EFFECT OF BORON AND F68 PLURONIC BLOCK COPOLYMER COMBINATION ON ADIPOGENIC DIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

by

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ABSTRACT

EFFECT OF BORON AND F68 PLURONIC BLOCK COPOLYMER COMBINATION ON ADIPOGENIC DIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

Obesity has become a crucial health problem in public worldwide, thereby making weight loss inevitable to recover for obese as well as overweight people. However, weight loss and weight control drugs provided by the current food industry have failed in the long-term maintenance of weight control. Therefore, it is a challenge in the field to find new types of drugs that could be valuable for the prevention and treatment of obesity. Since obesity is a consequence of the formation of new mature adipocytes from undifferentiated precursors, drugs that might control adipogenesis could be beneficial for the treatment of obesity. Therefore, developing new, safe, and effective complex for adipocyte inhibition has been a desirable field of research. In the current study, sodium pentaborate pentahydrate (NaB), pluronics F68 and their combination were investigated on adipogenic differentiation by using human adipose stem cells (HASC) in vitro. The data showed the suppressed expression of adipogenesis-promoting genes that are peroxisome proliferator-activated receptor-γ (PPARγ), fatty acid binding protein (FABP4) and adiponectin. In this report, we demonstrated that NaB and F68 combination prevented adipocyte differentiation by inhibiting the adipogenic transcriptional program leading to a decrease in lipid accumulation into the cell. Thus, our study using HASCs will be a useful guide for prevention of adipose tissue-associated diseases including obesity and diabetes.

ÖZET

BOR VE F68 BLOK KOPOLİMER KOMBİNASYONUN İNSAN ADİPOZ KÖK HÜCRELERİNİN YAĞ HÜCRELERİNE FARKLILAŞMASI ÜZERİNDEKİ ETKİSİ

Obezite dünya çapında çok önemli bir halk sağlığı sorunu oluşturmaktadır; bu yüzden hem obez hem de fazla kilolu insanları iyileştirmek için kilo vermelerini sağlamak kaçınılmaz hale gelmiş durumda. Ancak günümüzde gıda endüstrisi tarafından üretilen zayıflama ve kilo kontrol ilaçları kilo kontrolünün uzun süreli muhafazası konusunda başarısız olmuşlardır. Bu sebeple obezitenin önlenmesi ve tedavisinde kayda değer yeni ilaçlar bulabilmek zor ve bu yüzden obezite hastalığı oldukça uğraş verilmesi gereken bir alan. Obezite öncül yağ hücrelerinin farklılaşmasından meydana gelen olgun yağ hücrelerinin oluşumunun bir sonucu olduğundan adipogenezi kontrol edebilecek ilaçlar obezite tedavisinde de faydalı olabilir. Bu nedenle adipogenezi engellemek için yeni, güvenli ve etkili bir ilaç kompleksi geliştirmek araştırmacılar için çalışılması cazip bir araştırma alanı haline gelmiştir. Bu çalışmada sodyum pentaborat pentahidrat (NaB), Pluronik F68 ve bunların kombinasyonunun yağ farklılaşma üzerindeki etkisi insan yağ kök hücresi (HASC) kullanılarak laboratuvar ortamında incelenmiştir. Bulgular peroksizom proliferatör-aktive reseptörü-γ (PPARγ), yağ asidi bağlayıcı proteini (FABP4) ve adiponektin gibi adipogenez-destekleyici genlerin ekspresyonunun baskılandığını göstermiştir. Bu raporda NaB ve F68 kombinasyonunun hücre içerisinde yağ birikimine sebep olan adipojenik transkripsiyonel programını inhibe ederek engellediği gösterilmiştir. Bu yüzden, insan yağ kök hücrelerini kullanarak yaptığımız çalışmamız, obezite ve diyabet dahil tüm yağ dokusu kaynaklı hastalıklardan korunma yolunda faydalı bir rehber olabilir.

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LIST OF SYMBOLS / ABBREVIATIONS

αP2	Adipocyte protein 2
C/EBP	CCAAT/enhancer-binding protein
cDNA	Complementary deoxyribonucleic acid
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
HASC	Human Adipose Stem Cell
HSC	Hematopoietic stem cell
IBMX	3-isobutyl-1-methylxanthine
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-6	Interleukin-6
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LPL	Lipoprotein lipase
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Mikromolar
mg	Milligram
mM	Milimolar
nm	Nanometer
nM	Nanomolar

mL	Milliliter
mmol	Millimole
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MTS	Methyl tetrazolium salt
NCBI	The National Center for Biotechnology Information
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PSA	Penicillin streptomycin amphotericin B
RNA	Ribonucleic acid
rpm	Rotation per minute
UV	Ultraviolet

1. INTRODUCTION

1.1. GENERAL OVERVIEW OF STEM CELLS

Stem cells have a great potential to form into many different cell types in the body. They are undifferentiated cells that have the capacity of proliferation, differentiation, self-renewing and tissue regeneration. When a stem cell divides, each daughter cell has a capacity to remain as a stem cell or develop into another type of cell with a more specific function [1].

There are basically three types of stem cells which are embryonic stem cells (ES), adult (non-embryonic) stem cells and induced pluripotent stem cells (iPSC) (Figure1.1). Embryonic stem cells (ESCs) are pluripotent stem cells meaning that they can make all the different types of cells throughout the body. ESCs are derived from inner cell mass of a 5 or 6 day old human blastocyst [2]. They are capable of differentiating into primordial germ layers (ectoderm, endoderm and mesoderm). However, there are some limitations and disadvantages of using ECSs on research. It has been observed that pluripotent embryonic stem cells often form teratomas (a tumor-like, cancerous mass, resembling a self-fertilized cell). Teratoma formation have been observed when embryonic stem cells are injected into animals [3, 4]. Furthermore, despite their great differentiation potential, usage of ESCs raises ethical, religious and political debates since the isolation of ESCs involves the destruction of the embryo [5, 6].

In 2006, researchers discovered another improvement by describing conditions that could allow some specialized adult cells to be "reprogrammed" genetically to expect a stem cell-like capacity [7]. This reprogramming is achieved by being forced to express genes and transcription factors important for maintaining the defining properties of embryonic stem cells (Oct4, Sox2, Klf4, and c-Myc). These iPS cell lines show similar morphology and growth features as ES cells. However, there were two problems; low efficiency of iPS cell lines and observation of some alterations in gene expression profiling between iPS cells and ES cells. Moreover, the use of viral vector for iPS establishment is also an issue needed to be addressed [8, 9].

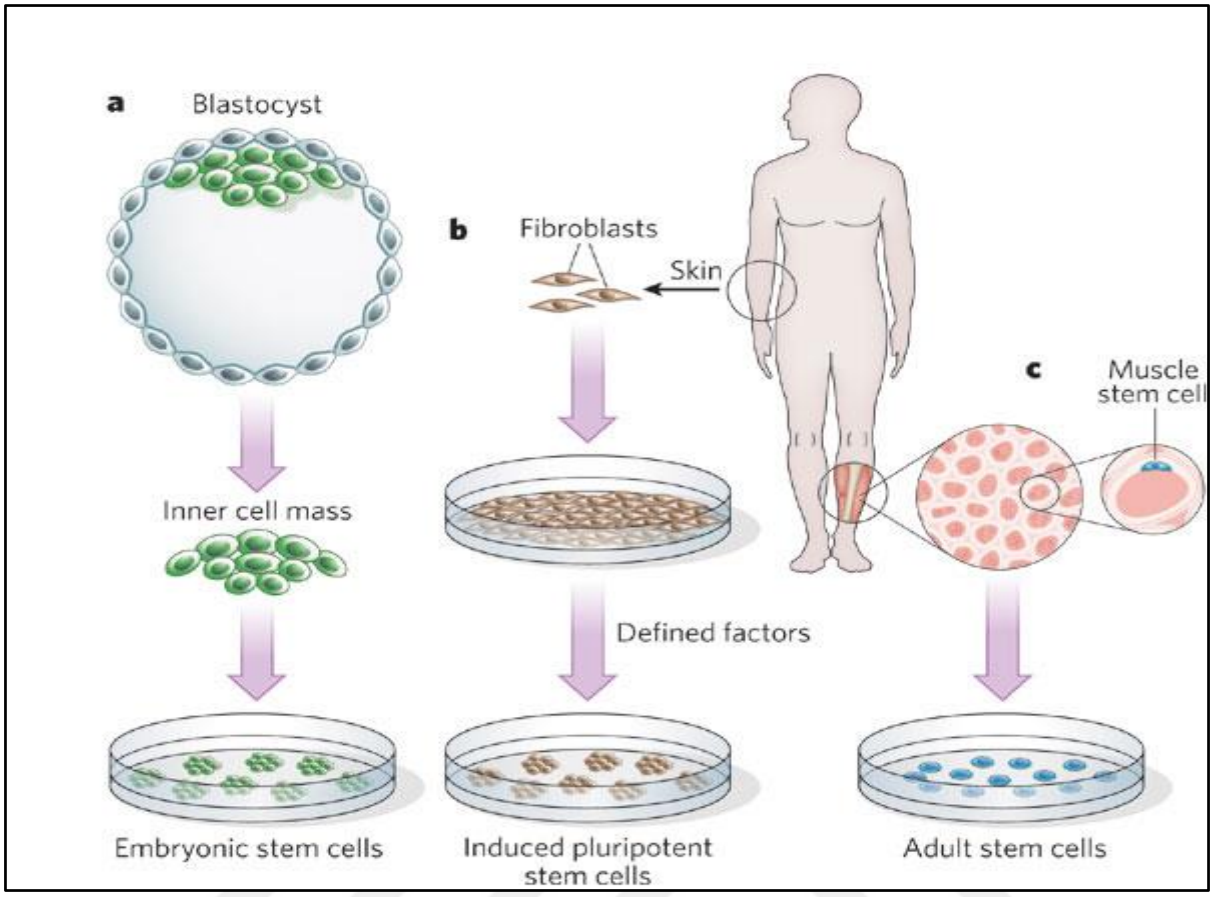

Figure 0.1.1. Origins and promises of stem cells [10]

Adult stem cells are considered as undifferentiated cells found among differentiated cells in a tissue or organ. The adult stem cell can renew itself and can differentiate into some or all of the major specialized cell types of the body. The major roles of adult stem cells in an organism are to maintain its survival and regenerate the damaged tissue in which they are found. Therefore, it is limited in its ability to differentiate. Unlike embryonic stem cells, the use of adult stem cells in research and therapy is not controversial because the production of adult stem cells does not require the destruction of an embryo. Adult stem cells also do not have the same immunological challenges as embryonic stem cells because they are taken from the patient [11,12].

Different types of adult stem cells have been identified in many organs and tissues. For instance, mesenchymal stem cells are nonhematopoietic adult stem cells that could generate fat cells, cartilage, bone, tendon, ligaments, muscle cells, skin cells and even nerve cells.

On the contrary, hematopoietic stem cells derived from bone marrow are able to give rise to only blood cells types (red cells, white cells and platelets), whereas neural stem cells generate the main phenotype of the nervous system [13, 14]. Bone marrow stem cells also have the ability to differentiate into a number of cell lineages and express tissue-specific proteins in a number of organs such as liver, brain, skeletal muscle, skin, heart, bone, cartilage and fat [15-17]. The existence of stem cells are also shown in adipose tissue, which are referred to as adipose stem cells. Adipose stem cells are similar, but not identical to bone marrow mesenchymal stem cells. They have been demonstrated to differentiate into a broad range of cell types including bone, liver, cartilage, neural and endothelium cells [18, 19].

1.2. BIOLOGY OF MESENCHYMAL STEM CELLS

First discovered and characterized by Friedenstein [24], mesenchymal stem cells are multipotent stromal and self-renewing progenitor cells with a high potential of differentiation. They are first defined as adherent colony-forming unit fibroblasts (CFU-U) [25]. It was only 20 years later that Caplan defined the terminology, MSCs [29]. MSCs are able to differentiate into a variety of cell types including osteoblasts, chondrocytes and adipocytes [26]. MSCs are initially isolated and characterized from bone marrow, but can be also obtained from other sources, such as the amniotic membrane, skin, hair follicles, dental pulp, adipose tissue, cord blood, the endometrium, amniotic fluid, fetal liver and the placenta [30]. MSCs have a distinct interest among other stem cells since they are easily isolated, cultured and manipulated.

There are no mesenchymal stem cell (MSC)-specific cellular markers, therefore their identification is achieved through their ability to adhere to plastic in vitro, through their multi-lineage differentiation potential in vitro and through a combination of positive expression or distinct lack of defined cell surface markers. These markers include CD105+, CD73+ and CD90+, whereas MSCs should lack CD45, CD34, CD14, CD19 and several other hematopoietic stem cell markers [27]. The correct balance between cell proliferation and differentiation is critical, especially for adult MSCs. The differentiation of MSCs in vitro largely depends on the culture conditions. Growth factors, such as the transforming growth factor-β family, result in chondrogenic differentiation [28].

Osteogenic differentiation of MSCs in vitro is induced by the presence of dexamethasone, ascorbic acid and β-glycerol phosphate [9], while MSCs cultured with dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin will differentiate down an adipogenic lineage [29]. Current studies have mainly focused on the adipogenic potential of MSCs in vitro and in vivo [30].

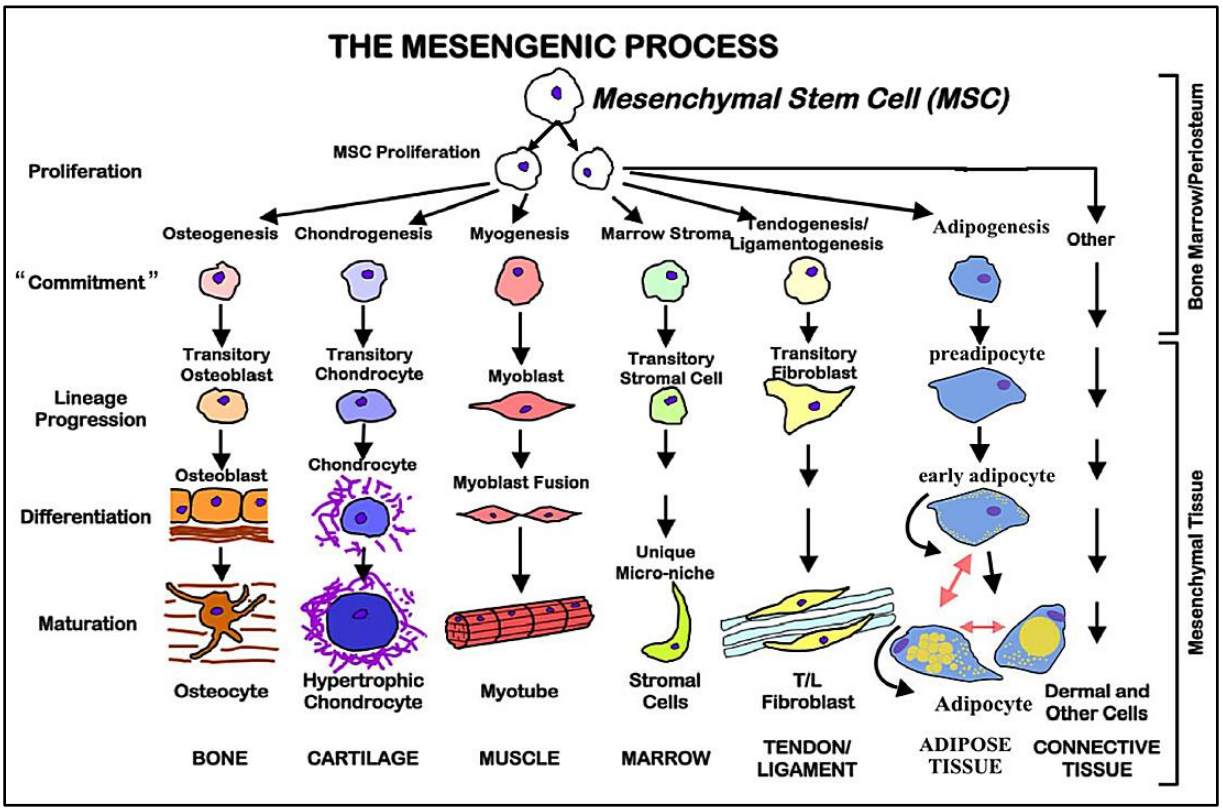

Figure 0.2. Process of mesenchymal stem cell differentiation [39]

However, to obtain more knowledge of differentiating and manipulating MSCs into different type of tissues is a crucial key for tissue repair and tissue engineering. Therefore, in order to determine the potential of MSCs in future clinical applications, it is necessary to build up more information into their differentiation capability and study the tissues formed by these cells at the morphological and gene expression levels.

1.3. ADIPOSE TISSUE

Adipose tissue is one of the largest tissues in the human body and total amount stored could have a destructive impact on regular body functions. Adipose tissue or body fat is loose connective tissue composed mostly of adipocytes within a structural network of fibers. It is found mainly under the skin; however, also in deposits among the muscles in the intestines and around the heart and many other places. In addition to adipocytes, adipose tissue contains the stromal vascular fraction (SVF) of cells including preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells [38, 39, 40]. Adipose tissue is derived from preadipocytes. Its main role is to store energy in the form of lipids while it also insulates the body. Adipose tissue is a major endocrine organ and plays a key role in energy homeostasis [41, 42]. The fat stored in this tissue comes from dietary fats or is produces in the body.

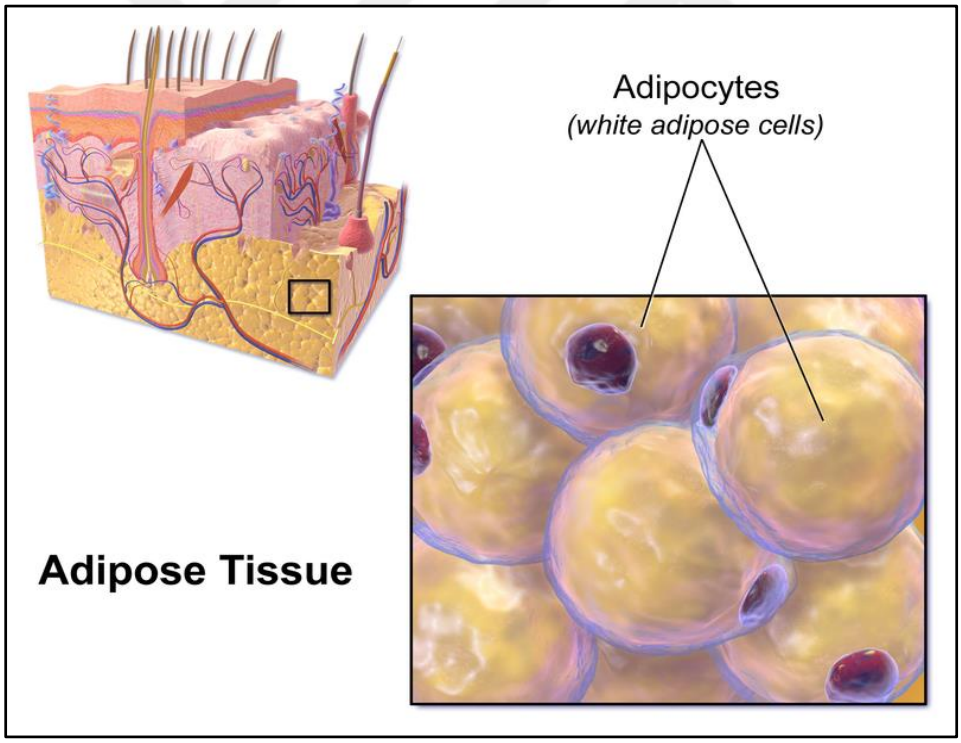

Figure 0.3. Microanatomy of adipose tissue

1.4. HUMAN ADIPOSE STEM CELLS

The potential of adipose tissue in regenerative medicine has been underestimated for a long time, being reduced to the simple function of energy storage. Since early studies, numerous studies have confirmed the efficacy of the isolation and application of adult adipose-derived stem cells (ASCs) in reconstructive medicine [43, 44, 45]. Detailed protein expression analyses proved a significant level of growth factors highlighting their exceptional regenerative capacity [46]. In recent years, studies using ASCs for various applications in tissue engineering and regenerative medicine have become widespread [47].

Adipose tissue is a source of multipotent adult stem cells and the advantages of using adipose tissue as a stem cell source are that it can be obtained in abundance, it has been found to have multi-lineage differentiation potential and it can be harvested easily with a minimally invasive method compared to bone marrow. Adipose-derived stem cells (ASC) can differentiate into adipogenic cells, chondrogenic cells, osteogenic cells [48] and neurogenic cells [49]. One of the ideal characteristics of stem cells is that they are be able to maintain their multi-lineage differentiation ability while they are in long-term culture or experience extensive subculture. Guilak et al. reported that adipose-derived human mesenchymal stem cells can undergo clonal expansion while maintaining their differentiation ability [50]. Additional factors, such as age and sex, have showed effects on the proliferation and differentiation capacities of ASCs. For example, ASCs from elderly donors (>60 years of age) display lower proliferation rates and impaired osteogenic and chondrogenic differentiation, whereas adipogenic differentiation is not dependent on donor age [51]. The differentiation potential and mechanical properties of ASCs also decline with extended cell passaging [52]. Therefore, many protocols and tissue engineering strategies utilize cells between passage numbers of 2 and 5.

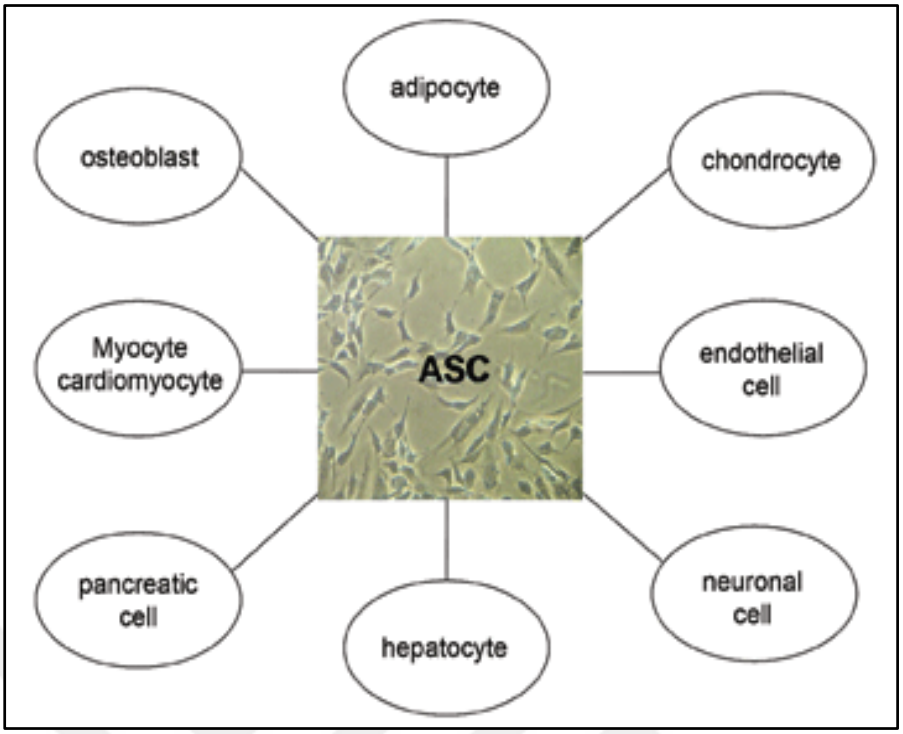

Figure 0.4. Differentiation capacity of adipose-derived stem cells [48]

1.5. ADIPOGENESIS

Adipogenesis is a process that stem cells differentiate into adipocytes. The function of adipocytes is to store triglycerides during energy excess and mobilize them during deprivation, which is controlled by the lipogenic and lipolytic processes through hormonal signals from the bloodstream [53]. The process of adipogenesis initiates with pluripotent mesoderm stem cells. During adipogenic differentiation, committed preadipocytes will withdraw from the cell cycle before undergoing adipose conversion as growth arrest is required for this differentiation [54]. Following this step, appropriate combination of mitogenic and adipogenic signals is required to proceed to the next step of the differentiation, which lead to the changes in morphological and biochemical characteristics of the mature adipocyte [55].

Adipocyte differentiation is a complex process involving coordinated expression of specific genes and proteins associated with each stage of adipogenesis [56, 57]. Peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) have been known to play a key role in the regulation of adipogenesis and in the modulation of fat cell function in adipose tissue. PPARγ was identified as a component of a differentiation dependent regulatory factor and a far-cell-specific enhancer of the adipocyte fatty acid-binding protein (FABP4) gene [58-61].

1.5.1. Peroxisome proliferator-activated receptor-γ (PPAR)

Peroxisome proliferator-activated receptor-γ (PPAR) is generally considered as the major regulator of adipogenesis and it maintains adipocyte specific functions such as lipid storage. PPARγ is an adipogenic transcription factor involved in the induction of adipocyte differentiation [64, 65, 66]. It is a ligand activated transcription factor highly expressed in the adipose tissues [67]. PPARγ is not expressed in preadipocytes but is activated during adipocyte differentiation. It is expressed prior to the expression of most adipocyte genes and regulate the expression of genes involved in generating and maintaining adipocytes including FABP4 [62, 63].

By binding to PPARγ-responsive regulatory elements as heterodimers with retinoid X receptor (RXR), PPARγ regulates the expression of networks of genes involved in adipogenesis, lipid metabolism, inflammation, and maintenance of metabolic homeostasis [68].

Initially named as PPARα [69], subsequent structural analogs PPARδ and PPARγ were since discovered. All three PPARs are found in mammals and are activated by polyunsaturated fatty acids, interacting with binding sites on targeted genes by forming heterodimers. While both PPARα and PPARδ are expressed during adipogenesis, PPARγ is adipocyte restricted and more rapidly increases in expression during early adipogenesis [70, 71].

1.5.2. Fatty acid binding protein (FABP4/aP2)

Fatty acid binding protein (aP2) also known as FABP4 plays an important role in the development of metabolic syndrome and the coordination of cholesterol trafficking as well as inflammatory activity [72, 73]. FABPs act as cytoplasmic lipid chaperones and play a role in the cellular trafficking of fatty acids and other lipid signals through their interaction with functional targets. [74] This protein may be an important regulator of insulin sensitivity and lipid and glucose metabolism [75]. FABP4 deficient mice prevented the development of hyperinsulinemia, hyperglycemia and insulin resistance in the terms of both dietary and genetic obesity [76, 77].

1.5.3. Adiponectin

Adiponectin (ADPN) is a 30 kDa adipokine hormone secreted from adipose tissue [78, 79]. It plays a fundamental role in lipid and carbohydrate metabolism. ADPN stimulates fatty acid oxidation, decreases plasma triglycerides, improves glucose metabolism and increases insulin sensitivity [80]. Studies also indicated that circulating adiponectin level was reduced in patients with insulin resistance, type2 diabetes, obesity, or cardiovascular disease [81-85].

All in all, PPARγ, FABP4 and adiponectin genes can be used as markers to study the progression of stem cells in adipogenic differentiation, especially in long-term culture.

1.6. OBESITY

Obesity has emerged as an important public health problem worldwide. It is well recognized that obesity is the major risk factor for certain diseases. Obesity is induced by the hypertrophy of adipocytes and the generation of new adipocytes from preadipocytes [31, 33]. Obesity in animals, including humans, is not dependent on the amount of body weight, but on the amount of body fat - specifically adipose tissue. Increased adipocyte production leading to obesity is considered to be a hallmark of metabolic syndrome (MetS) that includes arteriosclerosis, diabetes, hypertension and hyperlipidemia, and is a major public health problem in many countries [34, 35]. 300 million individuals worldwide are affected by obesity and this number is expected to increase in the following years, making obesity a priority in health problems [36, 37].

As a solution, weight loss is progressively admitted to have extreme health benefits for obese as well as for overweight people. However, weight loss and weight control drugs provided by the current food industry have failed in the long-term maintenance of weight control. Therefore, it is a challenge in the field to find new types of drugs that could be valuable for the prevention and treatment of obesity. Since obesity is a consequence of an increase in adipocyte size and the formation of new mature adipocytes from undifferentiated precursors, drugs that might control adipogenesis could be also beneficial for the treatment of obesity [86-89].

1.7. 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 [90].

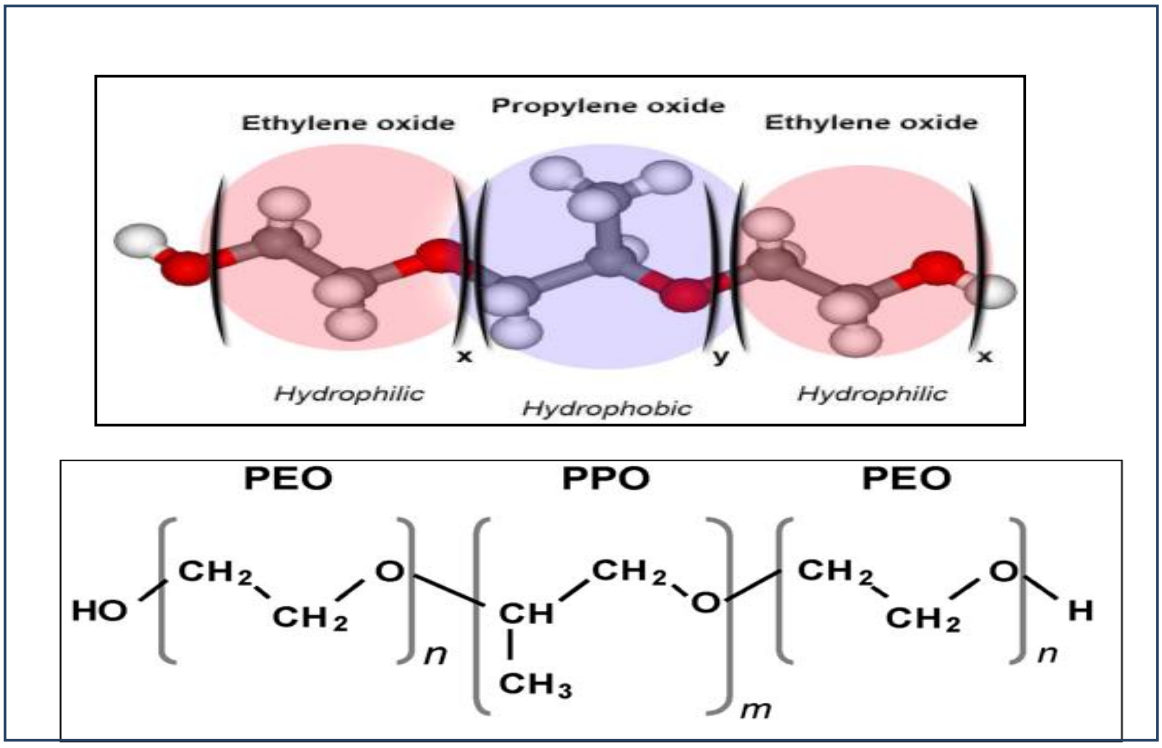

Figure 0.5. Pluronic molecule and its chemical structure [91]

Different types of pluronics can be found as commercial products and these pluronics differ depending on their numbers of PPO and PEO blocks (Figure 1.5.) [91, 92]. 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 [92, 93].

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 with biological membranes. Pluronic block copolymers are synthesized by the consecutive polymerization of polyethyleneoxide and polypropyleneoxide units with an alkaline catalyst [94].

Concentrations higher than critical micelle concentration (CMC) in aqueous solutions cause self-assembly of copolymers forming micelles. The diameter of pluronic micelles changes between 10 nm and 100 nm [95]. Hydrophobic PPO blocks generate the core of the micelles having the capacity absorbing different therapeutic reagents or drugs (Figure 1.6.).

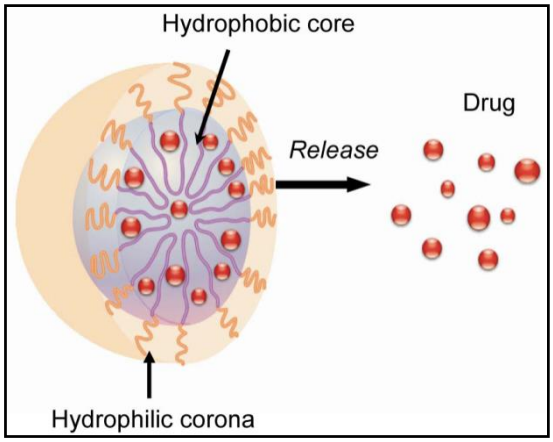

Figure 0.6. Pluronic micelle with a solubilized drug [91]

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 micelle forms in which PPO chains are hidden in the micellar core preventing pluronics from interacting with the cellular membranes [96].

Pluronic triblock copolymers can be used for different applications and have different effects on cell metabolism. When they are used as drug delivery agents they are inert and prevent drug from degradation and enhance the uptake of drug by tissues [97]. Interaction of pluronics with the plasma membrane causes inhibition of Pgp (P-glycoprotein) or MRP (Multidrug Resistant Protein) ATPase activity.

There is a complex mechanisms about pluronic effects on MDR (Multidrug Resistant) cells. It was shown that pluronic block copolymers; interact with membranes by altering microviscosity [91]; inhibit Pgp [98-100], multidrug resistance proteins (MRPs) [101] and breast cancer resistance protein (BCRP) [102, 103]; increase secretion of cytochrome C and reactive oxygen species (ROS) levels in the cytoplasm [91], trigger proapoptotic pathway and prevent anti-apoptotic mechanism in MDR cells [104]; inhibit the glutathione/glutathione S-transferase detoxification system [95]; inhibit drug sequestration within cytoplasmic vesicles [105]; and control shear stress in bioreactors increasing cell survival under stress conditions [106].

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 [107, 108]. In addition, pluronic block copolymers can be suitable agents for nonviral gene therapy by increasing the gene transfer 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. Studies revealed some pluronics acting as cell encapsulation agents and exerting positive effects on the tissue repair [109].

1.7.1. Pluronic F68

Pluronic F68, 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 [110].

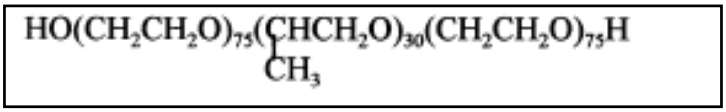

Figure 0.7. Structure of F68 [110]

Pluronic F68 is a white powder with 8350 Daltons molecular mass. It is soluble in water and has a neutral pH of 7. The percentage of polypropylene oxide (% hydrophobicity) is about 20. It disperses calcium and magnesium salts [145]. The critical micelle concentration (CMC) is 1.1 mM [111]. Pluronic F68 does not create micelles but produces two to three layers of the block polymers attaching to the membrane surface and prevent aggregation [110]. Up to this time, the general use of Pluronic F68 is to prevent cells from the effects of shear forces in culture situations (shaker and spinner cultures). This is achieved with the help of cell bubble interactions [112].

1.8. BORON

Boron is an essential element for plants, although the role and mechanism of boron is not yet well understood for human beings and mammalian systems. It was reported that boron is essential for optimal health in rats and that it also plays an important role in embryogenesis, bone growth and immune functions [113]. Furthermore, it was previously indicated that boron deprivation in the diet significantly affects both bone and teeth growth [114].

Nielsen and Stoecker have demonstrated that boron (as boric acid) deficiency in the diet exhibits significant decrease in bone volume fraction compared to 3-mg/kg boron diet in rats [115]. Moreover, it has been claimed that lack of boron supply would result in abnormal bone growth and impaired development [116]. Apart from these, boric acid has been proven to be involved in reducing periodontal inflammation and alveolar bone loss [117]. Therefore, sodium pentaborate pentahydrate is a good alternative as boron source in bone and tooth regeneration studies.

Apart from the studies related to bone regeneration, boron has also been investigated on lipid metabolism [118]. Sodium borate is known as a toxic material in veterinary and human medicine. The effect of sodium borate on lipid profiles was investigated in dogs fed a fatty diet in a previous study [119, 120] where it was suggested that sodium borate could be worth investigating as a drug to lower plasma lipid in humans and animals. Still, there is a lot to discover on boron's working principle and its effect on adipose differentiation.

1.9. AIM OF THE STUDY

In the present study, the object was to elucidate the effect of sodium pentaborate pentahydrate (NaB) and poloxamer F68 alone and in combination on adipogenic differentiation of human adipose stem cells (HASCs) in vitro. This is the first study that demonstrated the inhibitory effect of NaB together with F68 on adipogenic differentiation and fat accumulation.

MATERIALS AND METHODS

2.1. ISOLATION OF HASCs

Primary human ASCs used in this study were isolated from subcutaneous adipose tissue samples harvested from the abdomen and upper inner thighs of two healthy adult female donors undergoing liposuction procedure at Bezmialem University (age range: 26 to 59). Initially, the adipose tissue and collagenase solution were mixed at a ratio 1:1 and digested for 1h with continuous shaking at 170 rpm), at 37°C. The digested tissue was transferred into a 50 ml falcon tube and the rest of the tube was filled with the erythrocyte lysis buffer and then centrifuged at 2500 rpm for 7 minutes at room temperature. The supernatant was discarded and the pellet was resuspended with 2 ml of erythrocyte lysis buffer. The cell suspension was transferred into a new falcon tube and the volume was completed to 50 ml with erythrocyte lysis buffer. After 10 minutes of incubation at 37°C with continuous shaking at 170 rpm, the cell suspension was centrifuged at 1400 rpm for 7 min at room temperature. The cells in the pellet were washed with PBS (1X, without Ca/Mg) and subsequently centrifuged at 1400 rpm for 7 min at room temperature. Cell pellet was resuspended in 6-10 mL expansion Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) PSA (10.000 units/mL potassium penicillin, 10.000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B) (Invitrogen, Gibco, UK). Finally, the cells were filtered through 100 μm cell strainer and seeded on T150 flasks. The cells were maintained at 37ºC and 5% CO_2 in a humidified incubator. Cells from passages 1-5 were used in all experiments.

2.2. CHARACTERIZATION OF HASCs

The procedure previously published by our group was done for characterization of HASCs [121]. Cells were trypsinized and incubated with the primary antibodies which were prepared in PBS. For characterization, primary antibodies against CD29 (cat #BD556049), CD34 (cat #SC-51540), CD45 (cat #SC-70686), CD90 (cat #SC-53456), CD105 (cat #SC-71043), CD14 (cat # SC-9150), (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) and CD73 (cat # BD550256) (Zymed, San Francisco, CA, USA) were used. The cells were washed with PBS to remove the excess primary antibodies.

Thereafter, the cells were incubated with fluorescein-iso-thio-cynate (FITC)-conjugated secondary antibody (cat #SC-2989) at 4ºC for one hour except CD29 phyco-erythrin (PE)-red light-harvesting protein containing chromophore-conjugated monoclonal antibody was used. The flow cytometry analysis of the cells was done using Becton Dickinson FACS Calibur flow cytometry system (Becton Dickinson, San Jose, CA, USA). 20000 cells were counted for each sample.

2.3. PREPARATION OF PLURONIC F68 AND BORON

Pluronic block co-polymer F68 was purchased from BASF Corporation (Badische Anilin und Soda-Fabrik, Ludwigshafen-am-Rhein, Germany). F68 (BASF, USA, cat # 52389638) was prepared according to the protocols described by Exner et al. in 2005 [122]. Since pluronics dissolve at low temperature the preparation was performed on ice. 10% (w/v) concentration of F68 was prepared in PBS and filtered through a 0.2-µm filter (Sartorius AG, Göttingen, Germany). The solution was kept at 4ºC until use.

NaB was kindly obtained from National Boron Research Institute-BOREN (Ankara, Turkey) and prepared in Dulbecco's modified Eagle's medium using 10% fetal bovine serum (Invitrogen), and 1 % of penicillin, streptomycin, and amphotericin (Biological Industries, Beit Haemek, Israel). A total of 10 mg/ml concentration of boron were dissolved in DMEM. The stock solution was filtered through a 0.2-µm filter and subsequently diluted to 1 mg/ml in DMEM.

2.3. CYTOTOXICITY ASSAY OF BORON AND F68 PLURONIC

Effects of pluronics F68 and NaB were tested on the cell viability. F68 solutions with seven different concentrations (1%, 0.7%, 0.5%, 0.3%, 0.1%, 0.05%, 0.01% w/v) were prepared by dilution in DMEM. Moreover, seven separate concentrations between 5 and 300 μg/ml (5, 10, 20, 50, 100, 200 and 300) of NaB solution and negative control (NC) were prepared from the stock solution.

HASCs of passage number 3 were cultured onto 96-well plates (Corning Plasticware, Corning, NY, cat. no. CLS3360) at a concentration of 5.000 cells/well. The following day cells were treated with prepared F68 and NaB solutions of different concentrations. Cell viability was measured by the MTS assay (CellTiter96 AqueousOne Solution; Promega, Southampton, UK) 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 yellow tetrazolium salt which is catabolized to formazan by dehydrogenase enzyme in mitochondria of the living cells. Formazan is a purple compound and the detection in this assay is based on the measurement of formazan compounds by an ELISA plate reader [123, 124]. After incubating the cells for 24, 48 and 72 hours, 10 μl MTS reagent and 100 μl DMEM was given to the cells followed by 2 hours of incubation at 37°C. Thereafter, the absorbance at 490 nm was measured by an ELISA plate reader (BioTek Instruments, Inc., VT, USA).

2.4. DIFFERENTIATION PROCESS

HASCs were induced to differentiate into adipogenic cells. The cells were seeded on a six well plate for RNA isolation and 48 well plate (BIOFIL, TCP, Switzerland) for immunocytochemistry at a density of 100.000 cells/well and 8.000 cells/well respectively for each differentiation. For adipogenic differentiation, the cells were induced to differentiate into adipocytes using a previously published method [125]. The cells were seeded on a six well plate and given adipogenic differentiation medium, which is composed of DMEM, 10% (v/v) FBS, 1 μM dexamethasone, 100 μM indomethacin (Sigma, USA), 500 μM IBMX (Calbiochem, Merck Millipore, Germany) and 0.01 mg/mL insulin (Gibco, UK).

5 different groups were prepared including F68 only, NaB only, F68 and NaB combination, PC and NC. Adipogenic differentiation lasted 10 days in the adipogenic differentiation medium changing every 2 days. Positive control group was treated with adipogenic differentiation medium while negative control group was cultured only in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) PSA.

2.5. IMMUNOCYTOCHEMISTRY ANALYSIS

At the end of the differentiation procedures of HASCs, the cells were incubated with 2% (w/v) paraformaldehyde for 30 minutes at 4^{o}C for fixation. The cells were washed with PBS three times for 5 minutes by shaking the plates on a plate shaker. Later on, the cells were permeabilized by incubating with 0.1% (v/v) Triton X-100 (in PBS) for 5 min at room temperature followed by washing three times for 5 min. with PBS. The cells were incubated with 2% (v/v) goat serum (Sigma, USA) for 20 minutes at 4^{o}C for preventing non-specific binding of primary antibodies. The cells were again washed three times with PBS. The cells were incubated overnight at 4^{o}C with primary antibodies of PPAR-γ (ab8934, Abcam, UK), fatty acid binding protein (FABP4) (sc-30088, Santa Cruz Biotechnology, TX, USA) and adiponectin (ab22554, Abcam, UK). The cells were washed three times with PBS to remove the excess antibody after incubating with primary antibodies. Thereafter, the cells were treated with secondary antibodies (Goat anti rabbit IgG Alea Fluor 488, Goat anti mouse IgG Alea Fluor 488) and incubated for 2 hours at 4^{o}C followed by rinsing three times with PBS. DAPI (AppliChem, Germany) was used to stain the nuclei of the cells by incubating for 5 minutes at room temperature. The cells were then rinsed three times with PBS and observed under fluorescence microscope (Nicon Eclipse TE200).

2.6. TOTAL RNA ISOLATION AND QUANTITATIVE RT-PCR

Primer FABP4 was designed using nBLAST online software of The National Center for Biotechnology (NCBI). The other primers sequenced were used as previously described in the literature (Table 2.1).

Table 0.1. Primers used for Real Time PCR

Primer	**Sense (5'-3')**	**Antisense (5'-3')**	**Ref.**
Adiponectin	TATCCCCAACATGCCCATTCG	TGGTAGGCAAAGTAGTACAGCC	[126]
PPARγ	CCTATTGACCCAGAAAGCGATT	CATTACGGAGAGATCCACGGA	[126]
FABP4	AACCTTAGATGGGGGTGTCCT	TCGTGGAAGTGACGCCTTTC	[126]

Total RNA isolation after differentiation was performed using High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. cDNA synthesis from isolated RNA samples were done using High Fidelity cDNA Synthesis Kit (Roche, Germany). Real time PCR was performed using Maxima SYBR Green/ROX (Fermentas, USA) for the determination of expression levels of marker genes after differentiations. cDNAs of the differentiated cells incubated 5 groups were used as template and were mixed with primers and Maxima SYBR Green/ROX qPCR Master Mix (2X) (Table 2.2 and Table 2.3). Real time PCR experiments were performed using iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA).

Table 0.2. Reagents used for RT-PCR

Reagents	**Volume**
SYBRGreen	5 µl
Primer Forward (10 µM)	0.3 µl
Primer Reverse (10 µM)	0.3 µl
Distilled water	2.9 µl
Template (1000 ng/µl)	1.5 µl

Table 0.3. RT-PCR Protocol

Cycle 1	Step 1	50ºC	2 min
	Step 2	95ºC	15 min
Cycle 2 x40	Step 1	95ºC	15 sec
	Step 2	60ºC	1 min
	Step 3	72ºC	30 sec - ¤
Cycle 3	Step 1	95ºC	1 min
Cycle 4	Step 1	55 ºC	1 min
Cycle 5- Melt Curve	Step 1	55ºC -95ºC	10 sec/0.5ºC up

2.7. OIL RED OIL STAINING

Oil red oil solution was prepared by dissolving 0.5 gram oil red oil (Sigma, USA) in 100 mL isopropanol. The cells were then fixed with 2% (w/v) paraformaldehyde for 30 minutes followed by wash three times with PBS. The cells were then incubated with oil red oil solution diluted 6:4 in PBS for 1 hour for staining. Cells were washed with PBS and observed under the light microscope [127].

2.8. STATISTICAL ANALYSIS

All data are shown as the means ± standard errors. Graphics were drawn using GraphPad Prism 5 software. The results of real time PCR data were normalized to the mRNA level of GAPDH. The statistical analysis of the results were performed with unpaired t test using GraphPad Prism 5 software. Statistical significance was determined at $P < 0.05$. In addition, the results obtained from immunocytochemistry and oil red oil staining were also demonstrated in graphical representation by using Adobe Creative Suite 6 Programme.

RESULTS

3.1. CHARACTERIZATION OF HUMAN ADIPOSE STEM CELLS

Human adipose stem cells were analyzed to be characterized for the surface antigens CD14, CD29, CD34, CD44, CD45, CD73, CD90 and CD105 by flow cytometry. Cells were positive for CD29, CD44, CD73, CD90 and CD105 whereas they were negative for CD14, CD34 and CD45 (Figure 3.1). These data showed that HASCs were positive for MSC markers and negative for HSC markers.

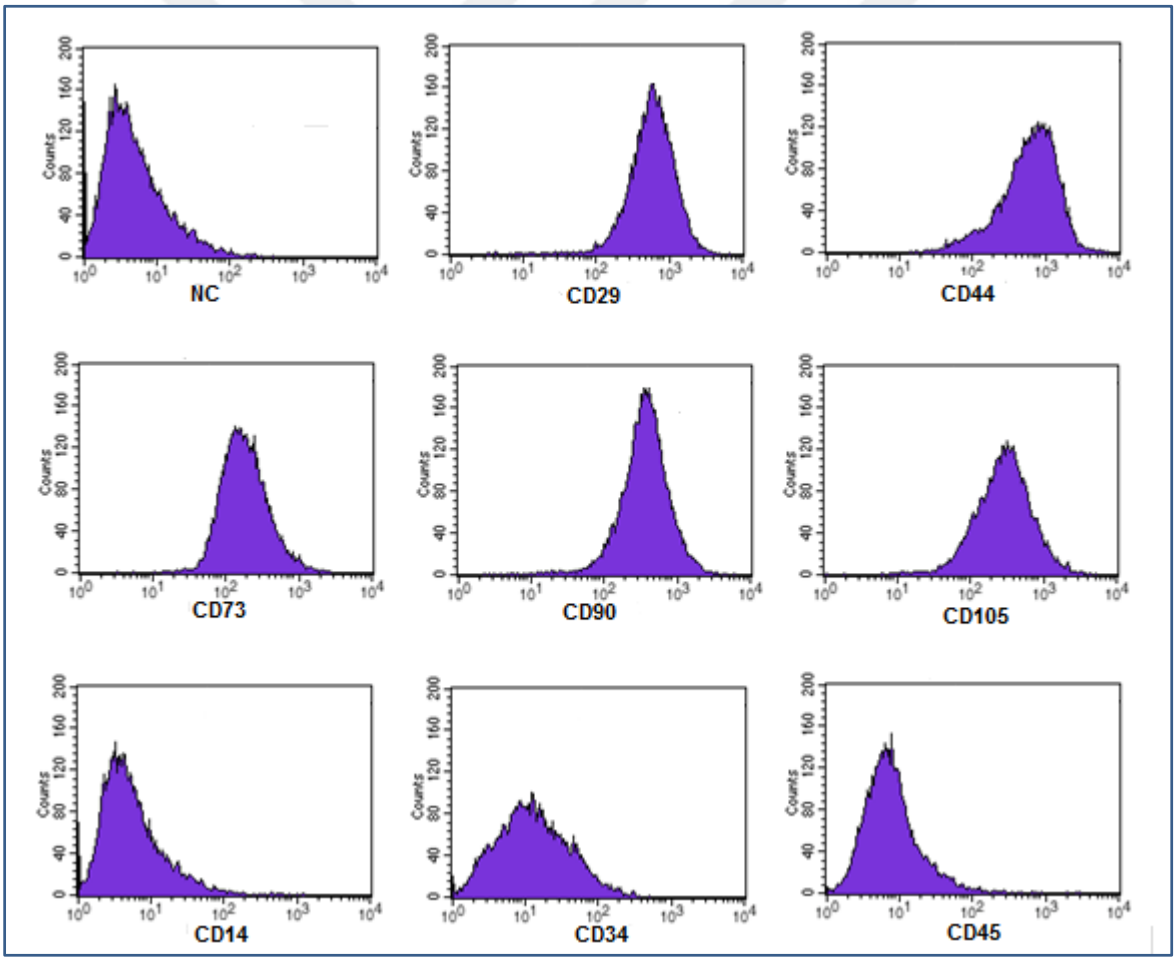

Figure 0.1. Flow cytometry analysis of HASCs

3.2. CYTOTOXICITY ASSAY FOR F68 AND BORON

Toxicity of F68 and NaB was measured by MTS assay at seven different concentrations for three days separately. The results showed that none of the concentrations were toxic to the cells (Figure 3.2). Moreover, 0.05% concentration of pluronic F68 is increased the cell viability of hASCs for 72 hours. 0.05% F68 raised the survival by 101%, 103% and 105% at day 1, 2 and 3 respectively whereas these numbers for NaB concentration 20 µg/ml are 101%, 106% and 109%.

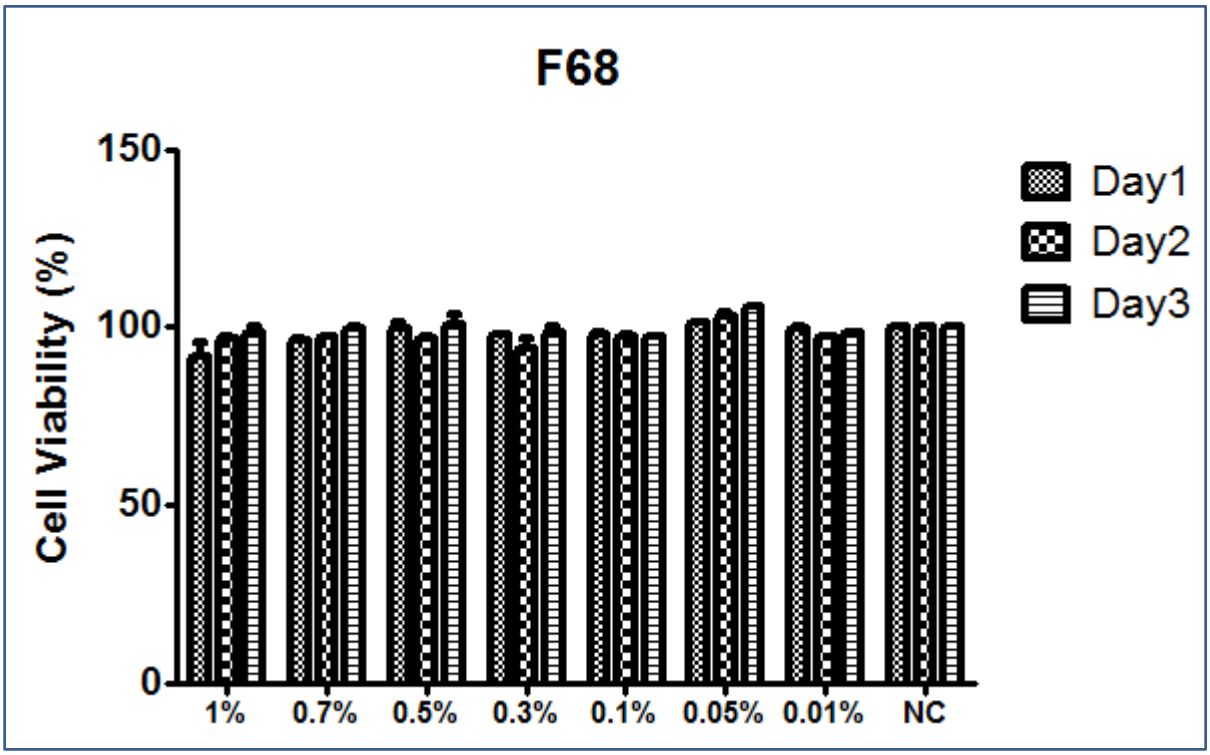

Figure 0.2. Toxicity results of different concentrations of F68. NC: Negative control.

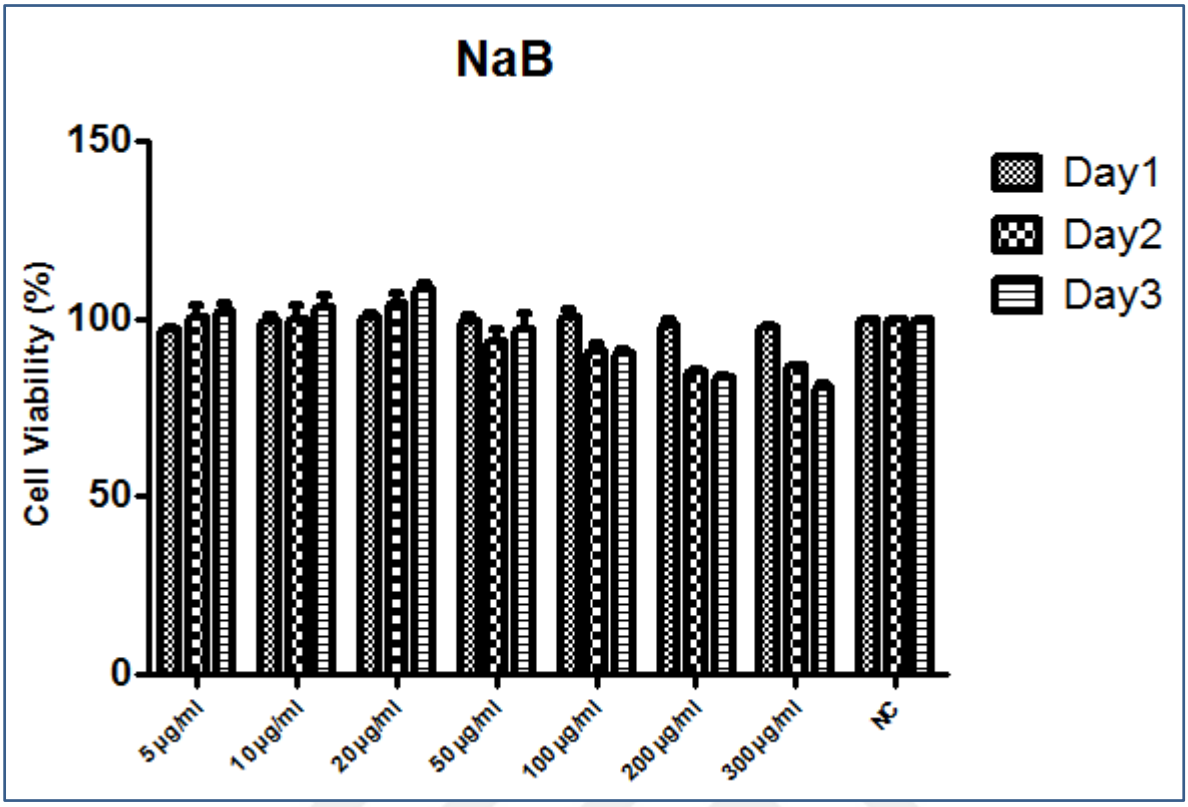

Figure 0.3. Toxicity results of different concentrations of NaB. NC: Negative control.

3.3. ADIPOGENIC DIFFERENTIATION

3.3.1. Effect of F68 and Boron Combination Treatment in Adipogenic Marker Genes

Adipocyte differentiation of expression levels of markers PPAR-γ, FABP4 and adiponectin were detected by real time PCR. Cells treated with F68 and NaB combination during adipogenic differentiation showed the lowest mRNA expressions of the all marker genes compared to positive control groups (Figure 3.4).

F68 and NaB combination decreased the expression of PPAR-γ, FABP4 and adiponectin whereas NaB alone increased this level in comparison with the positive control.

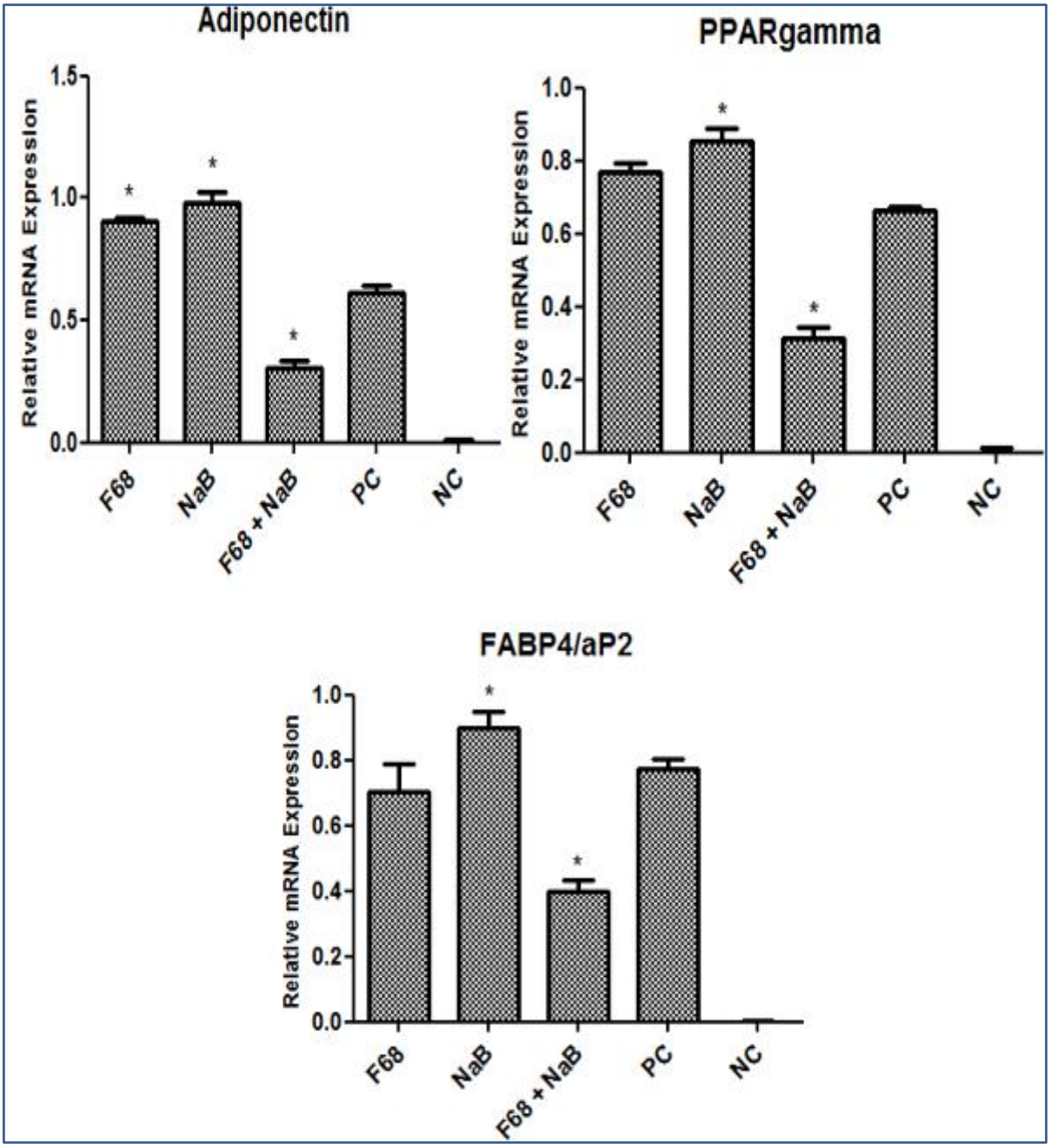

Figure 0.4. PPAR-γ, FABP4 and Adiponectin gene expressions in Boron, F68, their combination and control groups. NaB: sodium pentaborate pentahydrate, PC: Positive control, NC: Negative control. *P < 0.05 versus the positive control

3.3.2. Immunocytochemical Analysis of PPAR-γ, FABP4 and Adiponectin

For adipogenic differentiation, the expression of PPAR-γ, FABP4 and Adiponectin was checked by immunocytochemistry. Combination group was found to down-regulate the expression of these three markers while the positive control groups exhibit higher expressions of Adiponectin, FABP4 and PPAR-γ (Figure 3.5 and Figure 3.6).

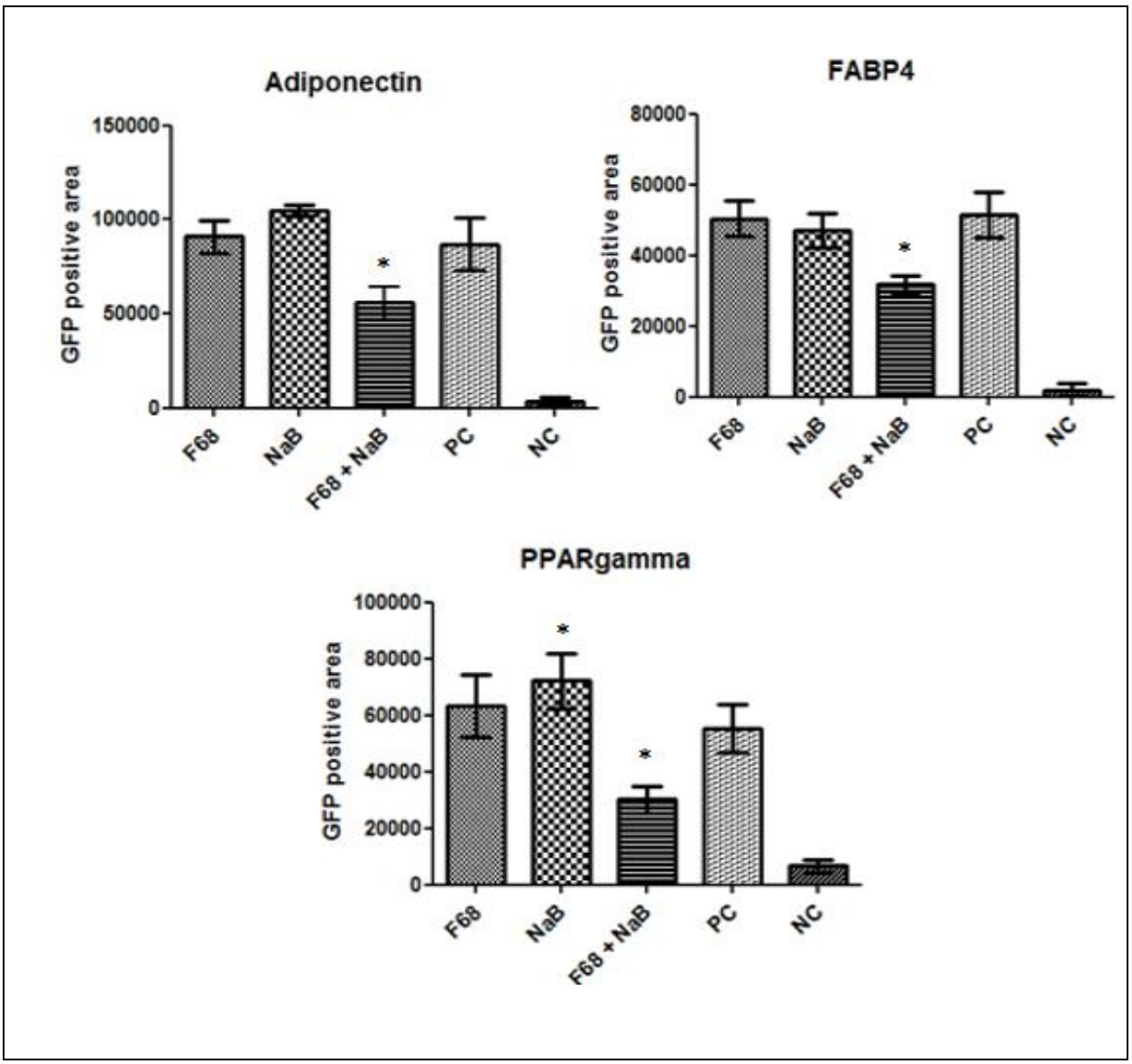

Figure 0.5. Immunocytochemistry analysis of Adiponectin, FABP4 and PPAR-γ by using Adobe Creative Suite 6 Programme. PC: Positive control, NC: Negative control. *P < 0.05 versus the positive control

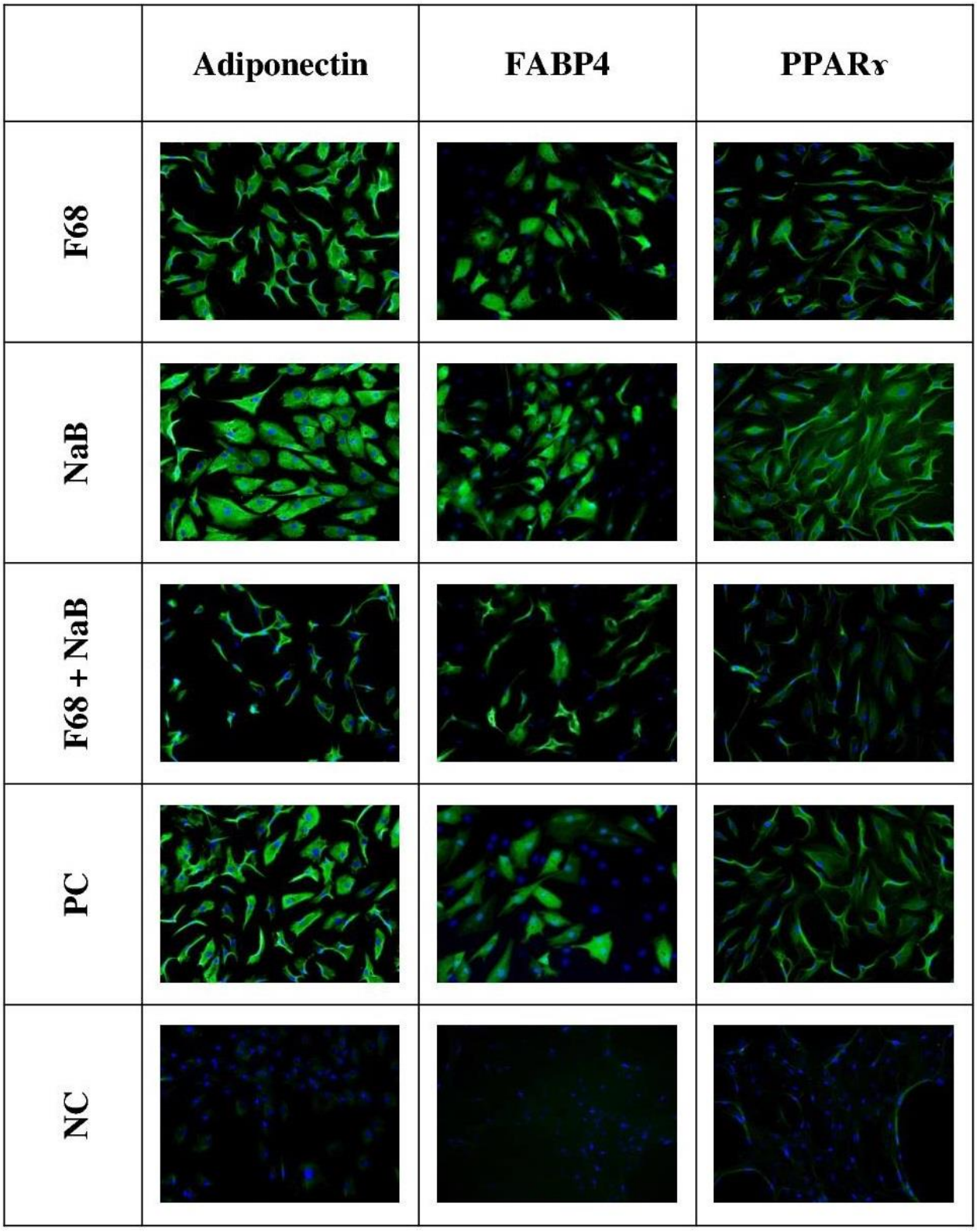

Figure 0.6. Immunocytochemistry results of Adiponectin, FABP4 and PPAR-γ. PC: Positive control, NC: Negative control.

3.3.3. Oil Red Oil Staining for Adipogenic Differentiation

Oil red oil is a lysochromediazo dye used to stain lipids and neutral triglycerides. Oil red oil staining was performed to visualize the intracellular lipid vesicles after adipogenic differentiation. Lipid vesicles were observed in the highest amount in NaB treated group while the amount showed the lowest effect on the combination group compared to positive control (Figure 3.7 and Figure 3.8).

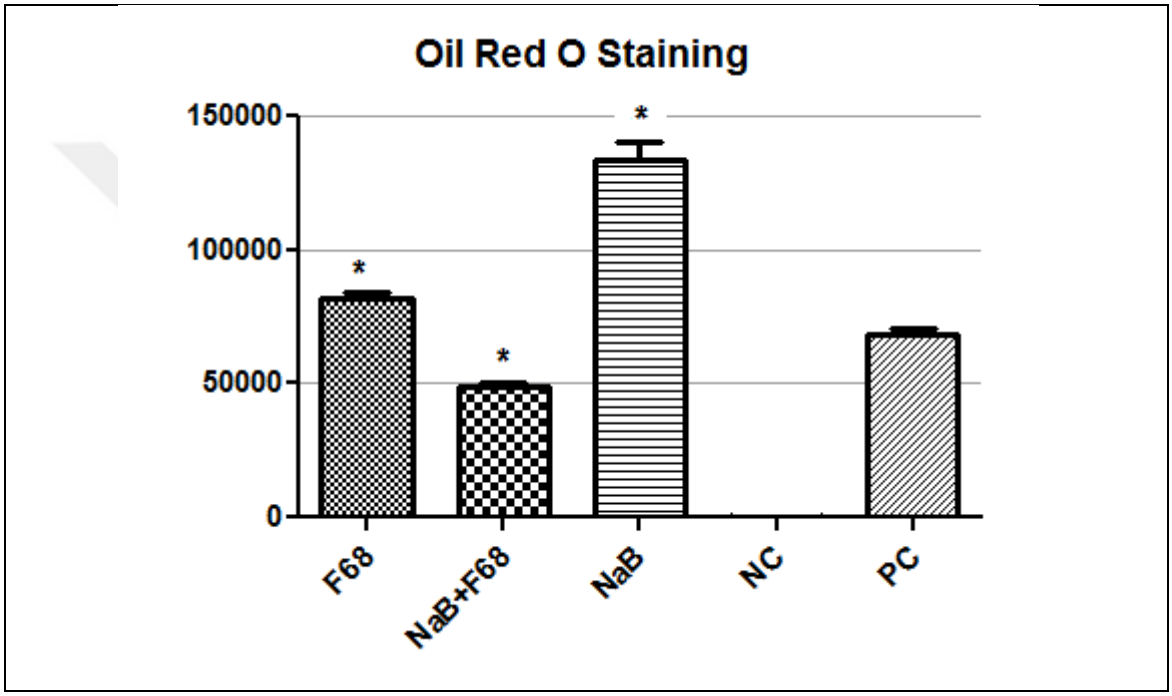

Figure 0.7. Oil red oil staining analysis by using Adobe Creative Suite 6 Programme. PC: Positive control, NC: Negative control. *P < 0.05 versus the positive control

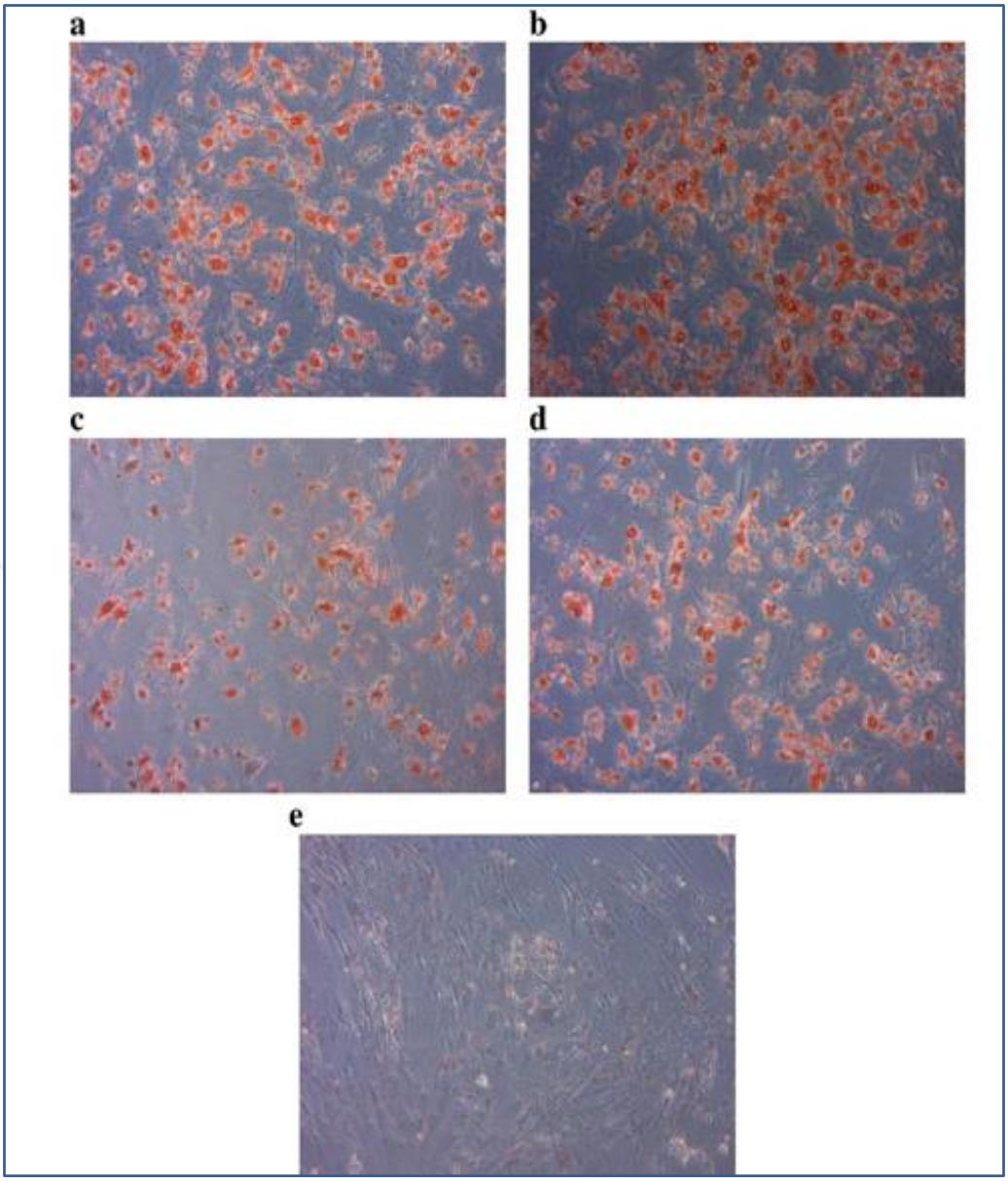

Figure 0.8. Oil red oil staining of a. F68, b. NaB, c. F68 + NaB, d. positive control and e. negative control.

DISCUSSION

Stem cells are a good candidate for the regenerative medicine because of their great proliferation and differentiation capacity [128]. The source of stem cell is of great importance; hence, one of the major issues to be addressed since adequate number of stem cell is required for therapeutic usage. The number of stem cells in the human body is limited, but numerous studies revealed their availability in some tissues such as bone marrow and adipose [129]. However, isolation from other sources like nervous system is considered as unfeasible. The ideal scheme should be to find a source of stem cell at sufficient number and with high proliferation and differentiation ability. Easiness of isolation from the body without any risk to the patient is also an important factor that should be taken into account. All of these requirements led scientists to search for stem cell sources with high proliferation, self-renewal and differentiation capability. Use of embryonic stem cells are limited due to the ethical concerns in spite of their huge pluripotency and differentiation ability.

Adult stem cells have gained interest for stem cell based therapies. They are a good choice since there are several sources in human body. Researchers have characterized various tissues possessing stem cells with the mesenchymal stem cell properties ever since bone marrow was identified as the first mesenchymal stem cell source [34, 130-135]. Bone marrow mesenchymal stem cells can be isolated and cultured *in vitro*. Thanks to their capacity of self-renewing and differentiation with mesenchymal properties rendered them a good candidate for various studies [136]. However, surgical procedures are required for the isolation of bone marrow stem cells, causing a huge risk for contamination. Furthermore, the isolation procedure is traumatic and the number of cells obtained is limited [137]. These issues associated with bone marrow mesenchymal stem cells led researchers to seek new adult stem cell sources and isolation methods.

All in all, adipose tissue seems to be a good candidate as an alternative stem cell source. MSCs derived from human adipose tissue are referred to as human adipose stem cells (HASCs) in this study, which exhibit an easily obtainable and available source for stem cell therapy and tissue engineering. They have been carried out in numerous animal and clinical trials [138, 139]. HASCs have been also used for identifying key molecular markers, transcription factors and various interactions that are required for adipogenesis [140-142].

Adipogenesis is the alteration of preadipocyte fate to mature adipocyte with organized changes in cell morphology and gene expression [143]. Thus, it is a very useful tool for an immediate screen and can help assess the adipogenic potential of various agents. Adipogenic cells can be easily distinguished by the change in cell shape where the fibroblastic cells changes to spherical shape cells after induction. Since obesity is a consequence of an increase in adipocyte size and the formation of new mature adipocytes from undifferentiated precursors, drugs that might control adipogenesis could be also beneficial for the treatment of obesity. It is a challenge in the field to find new types of drugs that could be valuable for the prevention and treatment of obesity.

This study investigates the effects of sodium pentaborate pentahydrate (NaB) combined with poloxamer F68 on adipogenesis of HASCs in vitro. In this report, we demonstrated that NaB and F68 combination prevented adipocyte differentiation by inhibiting the adipogenic transcriptional program leading to the reduction of lipid accumulation into the cell.

Before starting differentiation procedure, toxicity assay was performed to determine the optimum concentration of F68 and boron. F68 is a pluronic block copolymer which is widely used as a surfactant to protect cells from shear stress in bioreactors. In summary, it can be said that pluronic F68 is generally used to protect cells against toxic conditions and for drug delivery purposes. As a result of testing seven different values to measure its effect on survival for 3 days, none of the F68 concentrations exhibited toxicity for HASCs. Results are consistent with the literature that F68 is not toxic for cells at 0.05% (w/v) concentration which is mostly preferred concentration for pluronics.

In conclusion, there is no study describing the effect of F68 on differentiation of HASCs. Although there are some studies investigating the interaction between F127 (another block copolymer) [157] and mesenchymal stem cell differentiation, there is still a lot to discover on how F127 and F68 changes the expression of differentiation markers. In this study, the effect of F68 was investigated as a block coplymer for the first time on adipogenic differentiation of HASCs.

Boron is primarily a natural product and generally occurs in the environment as borates [144]. Borates are boron–oxygen compounds that result from the binding of boron with oxygen. When administered to animals, inorganic borates are biotransformed into boric acids and are absorbed from mucosal surfaces. More than 90% of the borate administered to mammalians including humans is excreted as boric acid. Recent studies on the biological significance of boron revealed that it is essential for humans and animals. In another study, it was suggested that sodium borate could be worth investigating as a drug to lower plasma lipid in humans and animals [145-148]. In addition, unpublished data obtained by our group revealed that high doses of NaB may inhibit adipocyte formation on pre-adipocyte cells. However, this is the first study showing the effect of boron solely and together with F68 on adipogenic differentiation of HASCs.

As a result of toxicity assay, among seven different concentration values between 5 and 300 μg/ml of NaB, 20 μg/ml was chosen to be used. The data demonstrated that as the concentration increases (starting with 100 μg/ml), the toxic effect of boron to the cells also increased. This is why, a higher dose of boron could not be selected since its combination with F68 would end up with a much more toxic impact on cell survival due to delivery potential of F68 through cell membrane.

Adipogenesis is regulated by several transcriptional factors [149, 150]. In this study, adipogenic differentiation of HASCs was confirmed by the detection of mRNA levels of adiponectin, FABP4 and PPAR-γ. According to the real time PCR results, positive control shows high expression levels for all of the selected marker genes indicating that adipogenic conversion was successfully performed. NaB treated group exhibited a higher mRNA levels of the marker genes compared to the positive control. Moreover, NaB and F68 combination significantly inhibited the expression level of marker genes.

PPARγ (peroxisome proliferator-activated receptor-γ) and CCAAT/enhancer-binding protein (C/EBP) are the master regulators of adipogenesis. They play a role in the initiation of adipocyte differentiation and induce the synthesis of various adipogenic genes [151]. In this project, cells in the presence of NaB showed the highest expression of adipogenic-related genes.

On the contrary, when we examined the effects of the NaB together with F68 on the gene expression of key adipogenesis activator during differentiation, there was a significant decrease in mRNA levels for PPARγ as compared with those of the untreated cells. As a result of PPARγ inhibition, adiponectin and FABP4 (aP2) expression was also decreased. The reason of this subsequent decline is that the increased level of PPARγ stimulates the mRNA expression of downstream target genes which are FABP4 (aP2) and adiponectin leading to the synthesis of several proteins required for intracellular lipid synthesis and storage [152-157]. Thus, adiponectin and FABP4 are called adipogenic markers. They are immensely produced in response to the insulin signaling pathway to promote cellular absorption of glucose which is eventually converted into lipid storage. Adiponectin is a prime marker for differentiated adipocytes [158]. It mediates glucose uptake, thereby promoting lipid synthesis in adipocytes. FABP4 (fatty acid binding protein-4) is a carrier of fatty acids that plays a supporting role in differentiation of the adipocytes [159, 160]. The down-regulated expression of this gene by the NaB and F68 combination indicated a decline in the ability of the cells to maintain and metabolize fatty acids. As a result, the intracellular lipid synthesis was decreased.

Apart from quantitative RT-PCR, immunocytochemistry of adiponectin, FABP4 and PPARγ and oil red oil staining was also performed as other indicators of adipogenesis. Oil red oil stain is used to dye and observe lipids and neutral triglycerides produced by the cells. In addition, the images taken for the immunocytochemistry and oil red oil staining were analyzed by using Adobe Creative Suite Program. 6 different images were used to calculate the average pixel values. As a consequence, lipid formation was observed to decrease in NaB/F68 combination group whereas it was increased in NaB treated group compared to the positive control. All these results including immunocytochemistry prove that the cells treated with NaB together with F68 had less intracellular lipid accumulation and fat droplet formation than the untreated controls.

In this study, only the effect of F68 was investigated as a block coplymer for the first time on adipogenic differentiation of HASCs. In the future studies, the impact of F127 on differentiation will also be tested. Our results indicated that NaB and F68 combination might suppress HASC adipocyte differentiation via inhibiting the expression of adipogenesis-related genes at transcriptional level. This can be a target for the discovery of drugs with potential efficacy for type 2 diabetes and obesity.

Consequently, this is the first study that demonstrated NaB together with F68 possessed an anti-obesity property. It acted directly on cells by inhibiting their differentiation through down-regulation of the key adipogenic transcription factor-PPARγ. Further study is necessary to evaluate the anti-obesity effect of NaB and F68 combination in experimental animals. We could next attempt to investigate a NaB containing hydrogel co-formulated with pluronics in vivo. In this way, NaB/F68 combination could be a new prescription to be used in pharmacology and be a future solution for obesity, but first, further studies should be conducted to explore its exact working principle and the effects of this formulation.

In conclusion, the current study proves that an appropriate concentration of NaB with F68 block copolymer contributes to inhibit the adipogenic lineage fate of HASCs. The outcomes are promising; on the other hand, much work remains to be undertaken for the analysis of the concept of obesity.

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