell types.

Stem cells are individuated from other cell types by two important characteristics:

- Stem cells are unspecialized cells capable of regenerating themselves during cell division,
- Under optimal conditions, they can be turn into tissue- or organ-specific cells.

Stem cells come from two main source; embryonic blastocyte and adult tissue. Embryonic stem cells are derived from the early mammalian embryo and they then can differentiate into many cell types [1].

Human embryonic stem cells (hES) derived from the inner cell mass of a developing blastocysts.and have the potential to become a variety of specialized cell types [2]. A hESs are self-renewing and pluripotent, they can differentiate into all 220 different cell types found in the human body.

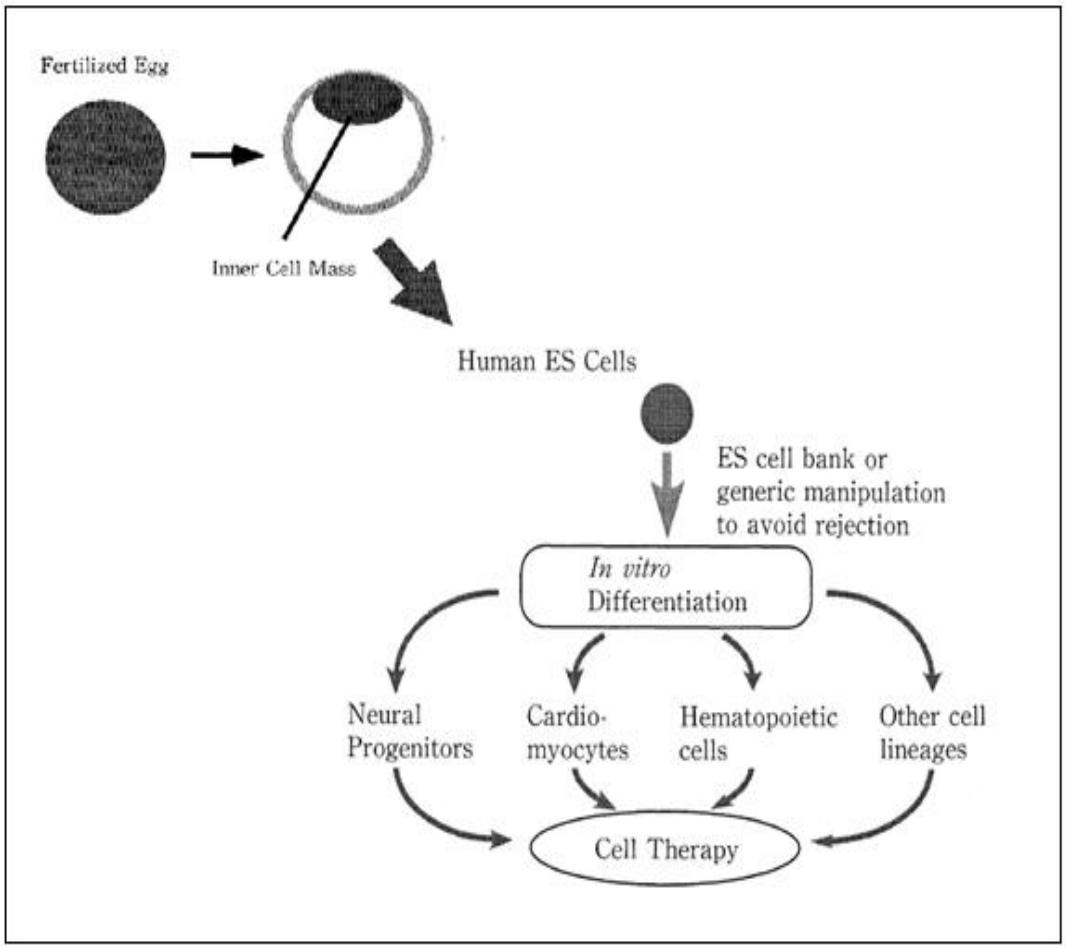

Figure 1.1. Establishment of embryonic stem cells. ESs derived from inner cell mass of blastocysts [3]

Adult stem cells are taken from different tissues of the developed organisms. Adult organisms remain this undifferentiated, or unspecialized, state. These adult stem cells can give rise to differentiate other cell types. Some of them can be, re implanted in the body.

1.2. SOURCE OF MESENCHYMAL STEM CELLS AND THEIR PROPERTIES

Mesenchymal stem cells (MSCs) can be isolated almost from every type of tissue such as bone marrow, lung, adipose tissue, dental tissues, cord blood and various fetal tissues [4]. MSCs are non-embryonic adult stem cells and can differentiate into a variety of tissues, including bone, tooth, cartilage, tendon, muscle and adipose when optimum conditions are provided. For example for tooth differentiation cells must be treated with chemicals,

cytokines and growth factors [5]. MSCs are currently under investigation for the tissue engineering applications, in particular bone, tooth, skin and cartilage repair [6, 7].

The first successful mesenchymal stem cell isolation is the isolation of fibroblast like colonies from the bone marrow, reported by Friedenstein about 4 decades ago [8]. The isolation method was based on the adherence of marrow-derived, fibroblast like cells to a plastic substrate of the cell culture plate, with a concomitant lack of adherence shown by the marrow derived hematopoietic cells [9, 10].

According to the criteria of The International Society for Cellular Therapy, Mesenchymal Stem Cells are defined by their surface markers should be negative for the hematopoietic cells surface markers such as CD14, CD34, CD45 [11-13] and be positive for the MSCs surface markers such as CD105, CD73, and CD90 [4]. The phenotypic identity of MSCs is not unique, sharing features of multiple cell lineages, including the mesenchymal, hematopoietic, endothelial, epithelial, and muscle cells [14, 15]. In addition to this phenotypic features, MSCs have display a variety of morphological characteristics [12]. To prevent acute graft-versus-host disease, immunosuppressive capacities of MSCs have been estimated in experimental autoimmune models, in humans [16]. Zappia and colleagues [17] were the first to report the therapeutic efficacy of MSCs in the experimental autoimmune murine model of the multiple sclerosis (MS).

1.3. IMMUNOMODULATORY PROPERTIES of MESENCHYMAL STEM CELLS

MSCs have immunomodulatory features that are within the most intriguing aspects of their biology. Francesco Dazzi reported that MSCs inhibit the cell division of stimulated T cells, by preventing their entrance into the S phase of the cell cycle, in a murine model [18].

MSCs also induced the arrest of T cell division in mixed lymphocyte reactions (MLRs); and this reaction was irrevocable on the removal of the MSCs. This reversibility models is observed in primate [19] and human [20, 21].

The strong inhibitory effects of MSCs on T cell proliferation only relatively minor and reversible effects on the T cell effector function. The T cell inhibition does not appear to be antigen specific [19], and it targets primary and secondary responses and works as HLA barriers [22]. On the other hand, the T cell proliferation was found to be inhibition of the across strain and species barriers. The immunomodulatory activity of MSCs *in vitro* [23] and *in vivo* [24] emphasized by Dennis McGonagle, may have differences between each other. There may be the importance of establishing the relationship between MSCs and inflammation *in vivo*. Furthermore, MSCs originated from synovial membranes differed in their expression of differentiation potential and transcription factors compared with MCSs derived from other sources such as skin fibroblasts or neural cells [25].

Braccini at.al. studied the expansion of MSCs in threedimensional scaffolds. Three dimensional (3D) culture system is an interesting new approach to tissue repair (for example, tooth, bone, adipoz and cartilage) and large scale commercial production because of the improved multypotency and differentiation capacity of MSCs [26]. Bocelli-Tyndall et. al., by asking the questions about the role of the antiproliferative effect of MSCs in the regulation of tissue organization and maintenance, has shown that mature chondrocytes are more anti proliferative than non differentiated chondrocytes [27].

1.4. HUMAN TOOTH GERM STEM CELLS

Human tooth germ stem cells (hTGSCs), including dental pulp stem cells (DPSCs) and dental follicle stem cells (DFSCs), obtained from neural crest and include both MSCs and neural stem cells (NSCs) part of the developed teeth [28]. Isolation of MSCs from dental tissue is cost effective, very easy and does not raise additional safety and ethical concerns, since they are collected during regular orthodontic procedures [29]. It was shown that hTGSCs have neuroprotective effects on the motor neurons in spinal cord [30] and the dopaminergic neurons [31].

There can be several advantages of using hTGSCs derived from the 3rd molar tooth germs:

- They begin to organogenesis at around age 6 [32] and therefore these cells remain developmentally and replicatively young;

- They are collected from teeth that are extracted because they are taken during the routine treatment;
- They are considered to be adult stem cells and, unlike ESCs, they do not cause controversy.

Stem cells isolated from the 3rd molar tooth germs were recently shown to be pluripotent and are able to give rise to functional cells originating from three germ layers: ectoderm, mesoderm and endoderm [33].

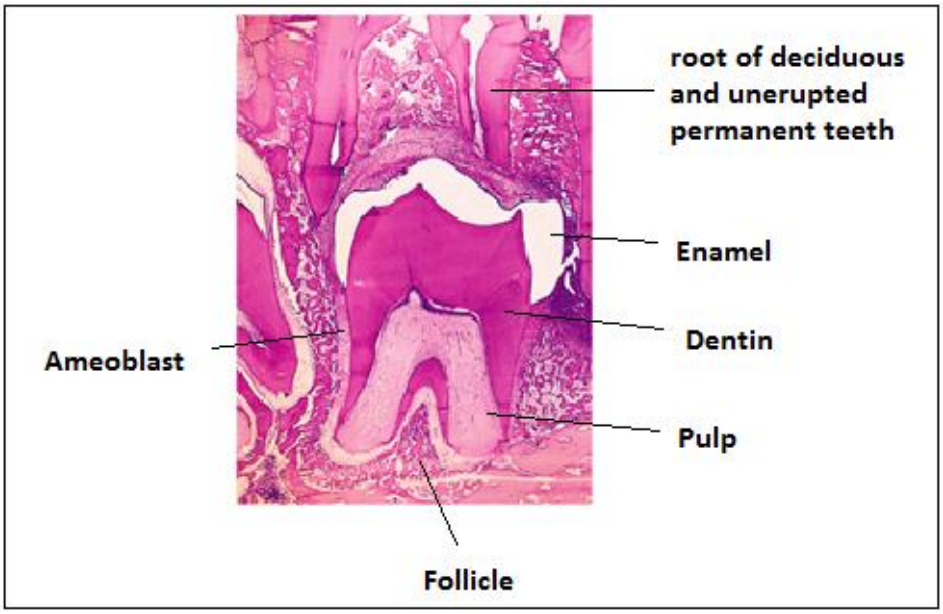

Figure1.2. Third molar teeth in a 10-year-old child. Perpendicular section through the roots of two deciduous teeth with underlying permanent molar tooth. Empty space was left by enamel that was dissolved by decalcification [34]

One of the major issues with tooth germ stem cells is their cryopreservation, since unsitable storage may decrease their cell viability and alter their multipotency capacity [35]. hTGSCs were reported to be successfully cryopreserved without change or loss of their characteristics and function upon thawing [35-37].

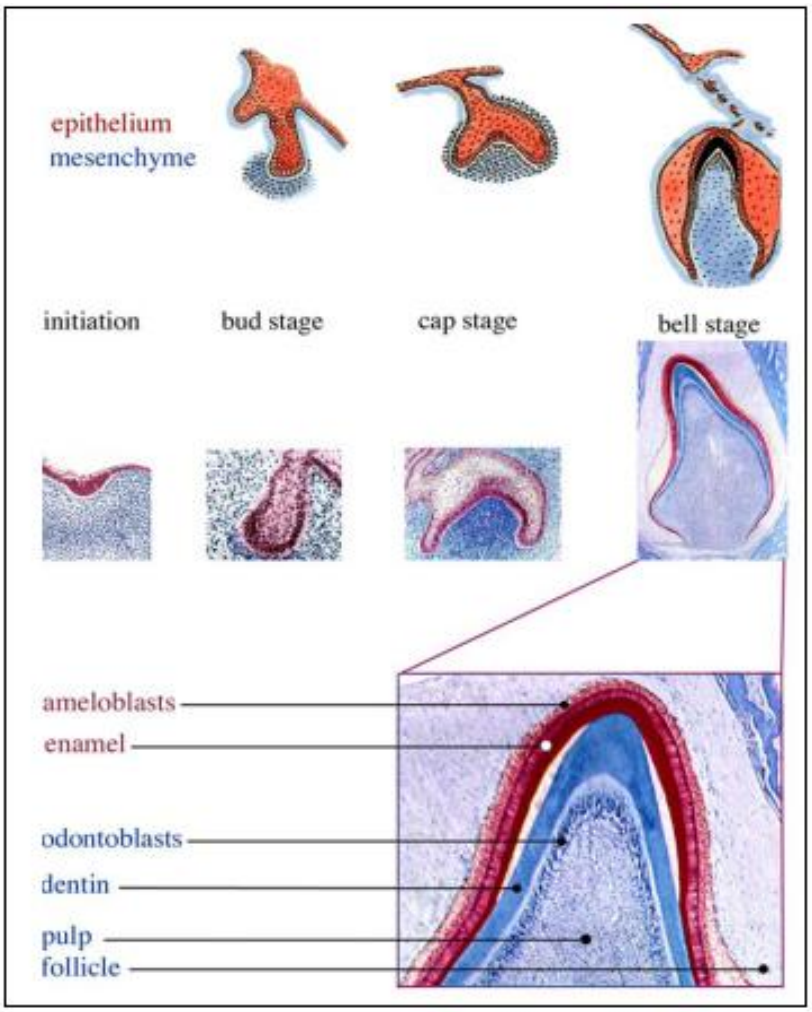

Figure 1.3. Histological staining of tooth in humans. Orange colour indicates epithelial tissues and epithelial derivatives (ameloblasts and enamel) while blue colour shows mesenchymal tissues and mesenchyme derivatives (odontoblasts, dentin, dental pulp and follicle) [38]

In case of dental injury, dental pulp is involved in a process called regenerative dentinogenesis, cells deposit and elaborate a new dentin matrix for the repair of the injured site [39]. It has been shown that adult dental pulp contains precursor cells capable of forming odontoblasts under appropriate signals such as growth factors and chemicals [40-46]. Dental pulp progenitor cells have not been completely identified but some studies suggest that pericytes, are able to differentiate into osteoblasts and odontoblasts [41, 47, 48]. Tooth repair is a long process and the *in vivo* therapeutic targeting of these stem cells remains to be explored.

1.5. ODONTOGENIC DIFFERENTIATION OF DENTAL STEM CELLS

DPSCs have gained great importance for use in the regenerative treatment of infected and defected dental tissues in the dentin-pulp complex [49, 50]. Recent stem cell based studies have revealed that progenitor cells are also present in dental pulp tissue [44, 45], which suggests the possible healing of dentin pulp complex, as against to the conventional treatments [51]. Stem cells derived from adult pulp tissue maintain the characteristics of stem cells, including multipotency and self-renewal [43]. Recent studies have shown that DPSCs can differentiate into a variety of cells such as adipocytes, osteocytes etc. [37, 52, 53].

Recent improvements in the development of biomaterials and chemicals have spurred the extended use of stem cells in tissue engineering [54, 55]. When formulated within the 3D scaffolding substrate or proper chemicals, stem cells better perform the biological roles involved in the regeneration process and can maintain the physiological stability [56, 57]. Some chemicals and bioactive scaffolds have shown promise in the regeneration of dental tissues, including dentin-pulp complex. One of the most popular groups of materials are calcium phosphate (CaP) ceramics, which have generally been shown to induce appropriate osteoblastic differentiation of stem/progenitor cells *in vitro* and bone formation *in vivo* [58]. Few studies have been conducted to examine the effects of these bone-regenerating materials on odontoblast behavior and dentin formation [59].

Collagen type I [60], dentin sialoprotein [61], bone morphogenic protein 2 and dentin matrix protein 1 are early marker for odontogenic differentiation. Odontogenicly differentiated cell express these markers at an early stage of differentiation. Type I collagen is the important participant of the collagenous proteins in dentin and formation of mineral [62-64]. Dentin matrix protein secreted by odontoblast cells, it is the major component of dental mineralized tissue [62]. Dentin matrix proteins secreted by odontoblast cells can be classified into primary, secondary or tertiary dentin based on the time and circumstances of its secretion [65-68]. DSP is synthesized by odontoblast cells and secreted through the odontoblastic process at the mineral formation [63, 64, 69]. DSP is strongly associated with the mineralization of dentinogenesis [63, 64, 69, 70]. BMP 7

and BMP 2 promote the odontogenic differentiation and dentin-like tissue formation both in vitro and in vivo [71, 72].

1.6. PLURONICS TRIBLOCK COPOLYMERS

Pluronics, also known by Ploxamers, are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene oxide (PPO) adjoining by two hydrophilic chains of polyoxyethylene oxide (PEO) [73] . Because of the hydrophobicity difference between the PPO and PEO blocks, they tend to micellize in the aqueous solution [74].

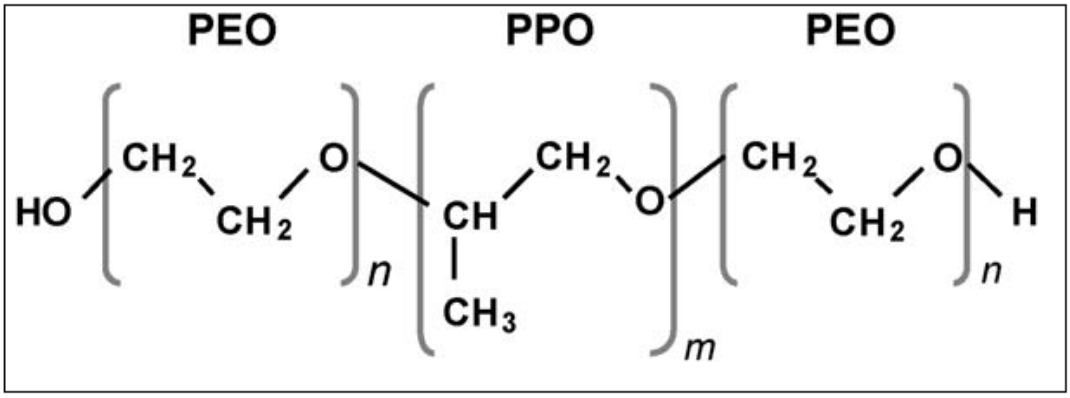

Figure1.4. Chemical structure of a poloxamer. It presents a central hydrophobic fragment of polyoxypropylene (PPO) and same hydrophilic chains of polyoxyethylene (PEO) at both sides [75]

Polypropylene oxide is the polymer of propylene glycol and chemically it is a polyether. The polypropylene oxide or PPO is reserved for low to medium range molar mass polymer when the nature of the terminal-group, which is usually a hydroxyl group, and the term "oxide" is used for high molar mass polymer when end-groups no longer affect polymer properties [76].

PEO is a polyether compound with many applications and it has also been known as polyethylene glycol (PEG) or POE, depending on its molecular weight [77].

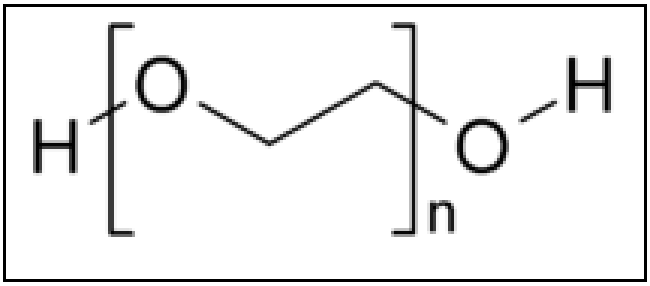

Figure 1.5. Formulation of Polyethylene Oxide (PEO)

Pluronics may display complex behaviors, which are influenced by the number and sequence of PEO and PPO blocks in the polymer. Increasing the PPO content or size of a Pluronics has been shown to cause reductions in both its critical micelle concentration and optimum micelle formation temperature [78] , and the micelle structures of different Pluronics, that are well characterized [79, 80] . Many recent studies have focused on the gel states formed by Pluronics at high concentration and temperature and these factors are important in the pluronics behavior [81]. In addition to this ability of Pluronics there is also confirmation that they are able to increase the effect of drugs [82, 83].

The choice of Pluronic appears to depend upon the involved drug concentration [84]. Some studies have investigated the change in micellar structure of Pluronic micelles on the uptake of drug molecules. Release profiles in vivo is hard to understand, because the aggregation number and size of the pluronic micelles is critical for determining the bioavailability of the drug and time of circulation in blood decrease, with no correlation to the characteristics of the drugs. Nevertheless, an increase in the core size was noted upon the addition of a wide range of drugs [85].

Pluronic micelles have been suggested as drug carriers because of a number of advantageous facilities that include:

- An appropriate size of pluronic micelles escape from the renal excretion, which allows the extravasation at the tumor site,
- In solid tumors, pluronic micelles provides selective accumulation for the encapsulated drugs which has an enhanced penetration and retention effect [86-88],

- A shielding effect that diminishes the drug interaction with healthy tissues [89-96],
- Release drug from micelles in a partially and temporally controlled by ultrasonic irradiation, chemicals or heat [91, 94, 97],
- Encapsulated micelles enhance intracellular uptake of drugs under the action of ultrasound [95-97],
- The uptake of drugs encapsulated in micelles via a fluid phase endocytosis rather than a passive diffusion.

The latter factor is important for overcoming drug resistance, which is a significant problem in cancer chemotherapy. Drug resistance and multidrug resistance (MDR) of the cells are attributed to the work of the ATP dependent efflux pumps localized in the cytoplasmic membranes. Drugs internalized by endocytosis bypass efflux pumps; [98-100] however, endocytosed drugs are sequestered in the cytoplasmic acidic vesicles, which create a second barrier on the way of the drug toward its target. For anticancer drugs, the targets are commonly located outside of the cytoplasmic acidic vesicles; in particular, for anthracyclin drugs, the DNA target is localized in the cell nuclei.

1.6.1. Pluronic P85

Pluronic P85 is an amphiphilic block copolymer. It has been reported to possess inhibitory activity against the multiple drug effusion transporters [101]. In vitro, cultured primary cultures of bovine brain micro vessel endothelial cells (BBMEC), membrane and trans cellular transport of many drugs enhanced by pluronic P85 [99, 102], as well as in cells genetically modified to over express the drug effusion transporters like P-gp [98, 103].

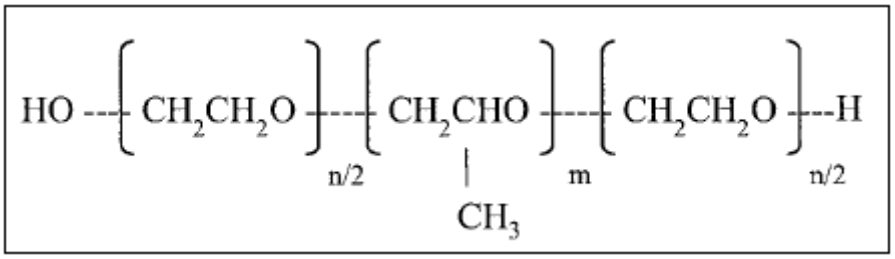

Figure1.6. Structure of Pluronic P85 n=52, m=40 mol.wt. 4600 block copolymer, containing two exterior hydrophilic PEO blocks and an interior PPO block [103]

The block copolymer micelles can incorporate drugs and it serve as carriers for drug delivery [104-106]. This micelle structure increases the solubility of drugs and protects drugs from the degradation. Pluronic micelles often increase the exposure of the drug to the target tissues due to the ability of the micelle structure to increase the drug residence time in the bloodstream [105, 107, 108].

1.6.2. Pluronic F68

Pluronic F68 is a FDA approved percipient under the trade name of Poloxamer 188. It is both water and organic solvent soluble. It has been primarily used in the pharmaceutical formulations as emulsifier.

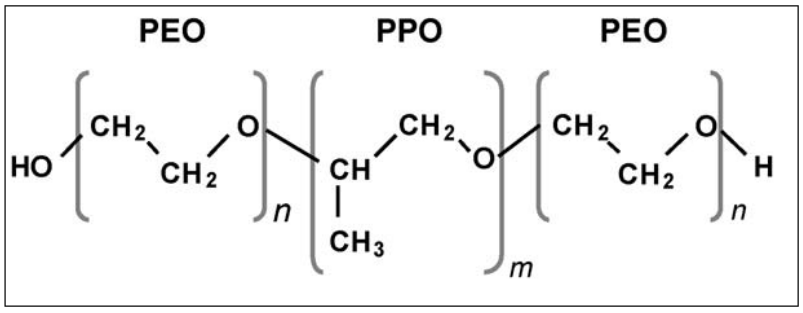

Figure 1.7. For the Pluronic F68, n = 75 PEO units and m = 30 PPO units

Pluronic F68 is a synthetic nonionic surfactant with a molecular weight of 8.4 kDa [109]. F68 is widely used to protect insect and mammalian cells from agitation and sparging related injuries in the aerated and agitated bioreactors [110, 111]. The pluronic block copolymer exists either in the form of micelle or unimer, depending on the concentration of pluronics [112].

To explain the cell protective effect of Pluronic F68, two mechanisms have been proposed:

- In sparged cultures, Pluronic F68 can increase the stability of foam layer on the top of the culture medium, in this manner reducing the hydrodynamic forces accompanying bubble breakup [113] and mammalian cells to bubble attachment [113-116];

- Pluronic F68 could also enhance the resistance of the cells to shear stresses at high agitation rates by its physical incorporation into the plasma membrane [110] and thus decreasing the plasma membrane fluidity of cells [117, 118].

Interestingly, Pluronic F68 was also shown to expand biological effects, absence of hydrodynamic stresses. In presence of pluronic F68, fibroblast attachment to plastic surfaces was enhanced [119] and cell growth was stimulated [120]. In addition, Gigout et al. showed that pluronic F68 was able to enter CHO cells and chondrocytes. It also accumulates in the endocytic pathway [121]. However, in the literature, very few data concerning the effect of pluronic F68 on the recombinant protein expression and cell metabolism and glycosylation produced by animal cells are available.

1.6.3. Pluronic F127

Pluronic F127 is an ABA type block copolymer and it is consisting of PEO units 70% as A and PPO blocks 30% as B. Commonly used pluronic for pharmaceutical and medical purposes. Pluronic F127 can carry various drugs have been used for treating patients. Recent studies reported that pluronic F127, suppository formulation of indomethacin, was the most suitable of three pluronics tested in rabbits due to its prolonged action and minimal side effects [122]. F127 based dental gel studies has been in use for several years in treatment of patients with sensitive gums and teeth [123]. Moreover, first clinical use of F127 was as a vehicle for the silver salts to treat the topical burns [124].

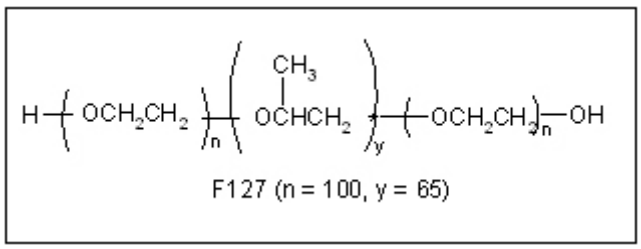

Figure 1.8. Formulation of Pluronic F127

Pluronic F127 has been employed for the topical delivery of lidocaine and benzocaine [125], the anticancer drugs such as 5-fluorouracil and adriamycin [126], and for the healing

of burn wounds [124, 127]. Some anti-inflammatory drugs have been prepared with F127 such as gels containing diclofenac and hydrocortisone [128]. In vivo studies, has been shown that F127 significantly enhance the brain penetration of the digoxin as a substrate of P-gp by inhibiting P-gp at the blood brain barrier [103].

1.7. AIM OF THE STUDY

In this study, it was aimed to show the effects of three pluronics, F68, F127 and P85 on the odontogenic differentiation of hTGSCs as a MSCs derived from the germ. Testing, effect of different polymers on MSCs, may open new ways to increase the efficiency of MSC based tissue engineering applications in the dental tissue regeneration.

2. MATERIALS AND METHODS

2.1. ISOLATION OF HTGSCS

hTGSCs were isolated from the wisdom teeth of 14 year old patient. Germ tissue was shredded into small pieces in 6 well plates (Zellkultur Testplatte, Switzerland, cat # 92006) with Dulbecco's modified essential medium (DMEM) (Invitrogen, Gibco, UK, cat # 31885) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Gibco, UK, cat # 10270-106) and 0,1mg/ml (w/v) PSA pH=7.4 (Invitrogen, Gibco, UK, cat # 15240-062). One week later cells started the diffuse on six well plate and a few days after the cell confluency reached 80%. Cells were, then detached from plates with 0,5% (v/v) tyrpsin-EDTA (Invitrogen, Gibco, UK, cat # 25200) treatment. Medium was added to inhibit the activity of tyrpsin and centrifugation was performed at 1000 rpm for 5 min at room temperature. After centrifugation supernatant was removed and cells were seeded on T-75 (Zelkultur Flaschen, Switzerland, cat # 90075) tissue culture flask.

2.2. CHARACTERIZATION OF hTGSCs

hTGSCs were characterized for the mesenchymal stem cells surface markers by flow cytometry. In this study anti-bodies against CD29 (cat # BD556049), CD14 (cat # SC-9150), CD34 (cat # SC-51540), CD44 (cat # SC-9960), 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) and CD73 (cat # BD 550256) (Zymed, San Francisco, CA, USA) were used. Cells were harvested and 5000 cells were transferred to the centrifuge tubes. 2 µl of conjugated antibodies were mixed with 100 µl PBS for each antibody. The mixture was then added to the cells and the cells were incubated for 1 hour at room temperature. Cells were rinsed with PBS. 500 µl of PBS and resusupended in before the flow cytometry analysis was done using the Becton Dickinson FACS Calibur flow cytometry system (Becton Dickinson, San Jose, CA, USA).

2.3. PREPERATION OF PLURONICS

The solutions of F68 (BASF, USA, cat # 52389638), F127 (BASF, USA, cat # 55401892) and P85 (BASF, USA, cat # 30085877) block copolymers were prepared in PBS at 10% (w/v) concentration. Since pluronics dissolve at low temperature the preparation was performed on ice. Pluronic solutions were then diluted in DMEM to different concentrations (0.01%, 0.02%, 0.05%, 0.1%) to be used in cell treatments.

2.4. TOXICITY ASSAY OF PLURONICS

F68, F127 and P85 were prepared at different concentrations (0.01%, 0.02%, 0.05%, 0.1% in PBS) (w/v) were dissolved in DMEM. Cells were seeded at a concentration of 4000 cell/well on 96 well plates (BIOFIL, TCP, Switzerland, cat # 011096). The following day cells were treated with the pluronic solutions for 3 days. For long term toxicity assay of P85 cells were treated with 0.05% P85 and 20% (v/v) DMSO for 13 days. MTS (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2- (4-sulfo-phenyl)-2H-tetrazolium) (CellTiter96 Aqueous One Solution, Promega, UK) solution was prepared according to the manufacturer's instructions and cells were incubated with the MTS solution for 2 hours. MTS is a yellow tetrazolium-salt which is catabolized to formazan by dehydrogenase enzyme in mitochondria of the living cells. Formazan is a purple compound and this assay is based on the detection of formazan compounds by ELISA plate reader (Biotek, model no: EL800). Therefore cell viability depending on the toxicity of pluronics at different concentrations and long term effect of P85 can be determined by this assay.

2.5. DIFFERENTIATION PROCESS

In this study, the effect of pluronics on odontogenic differentiation of hTGSCs was evaluated. Cells were seeded on 6 well plates at a concentration of 50000 cells/well. Odontogenic differentiation medium was prepared by conditioning the culture medium with 50 μg/mL ascorbic acid (Sigma, USA, cat # D1756), 50 nM β-glycerol phosphate (Sigma, USA, cat # G9891), and 10^{-8} M dexamethasone (DXM) (Sigma, USA, cat # A4544). The pluronics were freshly added to the odontogenic medium and cells were treated with this medium on every other day for 14 days. Two additional groups were

arranged; in the first group cells were treated with odontogenic differentiation medium (positive control) and in the cells were grown media (negative control).

2.6. ALP ACTIVITY ASSAY

Alkaline phosphatase (ALP) activity is a marker of odontogenic differentiation. After the process of odontogenic differentiation, ALP activity of the cells was determined. Medium was removed from the wells, and 0.2% (w/v) Triton-X-100 (BioBasicInc., cat # 9002-93-1) was added to cells for lysis. Cells were harvested from 6 well plates and mixed by vortexing for 20 minutes at room temperature. 10 µl of lysate was added to each well of 96 well plates, then 90 µl of ALP solution (BioAssay Systems, USA, cat # DALP-250) was added to lysates by multichannel pipette. After 15 min the absorbance at 405 nm was measured by ELISA plate reader to determine ALP activity.

2.7. VON KOSSA STAINING

von Kossa Staining assay is based on demonstrating the deposits of calcium or calcium salts. Cells were seeded on 6 well plates at a concentration of 50000 cells/well. Medium was removed from wells and the cells were rinsed with PBS for 3 times. Cells were fixed by 500 µl of paraformaldehyde (Sigma, US cat # 158127) for 20 minutes and rinsed with PBS for 3 times. After addition of 3% (w/v) silver nitrate, the cells were incubated under UV light for 1 hour at room temperature. Cells were rinsed with PBS for 3 times and 5% (w/v) sodium thiosulfate was added into each wells following an incubation for 2 minutes at room temperature, cells were rinsed with PBS for 3 times. Nuclear fast red was then into each well added for 5 minutes at room temperature, following another set of PBS wash. Cells were observed under the light microscope.

2.8. IMMUNOCYTOCHEMISTRY ANALYSIS

Dentin sialoprotein (DSP) (Santa Cruz, CA, USA, cat # SC-33586), bone morphogenic protein 2 (BMP 2) (Santa Cruz, CA, USA, cat # SC-73743), bone morphogenic protein 7 (BMP 7) (Santa Cruz, CA, USA, cat # SC-65346), dentin matrix protein 1 (DMP 1) (Santa Cruz, CA, USA, cat # SC-73633) and collagen type I (COL1A) (Santa Cruz, CA, USA cat

59772) are odontogenic differentiation markers. Immunocytochemistry analysis was performed to visualize to these markers. Medium was removed from wells and the cells were rinsed with PBS for 3 times for 5 min. Cells were fixed by 100 µl of 2% (w/v) paraformaldehyde for 20 min at +4^{o}C. Following a set of washing step with PBS, cells were incubated with 100 µl of 0.1% (v/v) Triton-X-100 5 min at room temperature. Cells were then rinsed with PBS for 3 times for 5 min, and incubated with 2% (w/v) goat serum (Sigma, USA, cat # G9023) for 20 min at +4^{o}C in order to block the nonspecific binding of antibodies. Cells were then rinsed with PBS for 3 times for 5 min and incubated with 1 µl primary antibody dissolved in 100 µl PBS for overnight at +4^{o}C. After rinsing the cell monolayer with PBS for 3 times for 5 min, cells were incubated with 1 µl secondary antibodies Goat anti rabbit IgG Alea Fluor 488 (Invitrogen, USA, cat # A11008), Goat anti mouse IgG Alea Fluor 488 (Invitrogen, USA, cat # A11001) diluted in PBS (1:100 dilution). Starting from this step the rest of the experiment was carried out in dark. Cells were then rinsed with PBS for 3 times for 5 min and stained with 4',6-diamidino-2-phenylindole (DAPI) (Applichem, Germany, cat # A40990010) visualize the nuclei of the cells. 0.5 µl (100:0.1 dilution) DAPI was added to 200 µl PBS and this solution was added to cells for 20 min at +4^{o}C. Cells were rinsed with PBS for 3 times for 5 min and observed under fluorescence microscope (Nicon Eclipse TE200, Germany, Model no: CCD1300B).

2.9. PRIMER DESIGNING

Primers for COL1A, DSPP, BMP 2 and BMP 7 genes were designed by using nBLAST online software of The National Center for Biotechnology (NCBI). These primers were ordered Invitrogen Company to be synthesized at 50 nmoles.

Table 2.1. Primers that were used in this study for the quantitative Real Time PCR analysis

Primer	Sense (5'-3')	Antisense (5'-3')	Base pair
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA	123
COL1A	CCACGCATGAGCGGACGCTAA	ATTGGTGGGATGTCTTCGTCTTGG	178
DSPP	CAGTAVAGGATGAGTTAAATGCC	TACTTCTGCCCACTTAGAGCC	134
BMP 2	TGAATTCATGGTGGCCGGGAC	ATTGTCGACGCGACACCCACA	175
BMP 7	TGGATCCATGCACGTGCGCTC	ATTGTCGACGTGGCAGCCACA	144

2.10. RT-PCR ANALYSIS

Total RNA isolation was performed using the High Pure RNA isolation kit (ROCHE, USA, cat # 11828665001) according to the manufacturer's instructions. cDNA synthesis from RNA samples was done using the High Fidelity cDNA synthesis kit (ROCHE, USA, cat # 05081955001) according to manufacturer's instructions. For the determination of gene levels, Real Time PCR was performed by using Maxima SYBR Green/ROX. cDNA synthesized was used as a template. 10 µl of Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, EU, cat # K0221), 0.3 µM of forward primer, 0.3 µM of reverse primer, 500 ng template and dH_2O were mixed in a final concentration of 20 µl. GAPDH (glycer-aldehyde-3-phosphate-de-hydrogenase) gene was used in this study as a housekeeping gene for, normalization. iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA, icycler iQ Optical Module) was used for the Real Time PCR.

Table 2.2. RT-PCR reagents

SYBRGreen 2X	10µl
Primer Forward (10µM)	1µl
Primer Reverse (10µM)	1µl
Distilled water	4µl
Template (100ng/ml)	4µl
Total Volume	20µl

Table 2.3. RT-PCR conditions

Cycle	Repeat	Step	Dwell Time	Set Point
1	1	1	5 min	95 °C
2	40	1	30 sec	95 °C
-		2	40 sec	58 °C
-		3	50 sec	72 °C
3	1	1	10 min	72 °C
4	110	1	12 sec	40 °C
5	1	1	-	4 °C

2.12. STATISTICAL ANALYSIS

All graphics were prepared using the Microsoft Office Excel and GraphPad Prism5 softwares. Standard errors and t-test values were calculated using GraphPad Prism5 software. For statistical analysis student t test was used in the GraphPad prism5 programs. P value less than 0.05 was considered as statistically significant.

3. RESULTS

3.1. CHARACTERIZATION OF HUMAN TOOTH GERM STEM CELLS

hTGSCs were characterized for mesenchymal stem cells surface markers CD29, CD34, CD45, CD44, CD90, CD105, CD133,CD14, CD166 and CD73 using Flow cytometry. hTGSCs were shown as a positive for CD29, CD105, CD44, CD90, CD73, CD166 and negative for CD34, CD45, CD133, CD14. This data prove that HTGSCs are positive for Mesenchymal Stem Cell surface markers and negative for Hematopoetic Stem Cell surface markers.

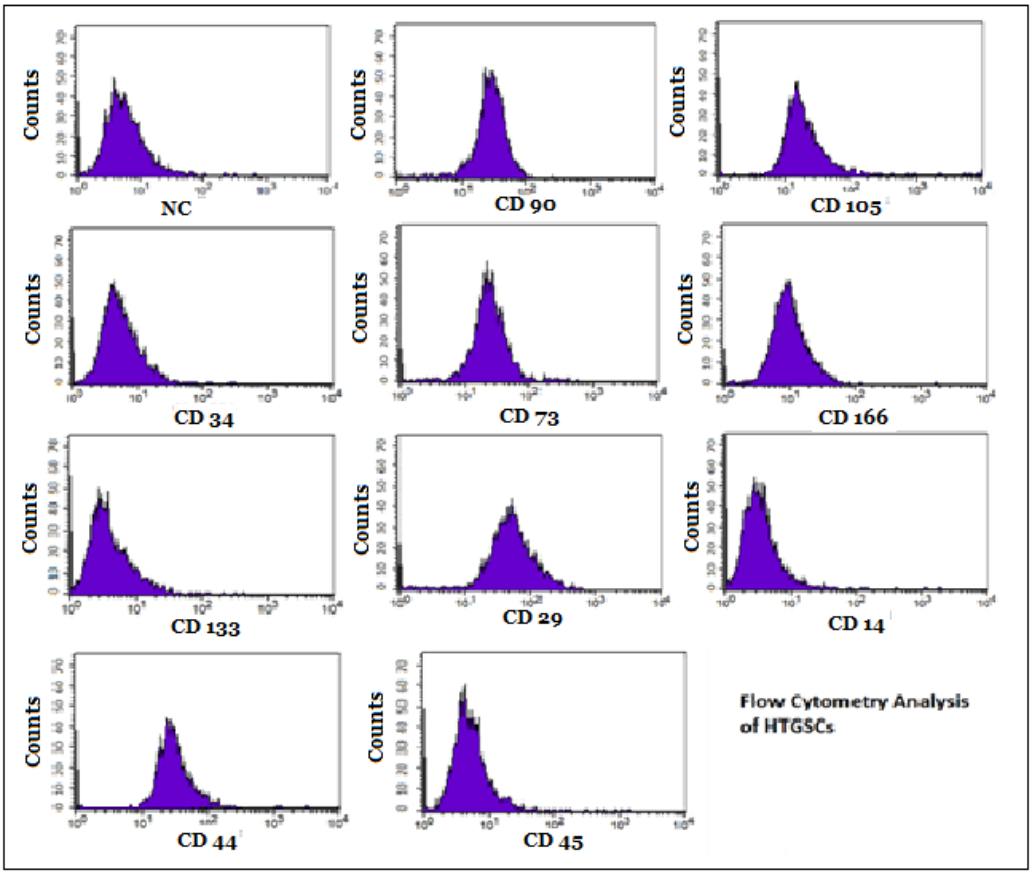

Figure 3.1. Flow cytometry analysis of hTGSCs

3.2. TOXICITY OF PLURONIC BLOCK COPOLYMERS

All three pluronics were prepared according to the manufacturer's instructions. Four different concentrations were prepared in PBS solution and cells were treated with these concentrations (% 0.01, % 0.02, % 0.05 and % 0.1) for 3 days.

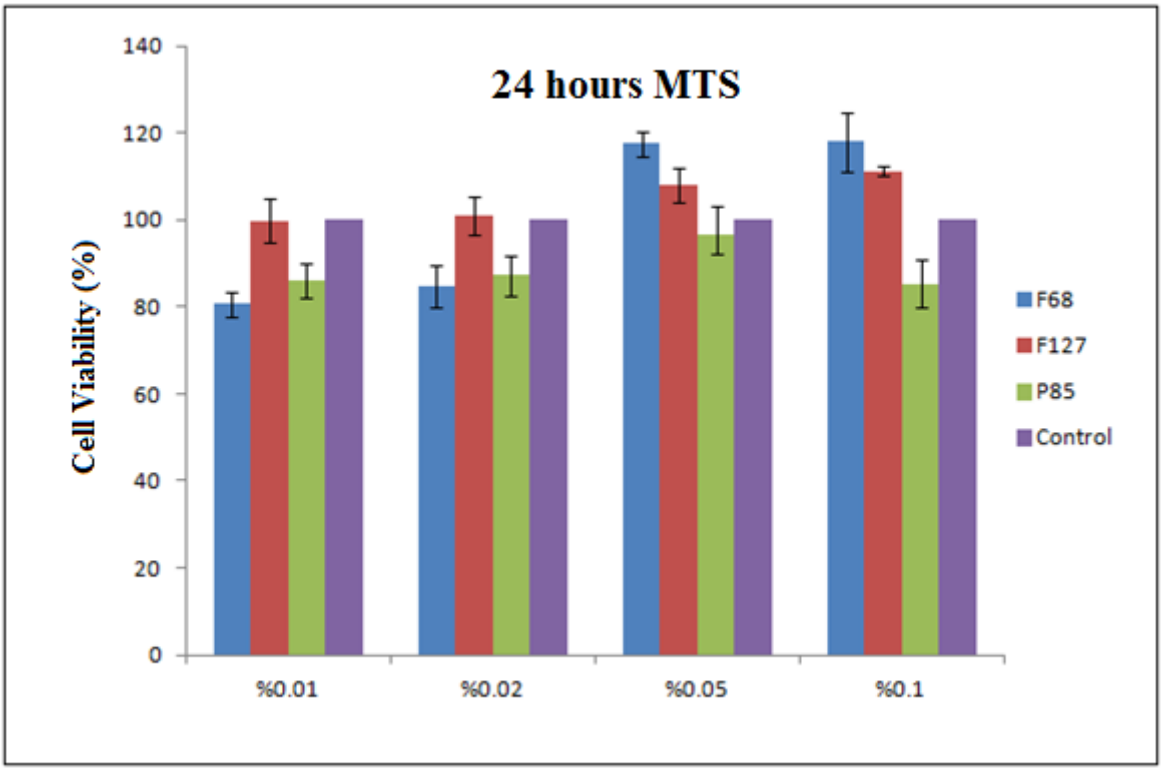

Figure 3.2.1. MTS assay of four concentration ofpluronics on hTGSCs for 24 hours

Four concentrations of pluronic F68, F127 and P85 were applied to the hTGSCs for 3 days. This data shown that non of concentration of F68 have toxic effect on hTGSCs, moreover 0.05% concentration of pluronic F68 is increased the cell viability of hTGSCs for 24 hours. All four concentration of F127 have no effect on hTGSCs during 24 hours. 0.1% concentration of P85 was shown toxic effect for hTGSCs, while other concentrations of P85 were not toxic.

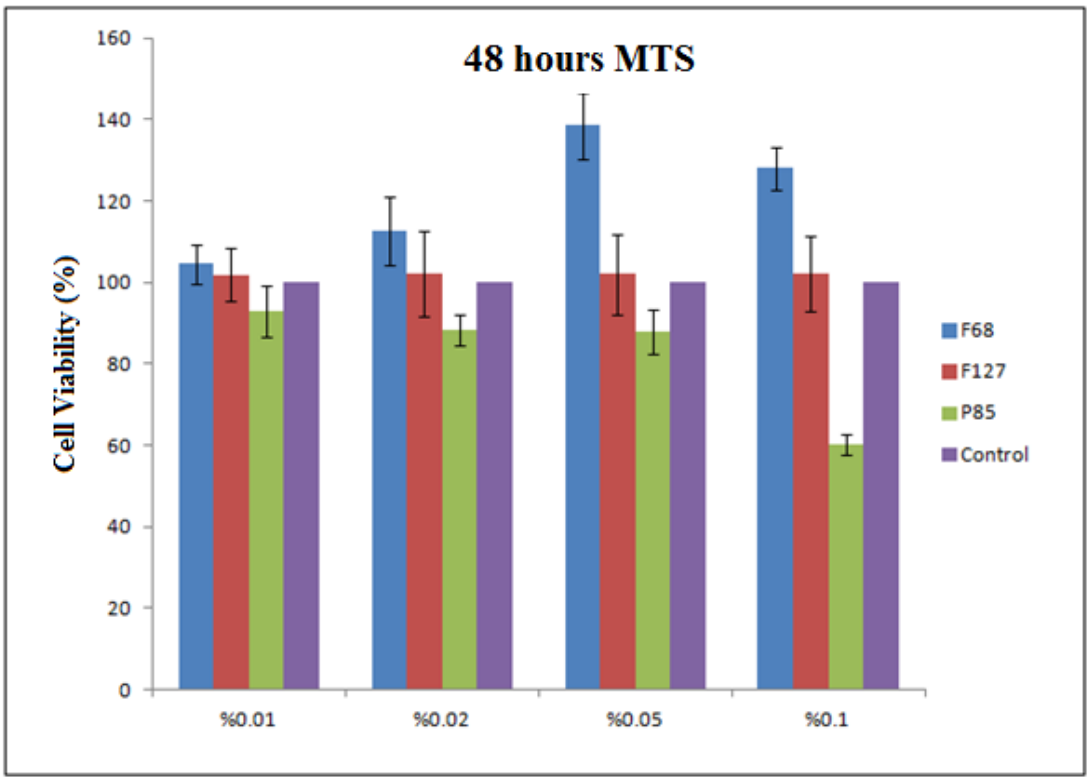

Figure 3.2.2. MTS assay of four concentration of pluronics on hTGSCs for 48 hours

This data shown that non of concentrations of F68 does not have any toxic effect on hTGSCs, moreover all four concentration of pluronic F68 were increased the cell viability of hTGSCs at 48 hours. F127 has no effect on hTGSCs at 48 hours to. P85 has higher toxic effect for hTGSCs for 48 hours than 24 hours treatment.

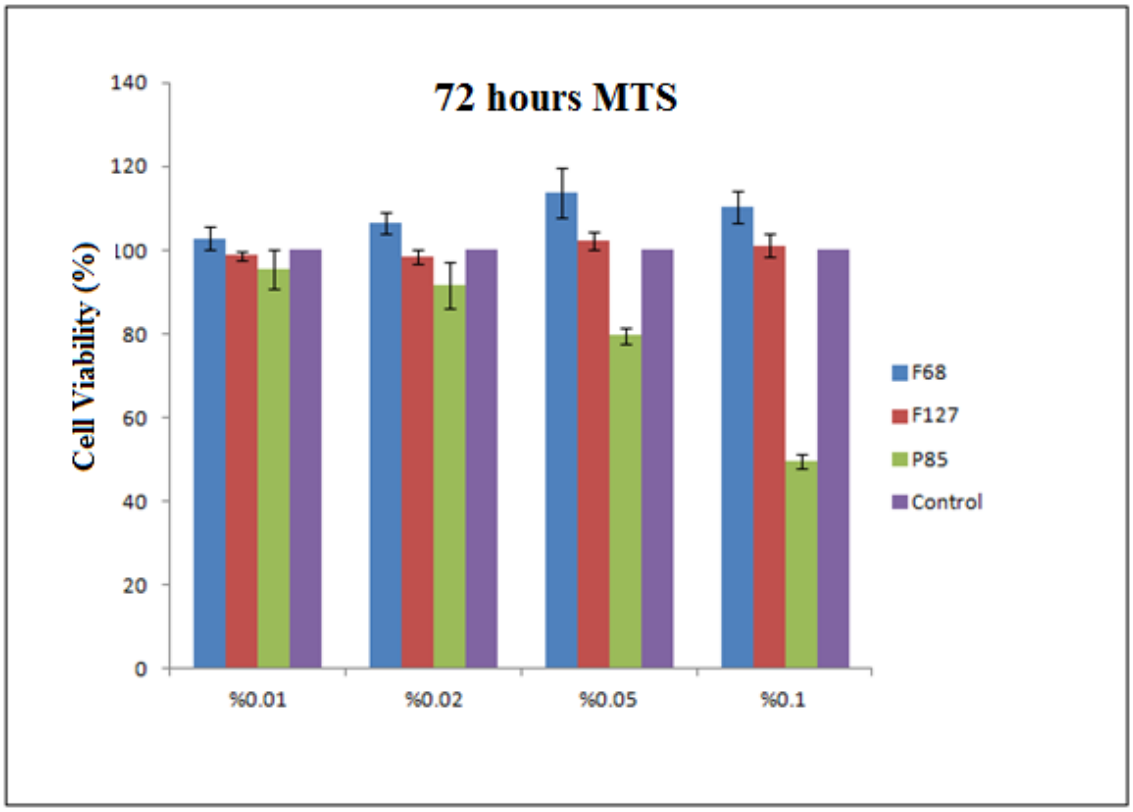

Figure 3.2.3. MTS assay of four concentration of pluronics on hTGSCs for 72 hours

The 72th hours of MTS results show that all four concentrations of F68 increase cell viability. F127 has no any effect of hTGSCs during 72 hours. Higher concentration of P85 such as % 0.05 or % 0.1, decrease the cell viability but it is not kill the all cells, at %0.05 concentration of P85, more than % 80 of the cells were remained.

3.3. LONG TERM TOXICITY OF PLURONIC P85

According to the 3rd day of MTS results we show that P85 has toxic effect on hTGSCs. Upon this we checked the cytotoxicity of P85 on hTGSCs at 1st, 3rd, 5th, 7th, 9th, 11th and 13th days. It was shown that P85 started to show toxic effects on the 3rd day of the MTS assay at 0.05% and 0.1% concentrations but end of the 13th day 0.05% concentration of P85 did not kill all the cells and 58% of the cells remained on the plate at 13rd day.

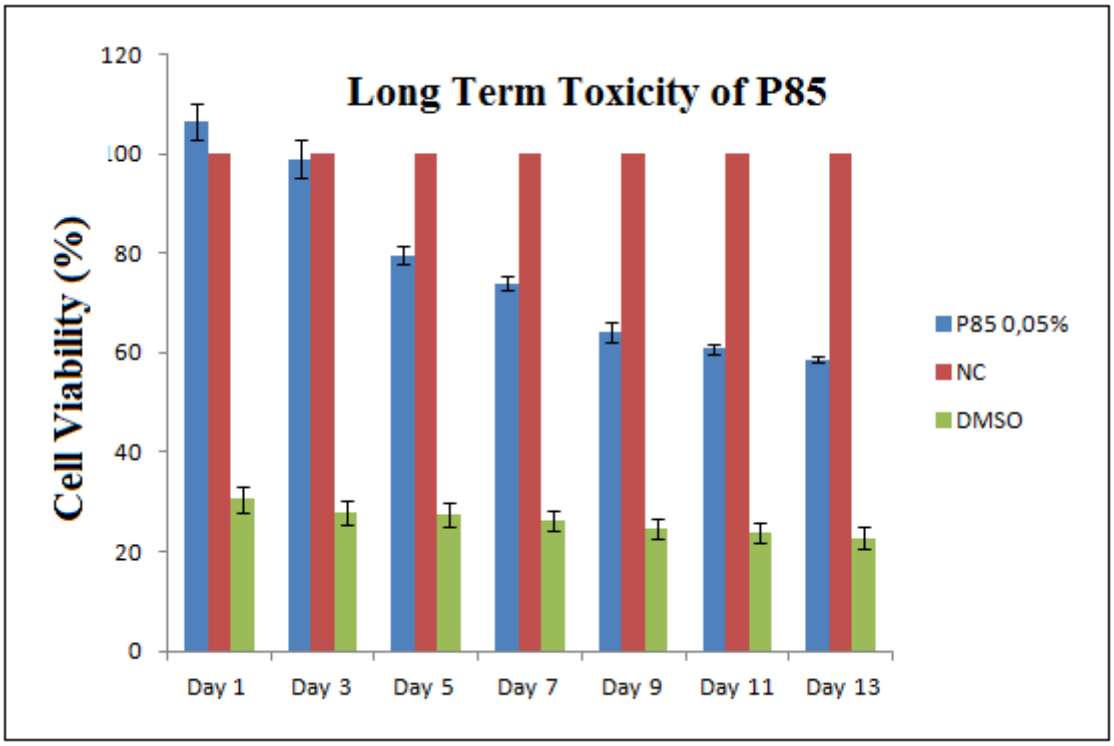

Figure 3.3. Long term toxicity of 0.05% (w/v) concentration of P85. 20% (v/v) DMSO treated group as a PC and NC as hTGSCs

Long term toxicity of P85 was shown to be toxic for hTGSCs. End of the 13th day 20% (v/v) DMSO treated cells all died but 0.05% (w/v) P85 treated cells remained to live. 60% of the P85 treated cells were alive compared to the NC as non differentiated hTGSCs.

3.4. ODONTOGENIC DIFFERENTIATION

3.4.1. ALP Activity

Determining to the odontogenic differentiation in Pluronic treated and non treated groups, level of ALP activity was determined using the ALP activity assay.

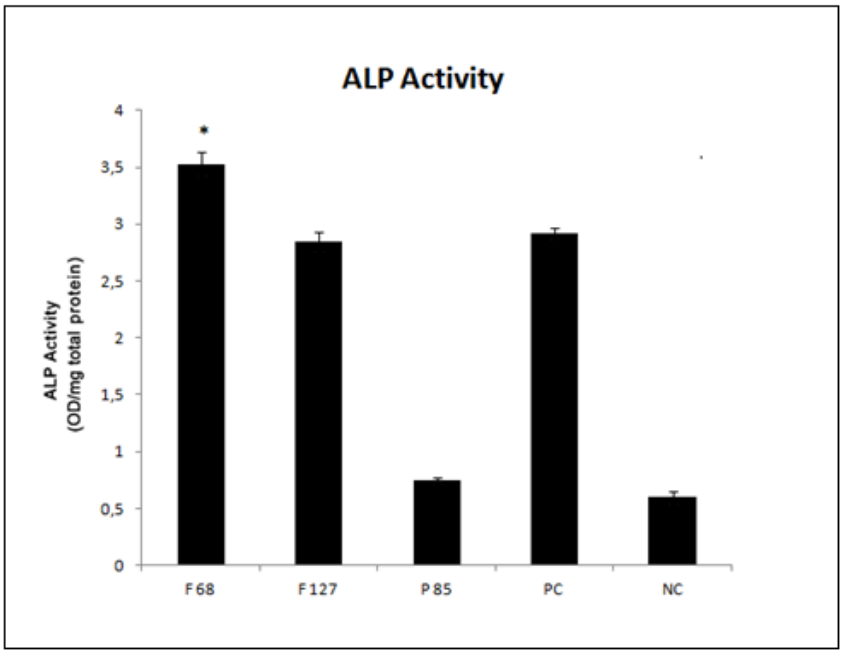

Figure 3.4. ALP activity of 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group, 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days (*$p<0.05$)

The results showed that all groups have higher level of ALP activity compared to the non-differentiated group as a NC as non differentiated hTGSCs, except P85 treated group. Moreover, F68 treated group increased ALP activity significantly comparing to the PC group. F127 treated group has no difference between PC as an odontogenic differentiation medium treated group. ALP activity level of P85 treated group is lower than PC significantly and higher than NC but not significantly.

3.4.2. von Kossa staining

von Kossa Staining is the marker for odontogenic differentiation and it shows calsium deposition in mineralized tissue. von Kossa Staining was performed to show the calsium deposition in odontogenicly differentiated groups. All differentiated groups show higher calcium deposition than NC.

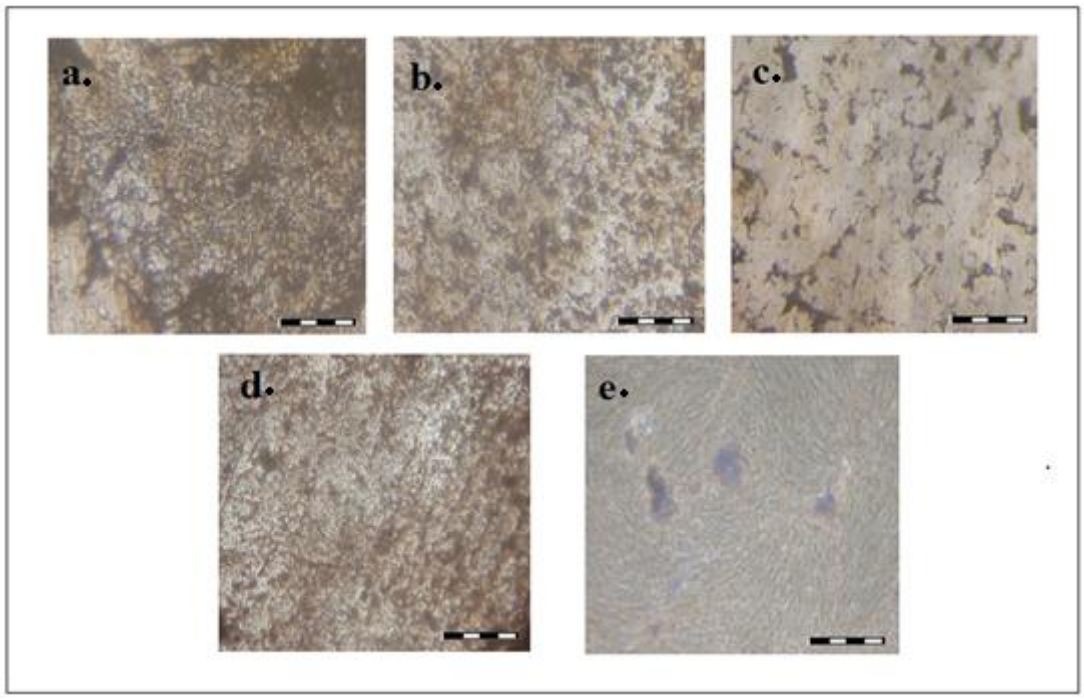

Figure 3.5. von Kossa Staining of a. 0.05% (w/v) F68 + odontogenic differentiation medium treated group, b. 0.05% (w/v) F127 + odontogenic differentiation medium treated group, c. 0.05% (w/v) P85 + odontogenic differentiation medium treated group, d. PC as an odontogenic differentiation medium treated group and e. NC as non differentiated hTGSCs for 14th days. Scale bar: 100 μm

3.4.3. Immunocytochemistry

Immunocytochemistry data show that expression of COL1A as an odontogenic marker. According to immunocytochemistry data 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group, 0.05% (w/v) P85 + odontogenic differentiation medium treated group and PC as an odontogenic differentiation medium treated group show COL1A expression higher than NC as a non differentiated hTGSCs, as it seen in the Figure 3.6.1.

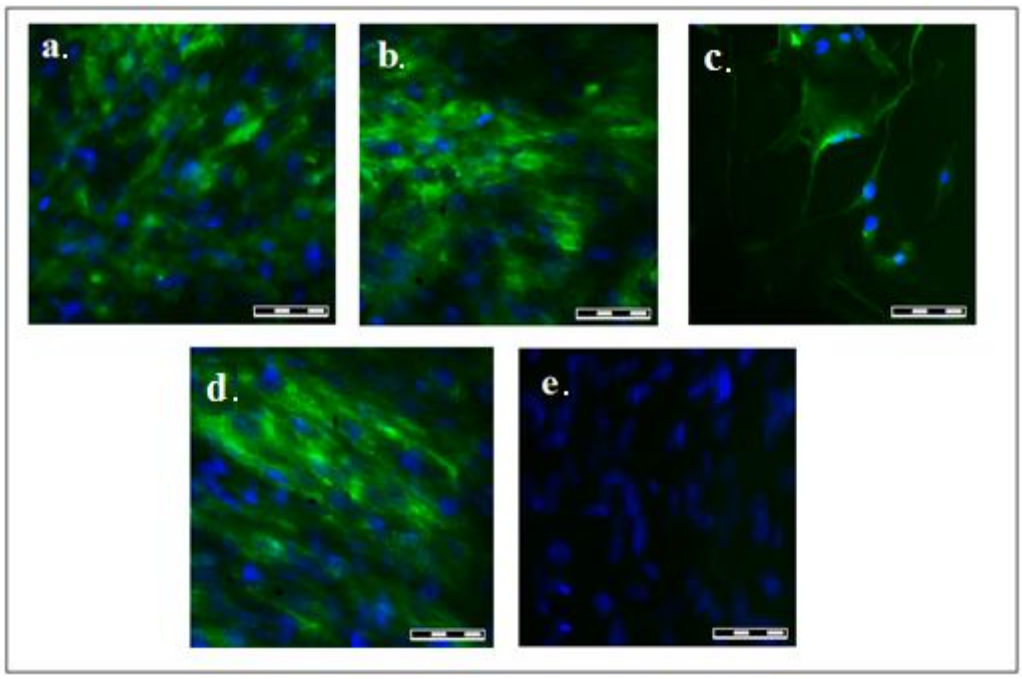

Figure 3.6.1. Immunocytochemistry of a. 0.05% (w/v) F68 + odontogenic differentiation medium treated group, b. 0.05% (w/v) F127 + odontogenic differentiation medium treated group, c. 0.05% (w/v) P85 + odontogenic differentiation medium treated group, d. PC as an odontogenic differentiation medium treated group and e. NC as non differentiated hTGSCs for 14th days with COL1A antibody. Scale bar: 100 µm

Immunocytochemistry data show that expression of DMP 1 as an odontogenic marker. According to immunocytochemistry data 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group, 0.05% (w/v) P85 + odontogenic differentiation medium treated group and PC as an odontogenic differentiation medium treated group show DMP 1 expression higher than NC as a non differentiated hTGSCs, as it seen in the Figure 3.6.2.

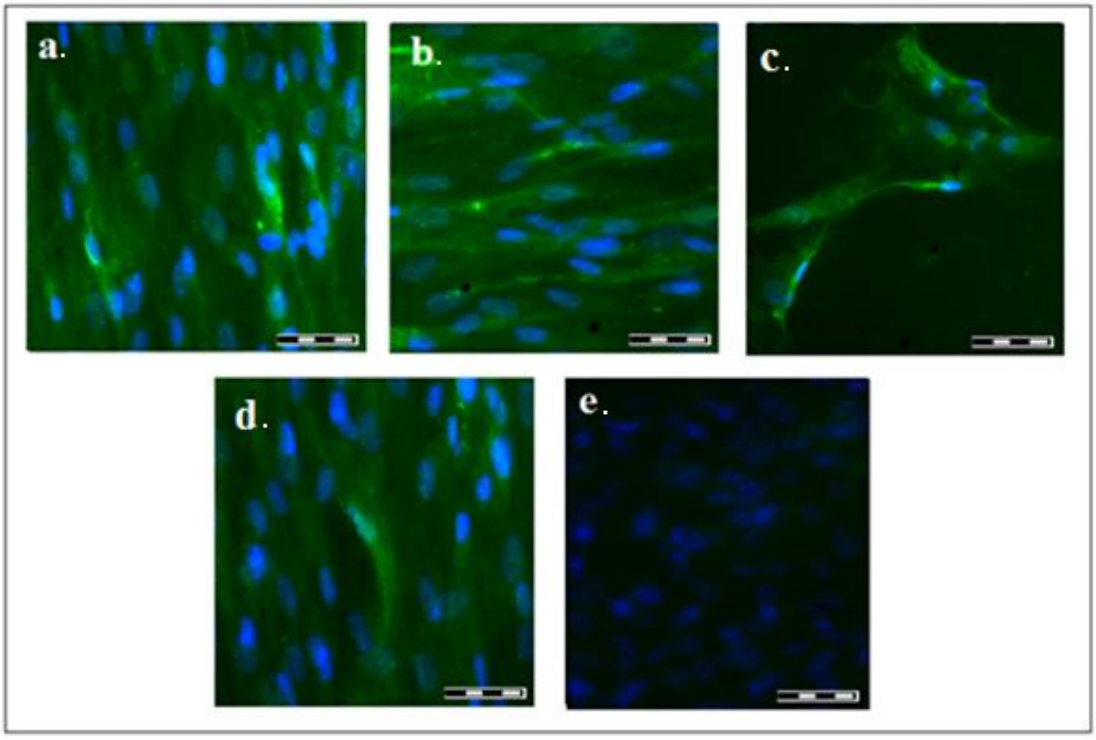

Figure 3.6.2. Immunocytochemistry of a. 0.05% (w/v) F68 + odontogenic differentiation medium treated group, b. 0.05% (w/v) F127 + odontogenic differentiation medium treated group, c. 0.05% (w/v) P85 + odontogenic differentiation medium treated group, d. PC as an odontogenic differentiation medium treated group and e. NC as non differentiated hTGSCs for 14th days with DMP 1 antibody. Scale bar: 100 μm

Immunocytochemistry data show that expression of DSP as an odontogenic marker. According to immunocytochemistry data 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group, 0.05% (w/v) P85 + odontogenic differentiation medium treated group and PC as an odontogenic differentiation medium treated group show DSP expression higher than NC as a non differentiated hTGSCs, as it seen in the Figure 3.6.3.

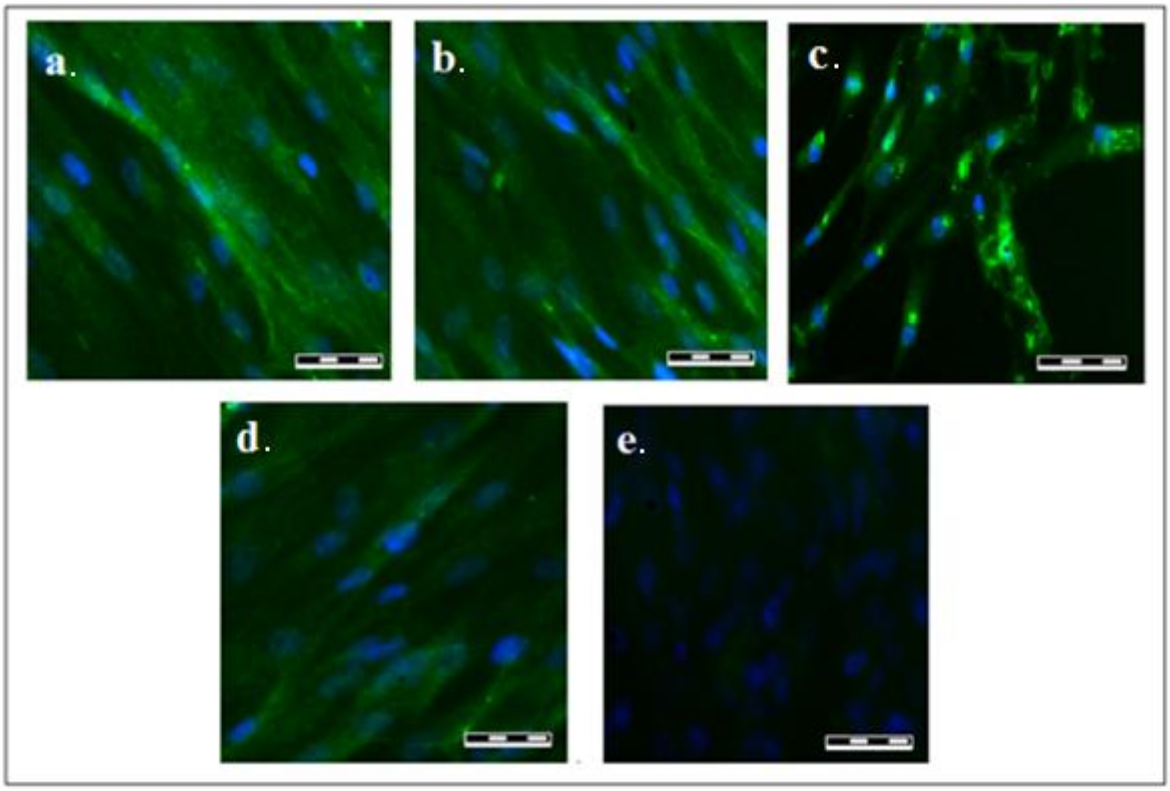

Figure 3.6.3. Immunocytochemistry of a. 0.05% (w/v) F68 + odontogenic differentiation medium treated group, b. 0.05% (w/v) F127 + odontogenic differentiation medium treated group, c. 0.05% (w/v) P85 + odontogenic differentiation medium treated group, d. PC as an odontogenic differentiation medium treated group and e. NC as non differentiated hTGSCs for 14th days with DSP antibody. Scale bar: 100 μm

Immunocytochemistry data show that expression of BMP 2 as an odontogenic marker. According to immunocytochemistry data 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group, 0.05% (w/v) P85 + odontogenic differentiation medium treated group and PC as an odontogenic differentiation medium treated group show BMP 2 expression higher than NC as a non differentiated hTGSCs, as it seen in the Figure 3.6.4.

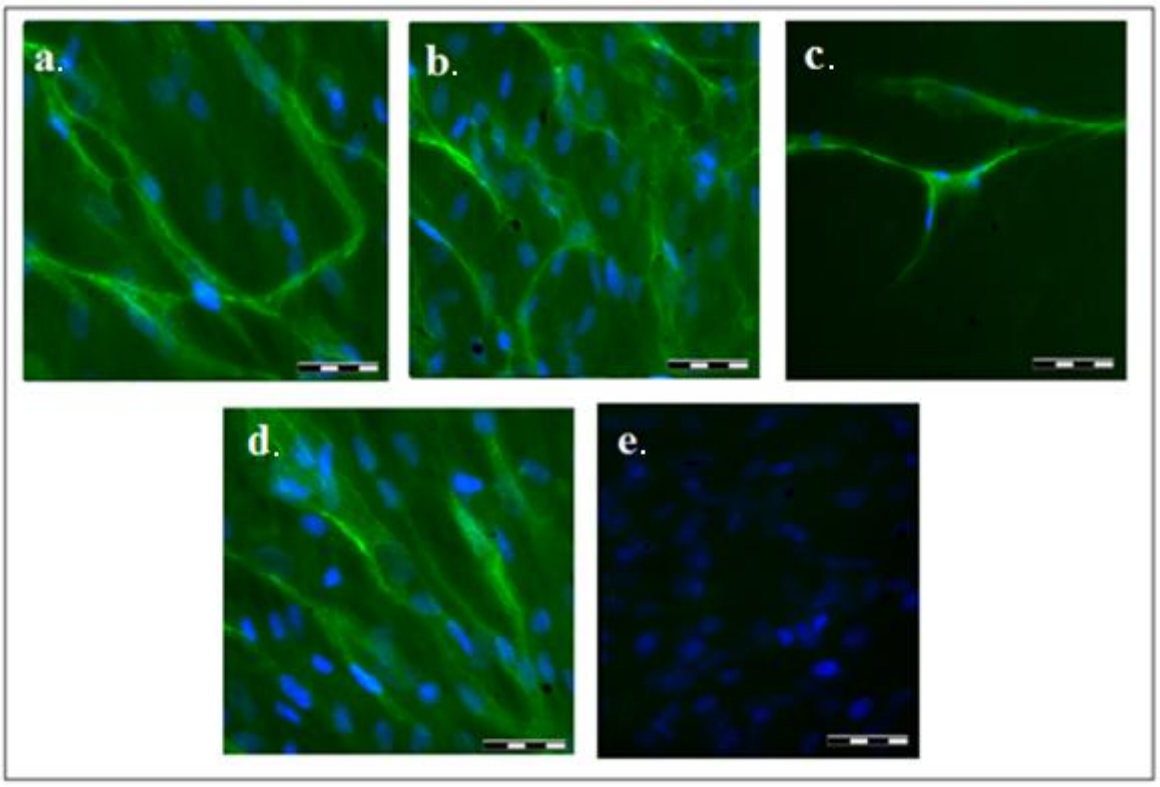

Figure 3.6.4. Immunocytochemistry of a. 0.05% (w/v) F68 + odontogenic differentiation medium treated group, b. 0.05% (w/v) F127 + odontogenic differentiation medium treated group, c. 0.05% (w/v) P85 + odontogenic differentiation medium treated group, d. PC as an odontogenic differentiation medium treated group and e. NC as non differentiated hTGSCs for 14th days with BMP 2 antibody. Scale bar: 100 μm

Immunocytochemistry data show that expression of BMP 7 as an odontogenic marker. According to immunocytochemistry data 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group, 0.05% (w/v) P85 + odontogenic differentiation medium treated group and PC as an odontogenic differentiation medium treated group show BMP 7 expression higher than NC as a non differentiated hTGSCs, as it seen in the Figure 3.6.5.

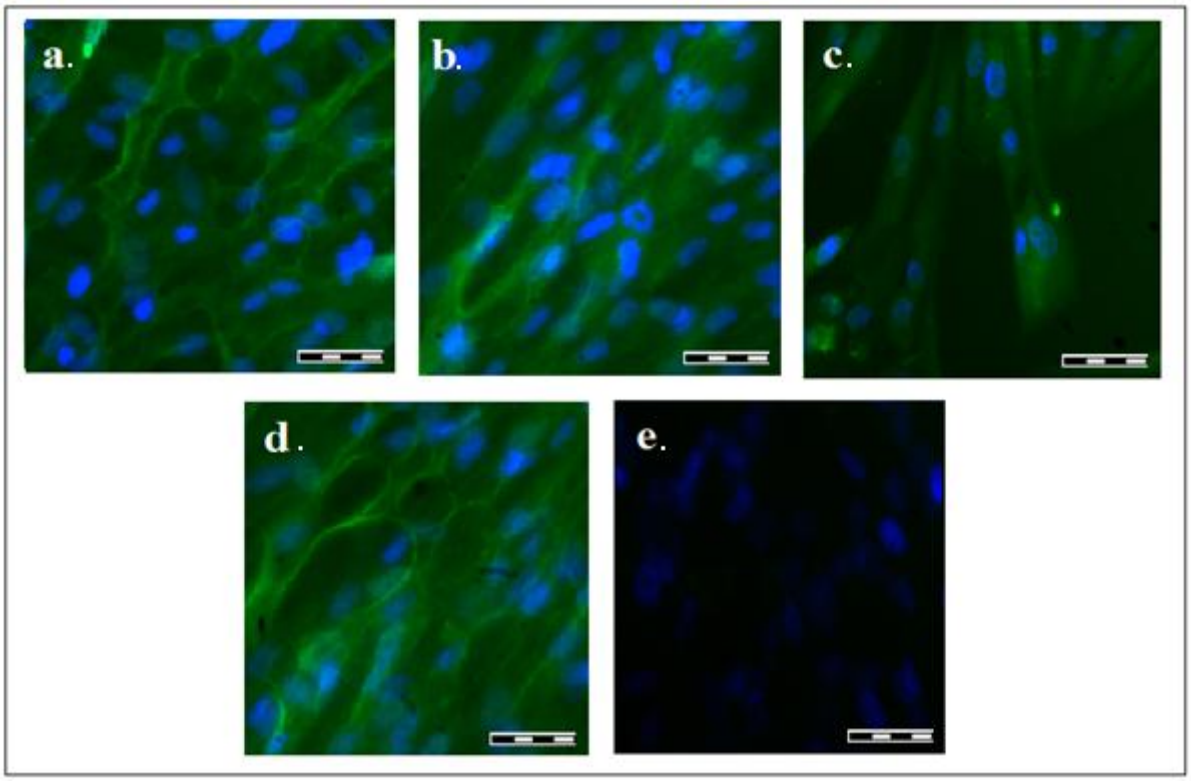

Figure 3.6.5. Immunocytochemistry of a. 0.05% (w/v) F68 + odontogenic differentiation medium treated group, b. 0.05% (w/v) F127 + odontogenic differentiation medium treated group, c. 0.05% (w/v) P85 + odontogenic differentiation medium treated group, d. PC as an odontogenic differentiation medium treated group and e. NC as non differentiated hTGSCs for 14th days with BMP 7 antibody. Scale bar: 100 μm

3.5. Real Time PCR

3.5.1. Relative mRNA Expressions of COL1A

This data shows mRNA expression levels of COL1A in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days. GAPDH gene was used as a house keeping gene to normalize RT-PCR results.

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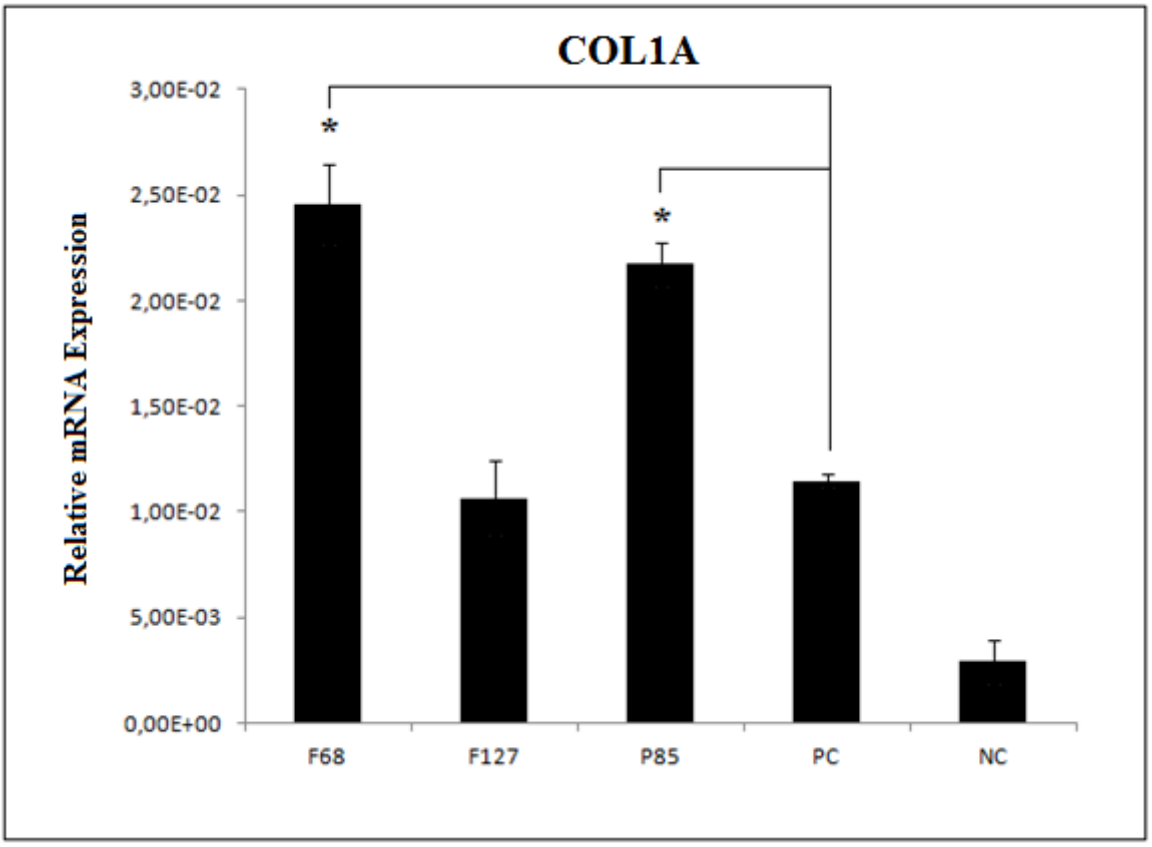

Figure 3.7. Expression levels of COL1A in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days. (*$p<0.05$)

0.05% (w/v) F68 + odontogenic differentiation medium treated group showed 2 fold higher expression level than PC and 0.05% (w/v) P85 + odontogenic differentiation medium treated group showed 1.8 fold higher than PC but 0.05% (w/v) F127 + odontogenic differentiation medium treated group had no effect compared to PC which was treated with odontogenic differentiation medium. All differentiated groups displayed significantly increased COL1A expression compare to NC which consisted of non differentiated hTGSCs, as it is seen in the Figure 3.7.

3.5.2. Relative mRNA Expressions of DSPP

This data shows mRNA expression levels of DSPP in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days. GAPDH gene was used as a house keeping gene to normalize RT-PCR results.

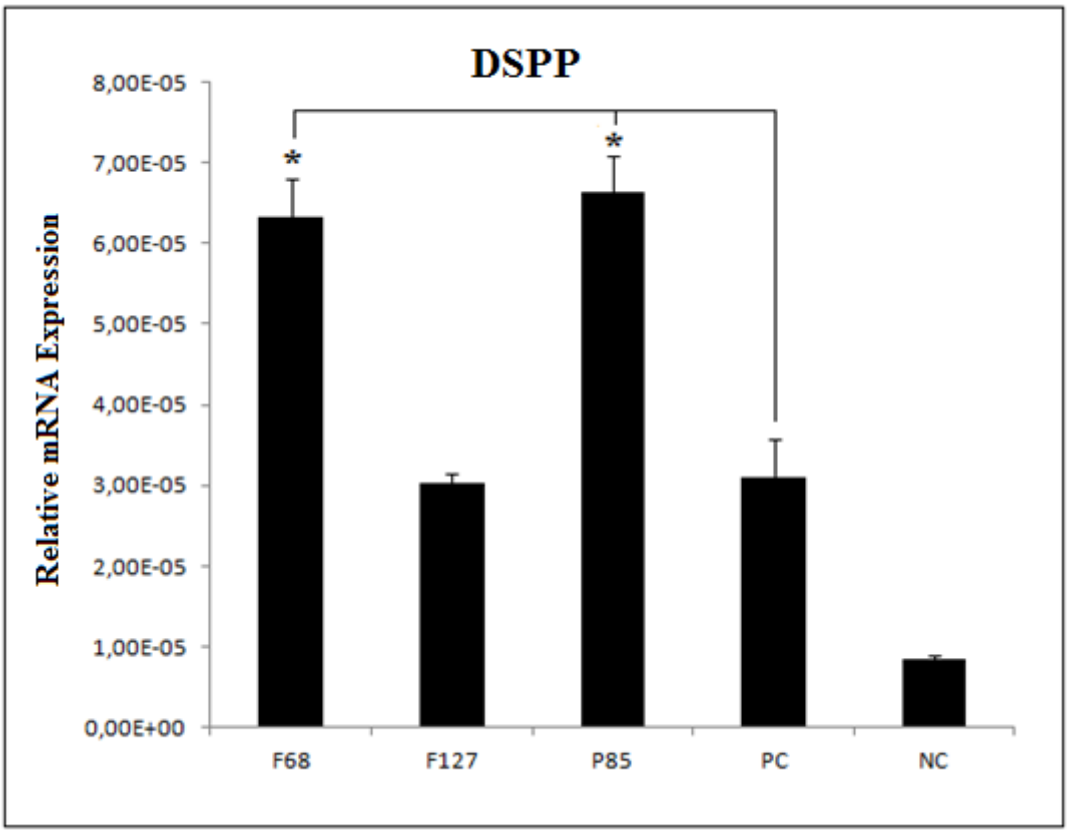

Figure 3.8. Expression levels of DSPP in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days. (*$p<0.05$)

0.05% (w/v) F68 + odontogenic differentiation medium treated group showed 2 fold higher expression level than PC and 0.05% (w/v) P85 + odontogenic differentiation medium treated group showed 2,2 fold higher than PC but 0.05% (w/v) F127 + odontogenic differentiation medium treated group had no effect compared to PC which was treated with odontogenic differentiation medium. All differentiated groups displayed significantly increased DSPP expression compare to NC which consist of non differentiated hTGSCs, as it is seen in the Figure 3.8.

3.5.3. Relative mRNA Expressions of BMP 2

This data shows mRNA expression levels of BMP 2 in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days, as it is seen in the Figure 3.9. GAPDH gene was used as a house keeping gene to normalize RT-PCR results.

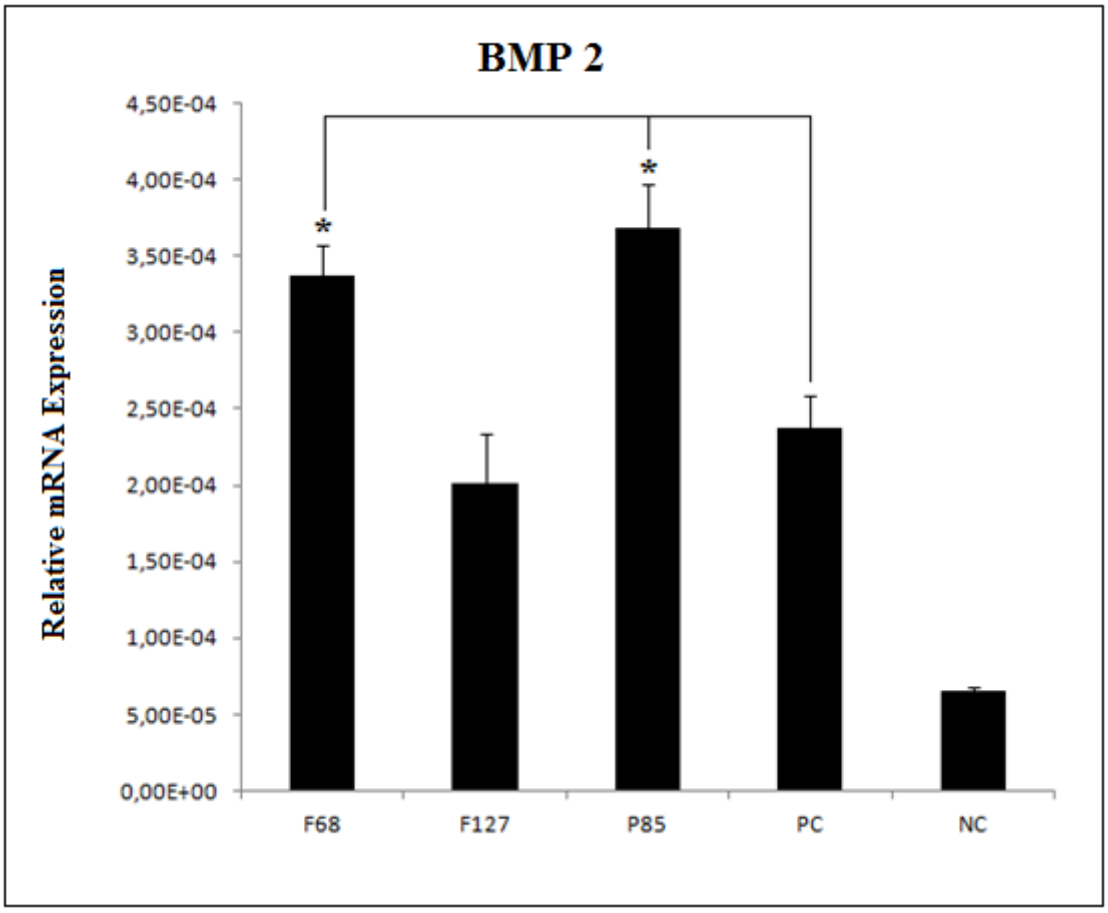

Figure 3.9. Expression levels of BMP 2 in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days. (*$p<0.05$)

0.05% (w/v) F68 + odontogenic differentiation medium treated group showed 2 fold higher expression level than PC and 0.05% (w/v) P85 + odontogenic differentiation medium treated group showed 2,3 fold higher than PC but 0.05% (w/v) F127 + odontogenic differentiation medium treated group had no effect compared to PC which was treated with odontogenic differentiation medium. All differentiated groups displayed significantly increased BMP 2 expression compare to NC which consist of non differentiated hTGSCs.

3.5.4. Relative mRNA Expressions of BMP 7

This data shows mRNA expression levels of BMP 7 in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days, as it is seen in the Figure 3.10. GAPDH gene was used as a house keeping gene to normalize RT-PCR results.

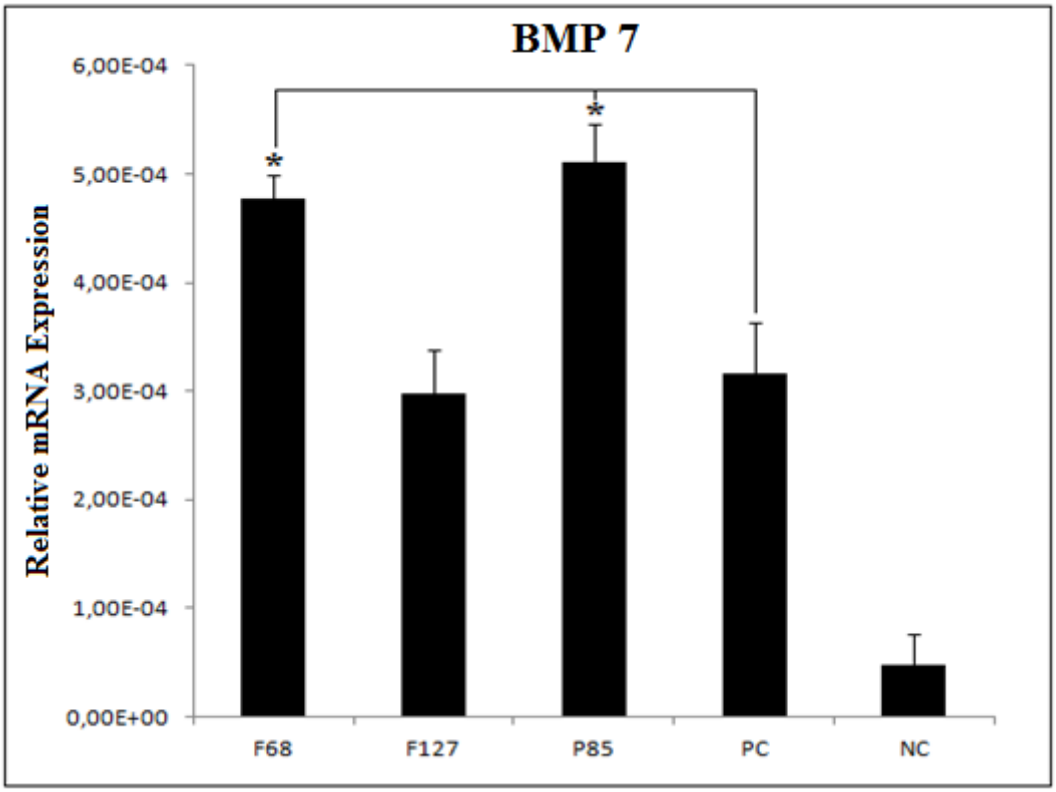

Figure 3.10. Expression levels of BMP 7 in 0.05% (w/v) F68 + odontogenic differentiation medium treated group, 0.05% (w/v) F127 + odontogenic differentiation medium treated group and 0.05% (w/v) P85 + odontogenic differentiation medium treated group, PC as an odontogenic differentiation medium treated group and NC as non differentiated hTGSCs for 14th days. (*$p<0.05$)

0.05% (w/v) F68 + odontogenic differentiation medium treated group showed 2 fold higher expression level than PC and 0.05% (w/v) P85 + odontogenic differentiation medium

treated group showed 2,3 fold higher than PC but 0.05% (w/v) F127 + odontogenic differentiation medium treated group had no effect compared to PC which was treated with odontogenic differentiation medium. All differentiated groups displayed significantly increased BMP 7 expression compare to NC which consist of non differentiated hTGSCs, as it is seen in the Figure 3.10.

As a conclution, in this experiment we used differentiation medium and these medium contains different chemicals such as dexamethasone, ascorbic acid and β-glycerol phosphate, but dexamethasone is also used as a chemotherapeutic drug, that's why P85 may have effect transport of chemicals in differentiation medium including dexamethasone to the cells excessively leading cells death. However, F68 and F127 did not show the same effect during odontogenic differentiation process of hTGSCs.

5. DISCUSSION

A commonly known definition of tissue engineering is “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ” [55]. Tissue engineering has also been defined as “understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use” [129]. Tissue engineering aims to stimulate the body either to regenerate tissue on its own or to grow tissue outside the body which can then be implanted as the natural tissue.

In the field of dental tissue and tooth engineering, studies have been made to explore MSCs such as stem cells from the apical part of the papilla (SCAPs), human exfoliated deciduous teeth (SHEDs), stem cells from the dental follicle (DFSCs), adult dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), bone marrow derived mesenchymal stem cells (BMSCs) and epithelium originated dental mesenchymal stem cells [38]. This various MSCs offer many advantages for dental pulp tissue regeneration or tooth engineering.

Tooth development is the cumulative result of repeater signaling of growth factor family members and also initial signals in the dental epithelium to induce the gene expression in the dental MSCs [130]. Signaling pathway between the epithelial and mesenchymal tissues continues throughout the tooth development, tooth position within the jaw, cause formation of a tooth of specific size and shape [131]. Dental MSCs differentiates into pulp and dentin, and the epithelial tissues of the enamel organ produce dental enamel. The cellular and molecular characteristic of enamel, dentin, and cementum development has been characterized by bioengineers [62, 130, 132-139].

Recently, appropriate biomaterials which must be non toxic, biocompatible and bio absorber used in dental tissue engineering [127]. Tissue engineering is a promising approach for regenerative therapy, and biopolymers are indispensable tools for the cell based tissue engineering. Dental tissue injuries, traumas or looses are very serious problems and it should have capability of regenerate itself. In order to create the optimum

conditions for cells, it is necessary to use appropriate biomaterials in the tissue engineering applications [140]. The regenerative capacity of human tissues such as MSCs varies depending on the tissue type. For instance, epithelial tissue can repair the injuries and regenerate the defected area through the life. However, connective tissues including the tooth has limited regenerative capacity when compared to the epithelial tissues.

Recent studies have shown that the use of MSCs in 3D scaffold is becoming an attractive issue in the tissue engineering and regenerative medicine. Bone and tooth grafting as a biological mechanism and the increased combination of endothelial cells into a scaffold is also considered as a critical requirement for the survival of a tissue engineered bone like constructs [141-143].

Pluronics F68, F127 and P85 were shown to be non toxic in the literature at small concentrations however at high concentrations they can be toxic for the cells [144]. In this study, MTS assay as a toxicity assay. Results supported the data in the literature suggesting that F68, P85 and F127 are not toxic for cells at 0.05% (w/v) concentration which is mostly preferred concentration for pluronics [83, 113, 145-149]. Exceptionally P85 showed toxic effect for cells during the odontogenic differentiation process at both 0.05% (w/v) and 0.01% (w/v) concentrations, but this toxic effect did not kill all the cells in culture plate. P85 has a great interaction and permeabilization ability through the membrane and due to this characteristic it is a suitable agent as an inhibitor of drug efflux systems especially in combination with drugs [102]. In our experiment we used differentiation medium and these medium contains different chemicals such as dexamethasone, ascorbic acid and β-glycerol phosphate. Dexamethasone is also used as a chemotherapeutic drug, that's why P85 may have effect on the transport of chemicals in differentiation medium including dexamethasone in to the cells excessively leading to cell death. However, F68 and F127 did not show the same effect during odontogenic differentiation process of hTGSCs.

Odontogenic differentiation was confirmed by ALP activity, von Kossa staining, immunocytostaining with DMP1, COL1A, DSP, BMP 7 and BMP2 antibodies, as well as COL1A, DSPP, BMP 7 and BMP2 mRNA expression levels by quantitative real time PCR.

ALP which is an early and common marker for the odontogenic differentiation was chosen in this study to determine the odontogenic differentiation. Role of ALP is the mineralization of calcifying tissues such as odontogenic and osteogenic cells [175]. Our results showed that wells treated with the odontogenic medium, odontogenic medium+Pluronic F68 and odontogenic medium+Pluronic F127 possessed significantly higher ALP activity than the negative control the non differentiated group. Pluronic F68 treated group showed the highest ALP activity compared with the positive control, P85 and F127 treated groups. There is important thing that we should becareful about, to measure ALP activity, number of cells in each grup must be the same. According to the long term toxicity results of P85, end of the 13^{th} day 60% cells were remained comparing to the NC. That's why ALP activity of P85 treated cells can not compared with other groups. Nevertheless, ALP activity of P85 treated group was shown to have higher ALP activity compared to the NC but not significantly.

Another marker for the odontogenic differentiation is the calcium deposits visualized by the qualitative von Kossa staining method [72]. F68, P85, F127 treated groups and PC were shown to form calcium depositions with von Kossa staining but the non differentiated group as a negative control has shown no mineralization.

Collagen type I [60], dentin sialoprotein [61], bone morphogenic protein 2 and dentin matrix protein 1 are early marker for odontogenic differentiation. These proteins are considered to be odontogenic differentiation markers. Type I collagen is one of the important participant of the mineralized tissue in dentin and formation of hard tissue [62-64]. Densialophosphoprotein is the proprotein of dentin matrix protein and dentinsialoprotein and, it plays important role on mineralization of dentin in tooth. Dentin matrix protein secreted by odontoblast cells and, it is the major component of dental mineralized tissue [62]. Dentin matrix proteins secreted by the odontoblast cells can be classified into primary, secondary or tertiary dentin based on the time and circumstances of its secretion [65-68]. Dentinsialoprotein synthesized by odontoblast cells and secreted through the odontoblastic process during the mineral formation [63, 64, 69]. DSP is strongly associated with the mineralization of dentinogenesis [63, 64, 69, 70]. Bone morphogenic protein 7 and bone morphogenic protein 2 promote the odontogenic differentiation and dentin-like tissue formation both in vitro and in vivo [71, 72]. In this

study, it was demonstrated that COL1A, DSP, DMP1 and BMP2 are expressed in all groups after odontogenic differentiation which was confirmed by immunocytochemisty anaylsis. Odontogenic differentiation can determine by level of these odontogenic markers. All differentiated groups were shown higher level of odontogenic markers compared to the negative control.

mRNA expression levels of COL1A, DSPP, BMP 2 and BMP 7 were performed by quantitative real time PCR. Expression of COL1A, DSPP, BMP 2 and BMP 7 in F68, F127, P85 treated groups and PC were shown significantly higher than NC as a non differentiated group. This data confirmed that differentiation of groups. Expression of COL1A in F68 and P85 treated cells showed significantly higher mRNA levels but F127 treated cells shown no difference compare to the PC. Expression of DSPP in F68 and P85 treated cells exerted higher mRNA levels but F127 treated cells shown no difference compare to PC, significantly. BMP 2 and BMP 7 expressions in F68 and P85 treated groups shown higher expression levels compare to PC, significantly. But F127 treated groups shown no effect compare to PC. This data confirmed that F68 and P85 increased odontogenic differentiation capability. Even though, P85 is toxic for the cells, P85 increased odontogenic differentiation. F127 has no effect on differentiation.

In conclusion, this study revealed that F68 and P85 are a proper pluronic block copolymers which might be used in tissue engineering of dental tissue. P85 toxicity may cause some problems, but if these problems could be solved in the future, P85 may be used in tissue engineering. F127 has no effect on odontogenic differentiation of hTGSCs.

In this study, for the first time, effects of these three pluronics were investigated on odontogenic differentiation of hTGSCs which were proven to be MSCs having the ability to differentiate into odontogenic cells. Pluronics may play important role on tooth regeneration. With proper scaffolds and chemicals, and optimum conditions; hTGSCs can form a tooth structure. We showed that *in vitro* study of pluronics can increase odontogenic differentiation capacity. Since we have achieved this, we are going to perform *in vivo* dental regeneration for tooth formation.

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