THE ROLE OF PLURONIC F68 AND F127 COMBINED GROWTH FACTORS AND STEM CELLS IN REDUCING KETAMINE INDUCED NEUROTOXICITY

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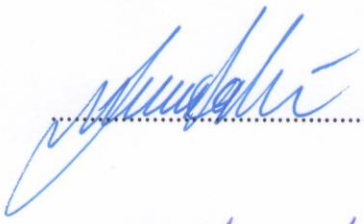
Prof. Dr. Fikrettin ŞAHİN ..... 
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ABSTRACT

THE ROLE OF PLURONIC F68 AND F127 COMBINED GROWTH FACTORS AND STEM CELLS IN REDUCING KETAMINE INDUCED NEUROTOXICITY

Ketamine is an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist which is used as human and animal medicine as an injectable anesthetic. Recently it was shown that Ketamine gives rise to neurotoxicity and cell death during surgical operations. Furthermore, long-term Ketamine administration increases the number of hyperphosphorylated tau positive cells, which is a hallmark of Alzheimer's Disease. In this study, we aimed to decrease the Ketamine induced neurotoxicity on SH-SY5Y neuroblastoma and primary mix brain culture cells applying several growth factors, namely; Fibroblast Growth Factor-2 (FGF-2) Insulin-Like Growth Factor-1 (IGF-1), Brain-Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Vascular Endothelial Growth Factor (VEGF) and stem cell conditioned medium in the presence of Pluronic F68 and F127. Moreover the effect of stem cells on this toxicity was investigated using stem cell co-culture system. Pluronics used in this study were reported to act as drug delivery reagents and membrane stabilizers which are important agents in the treatment neurodegenerative diseases. The stem cells used in this study were derived from Human Tooth Germs and not only comprise mesenchymal stem cell properties but also possess the capacity to differentiate into neurogenic cells. Our results demonstrated that the administration of either stem cell conditioned medium or direct stem cells in co-culture system protect cells from the Ketamine induced toxicity. The growth factors tested were also protective on SH-SY5Y and primary mix brain culture cells to some extent. Both stem cells and growth factors administered in combination with the Pluronics F68 and F127 were much more effective in reducing the neurotoxicity. These data suggested that stem cells and growth factors play an important role in ameliorating the adverse effects of Ketamine and the administration of Pluronics might increase the protection and healing of cells from the Ketamine induced stress. Pluronics can also provide an alternate approach for the treatment of other neurodenerative diseases.

ÖZET

PLURONİK F68 VE F127 İLE BİRLEŞTİRİLMİŞ BÜYÜME FAKTÖRLERİNİN VE KÖK HÜCRELERİN KETAMİNİN NEDEN OLDUĞU NÖROTOKSİSİTEYİ AZALTMADAKİ ROLÜ

Ketamin insan ve hayvana enjekte edilebilen bir unkompetitif NMDA reseptör antagonistidir. Son günlerde, ameliyat süresince kullanıldığında nörotoksisisteye ve hücre ölümüne neden olduğu gösterilmiştir. Ayrıca uzun süreli Ketamin alımı Alzheimer hastalığının ayırıcı özelliği olan hiperfosforile tau pozitif hücre sayısını arttırmaktadır. Bu çalışmada, çeşitli büyüme hormonlarının (Fibroblast büyüme faktörü-2, insülin benzeri büyüme faktörü-1, beyin kökenli nörotropik faktör, sinir büyüme faktörü, vasküler endotelial büyüme faktörü) ve kök hücrelerin besi ortamından alınan medyumun Pluronik F68 ve F127 varlığında Ketamin tarafından SH-SY5Y nöroblastoma ve primer beyin kültürü hücrelerinde oluşturulan nörotoksisiteyi azaltması amaçlanmıştır. Ayrıca kök hücrelerin bu toksisiteyi azaltmadaki etkisi kök hücre ko-kültür sistemi kurularak incelenmiştir. Bu çalışmada kullanılan pluroniklerin nörodejeneratif hastalıkların tedavisinde önemli biyolojik faktörler olan ilaç taşıyıcımada ve membran stabilizatörlüğünde rol aldığı bildirilmiştir. Bu çalışmada kullanılan kök hücreler insan diş germinden kökenlenmiştir ve sadece mezenkimal kök hücre özelliği taşımamakla birlikte aynı zamanda nörojenik hücrelere farklılaşabilme kapasitesine de sahiptirler. Sonuçlarımız kök hücrelerin besi ortamından alınan medyumun ya da direct olarak kök hücrelerin ko-kültür sisteminde hücreleri Ketamin'in neden olduğu nörotosisiteden koruduğunu kanıtlamıştır. Test edilen büyüme faktörleri de SH-SY5Y nöroblastoma ve primer beyin kültürü hücrelerinde bir miktar koruyucu olmuştur. Hem kök hücrelerin besi ortamından alınan medyumun hem de büyüme faktörlerinin Pluronic F68 ve F127 ile birleştirilerek uygulanması nörotoksisiteyi çok daha etkili bir şekilde azaltmıştır. Veriler kök hücrelerin ve büyüme faktörlerinin Ketaminin yan etkilerini iyileştirmede önemli rol oynadığını ve Pluronik uygulamasının hücreleri, Ketamin'in neden olduğu stresten korumayı ve iyileşmeyi arttırdığını göstermektedir. Pluronik'ler ayrıca diğer nörodejeneratif hastalıkların tedavisinde de alternatif bir yaklaşım sağlamaktadırlar.

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LIST OF SYMBOLS/ABBREVIATIONS

AIF	Apoptosis-Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
BBB	Blood Brain Barrier
BCSFB	Blood Cerebrospinal Fluid Barrier
BDNF	Brain-Derived Neurotrophic Factor
cDNA	Complementary Deoxyribonucleic Acid
CM	Conditioned Medium
CMC	Critical Micelle Concentration
CMT	Critical Micelle Temperature
CNP-ase	Cyclic Nucleotide Phosphodiesterase
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DFSCs	Dental Follicle Stem Cells
DMEM	Dulbecco's Modified Essential Medium
DNA	Deoxyribonucleicacid
DPSCs	Dental Pulp Stem Cells
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EO	Ethylene Oxide
ESC	Embryonic Stem Cells
F127	Pluronic F127
F68	Pluronic F68
FBS	Fetal Bovine Serum
FGF-2	Fibroblast Growth Factor-2
FITC	Fluorescein Isothiocyanate
G	Gram
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GCSF	Granulocytecolony Stimulation Factor
GDNF	Glial Cell Derived Neurotrophic Factor
GF	Growth Factor

GFAP	Glial Fibrillary Acidic Protein
GFs	Growth Factors
Glu	Glutamate
Gly	Glycine
h	Hour
HBSS	Hank's Buffered Salt Solution
HCL	Hydrochloric Acid
hESCs	Human Embryonic Stem Cells
HLB	Hydrophilic-Lipophilic Balance
HSC	Hematopoietic stem cell
hTGSCs	Human Tooth Germ Stem Cells
IGF-1	Insulin-Like Growth Factor-1
K	Ketamine
M	Molar
MAP2	Microtubule Associated Protein 2
MAPK p38	Mitogen-Activated Protein Kinase P38
MDR	Multi Drug Resistant
min	Minute
mg	Miligram
ml	Mililiter
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cell
MTBE	Methl-Tert Butyl Ether
MTS	Methyl Tetrazolium Salt
MW	Molecular Weight
N	Normal
NaOH	Sodium Hydroxide
NC	Negative Control
NF-L	Neuro-filament
ng	Nanogram
NGF	Nerve Growth Factor
nm	Nanometer
NMDA	N-methyl-D-aspartate

nNOS	Neuronal Nitric Oxide
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffered Saline
PC	Positive Control
PCR	Polymerase Chain Reaction
PDLSCs	Periodontal Ligament Stem Cells
PE	Phyco-erythrin
Pgp	P-glycoprotein
pH	Negative log of hydrogen ion concentration
PO	Propylene Oxide
PSA	Penicilin/Streptomycin/Amphotericin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCAP	Stem Cells from Apical Papilla
SCF	Stem Cell Factor
SHEDs	Stem Cells from Human Exfoliated Deciduous Teeth
v	Volume
VEGF	Vascular Endothelial Growth Factor
w	Weight
YUDETAM	Yeditepe University Experimental Research Center
µl	Microliter
µM	Micromolar

1. INTRODUCTION

1.1 STEM CELLS

Stem cells are unspecialized cells which can differentiate into different kind of cell types such as nerve cells, hearth cells, skin cells etc. with acquiring specialized functions when they are cultured in the right conditions [1, 2]. Mainly, until a stem cell gets a signal in order to generate specialized cell, it stays undifferentiated. In this context, they have important roles in the regenerative medicine because they have differentiation potential to other cell types that is why they can be used like a repair machine. Figure 1.1 and Figure 1.2 depict aims and properties of stem cells [3].

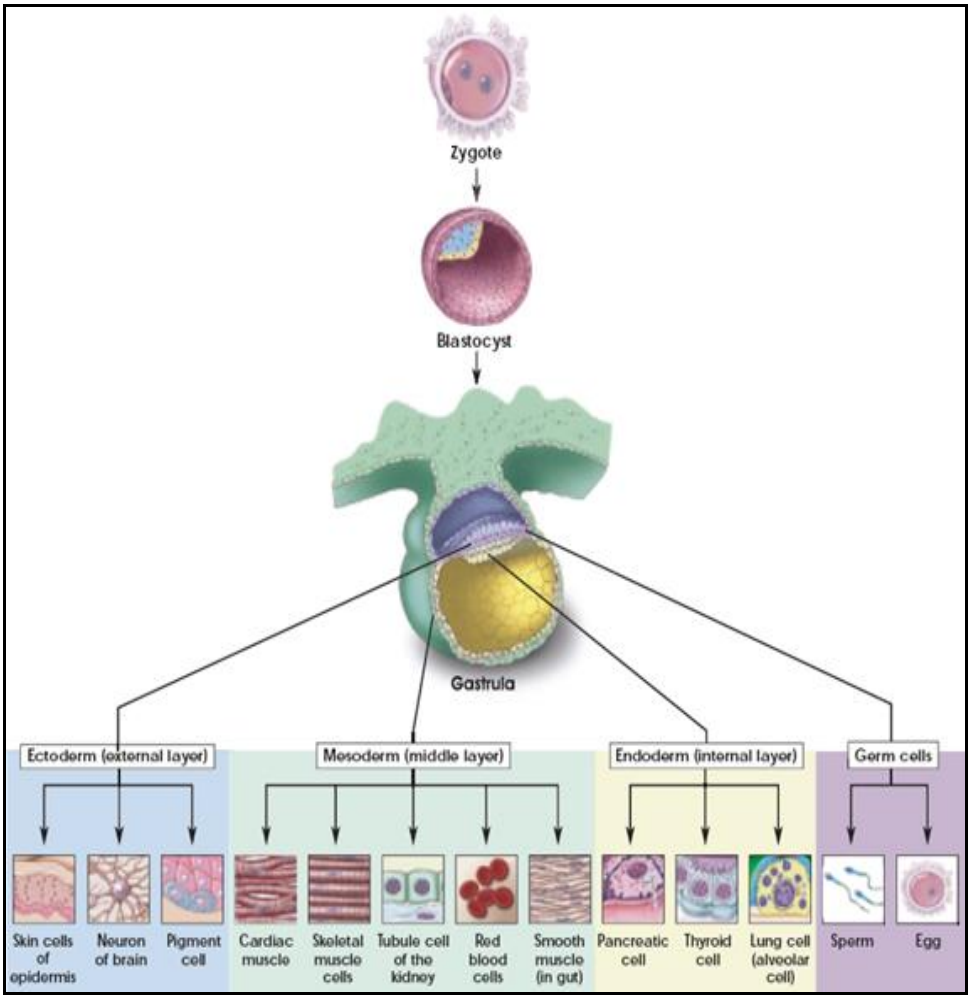


Figure 1.1. Differentiation potential of stem cells to other cell types [1]

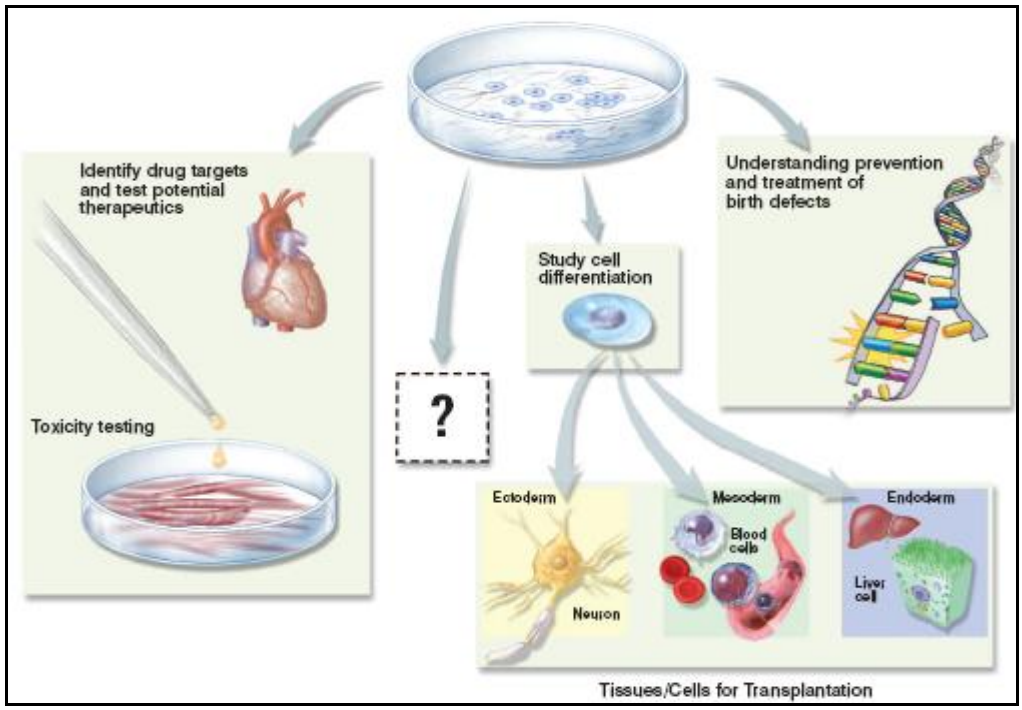


Figure 1.2. Aim of stem cell research [4]

A great deal of term is used in order to describe stem cells depending on their *in vivo* and *in vitro* features. A fertilized egg (zygote) is defined as totipotent. Totipotent stem cell has the ability to generate every cell type which is in the body [1, 5]. At day 5 after fertilization, totipotent stem cells constitute blastocyst which has a number of cell known as inner cell mass. Actually, this inner cell mass is pluripotent in nature and have the capacity to give rise to cells all of three embryonic germ layers, mesoderm, endoderm, and ectoderm. Morever, pluripotent stem cells are different from totipotent stem cells because pluripotent stem cells do not form a complete organism. Also they can not contitute the placenta or tissues that are vital for the foetal development [1, 6]. A multipotent stem cell can generate other cell types with limited capacity. For example, bone marrow stem cells can differentiate into the blood cell types but can not give rise to the brain cells [1, 2]. Unipotent stem cell which possesses lowest differentiation potential has the ability to specialize to only one type of cell [1].

Generally, stem cells are categorized as mainly two groups which are indicated in Figure 1.3 They are embryonic and adult stem cells. Adult stem cells can divide into 3

classes according to their origins, namely; hematopoietic stem cell, mesenchymal stem cells and other stem cells which are settled in the organs [3].

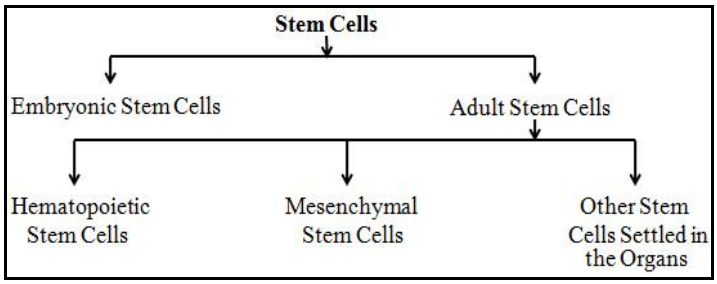


Figure 1.3. Categorization of human stem cells

1.1.1. Embryonic Stem Cell

After a human oocyte is fertilized, development starts. At 18 to 24 hours after *in vitro* fertilization of the oocyte is day 1. By day 2 (24 to 25 hours) the zygote achives the first division in order to generate 2 cell embryo. By day 3 (72 hours), the embryo attains the 8 cell stage which is defined as a morula. By day 4, the cells of the embryo adhere tightly to each other and this phase is called as compaction. By day 5, blastocyst cavity is completed as shown in Figure 1.4. The inner cell mass begins to separate from the outer cells, which becomes the trophectoderm that surrounds the blastocyst and this is the first cell differention event in the embryo [1, 4].

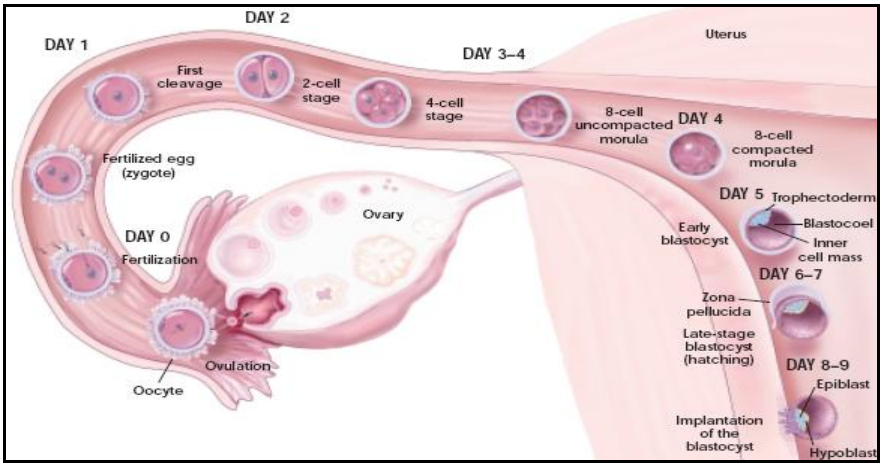


Figure 1.4. Development of zygote in humans [1]

Embryonic stem cells (ESCs) indicated in Figure 1.5 which are pluripotent are derived from the inner cell mass of the 5 to 6 day old blastocyst shown in Figure 1.6 [3]. At this stage, a normal human embryo contains 200 to 250 cells. Many of them form trophectoderm and in order to gain inner cell mass which consists of 30 to 34 cells in this step, the trophectoderm is taken away by microsurgery or immunosurgery. *In vitro* circumstances for growing of a human embryo to the blatocyst stage alter among the *in vitro* fertilisation clinics [1].

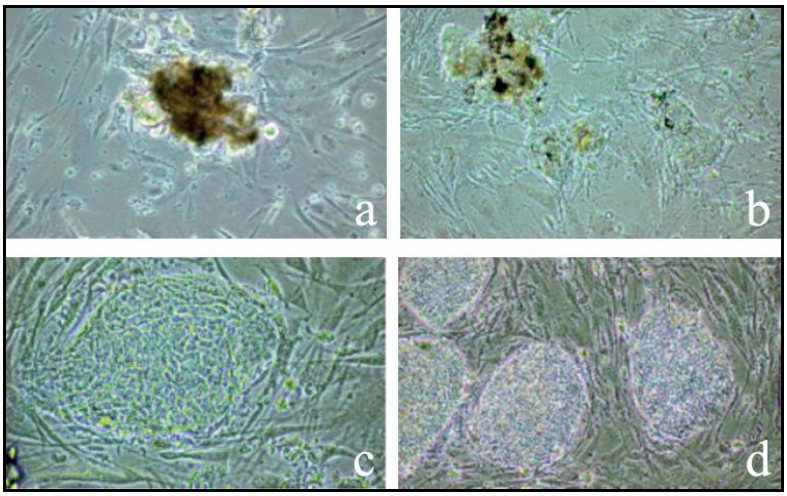


Figure 1.5. View of human embryonic stem cells (hESC) under phase contrast microscope [3]. (a) Blastocyst outgrowth take a couple of days; (b) On the right side of the picture, hESC-like clumps can be seen; (c) Generally hESC colonies are observable by the first passage; (d) hESC colonies are described with small, tightly packed cells with a high nucleus to cytoplasm ratio and distinctive nucleoli [7].

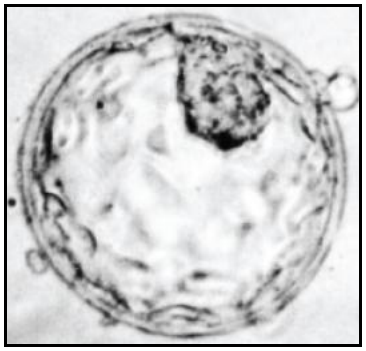


Figure 1.6. Human blastocyst which represents inner cell mass and trophectoderm [1]

ESCs are important in the regenerative medicine. They can reproduce unlimitedly and they can also generate wide range of cell types as shown in Figure 1.7. These provide big benefits as they can be used as a material for the testing drugs in terms of their safety and efficacy. For example, new drugs which is related to heart can not be examined directly *in vivo* on human because it can be dangerous. In this point, animal models are used but spesific differences can exist between in animal and human heart. Moreover, while a drug may not cause any toxicity in an animal model, same drug can cause toxicity and it can be lethal. Thereby, hESC derived cells are important in testing drugs before they are used in clinal trials also this procedure is safer and more effective [4]. Another important topic which is about advantages of ESCs is the cell replacement therapies. There are some diseases which occur due to the loss of cells or function of cells such as diabetes, Parkinson's disease, stroke, arthritis, multiple sclerosis, heart failure and spinal cord lesions etc. hESCs can help teratment of them due to their property to generate a variety of cell types such as neural, cardiac, skeletal muscle, pancreas and liver cells [8].

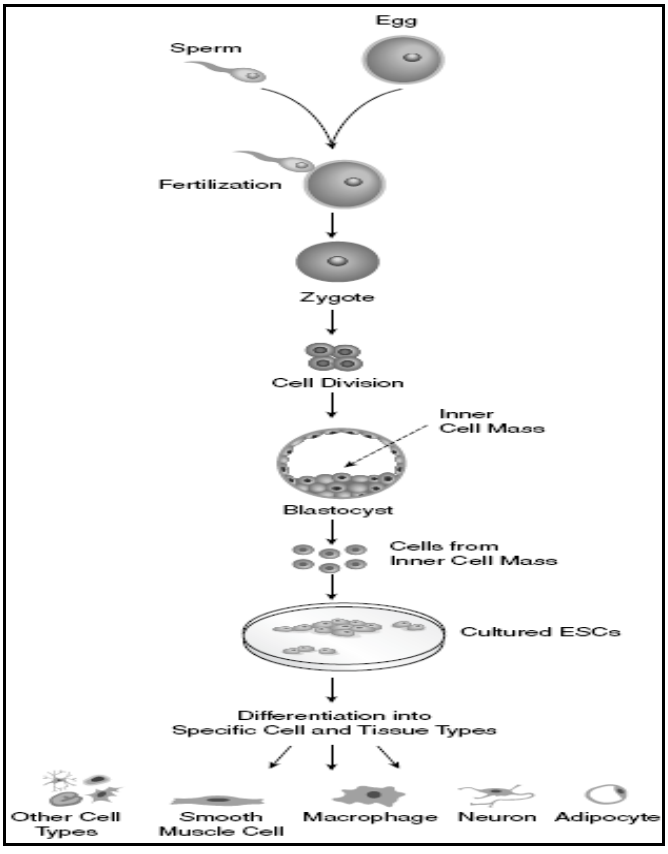


Figure 1.7. ESCs can generate wide range of cell types under appropriate conditions [2]

1.1.2. Adult Stem Cells

Adult stem cells are really common. Their basic tasks are to resume the homeostasis of tissue by as replacing the cells which die because of disease or injury [1]. Furthermore, they have at least two properties like the other stem cells. First of all, they are capable of long term self renewal ability that means they are able to generate identical copies of themselves for a long time. Secondly, they can make mature cell types which possess particular shapes and specialized assingments [9].

1.1.2.1. Hematopoietic Stem Cells

Hematopoiesis contains the generation and continuation of blood stem cells and their proliferation and differentiation into the cells of peripheral blood. The hematopoietic stem cell (HSC) shown in Figure 1.8 which is originated from mesoderm has multipotent feature so that it can differentiate into lots of cell types in appropriate conditions *in vivo* or *in vitro* [3]. Bone marrow, peripheral blood and umbilical cord blood are the sources of HSCs. In order to identify of HSCs some markers should be used. For instance, human HSCs have been defined with staining for Lin, CD34, CD38, CD43, CD45RO, CD45RA, CD59, CD90, CD109, CD117, CD133, CD166 and HLA DR (human) antigens [10].

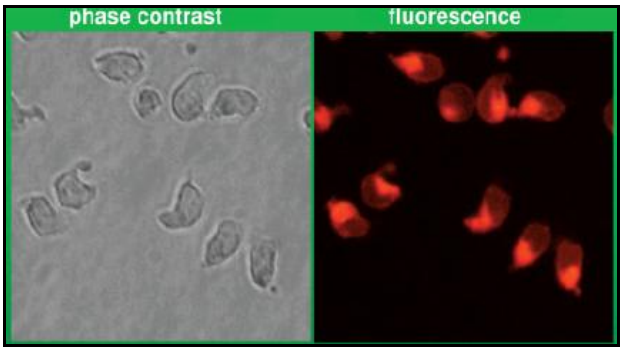


Figure 1.8. Hematopoietic stem cells which is labeled by the red flourescent membrane dye called PKH26 [11]

Studies show that there are two kinds of HSCs which are named long-term stem cells and short-term progenitor or precursor cells. When bone marrow cells which are taken from a mouse are transplanted to other mouse which received sufficient dose of irradiation

in order to destroy its own blood generating cells and if these cells repair its hematopoietic system within some months, they are evaluated as long term stem cells which have the ability of self renewal. Other bone marrow cells which are called as a short-term progenitor or precursor cells can renew a variety of blood cells. Also, they can support the hematopoiesis for three to four months in mouse. Progenitor or precursor cells which exist in same tissue type which is formed by fully differentiated cells are relatively immature cells. Although they can proliferate, they are not capable of differentiating into more excessive than one cell type as HSCs do [12]. HSCs have four peerless features. First of all, they possess self renewal propety however they are difficult to grow in culture restricting their application. The second characteristic which is very important in order to generate mature hematopoietic cells is the differentiation capacity shown in Figure 1.9. The third feature is the migration which happens at a definite time during development (seeding of fetal liver, spleen) and under particular conditions (cytokine induced mobilization) later in life [10]. The fourth hallmark of HSCs is the apoptosis which arranges the numbers of stem cells. For example in a transgenic mouse experiment it was shown that HSC numbers increase as two folds when the apoptosis threshold was risen [13].

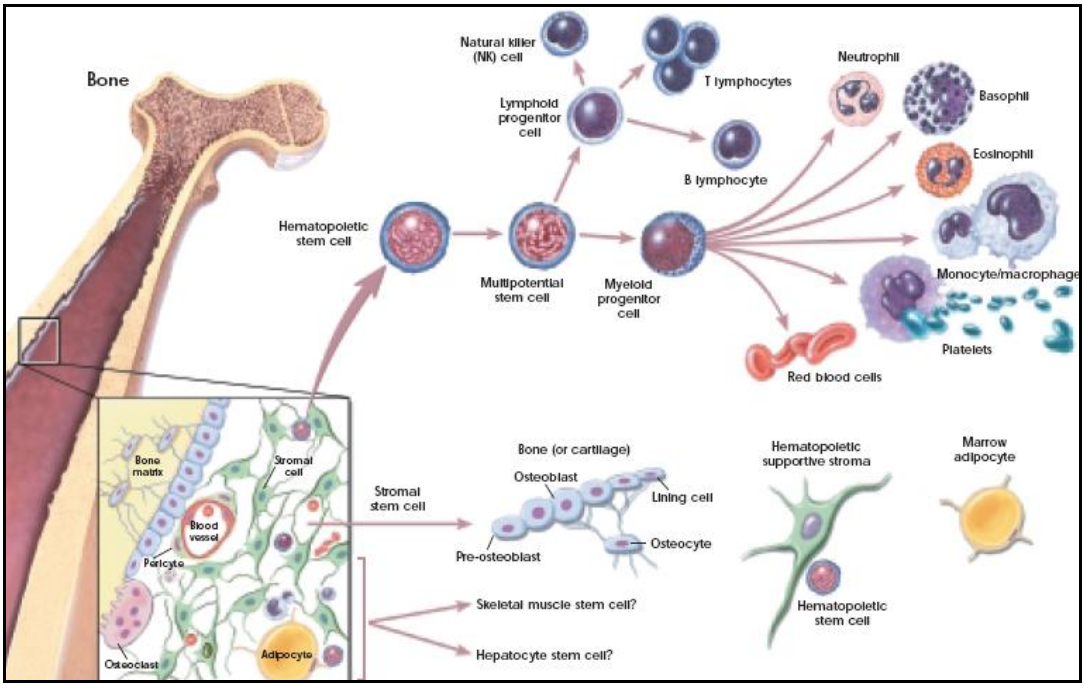


Figure 1.9. Differentiation of hematopoietic and stromal stem cell [1]

Bone marrow stem cells which are well known sources of HSCs are used in the treatment of disease but they have some limitations because of their harvesting procedure which entails general anesthesia which is undesirable for the patient. Because of these reasons umbilical cord blood and peripheral blood stem cells become more popular. Normally most of HSCs exist in the bone marrows of adults but because of cytokine mobilization, wide range of HSCs is released into blood providing means to collect the HSCs from the peripheral blood [10]. Umbilical cord blood is another source and harvested at birth [14]. Longer telomere lengths ,which is related to self renewal activity, showing better stability in time, less oncogenic potential and containing equal or larger quantities of HSCs when compared with the bone marrow stem cell, make the umbilical cord stem cells more convenient than the bone marrow stem cell [15, 16]

1.1.2.2. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) which have multipotent property can differentiate into a wide range of cell types such as osteocytes, chondrocytes, adipocytes, even hepatocytes and neurons which are nonmesodermal origin if the suitable microenvironment is provided. They take an important place in regenerative medicine because of four reasons. Firstly, when they are compared with other stem cells, these cells can be obtained from different kind of tissues such as bone marrow, umbilical cord blood, adipose tissue, synovial membrane, skeletal muscle, dermis, pericytes, trabecular bone, lung, dental pulp, amniotic fluid, fetal liver, and even peripheral blood [11, 17, 18]. Secondly, they can be maintained *in vitro* without loosing their self renewal capacity. The other property is their differentiation capacity. They can give rise to bone, cartilage, muscle, tendon, fat, cardiomyocytes, hepatocytes and neurons *in vitro* as depicted in Figure 1.10. Furthermore, together with HSCs, when allogenic MSCs are tranplanted, any graft rejection or major toxicities will not occur [11, 19]. Moreover, studies showed that MSCs can also migrate where the injury exist in order to repair the tissue. This property is important in terms of their usage as a tool for the site specific theraphy [20].

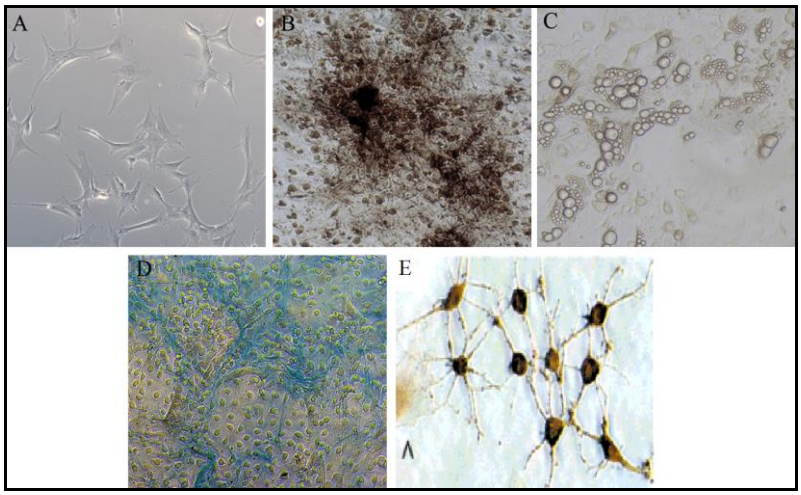


Figure 1.10. Differentiation capacity of MSCs. MSCs (A) can differentiate into osteocytes (B), adipocytes (C), chondrocytes (D), neurons (E) [19, 20]

1.1.2.3. Dental Stem Cells

Dental originated mesenchymal stem cells which have been studied widely in the last years play an important role in tooth regeneration and repair [21, 22]. Tooth germs are formed during embriyonic progress as a consequence of ecto-mesodermal interactions that generate neural crest cells having self renewal ability and capacity of multi-lineage differentiation [23, 24]. Some of these progenitor cells exist in the dental tissues because of the retarded state of tooth development. For instance, the dental follicle cells which enclose the developing tooth germ have been demonstrated to give rise to osteocytes, adipocytes and chondrocytes because of their progenitor (stem) cell property [25- 28]. Moreover, dental pulp cells also differentiate into various cell types such as osteocytes, neurons, adipocytes and chondrocytes [29, 30].

Dental derived mesencymal stem cells can divide into 5 groups as the dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells (DFSCs) from human third molars [22] and the human tooth germ stem cells (hTGSCs) [31].

Dental pulp stem cells (DPSCs) shown in Figure 1.11 are originated from dental pulp. They keep silent and exist in a specific perivascular microenvironment where they resume their stem cell properties [21,32]. If there is a dental injury in the dental pulp,

dentinogenesis will start. This process includes the deposition of new matrix around the cells in order to repair the injured site. It indicates that DPSCs can generate odontoblast under convenient signals [33].

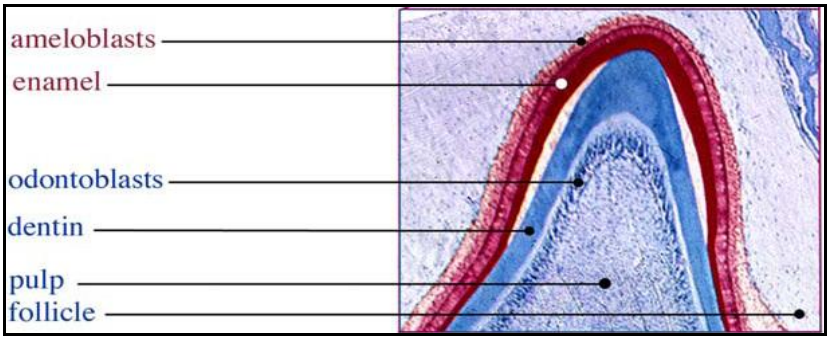


Figure 1.11. Histological sections of human tooth [21]. Histological sections of human tooth were stained with haematoxylin. Whereas blue colour indicates mesenchymal tissues and mesenchyme derivatives (odontoblasts, dentin, dental pulp and follicle), orange colour demonstrates epithelial tissues and epithelial derivatives (ameloblasts and enamel) [21]

DPSCs which have multipotential differentiation capacity are really important in the regenerative medicine. It was demonstrated that β-amyloid peptide and 6-OHDA induced neurotoxicity on primary hippocampal and ventral mesencephalic neuron cultures were reduced after they were co-cultured with DPSCs [34]. Moreover, it was shown that they also gave rise to functionally active neurons under the convenient conditions [35].

Stem cells from human exfoliated deciduous teeth (SHEDs) reside in the pulp tissue of human exfoliated deciduous teeth. Figure 1.12 is illustrated SHEDs. They are defined as widely proliferative clonogenic cells and able to differentiate into adipocytes, odontoblasts, osteoblasts, neuronal cells. When it is necassary, they can generate a considerable amount of alveolar and orofacial bone for the tissue regeneration [22, 32, 36]. Studies showed that *in vitro* cultured SHEDs which were isolated from the residual pulp tissues of the exfoliated teeth from 7-8 years old children, were highly proliferative and already express neural and glial markers such as microtubule associated protein 2 (MAP2), light neuro-filament (NF-L), glial fibrillary acidic protein (GFAP), nestin (type IV intermediate filament), tau (micro-tubule associated protein),cyclic nucleotide phosphodiesterase (CNP-ase), and neuron-specific class III β-tubulin (TuJ1) [36, 37].

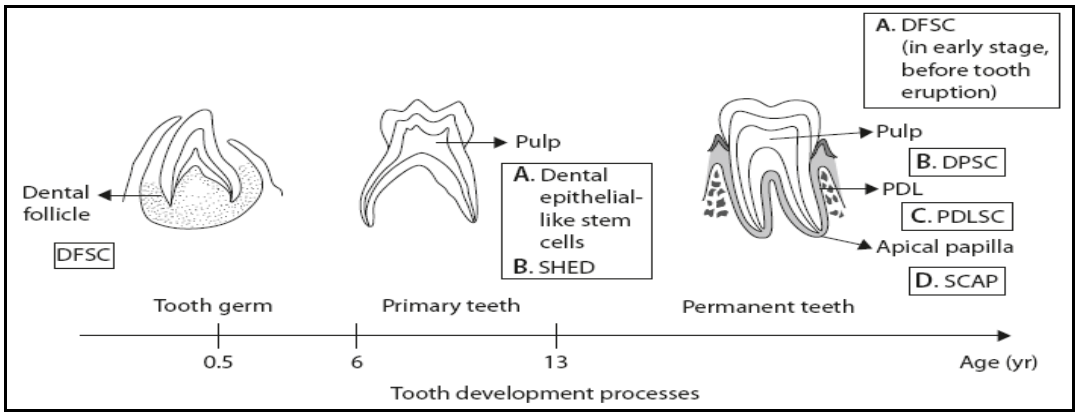


Figure 1.12. Phase of tooth development and dental derived stem cells [22].
Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells (DFSCs), stem cells from apical papilla (SCAP) [22]

Periodontal ligament stem cells (PDLSCs) which exist between the cementum and alveolar bone play an important role on the support and maintenance of the teeth and periodontal repair. They can differentiate into cementoblast like cells, osteocyte, chondrocyte and adipocyte [22, 38].

Dental follicle stem cells (DFSCs) which have been isolated from the follicle of human third molars after the removal of wisdoom tooth. Schematic and histological representation of dental pulp tissue are taken part in Figure 1.13 and Figure 1.14. They have serious assignments in tooth eruption by means of regulating the osteoclastogenesis and osteogenesis [21, 25, 39- 41]. After tooth eruption dental follicle gives rise to the cells of the periodontium, alveolar osteoblasts, the periodontal ligament, fibroblasts and cementoblasts [42]. Moreover, they can differetiate into other cell types such as neurons and adipocytes. While neurogenic differentiation was shown using neural progenitor markers such as notch-1 and nestin, adipogenic differentiation was indicated by cultivating the DFSCs with an adipogenesis medium [43, 44]

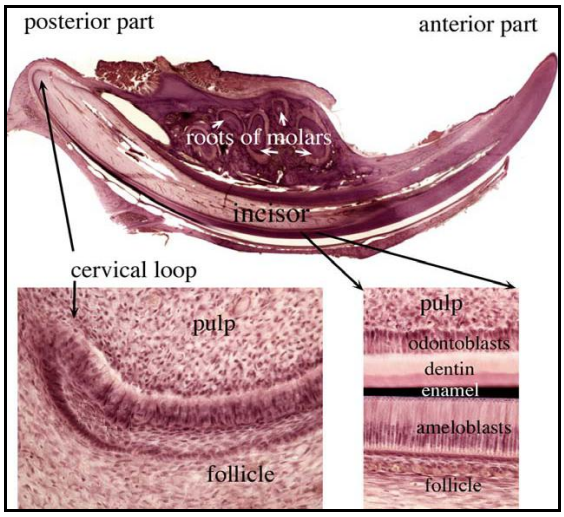


Figure 1.13. Mandibular rodent incisor was shown as histological sections. Sections were stained with haematoxylin/eosin [21]

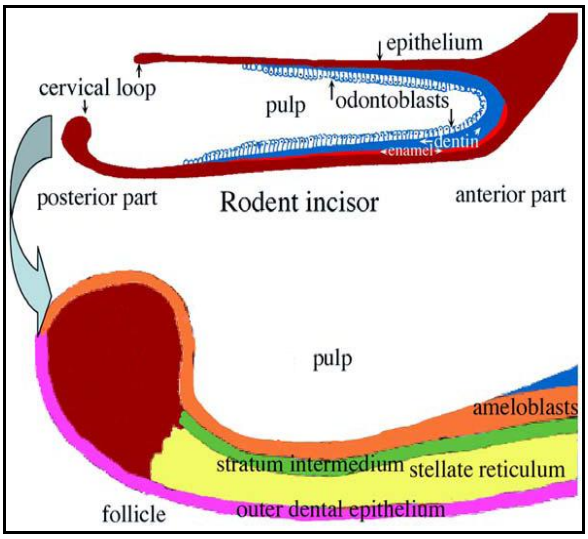


Figure 1.14. Schematic representation of a mandibular rodent incisor [21]

Human tooth germ stem cells (hTGSCs) was firstly isolated by Yalvac *et al*, 2009. In order to isolate hTGSCs whole tooth germ tissue including dental mesencyme residing in the developing crown and its surrounding follicle was taken. Figure 1.15 shows human tooth germ in the chin and Figure 1.16 represents day 10 of hTGSCs culture under the light microscope.

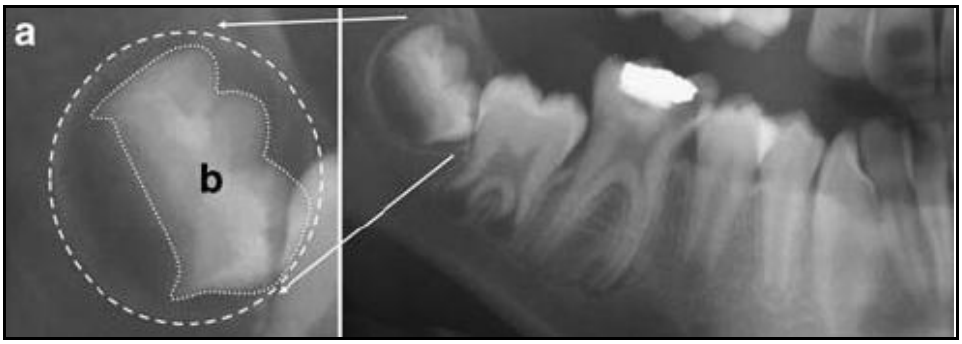


Figure 1.15. Isolation of hTGSCs from an impacted third molar tooth germ. a. Whole tooth germ was extracted (dashed line) and b. the crown part (dotted line) was removed [31]

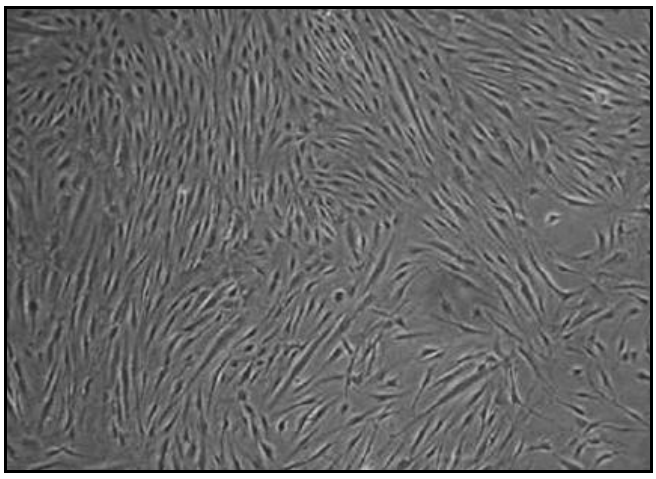


Figure 1.16. Day 10 of hTGSCs culture [31]

Actually third molar tooth germs are removed because of orthodontic reasons and enucleated tooth germs are generally thrown away. These tooth germs which have undifferentiated ectodermal and mesodermal content can be used as an important stem cell source but there is a crucial point. Until age 6, undifferentiated embryonic tissues which generate whole tooth exist in the human tooth germs because of this reason human tooth germs should be isolated from young individuals. hTGSCs can differentiate into osteogenic, adipogenic and neurogenic cells. Furthermore, they can form tube like structures when they were cultured on Matrigel. It also shows their angiogenic potential. They are depicted in Figure 1.17 [31, 45].

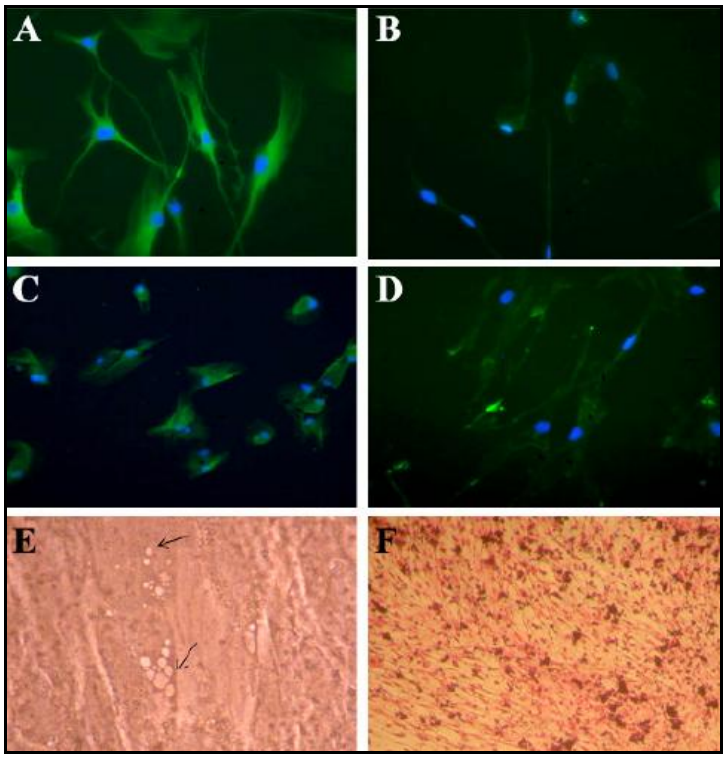


Figure 1.17. Differentiation capacity of hTGSCs [45]. hTGSCs were stained positive for neurogenic markers β3-tubulin (A), neuro-filament protein (B), netsin (C), osteogenic marker collagen type-I (D). Lipid vesicles (E, arrows) were indicated in order to detect adipogenic differentiation and calcium depositions stained black (F) were also determined in order to show osteogenic differentiation [45]

Capability of differentiation, proliferation and viability can reduce on stem cells because of improper cryopreservation. Furthermore, the study proved that hTGSCs can be cryo-preserved at -80^{o}C for at least 6 months without a loss in their functions [45].

1.2. PLURONIC TRIBLOCK COPOLYMERS

It is considered that as much as 1.5 billion people suffer from a variety of neurodegenerative diseases and if effective treatments will not be found, this number will be nearly 1.9 billion by 2020 [46]. Essentially main problem is the targetting and sending of the drugs to the central nervous system due to presence of several protective barriers such as blood brain barrier (BBB), blood cerebrospinal fluid barrier (BCSFB) etc. which limit the transition of foreign particles into the brain [47]. In view of this reason the development of effective drug delivery systems has taken enormus attention in recent years. There is a significant and promising drug delivery agent named Pluronic block copolymers (also known under their non-proprietary name 'poloxamers'). Pluronic block copolymers consist of ethylene oxide (EO) having hydrophilic property and propylene oxide (PO) having hydrophobic characteristic. These blocks are arranged in a basic A-B-A structure: EO_x-PO_y-EO_x [48, 49]. Figure 1.18 represents Pluronic block copolymer molecule and structure. Synthesis of Pluronic block copolymers takes place by the sequential addition of PO and EO groups in the presence of an alkaline catalyst such as a potassium or sodium hyroxide [50]. This specific formation provides the structure an amphiphilic property and because of their amphiphilic property they present surfactant characteristics and can make interactions with hydrophobic surfaces and biological membranes.

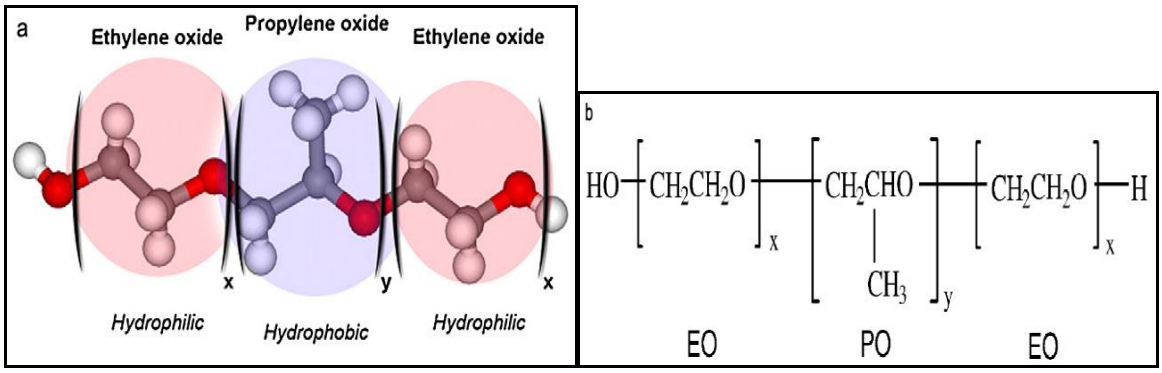


Figure 1.18. Pluronic block copolymer molecule and structure.
a. Pluronic triblock copolymer molecule [48] and b. molecular Formula [49]

The number of EO and PO units can change so different pluronic copolymers, whose size, hydrophilicity and lipophilicity vary, are formed. In addition to this, pluronic block copolymers have different hydrophilic-lipophilic balance (HLB) depends on the number of EO and PO groups [51].

A promising property of Pluronic block copolymers is the formation of micelles in aqueous solutions upon critical micelle concentration (CMC) so with this property they can be used as a microcontainer in order to incorporate drug molecules and carry them into the cell as shown in Figure 1.19. Before the micellization these amphiphilic block copolymers are in unimers which are described as polymer single chains [52- 54]. Unimers play an important role as biological response modifiers: They can bind to the cell membrane, change membrane microviscosity (membrane fluidization), inhibit selected membrane proteins, especially drug efflux transport ptotein, namely P-glycoprotein (Pgp) [55- 57].

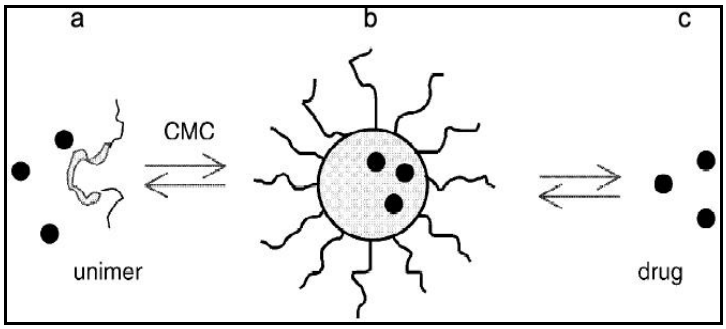


Figure 1.19. Drug release from the micelle [49]. a. Below CMC, Pluronic stays on as unimer, b. Above CMC, micellization occurs and drug can be incorporated into micelle, c. Drug releasing [49]

Because of having hydrophobic PO groups, Pluronic unimers are able to adsorb on surfaces, interact with the lipid membranes and move inside the cells [55, 57]. Pluronics cause structural alterations in the cell membrane such as decrease in the membrane microviscosity [55], increase in the rate of the lipid ‘’flip-flop’’ [58] and the enhancement of transmembrane transport of ions and solutes accompanied by binding to the cell membranes [58- 60]. In aquous solutions above a specific CMC, unimers self assemble into micelles whose diameter can change from 10 nm to 100 nm [61]. Pluronic micelles,

having a hydrophobic PO core and a hydrophilic EO shell, can be spherical, rod-like or lamellar shape depending on the length of the PO and EO groups [62].

Furthermore, in micellization not only CMC is important but also critical micelle temperature (CMT) is significant. Below room temperature PO and EO groups are hydrated and relatively soluble in water. In addition, PO group is dehydrated and turns out to be insoluble in water when the temperature increases and micellization starts. In this point CMT is defined as a temperature which is essential for micelle formation. For a great number of Pluronic the CMT values change from 25 to 40^{o}C, i.e. below or near body temperature [49]. Generally, Pluronic block copolymers which are used for drug delivery have a CMC range from 1 μM to 1 mM at body temperature (37^{o}C) [63, 64].

Pluronic block copolymers also have gel formation property. When the concentation of Pluronic increases, formed micelles generate tighter packaged structures and upon a specific threshold concentration the gels are formed as presented in Figure 1.20. [50, 65, 66, 67, 68, 69]. Moreover, they are thermosensitive similar to micelles. In a like manner, generation of the gels is seen only in the gelation temperature [70]. These gels possess microheterogeneus structure and the property of incorporating biomacromolecules even into the living cells. In addition, they are biocompatible [50, 65- 69].

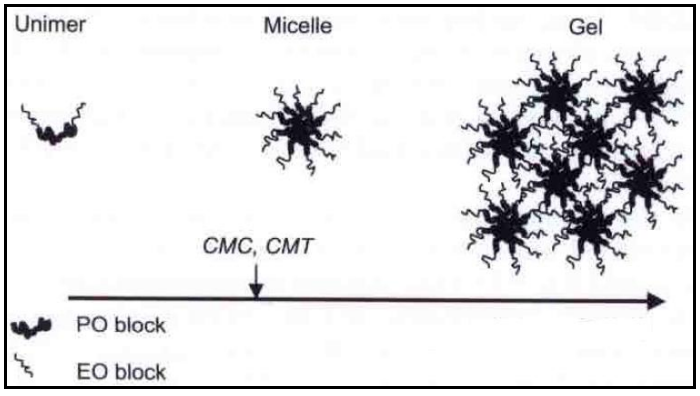


Figure 1.20. Formation of micelles from unimers under suitable conditions and gel formation as a result of copolymer concentration [68]

Pluronic triblock copolymers can be used for different applications. Effect of Pluronic on multi drug resistant (MDR) cancer cells is well studied. It is proved that Pluronics have the following futures;

- incorporation into cell membranes by altering its fluidization (microviscosity)
- causing a great decline of ATP levels in cancer and barrier cells
- inhibition of drug efflux transporters such as Pgp [55, 71- 74], multidrug resistance proteins [75], breast cancer resistance protein [76, 77]
- causing release of cytochrome C and enhance amount of reactive oxygen species (ROS) in the cytoplasm
- inducing pro-apoptotic signaling and reduce anti-apoptotic signaling in MDR cells [78]
- prevention of the glutathione/glutathione S-transferase detoxification system [55, 75]
- inhibiting drug sequestration within cytoplasmic vesicles [79]

Effects of pluronics on the cell are summarized in Figure 1.21.

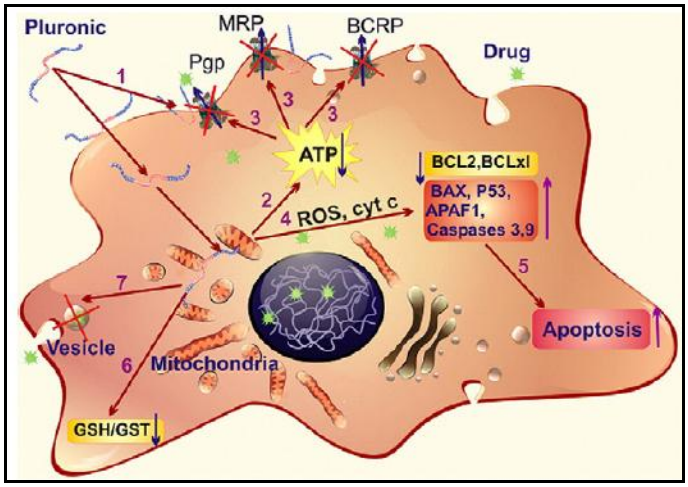


Figure 1.21. Effects of Pluronic block copolymers on MDR cells [48]

Pluronic block copolymers can protect biological materials from degradation, maintain exposure of biological materials to tissues and increase the transport efficiency of biological agents into cells [80]. In addition, Pluronics can be used for gene delivery and it is demonstrated that they increase the expression of transgenes delivered to the cells such

as naked DNA [81-87], and DNA polycation complexes [88- 92]. Other advantage of Pluronics is about sealing property. Pluronics enhance the cell membrane resealing and also diminish trauma after the electroporation [83, 93]. Additionally, Pluronics are promising agent for the neurodegenerative diseases because their usage provides a great benefit to increase drug penetration into the brain [51].

1.2.1. Pluronic F68 (Poloxamer 188)

Pluronic F68 has a nonproprietary name called Poloxamer 188. Its structure is shown in Figure 1.22. Since 2000, it is listed in the US Pharmacopoeia. Pluronic F68, which consists of PO and EO groups, is a nonionic, low foaming, relatively nontoxic, water soluble surfactant [94- 96] . It is a white powder having 8400 Daltons molecular mass. It has neutral pH (pH 7). Its CMC is $4.8.10^{-4}$M [97]. Physicochemical properties of Pluronic F68 are stated in Table 1.1.

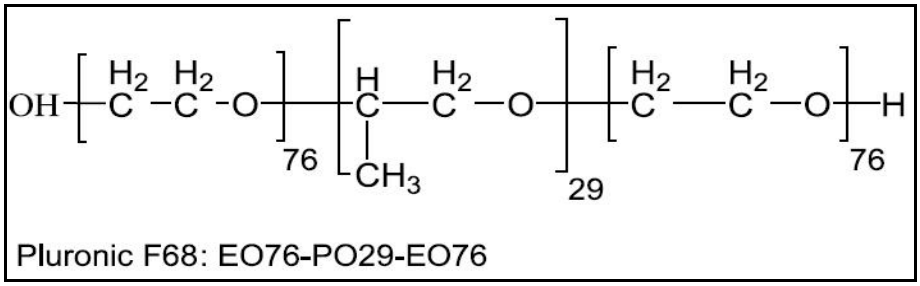


Figure 1.22. Structure of F68 [98]

Table 1.1. Physicochemical properties of Pluronic F68 [97]

Copolymer	**MW[a]**	**Average No. of EO Units[b]**	**Average No. of PO Units[b]**	**HLB[c]**	**CMC, M[d]**
F68	8400	152,73	28,97	29	$4,8.10^{-4}$

[a]MW: Molecular Weight, [b]The average numbers of EO and PO units

[c]HLB: Hydrophilic-Lipophilic Balance, [d]CMC: Critical Micelle Concentration (M)

1.2.2. Pluronic F127 (Poloxamer 407)

Pluronic F127 has a nonproprietary name called Poloxamer 407. Its structure is shown in Figure 1.23. As from 2000, it is listed in the US Pharmacopoeia. Pluronic F127, which consists of PO and EO groups, is a nonionic, relatively nontoxic, and water soluble surfactant [95, 99, 100]. Due to efficient solvation and hydrogen bonding at lower temperatures, Pluronic F127 can dissolve more in cold water when it is compared with in the hot water [101]. It is a white powder having 8400 Daltons molecular mass. Its CMC is 4.8.10-4M [97]. Physicochemical properties of Pluronic F127 are stated in Table 1.2.

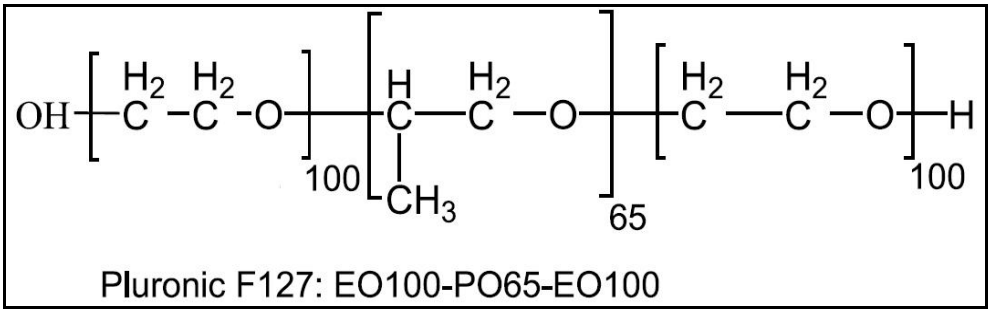


Figure 1.23. Structure of F127 [98]

Table 1.2. Physicochemical properties of Pluronic F127 [97]

Copolymer	**MW[a]**	**Average No. of EO Units[b]**	**Average No. of PO Units[b]**	**HLB[c]**	**CMC, M[d]**
F127	12600	200,45	65,17	22	$2{,}8.10^{-6}$
[a]MW: Molecular Weight, [b]The average numbers of EO and PO units [c]HLB: Hydrophilic-Lipophilic Balance, [d]CMC: Critical Micelle Concentration (M)					

1.3. KETAMINE

Ketamine, used as a general anesthetic for surgical operations ia an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist. In addition Ketamine is a nonbarbiturate and rapid dissociative anesthetic [102]. Ketamine is also illegaly used as a recrearional drug among especially young adults and adults. It is often taken at night dance parties called raves in nightclubs [103- 105]. Studies showed that in 5 years people who used Ketamine for illegal reasons are increased [106]. Due to its anesthetic and hallucinate effects, the user may not realize injury or related physical assaults including strange behaviour that could be fatal [103- 105].

Ketamine is applied epidurally and intrathecally for the treatment of postoperative, chronic cancer pain and neuropathic pain etc. [107, 108]. In order to use it as a general anesthetic the doses suitable for implementations are 1–2 mg/kg intravenously or 5–10 mg/kg intramuscularly. Recommended doses for sedation or analgesia are 200–750 μg/kg intravenously or 2–4 mg/kg intramuscularly [109]. Recent studies have indicated that Ketamine induces apoptosis in the immature rat brain [110, 111]. It is demonstrated that long term Ketamine administration such as over days and weeks causes neurotoxicity [112- 114]. Animal models and patients showed that when Ketamine is used intrathecally it impairs the white and grey matter of the spinal cord, most lesions exists subpial and around the spinal canal [114, 115]. Ketamine application on neuronal cells for 6 and 12-24 hours gives rise nearly 30% and 50-70% loss in cell viability, respectively [116]. Also, it was shown that long-term Ketamine administration on brains of mice and monkeys increases hyperphosphorylated tau positive cells, which is a hallmark of Alzheimer's disease [117]. Ketamine induced neurotoxicity is related to the calcium overload by means of glutamatergic sitimulation of the overactivated NMDA receptor [111, 118] and upregulation of NMDA receptor leads necrosis [119] and apoptosis [120].

NMDA receptors have a crucial role in a wide range of physiological and pathological processes, including neuronal development [121], acute neuropathologies, such as stroke and other trauma-related events [122], epileptiform seizures, synaptic plasticity [123], memory and learning [124], chronic neuropathologies such as Alzheimer's

[125], Parkinson's and Huntington's diseases [123, 126], and mental illnesses such as schizophrenia and anxiety disorder [123].

NMDA receptor is a member of glutamate-gated ion channel. It is a heteromeric complex and formed by three subunits which are known as NR-1, NR-2, NR-3. NR-1 have eight different versions due to alternative splicing from a single gene; NR-2A-D, and (in some cases) NR-3A-B which are originated from six different genes [127, 128]. Generally NMDA receptors include two NR-1 and two NR-2 subunits [128]. In order to activate NMDA receptor glutamate, the endogenous agonist, must bind to the NR-2 subunit of the receptor and glycine which is necessary as a co-agonist must bind to NR-1 (or NR-3) subunit of the receptor at the same time. Figure 1.24 shows NMDA receptor [129].

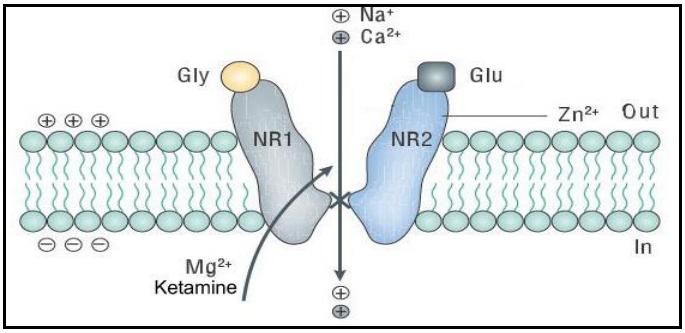


Figure 1.24. Schematic diagram of NMDA receptor [129]. Glycine (Gly) and Glutamate (Glu) are agonists of the NMDA receptor. Furthermore it has pore blockers such as endogenous Mg^{2+} and Ketamine. Zn^{2+} binding site is also shown [129].

NMDA receptor play an important role in synaptic transmission. Under normal circumstances Mg^{2+} sits in the pore of the receptor. When glutamate and glycine bind to their binding sites, receptor gets activated and cell becomes depolarized to remove the Mg^{2+} block. After the channel opens Ca^{2+} and Na^{+} enter into the cell and K^{+} moves out of the cell in order to carry out physiological actions [129, 130]. In this manner, synaptic activity proceeds along with entring Ca^{2+} to postsynaptic cell via NMDA receptor [131]. Figure 1.25 (1) shows NMDA receptot under normal circumstances.

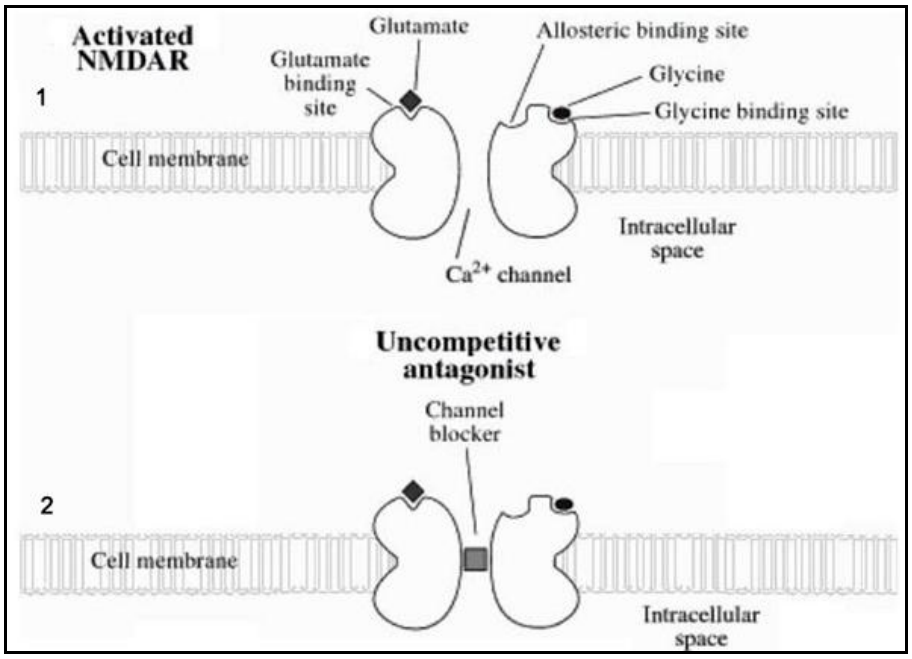


Figure 1.25. Activated NMDA receptor (1) and NMDA receptor exposed Ketamine (2) [132]

Under pathological states the receptor becomes overactivated. Figure 1.25 (2) shows NMDA receptor under normal circumstances. Upregulation of the receptor gives rise to increased level of Ca^{2+} into the neuron and along with it, variety of events inducing apoptosis, necrosis or dendritic/synaptic damage take place. As a result of excessive Ca^{2+} influx: mitochondria become overloaded, buffering capacity of mitochondria reduce the membrane potential, overproduction of reactive free radical superoxide anion O_2^- takes place because of the impairment of the electron transport. Caspases are activated and the releasing of apoptosis inducing factor happens [130, 133]. Moreover Ca^{2+}-dependent activation of neuronal nitric oxide synthase (nNOS), gives rise to the overproduction of nitric oxide (NO) and the formation of the toxic peroxynitrite ($ONOO^-$) and the S-nitrosylated glyceraldehyde-3-phosphate dehydrogenase (GAPDH); and the stimulation of mitogen-activated protein kinase p38 (MAPK p38), which activates transcription factors that can go into the nucleus to influence neuronal injury and apoptosis [130]. Excitotoxic neuronal necrosis is also induced by the upregulation of the NMDA receptor. Activation of calpain, a calcium dependent enzyme, can occur and this event causes the proteolysis of neuronal cytoskeleton [134, 135]. Another necrotic mechanism related to the overactivation of the receptor is the entrance of excessive Ca^{2+}, Na^+ together with Cl^- and

water. As a consequence, cell swelling and death occur [119]. Events are illustrated in Figure 1.26.

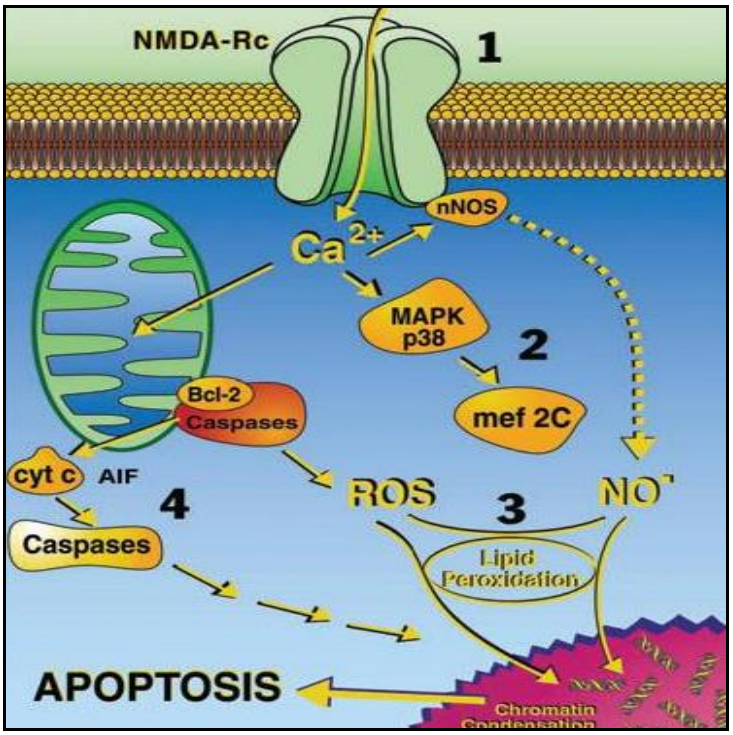


Figure 1.26. Apoptotic pathways induced by overactivated NMDA receptor [130]. 1. Overactivation of NMDA receptor. 2.Activation of p38 mitogen activated kinase (MAPK)–MEF2C (transcription factor) pathway (Afterwards, caspases cleaves MEF2 in order to generate an endogenous dominant-interfering form that make contribution to neuronal cell death. 3. Toxic effects of free radicals such as nitric oxide (NO) and reactive oxygen species (ROS). 4. Activation of caspases and apoptosis-inducing factor (AIF). nNOS: nitric oxide synthase; cyt c: cytochrome c [130]

1.4. AIM OF THE STUDY

In this study, it was aimed to demonstrate the effect of Pluronic F68 and F127 combined growth factors and stem cells in reducing Ketamine induced neurotoxicity on SH-SY5Y neuroblastoma and primary mix brain culture cells. By testing these polymers on Ketamine exposed cells it was expected to create a new strategy for increasing the effectiveness of drugs which might present a new insight into the treatment of neurodegenerative diseases. We have also examined the effect of stem cells on Ketamine triggered toxicity and aimed to suggest a new therapy strategy for patients who suffer from neurodegenerative diseases.

2. MATERIALS AND METHODS

2.1. ISOLATION OF hTGSCs

Human tooth germs were isolated from the wisdom teeth of 12 years old patient. Tooth germs, were minced into small pieces with a sterile scapel and placed into six well plates containing Dulbecco's modified essential medium (DMEM) (Invitrogen, Gibco, UK, cat # 31885) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Gibco, UK, cat # 10270-106) and 100 units/ml penicillin, 100 µg/ml streptomycin, 0,25 µg/ml amphotericin (1% (v/v) PSA) (Invitrogen, Gibco, UK, cat # 15240-062). After eight days of incubation in incubator (Thermo, US, model no: 3131) at 37 °C with 5% CO_2 and 95% humidity, cells whose medium changed every day reached confluency (80%). At this point, cells were lifted from six well plate using (BIOFIL, TCP, Switzerland, cat # 011006) 0.25% (w/v) trypsin-EDTA (Invitrogen, Gibco, UK, cat # 25200). After incubation with trypsin-EDTA solution for two minutes, medium was added to block the activity of trypsin. After centrifugation at 1500 rpm for 5 minutes, cell pellet was suspended in fresh medium and seeded to a T-75 (Zelkultur Flaschen, Switzerland, cat # 90075) flask. Cells were cultured in an incubator at 37 °C with 5% CO_2 and 95% humidity and passaged every other day [136].

2.2. CHARACTERIZATION OF hTGSCs

hTGSc were characterized according to the procedure used by our group [31]. Cells were lifted from flask using trypsin-EDTA and incubated with primary antibodies prepeared in PBS (Invitrogen, Gibco, UK, cat # 10010, pH 7,4) for one hour. Used primary antibodies for this study were anti- CD29 (cat#BD556049), CD34 (cat # SC-51540), CD45 (cat # SC-70686), CD90 (cat # SC-53456), CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat # SC-53551) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA, 100 testes in 2ml) and CD73 (cat # BD 550256) (Zymed, San Francisco, CA, USA) at 1:100 dilution. After cells were washed with PBS (Invitrogen, Gibco, UK, cat # 10010, pH 7,4) in order to clear away the excess primary antibodies, cells were incubated with the

fluorescein-iso-thio-cyanate (FITC)-conjugated chicken antimouse secondary antibodies (cat # SC-2989, 200µg/0.5ml) at 4^{o}C for one hour at dark, except for the CD29 which phyco-erythrin (PE)–red light-harvesting protein containing chromophore–conjugated monoclonal antibody. The flow cytometry analysis of the cells were achieved using Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA, USA, model no: 342975) flow cytometry system. 10000 cells were counted for each sample.

2.3. PREPARATION OF CONDITIONED MEDIUM (CM) OF hTGSCs

hTGSCs under the passage #5 (<#5) were in T-75 flasks with growth medium (DMEM). When they reached 70% confluency, the medium was changed to serum free medium follwed by collection of, conditioned medium (CM) after 12-24 hours. Then CM was, filtered using 0.2 µm filter (Sartorius, minisart, Germany, cat # 16534) in order to prevent any cell debris, and divided into 2 ml aliquots in 2 ml sterile plastic tubes, which were stored at -20^{o}C until use [45].

2.4. CULTURE OF SH-SY5Y CELLS

SH-SY5Y, human derived neuro-blastoma cell line was used in our experiments as *in vitro* model of neurons because of its neuronal origin as previousy reported. SH-SY5Y culture was maintained in DMEM containing 10% (v/v) FBS and 1% (v/v) PSA solution. Cells were grown in T-75 flasks with 15 ml medium at 37ºC in 5% CO_2 incubator until they reach to the confluency. They were usually passaged every 2-3 days.

2.5. PREPARATION OF PRIMARY MIX BRAIN CULTURE

Before the isolation, selected culture dishes (96 well plate and 48 well plate) suitable for the experiment were coated with poly L-lysine which was the most common method for enhancing the adhesion of neurons. It was prepared according to the protocol described by He and Baas [137]. First of all, borate buffer was prepared. 190 mg borax (Sigma, USA cat # 221732) and 124 mg boric acid (Bio Basic Inc Canada cat # BB0044) were dissolved into 40 ml dH_2O. In order prepare 200 µg/ml stock solution, 5 mg poly-L-lysine (Sigma, USA cat # P6282) dissolved in 25 ml borate buffer. After that poly-L-lysine solution was

filtered using a 0.2 μm filter and stored in 4°C at dark until use. One day before the cell isolation, 96 (BIOFIL, TCP, Switzerland, cat # 011096) and 48 well plates (BIOFIL, TCP, Switzerland, cat # 011048) were coated with the poly-L-lysine solution by placing 55 μl and 100 μl of poly-L-lysine into each well respectively and incubated at 4°C in dark. On the day of cell isolation, the wells were rinsed with sterile dH_2O five times for 5 minutes and left for air dry in hood until completely dried.

Before the isolation, forceps, scalpel handles and scissors were sterilized by using 70% ethanol and fire. Day 9, five Spraque-Dawley Rats were taken from Yeditepe University Experimental Research Center (YUDETAM) (Ethical approval was obtained for animal use in the experimental research from Research Ethics Committee, Yeditepe University, Istanbul, Turkey in 2010). Rats were decapitated by using sterile blade and the heads of the animals were disected to obtain their brains. Extracted brains were put into sterile Hank's Buffered Salt Solution (HBSS) (Invitrogen, Gibco, UK, cat # 24020-091). Then, brains were transferred into new dish containing Neurobasal Medium (Invitrogen, Gibco, UK, cat # 21103-049) supplemented with 2% (v/v) B-27 (Invitrogen, Gibco, UK, cat # 17504-044) and 1% (v/v) PSA. Brains were minced into small pieces with a sterile scapel. Figure 2.1 shows the isolation steps until this stage. In order to dice the brain pieces into smaller size, 10 ml syringe was used. Afterward all brain pieces were put into 15 ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes. After centrifugation cell pellet was suspended in neurobasal medium supplemented with 2% (v/v) B-27 and 1% (v/v) PSA and seeded into poly-L-lysine coated 96 well and 48 well plates. Cells were expanded in an incubator at 37°C with 5% CO_2 and 95% humidity. The following day their medium were changed with fresh medium.

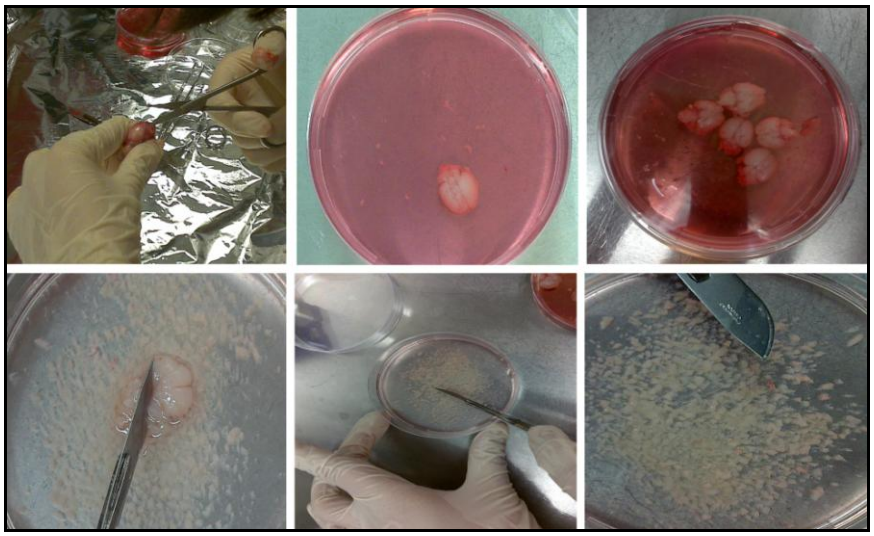


Figure 2.1. Preparation of primary mix brain culture

2.6. PREPARATION OF PLURONICS

Pluronic F68 (BASF, USA, cat # 52389638) and F127 (BASF, USA, cat # 55401892) were dissolved in PBS at 0.1 M concentration (stock solution) by vortexing. 0.1 M stock solutions were diluted to 80 μM, which is the concentration of Pluronics used in the experiments both for Pluronic F68 and F127, in PBS and sterilized with 0.2 μm filter (Sartorius, minisart, Germany, cat # 16534). Solutions were kept at 4ºC until use.

2.7. DETECTION OF NONTOXIC CONCENTRATION OF PLURONICS VIA CELL VIABILITY ASSAY

Five concentrations (20 μM, 40 μM, 60 μM, 80 μM, 100 μM) of Pluronic F68 and F127 were prepared from stock solutions (0.1 M) prepared in PBS. SH-SY5Y cells were seeded into 96 well plates (BIOFIL, TCP, Switzerland, cat # 011096) at a concentration of 5000 cells/well and treated with five concentrations of the two pluronics. Cells were incubated with pluronics for 24 hours. The cell viability was measured by the MTS assay (CellTiter96 Aqueous One Solution, Promega, UK, cat # 2587530) according to the manufacturer's instructions. MTS (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2- (4-sulfo-phenyl)-2H-tetrazolium) is a tetrazolium-salt based colori-metric assay for detecting the activity of enzymes (mostly in the mitochondria) that

reduce MTS to formazan, giving a purple color [138]. In short, after 24 hours incubation of cells together with Pluronic F68 and F127 seperately, 10 µl MTS reagent with 100 µl growth medium was added to each well and incubated for two to three hours followed by absorbance measurement at 490 nm using an ELISA (Biotek, model no: EL800) plate reader.

2.8. DETECTION OF TOXIC CONCENTRATION OF KETAMINE VIA CELL VIABILITY ASSAY

Five concentrations (100 µM, 200 µM, 300 µM, 400 µM, 500 µM) of Ketamine (Richter Pharma AG, Austria, serial number 1208456) were prepared from stock solution. Dilutions were prepared in serum free medium (DMEM) containing 1% (v/v) PSA. SH-SY5Y cells were seeded into 96 well plates at a concentration of 5000 cells/well and five concentrations of Ketamine were added on the cells. Cells were incubated with pluronics for 24 hours. The cell viability was measured by the MTS assay according to the manufacturer's instructions. In short, after 24 hours incubation of cells together with Ketamine, 10 µl MTS reagent with 100 µl growth medium was added to each well and they were incubated for two to three hours followed by reading absorbance at 490 nm using an ELISA (Biotek, model no: EL800) plate reader.

2.9. NEUROPROTECTION ASSAYS

2.9.1. Assay for The Detection of The Effect of CM Combined Pluronic F68 and F127

SH-SY5Y and primary mix brain culture cells were seeded according to experiment (Cell viability assay, RNA isolation, fatty acid profiling assay etc.) into multiwell culture plates containing DMEM supplemented with 10% (v/v) FBS and 1% (v/v) PSA, and Neurobasal medium supplemented with 2% (v/v) B-27 and 1% (v/v) PSA, respectively. The next day both of SH-SY5Y and primary mix brain culture cells were exposed to different types of solutions for 24 hours. There were seven different groups stated in Table 2.1. All solutions were prepaired in 1% (v/v) PSA supplemented serum free medium (DMEM) for SH-SY5Y and 2% (v/v) B-27, 1% (v/v) PSA supplemented Neurobasal

medium for primary mix brain culture cells. At third day, according to experiment related procedure was performed.

Table 2.1. Groups for CM combined Pluronics assays

	Groups
1	Growth medium with 200 µg/ml Ketamine and 20% CM
2	Growth medium with 200 µg/ml Ketamine, 20% CM and 80 µM Pluronic F68
3	Growth medium with 200 µg/ml Ketamine, 20% CM and 80 µM Pluronic F127
4	Growth medium with 200 µg/ml Ketamine and 80 µM Pluronic F68
5	Growth medium with 200 µg/ml Ketamine and 80 µM Pluronic F127
6	Growth medium (Negative control) (NC)
7	Growth medium with 200 µg/ml Ketamine (Positive Control) (PC)

2.9.2. Assesing The Effect of Growth Factor (GF) Combined Pluronic F68 and F127

SH-SY5Y and primary mix brain culture cells were seeded according to experimental set up (Cell viability assay, RNA isolation, etc.) into multiwell culture plates containing DMEM supplemented with 10% (v/v) FBS and 1% (v/v) PSA, and Neurobasal Medium supplemented with 2% (v/v) B-27 and 1% (v/v) PSA, respectively. The next day both of SH-SY5Y and primary mix brain culture cells were incubated with GFs (Fibroblast Growth Factor-2 [FGF-2] (Invitrogen, USA, cat #PHG0261), Insulin-Like Growth Factor-1 [IGF-1] (Invitrogen, USA, cat # 13245-063), Brain-Derived Neurotrophic Factor [BDNF] (Invitrogen, USA, cat # 10908-010), Nerve Growth Factor [NGF] (Invitrogen, USA, cat # 13257-019) and Vascular Endothelial Growth Factor [VEGF] (Sigma, USA, cat # V7259)) which were applied alone or in combination with Pluronic F68 and F127 for 24 hours. There were two assay set and each set contains seven different groups of culture media as shown in Table 2.2 and Table 2.3.

Table 2.2. Groups for GFs combined Pluronics assays (First Set)

	Groups
1	Growth medium with 200 µg/ml Ketamine, 20 ng/ml FGF-2 and 80 µM Pluronic F68/F127
2	Growth medium with 200 µg/ml Ketamine, 20 ng/ml IGF-1 and 80 µM Pluronic F68/F127
3	Growth medium with 200 µg/ml Ketamine, 20 ng/ml VEGF and 80 µM Pluronic F68/F127
4	Growth medium with 200 µg/ml Ketamine, 10 ng/ml BDNF and 80 µM Pluronic F68/F127
5	Growth medium with 200 µg/ml Ketamine, 10 ng/ml NGF and 80 µM Pluronic F68/F127
6	Growth medium (Negative control) (NC)
7	Growth medium with 200 µg/ml Ketamine (Positive Control) (PC)

Table 2.3. Groups for GFs combined Pluronics assays (Second Set)

	Groups
1	Growth medium with 200 µg/ml Ketamine, 20 ng/ml FGF-2
2	Growth medium with 200 µg/ml Ketamine, 20 ng/ml IGF-1
3	Growth medium with 200 µg/ml Ketamine, 20 ng/ml VEGF
4	Growth medium with 200 µg/ml Ketamine, 10 ng/ml BDNF
5	Growth medium with 200 µg/ml Ketamine, 10 ng/ml NGF
6	Growth medium (Negative control) (NC)
7	Growth medium with 200 µg/ml Ketamine (Positive Control) (PC)

All solutions were prepaired in 1% (v/v) PSA supplemented serum free media (DMEM) for SH-SY5Y and 2% (v/v) B-27, 1% (v/v) PSA supplemented Neurobasal medium for the primary mix brain culture cells. At third day, according to experiment the related procedure was performed.

2.10. IMMUNOCYTOCHEMISTRY ANALYSIS

SH-SY5Y and 10 day rat brain mix culture cells were fixed with 2% (w/v) paraformaldehyde (Sigma, US cat # 158127) by incubating for 30 mins at 4ºC. The cells were rinsed three times for 5 minutes with PBS by gentle shaking on a plate shaker (Heidolph, Titramax 100, Germany No: 544-11200-00-3). After washing with PBS, cells were permeabilized by incubation with 0.1% (v/v) Triton-X 100 in PBS, pH 7.4 for 5 minutes at room temperature. The cells were washed again with PBS three times for 5 minutes by gentle shaking. Thereafter cells were incubated with 2% (v/v) goat serum (Sigma, US cat #G9023) diluted in PBS placed on for 20 minutes at 4ºC in order to prevent the non-specific binding of antibodies. Every sample was rinsed with PBS three times for 5 minutes by gentle shaking. Cells were incubated with primary antibodies (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4ºC. NR-1 (p-NMDAζ1 (Ser 896)) (Santa-Cruz, sc-31669, 200µg/ml), beta-III tubulin (Santa-Cruz, sc-80016, 200µg/ml), and Glial Fibrillary Acidic Protein (GFAP) (Santa-Cruz, sc-9065, 200µg/ml) primary antibodies were used. When the incubation of the cells with antibodies was completed, the cells were rinsed with PBS three times for 5 minutes in order to remove unbound primary antibodies. After washing, goat anti mouse IgG Alexa Fluor 488 (Invitrogen, USA, cat #A11001) for β3 tubulin, goat anti rabbit IgG Alexa Fluor 488 (Invitrogen, USA, cat # A11008) for NR-1 and GFAP secondary antibodies were added to cells and incubated for 1 hour at 4 ºC at dark. After washing the cells with PBS three times for 5 minutes, DAPI (4',6-diamidino-2-phenylindole) (Applichem, Germany, cat #A40990010) was used in order to stain nuclei of the cells and for this aim cells were incubating for 20 minutes at 4ºC in dark. When the incubation of the cells with DAPI was completed, the cells were rinsed with PBS three times for 5 minutes. The stained cells were obseved under the fluorescence microscope (Nikon Eclipse TE200, Germany, Model no: CCD1300B).

2.11. FATTY ACID ISOLATION

Fatty acid isolation was performed in order to show the effect of Pluronic combined CM on the membrane fatty acid profile. Isolation of fatty acids from cells was done according to the protocol described by our group previously [139]. SH-SY5Y cells were counted and seeded in petri dishes at a concentration of 3 million cells/dish. The next day cells were exposed to different type of growth solutions for 24 hours. There were nine different groups represented in Table 2.4.

Table 2.4. Groups for CM combined Pluronics for fatty acid profiling assay

	Groups
1	Growth medium with 200 µg/ml Ketamine and 20% CM
2	Growth medium with 200 µg/ml Ketamine, 20% CM and 80 µM Pluronic F68
3	Growth medium with 200 µg/ml Ketamine, 20% CM and 80 µM Pluronic F127
4	Growth medium with 200 µg/ml Ketamine and 80 µM Pluronic F68
5	Growth medium with 200 µg/ml Ketamine and 80 µM Pluronic F127
6	Growth medium with and 80 µM Pluronic F68
7	Growth medium with and 80 µM Pluronic F127
8	Growth medium (Negative control) (NC)
9	Growth medium with 200 µg/ml Ketamine (Positive Control) (PC)

At third day, the cells were trypsinized and cell pellets were collected after centrifugation at 1000 rpm for 5 minutes. The cell pellets were transfered to the glass screw cap tubes. 1 ml of 1.2 M NaOH dissolved in 50% aqueous methanol was added to pellets and incubated at 100ºC for 30 minutes in a water bath. Samples were cooled at room temperature for 25 minutes, then treated with 2 ml of 54% (w/v) 6 N HCL prepared in 46% (v/v) aqueous methanol and incubated at 80ºC for 10 minutes in a water bath. After a rapid cool on ice, methylated fatty acids were extracted with 1.25 ml solution containing 50% (v/v) methl-tert butyl ether (MTBE) in hexane leading to formation of a bottom and top phases in the tube. The bottom phase was removed with a pasteur pipette. The top phase was mixed with 3 ml of 0.3 M NaOH and cleaned with the anhydrous sodium sulfate

followed by the transfer into a 1 ml clean glass vials for analysis in MIDI Fatty Acid Analysis System (Agilent Technologies, USA, model no: 6890-N)

2.12. CO-CULTURE ASSAY

hTGSCs and SH-SY5Y cells were labeled using PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling (Sigma, US cat # midi67-1KT) , according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO, USA). Briefly, cells were trypsinized and resuspended in diluent-C buffer and mixed with 2x dye working solution, consisting of diluent-C and fluorescent dye PKH67 at dark. Cell suspension was mixed immediately by gentle pipetting and incubated for 5 minutes at room temperature in dark. Labeling was terminated by the addition of FBS and after the centrifugation at 1500 rpm for 5 minutes cell pellet was suspended in 1 ml fresh medium. Cells for each cell line were counted [136]. hTGSCs and SH-SY5Y cells were seed into 24 well plates in 1:1 and 3:1 ratio, respectively (For 1:1 ratio, 28.000:28.000 cells/well) (For 1:3 ratio, 28.000:84.000 cells/well). Untreated (without Ketamine) PKH67 green fluorescent labeled SH-SY5Y cells and unlabeled SH-SY5Y cells were seeded together and used as a control group. After co-culturing labeled cells with unlabeled SH-SY5Y cells in the presence and absence of Ketamine induced stress for 24 hours, cell viability of unlabeled SH-SY5Y cells were analyzed by flow cytometry for all co-culture assays.

2.13. PRIMER DESIGNING AND RT-PCR ANALYSIS

Primers for Caspase-3 and NR-1 genes were designed using Primer BLAST online software of The National Center for Biotechnology (NCBI) and ordered from Invitrogen company to be synthesized at 50 nmoles. Table 2.5 shows primers used in this study.

Table 2.5. Primers used in this study

Primer	**Forward (5'-3')**	**Reverse (5'-3')**	**Product Length**	**Ref.**
NR-1	AGCGGGTGAACAACAGCAAC	GGGAATCTCCTTCTTGACCAGAATAG	179 bp	Custom made
Casp-3	GAGGCGGTTGTAGAAGAGTTTCGTG	TGGGGGAAGAGGCAGGTGCA	177 bp	Custom made
B-actin	GCGAGAAGATGACCCAGGATC	CCAGTGGTACGGCCAGAGG	141bp	[31]

Total RNAs were isolated from SH-SY5Y cell using High Pure RNA isolation kit (ROCHE, USA, cat # 11828665001) according to the manufacturer's instructions. cDNA was synthesized using cDNA High Fidelity cDNA synthesis kit (ROCHE, USA, cat # 05081955001). Detection of gene levels was performed using SYBRgreen real time PCR method. cDNAs of samples were mixed with primers and SYBR Premix Ex Taq (TAKARA, Japan, cat# RR041) in a final volume of 20 µl. Table 2.6 shows RT-PCR reagents and their amounts for the study. β-actin gene was used as the reference house-keeping gene for normalization of the data. All RT-PCR experiments were done using iCycler RT-PCR (Bio-Rad, Hercules, CA, USA, icycler iQ Optical Module) detection system. PCR conditions were shown in Table 2.7.

Table 2.6. PCR reagents used in this study

Reagents	**Volume**
SYBER Green	10 µl
Primer Forward (10 µM)	0,4 µl
Primer Reverse (10 µM)	0,4 µl
Template	5 µl
Distilled Water	4,2 µl

Table 2.7. PCR conditions

Cycle	**Repeats**	**Step**	**Dwell Time**	**Set Point**
1	1	1	3 min	93 ^{o}C
2	40	1	30 sec	93 ^{o}C
-	-	2	40 sec	60 ^{o}C
-	-	3	45 sec	72 ^{o}C
3	1	1	10 min	72 ^{o}C
4	110	1	12 sec	40 ^{o}C
5	1	1	-	4 ^{o}C

2.14. STATISTICAL ANALYSIS

In order to draw the graphichs Microsoft Office Excel and analyze the data GraphPad Prism5 (Version 5.03) softwares were used. For statistical analysis, one way ANOVA followed by Dunnett's Multiple Comparison Test was used. *P* values <0.05 are considered statistically significant.

3. RESULTS

3.1. CHARACTERIZATION OF HUMAN TOOTH GERM STEM CELLS

hTGSCs were characterized with the surface antigens, CD29, CD34, CD45, CD73, CD90, CD105, CD133 and CD166 by using flow cytometry. Flow cytometry analysis showed that cells express mesenchymal stem cell markers CD29, CD73, CD90, CD105 and CD166 but not express hematopoietic markers such as CD34, CD45 and CD133. Results were shown in Figure 3.1. This data demonstrated that hTGSCs used this study possess mesenchymal stem cell properties.

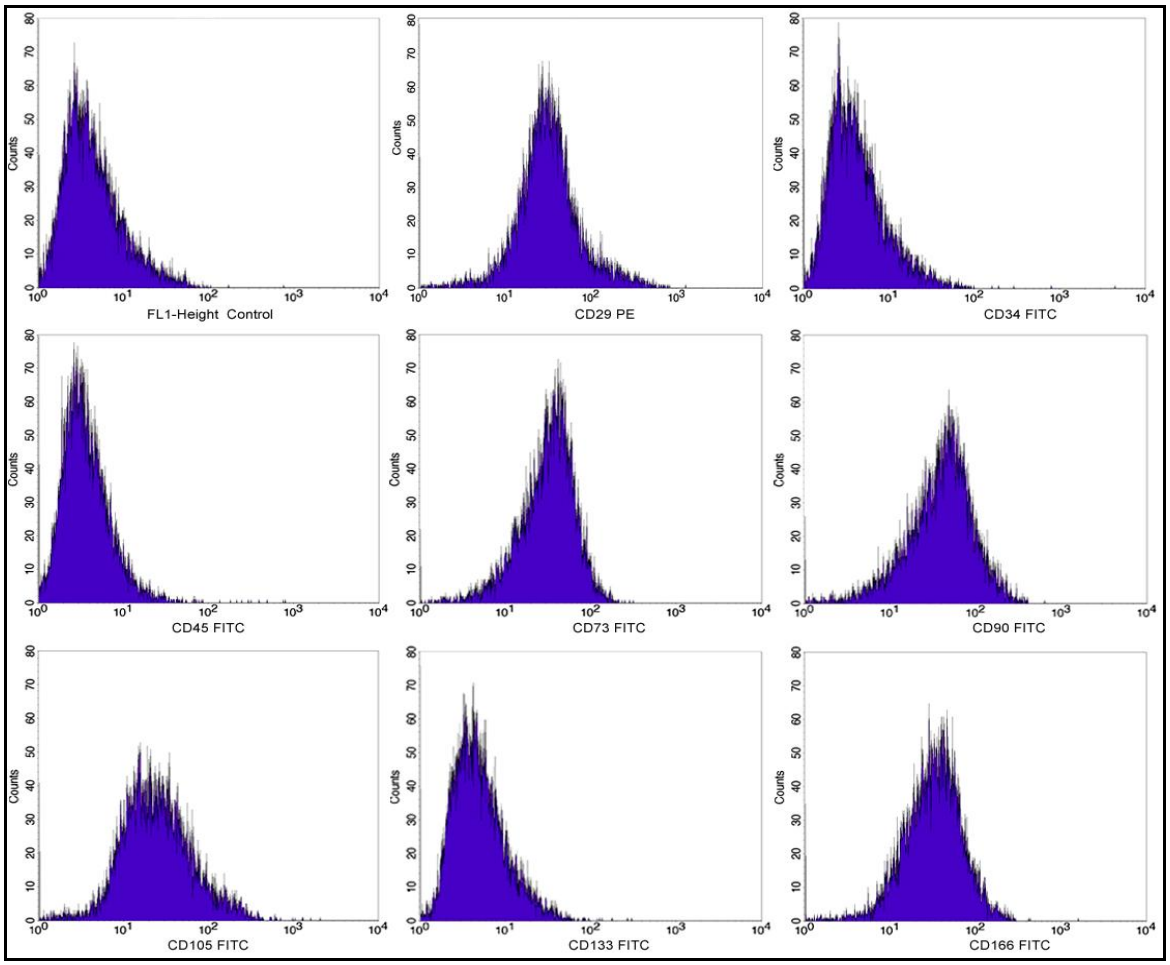


Figure 3.1. Flow cytometry analysis of hTGSCs

3.2. IMMUNOCHEMICAL ANALYSIS OF PRIMARY MIX BRAIN CULTURE CELLS

The following day of the isolation, the presence of neurons was observed under the light microscope. Figure 3.2 shows the light microscope images of neurons.

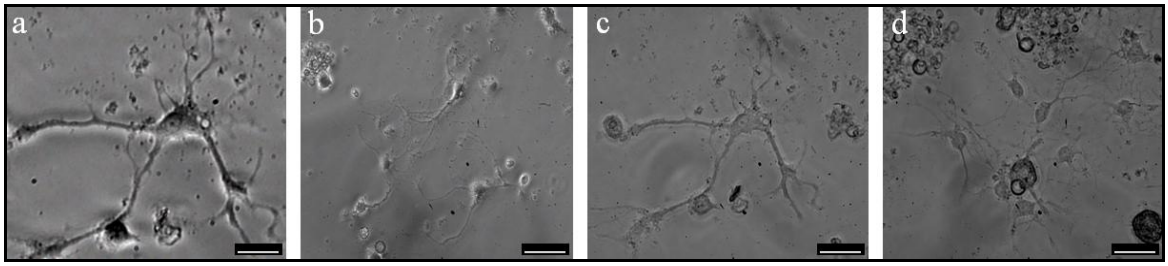


Figure 3.2. Light microscope images of neurons. a and b are 20x images (scale bar, 100 μm) and c and d are 40x images (scale bar, 50 μm)

The immunofluorescent staining of β3 Tubulin, a microtubule protein specific to neurons, was used in order to show presence of neurons in the culture. Furthermore GFAP a glial marker, indicates the existence of astrocytes. Figure 3.3.a and Figure 3.3.b illustrates the neuron-specific staining of cultured cells with β3 Tubulin and GFAP-labeled astrocytes, respectively.

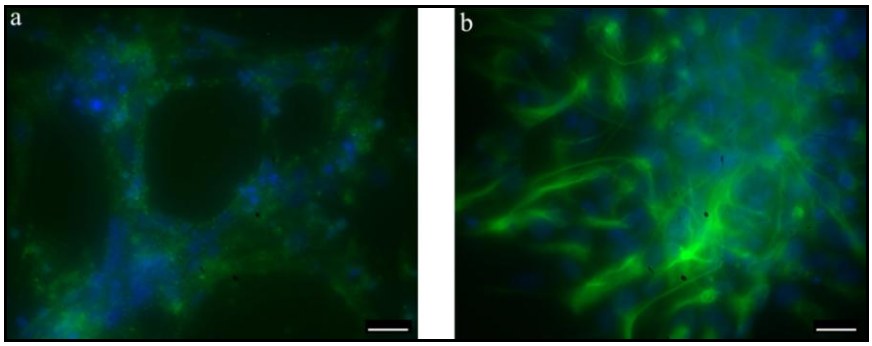


Figure 3.3. Cells were stained positive for β3 Tubulin (a) and GFAP (b) (20x) (scale bar, 100 μm)

3.3. DETECTION OF NONTOXIC CONCENTRATION OF PLURONICS

Based on the literature, toxicity assay was performed on SH-SY5Y cell line treated with five concentrations of two pluronics for 24 hours. The cell viability was measured using MTS test. The results showed that none of the pluronics were toxic for the cells. These are indicated in Figure 3.4 and Figure 3.5. In the presence of 80 µM Pluronic F68 and F127, the best cell viability was determined as 127% and 124%, respectively when compared to negative control which contains only growth medium (DMEM). Taken together out data indicated that for both Pluronic F68 and F127, 80 µM was detected as a suitable concentration for the study.

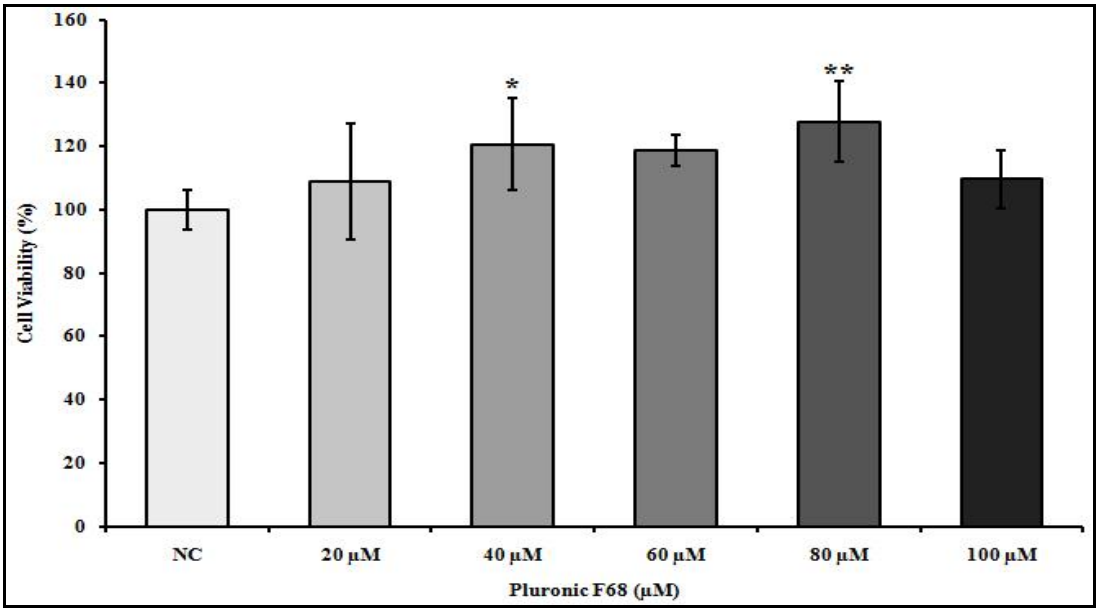


Figure 3.4. Toxicity results of Pluronic F68 for 24 hours (NC: Negative Control) (*P<0.05/**P<0.01 compared with negative control)

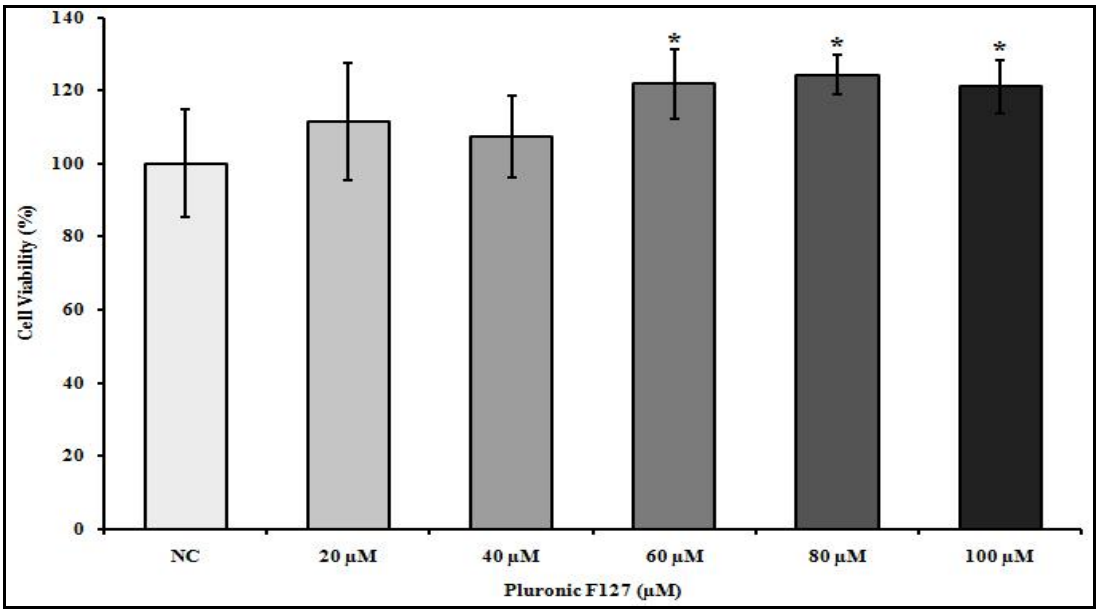


Figure 3.5. Toxicity results of Pluronic F127 for 24 hours (NC: Negative Control) (*P<0.05 compared with negative control)

3.4. DETECTION OF TOXIC CONCENTRATION OF KETAMINE

Based on the literature, toxicity assay was performed on SH-SY5Y cell line treated with five concentrations of Ketamine for 24 hours. The cell viability was measured by using MTS test. The results showed that all concentrations of Ketamine tested were toxic for the cells. Figure 3.6 demonstrates the results. In the presence of 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500 µg/ml Ketamine, the cell viability reduced by 15%, 22%, 17%, 24%, 30%, respectively when compared to negative control which contains only growth medium (DMEM). 200 µg/ml was detected as a suitable concentration for the study.

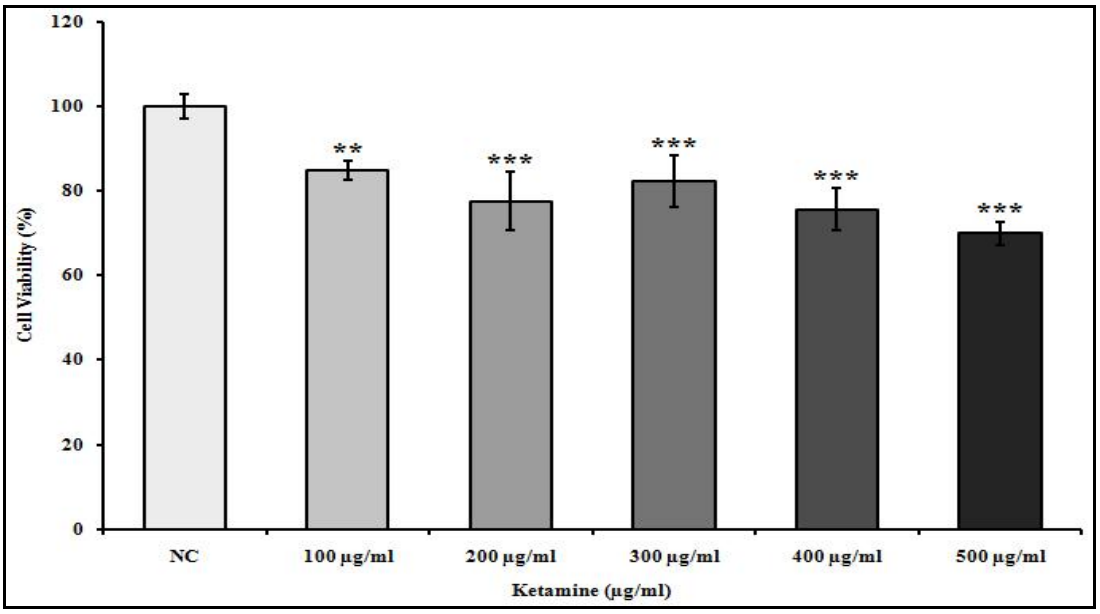


Figure 3.6. Toxicity results of Ketamine for 24 hours (NC: Negative Control) (**P<0.01/***P<0.001 compared with negative control)

3.5. EFFECT OF CM COMBINED PLURONICS ON SH-SY5Y CELL LINE AGAINST KETAMINE INDUCED NEUROTOXICITY

After incubation of cells exposed to different types of solutions for 24 hours, cell viability was measured by using MTS test. First experiment group of CM combined Pluronics was related to effect of Pluronic F68. In the presence of growth medium with CM; CM and Pluronic F68; Pluronic F68, the cell viability improved under the stress condition induced by Ketamine by 8%, 23%, 7%, respectively when compared to Ketamine treated cells (positive control). Second experiment group of CM combined Pluronics was related to effect of Pluronic F127. In the presence of growth medium with CM; CM and Pluronic F127 and Pluronic F127, the cell viability improved under the stress condition induced by Ketamine by 9%, 30%, 7%, respectively when compared to positive control. The results showed that CM combined Pluronic F68 and F127 increased the efficiency of CM and reduced the Ketamine induced neurotoxicity. Results were shown in Figure 3.7 and Figure 3.8.

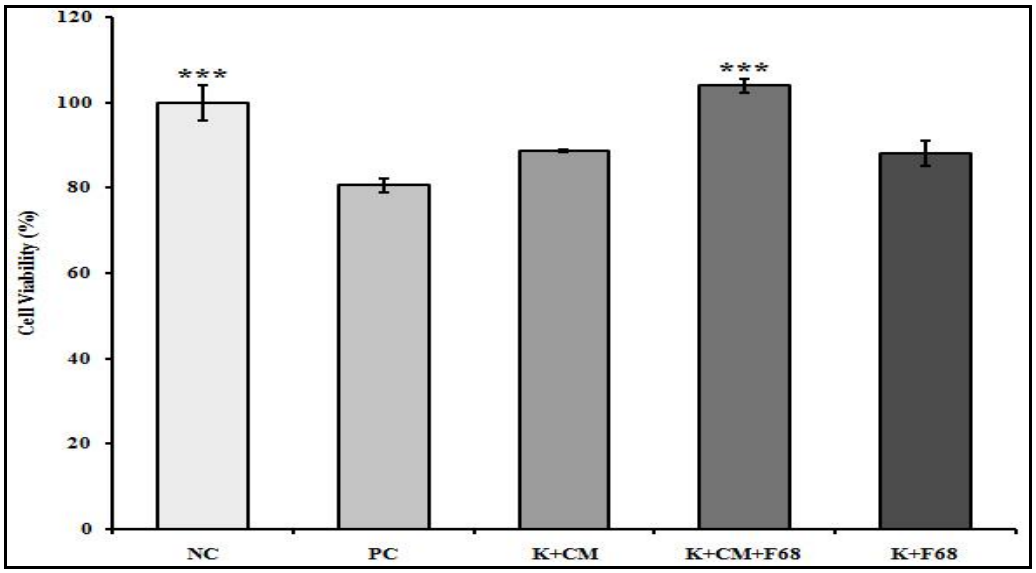


Figure 3.7. Effect of CM combined Pluronic F68 on SH-SY5Y cell line against Ketamine induced neurotoxicity (K: Ketamine, CM: Conditioned Medium, F68: Pluronic F68, NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, K+CM: Growth medium with CM and Ketamine, K+CM+F68: Growth medium with CM, Pluronic F68 and Ketamine, K+F68: Growth medium with Pluronic F68 and Ketamine) (***P<0.001 compared with positive control)

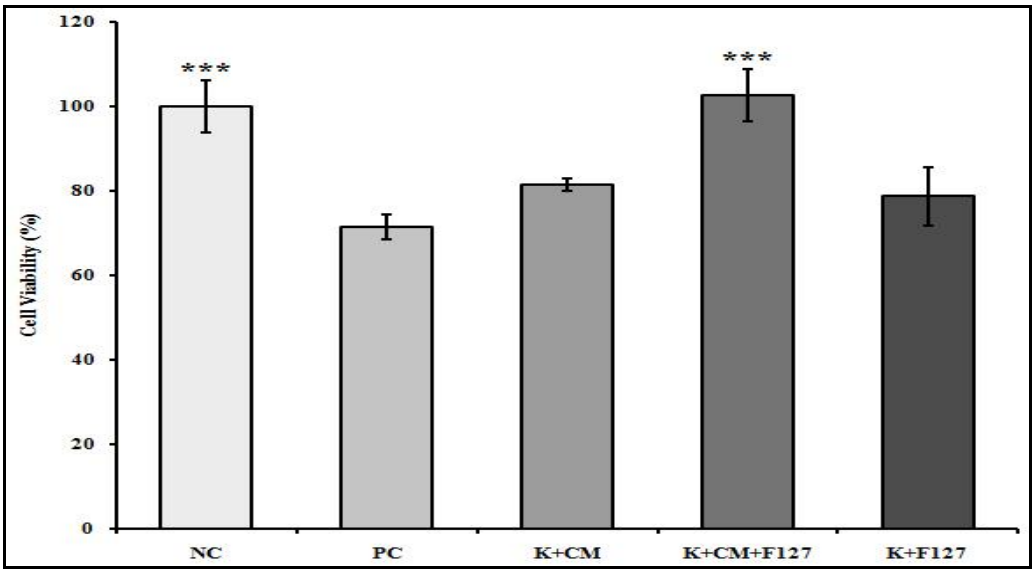


Figure 3.8. Effect of CM combined Pluronic F127 on SH-SY5Y cell line against Ketamine induced neurotoxicity (K: Ketamine, CM: Conditioned Medium, F127: Pluronic F127, NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, K+CM: Growth medium with CM and Ketamine, K+CM+F127: Growth medium with CM, Pluronic F127 and Ketamine, K+F127: Growth medium with Pluronic F127 and Ketamine) (***P<0.001 compared with positive control)

3.6. EFFECT OF CM COMBINED PLURONICS ON 9 DAY RAT PRIMARY MIX BRAIN CULTURE AGAINST KETAMINE INDUCED NEUROTOXICITY

After the treatment of cells with different types of solutions for 24 hours, cell viability was measured using the MTS test. In the presence of growth medium with CM; CM and Pluronic F68; CM and Pluronic F127; Pluronic F68 and Pluronic F127, the cell viability improved under the stress condition induced by Ketamine by 22%, 27%, 28%, 8%, 5%, respectively when compared to Ketamine treated cells (positive control). The results showed that CM combined Pluronic F68 and F127 increased the efficiency of CM and reduced the Ketamine induced neurotoxicity. Results were shown in Figure 3.9.

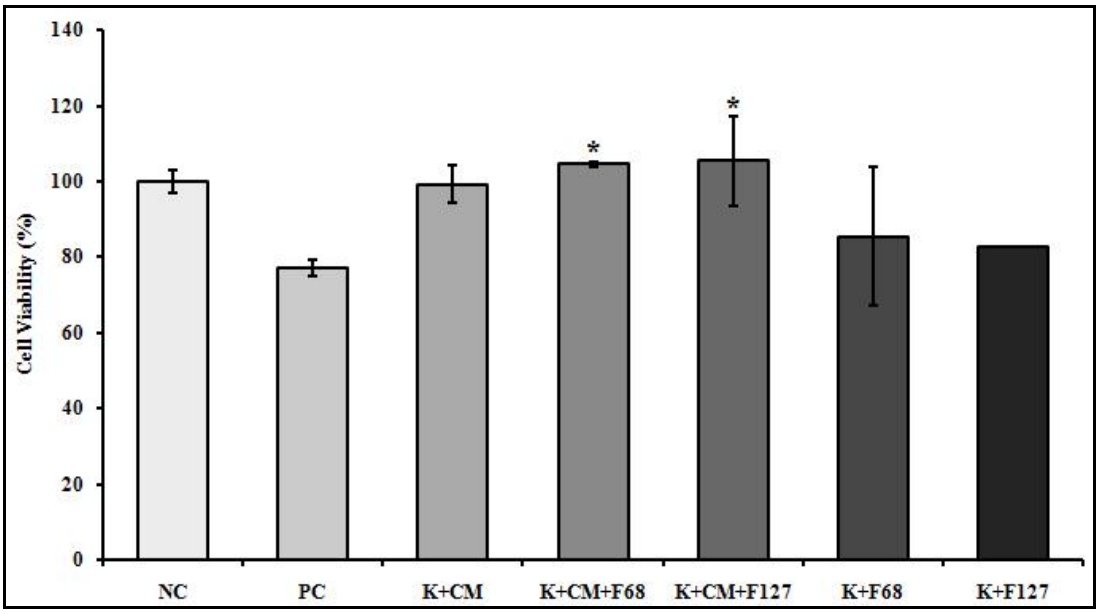


Figure 3.9. Effect of CM combined Pluronic on 9 day rat primary mix brain culture against Ketamine induced neurotoxicity (K: Ketamine, CM: Conditioned Medium, F68: Pluronic 68, F127: Pluronic F127, NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, K+CM: Growth medium with CM and Ketamine, K+CM+F68: Growth medium with CM, Pluronic F68 and Ketamine, K+CM+F127: Growth medium with CM, Pluronic F127 and Ketamine, K+F68: Growth medium with Pluronic F68 and Ketamine, K+F127: Growth medium with Pluronic F127 and Ketamine) (*P<0.05 compared with positive control)

3.7. EFFECT OF GFs COMBINED PLURONICS ON SH-SY5Y CELL LINE AGAINST KETAMINE INDUCED NEUROTOXICITY

After incubation of cells with different types of solutions for 24 hours, cell viability was measured by using MTS test. In the presence of growth medium and Pluronic F68 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], Brain-Derived Neurotrophic Factor [BDNF] and Nerve Growth Factor [NGF], the cell viability improved under the stress condition induced by Ketamine by 22%, 19%, 17%, 15%, 15%, respectively when compared to Ketamine treated cells (positive control). In the presence of growth medium without Pluronic F68 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], Brain-Derived Neurotrophic Factor

[BDNF] and Nerve Growth Factor [NGF], the cell viability increased under the stress condition induced by Ketamine by 15%, 3%, 12%, 8%, 6%, respectively when compared to Ketamine treated cells (positive control). Results showed that Pluronic F68 combined GFs increased the efficiency of FGF-2, IGF-1, VEGF, BDNF and NGF when it was compared with effect of GFs against Ketamine induced neurotoxicity. Results were shown in Figure 3.10 and Figure 3.11.

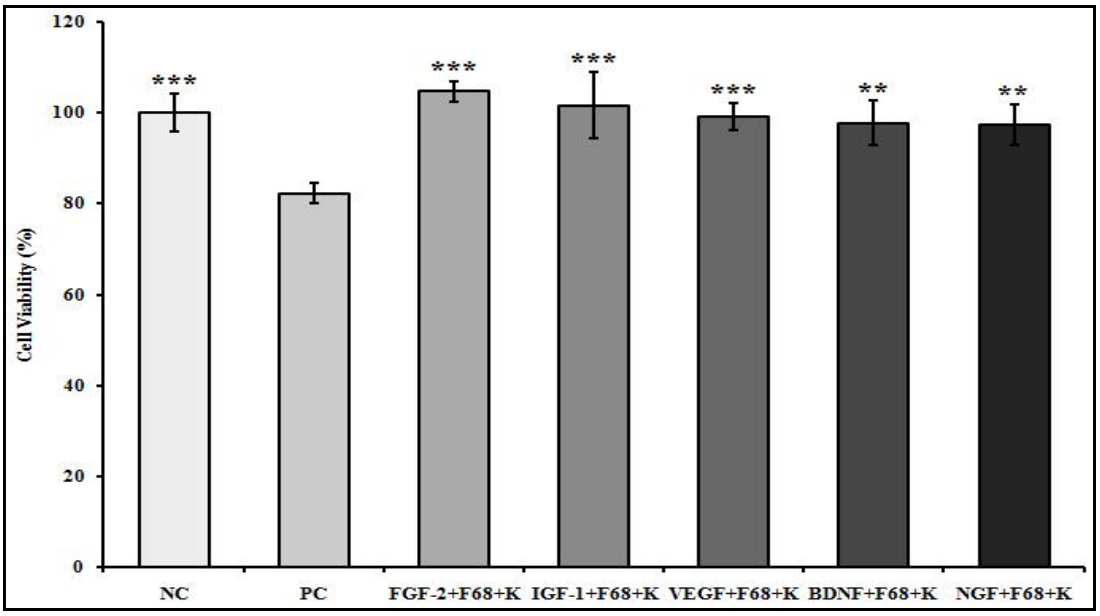


Figure 3.10. Effect of GFs combined Pluronic F68 on SH-SY5Y cell line against Ketamine induced neurotoxicity (K: Ketamine, F68: Pluronic 68, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+F68+K: Growth medium with FGF/IGF/VEGF/BDNF/NGF, F68 and Ketamine)
(**P<0.01/***P<0.001 compared with positive control)

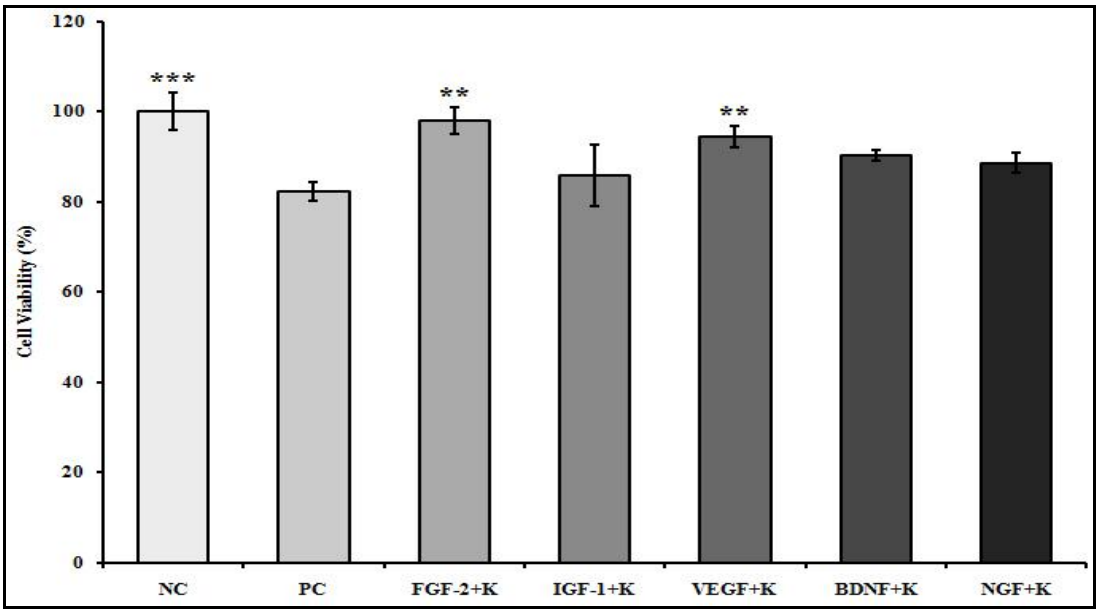


Figure 3.11. Effect of GFs on SH-SY5Y cell line against Ketamine induced neurotoxicity (K: Ketamine, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+ K: Growth medium with FGF/IGF/VEGF/BDNF/NGF and Ketamine) (**P<0.01/***P<0.001 compared with positive control)

In the presence of growth medium and Pluronic F127 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], Brain-Derived Neurotrophic Factor [BDNF] and Nerve Growth Factor [NGF], the cell viability improved under the stress condition induced by Ketamine by 14%, 18%, 34%, 25%, 29%, respectively when compared to Ketamine treated cells (positive control). In the presence of growth medium without Pluronic F127 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], Brain-Derived Neurotrophic Factor [BDNF] and Nerve Growth Factor [NGF], the cell viability increased under the stress condition induced by Ketamine by 47%, 13%, 10%, 23%, 5%, respectively when compared to Ketamine treated cells (positive control). Furthermore, Pluronic F127 combined GFs increased the efficiency of IGF-1, VEGF, BDNF and NGF when it was compared with the effect of GFs

against the Ketamine induced neurotoxicity. Results were shown in Figure 3.12 and Figure 3.13.

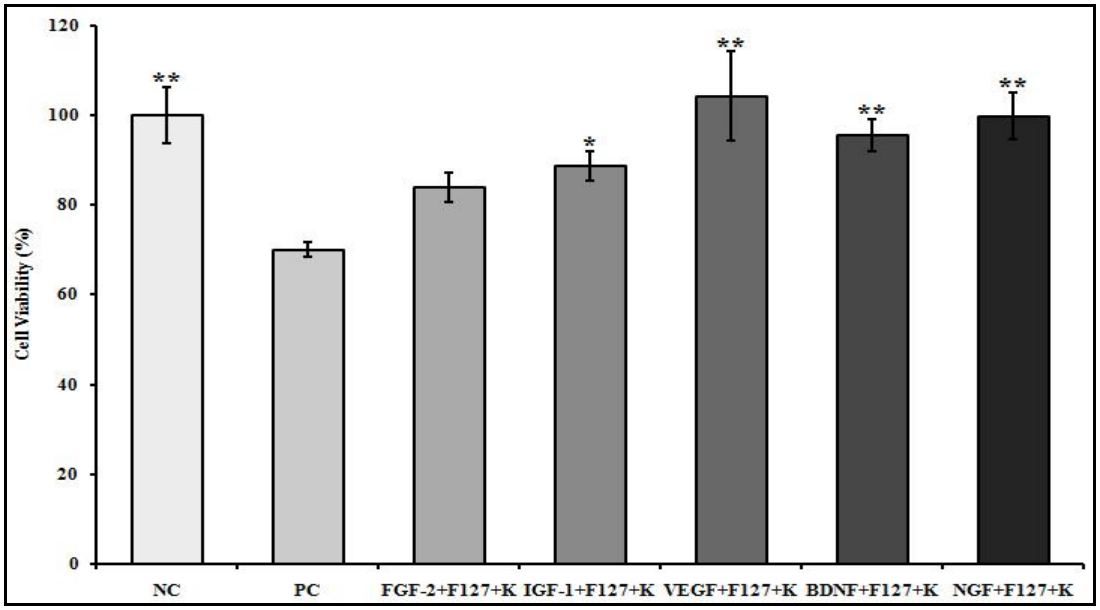


Figure 3.12. Effect of GFs combined Pluronic F127 on SH-SY5Y cell line against Ketamine induced neurotoxicity (K: Ketamine, F127: Pluronic 127, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+F127+K: Growth medium with FGF/IGF/VEGF/BDNF/NGF, F127 and Ketamine)
(*P<0.05/**P<0.01 compared with positive control)

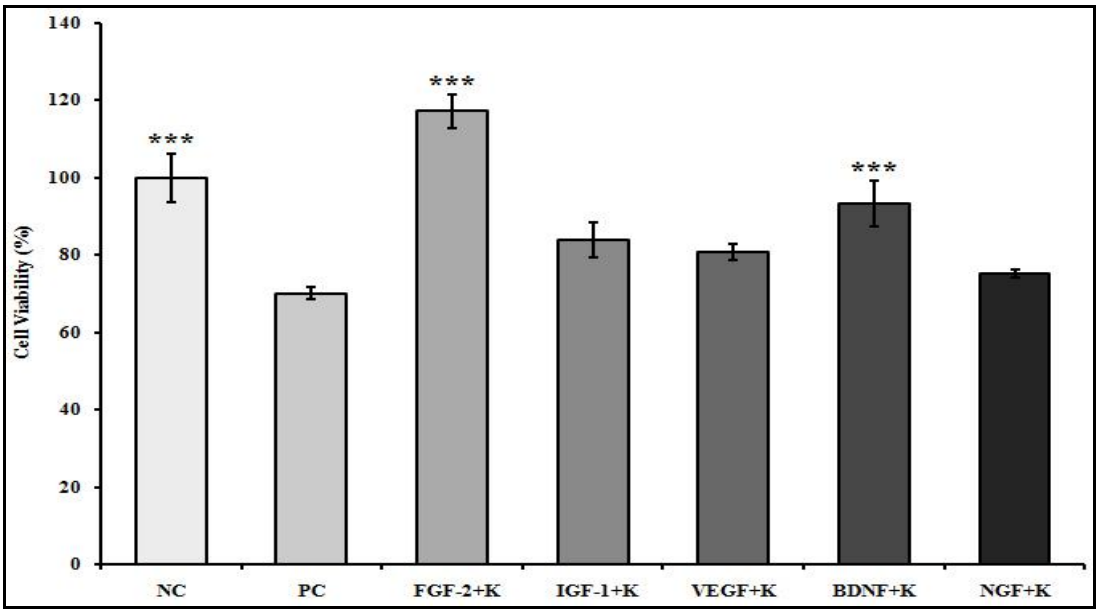


Figure 3.13. Effect of GFs on SH-SY5Y cell line against Ketamine induced neurotoxicity (K: Ketamine, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+ K: Growth medium with FGF/IGF/VEGF/BDNF/NGF and Ketamine) (***P<0.001 compared with positive control)

3.8. EFFECT OF GFs COMBINED PLURONICS ON 9 DAY RAT PRIMARY MIX BRAIN CULTURE AGAINST KETAMINE INDUCED NEUROTOXICITY

After incubation of cells with different types of solutions for 24 hours, cell viability was measured by using MTS test. In the presence of growth medium and Pluronic F68 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], Brain-Derived Neurotrophic Factor [BDNF] and Nerve Growth Factor [NGF], the cell viability improved under the stress condition induced by Ketamine by 10%, 35%, 25%, 23%, 16%, respectively when compared to Ketamine treated cells (positive control). In the presence of growth medium without Pluronic F68 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], Brain-Derived Neurotrophic Factor [BDNF] and Nerve Growth Factor [NGF], the cell viability increased under the stress condition induced

by Ketamine by 13%, 17%, 0.7% 2%, 2%, respectively when compared to Ketamine treated cells (positive control). Results showed that Pluronic F68 combined GFs increased the efficiency of IGF-1, VEGF, BDNF, NGF when it was compared with effect of GFs against the Ketamine induced neurotoxicity. Results were shown in Figure 3.14 and Figure 3.15.

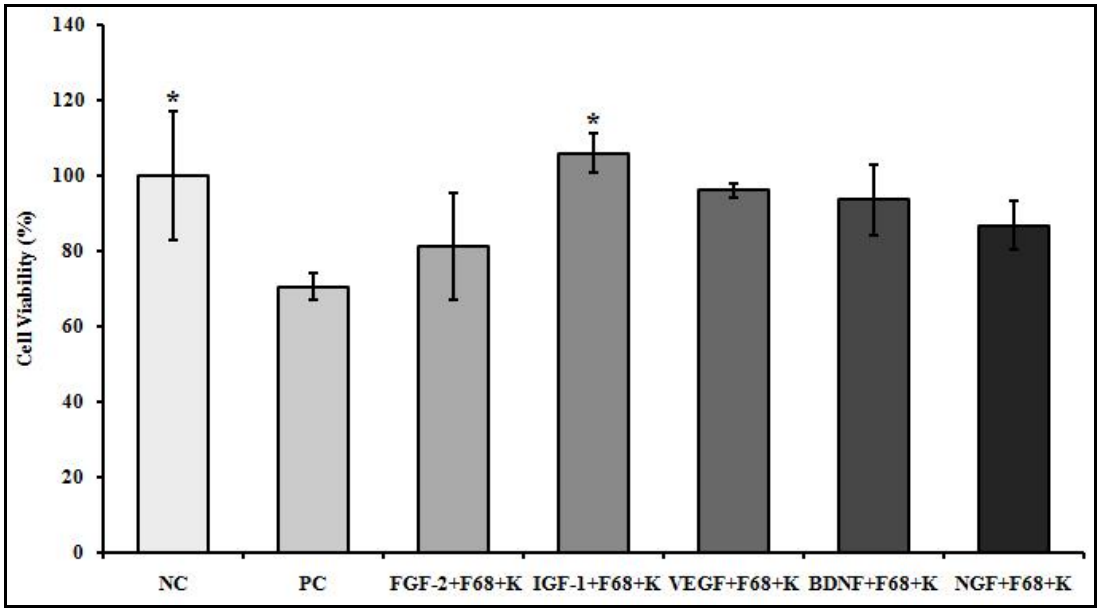


Figure 3.14. Effect of GFs combined Pluronic F68 on 9 day rat primary mix brain culture against Ketamine induced neurotoxicity (K: Ketamine, F68: Pluronic 68, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+F68+K: Growth medium with FGF/IGF/VEGF/BDNF/NGF, F68 and Ketamine)
(*P<0.05 compared with positive control)

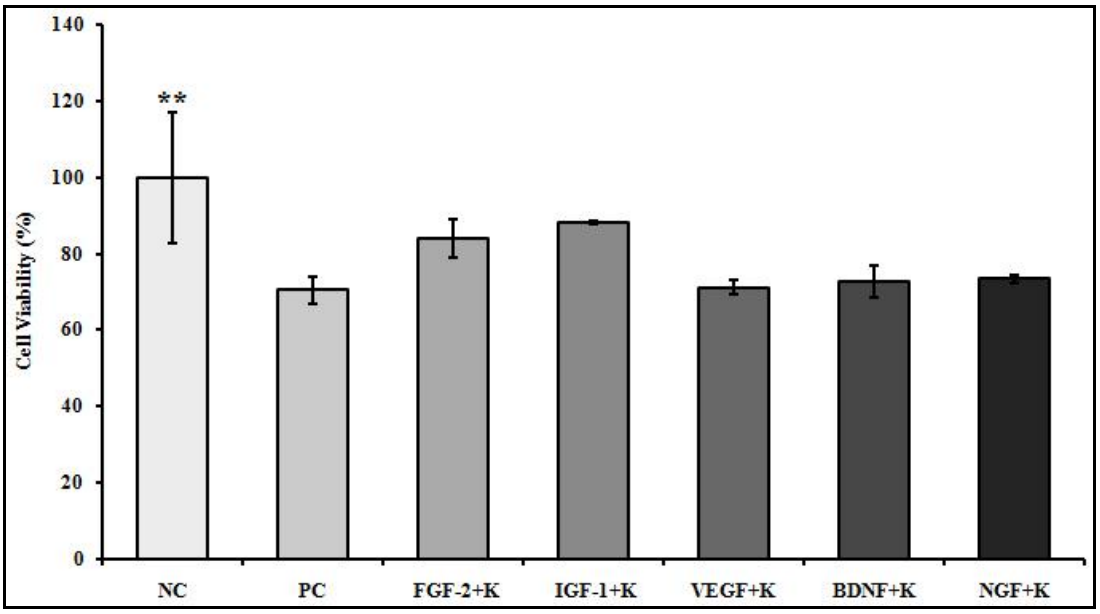


Figure 3.15. Effect of GFs on 9 day rat primary mix brain culture against Ketamine induced neurotoxicity (K: Ketamine, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+ K: Growth medium with FGF/IGF/VEGF/BDNF/NGF and Ketamine) (**P<0.01 compared with positive control)

In the presence of growth medium and Pluronic F127 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Vascular Endothelial Growth Factor [VEGF], and Nerve Growth Factor [NGF], the cell viability improved under the stress condition induced by Ketamine by 1%, 26%, 26%, 2%, respectively when compared to Ketamine treated cells (positive control). In the presence of growth medium without Pluronic F127 with Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1] and Nerve Growth Factor [NGF], the cell viability increase under the stress condition induced by Ketamine by 11%, 9%, 2%, respectively when compared to Ketamine treated cells (positive control). Furthermore, Pluronic F127 combined GFs increased the efficiency of IGF-1, VEGF, BDNF and NGF when it was compared with the effect of GFs against the Ketamine induced neurotoxicity. Results were shown in Figure 3.16 and Figure 3.17.

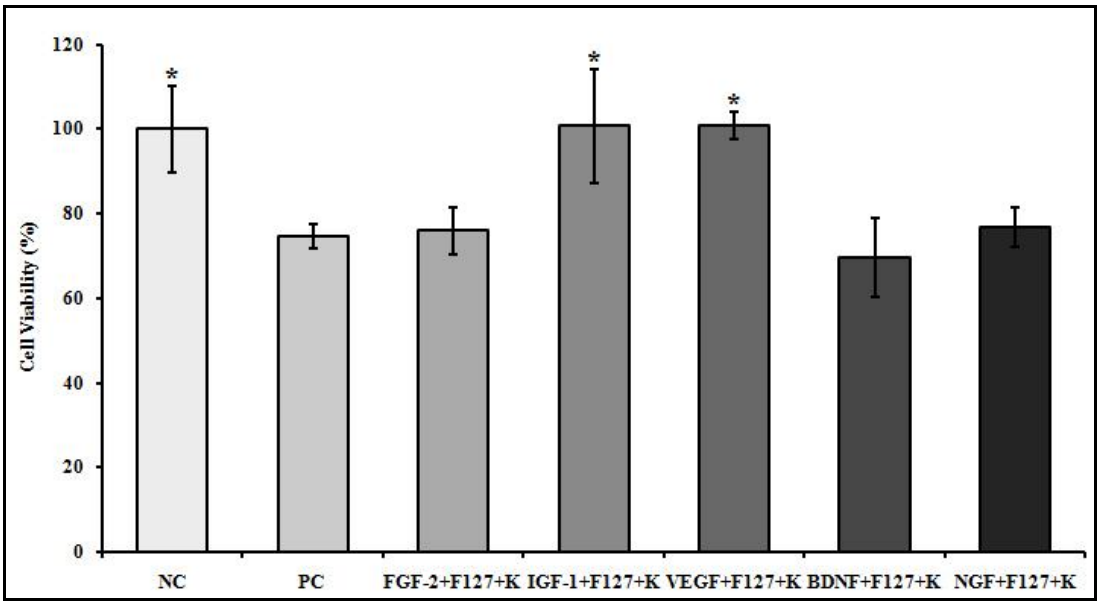


Figure 3.16. Effect of GFs combined Pluronic F127 on 9 day rat primary mix brain culture against Ketamine induced neurotoxicity (K: Ketamine, F127: Pluronic 127, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+F127+K: Growth medium with FGF/IGF/VEGF/BDNF/NGF, F127 and Ketamine)
(*P<0.05 compared with positive control)

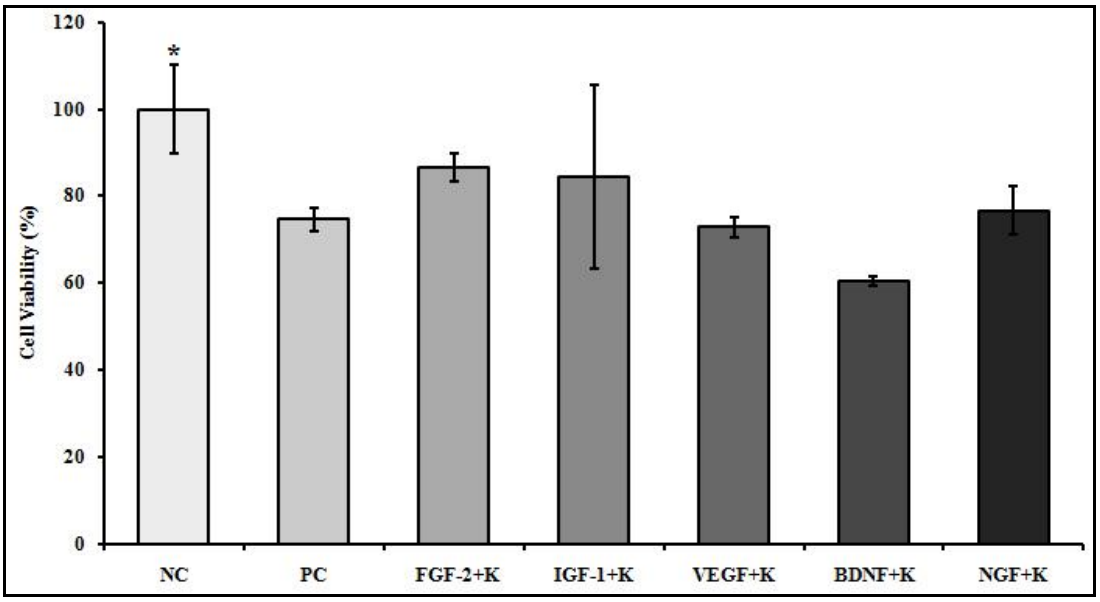


Figure 3.17. Effect of GFs on 9 day rat primary mix brain culture against Ketamine induced neurotoxicity (K: Ketamine, Fibroblast Growth Factor-2 [FGF-2], Insulin-Like Growth Factor-1 [IGF-1], Brain-Derived Neurotrophic Factor [BDNF], Nerve Growth Factor [NGF], and Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/IGF/VEGF/BDNF/NGF+ K: Growth medium with FGF/IGF/VEGF/BDNF/NGF and Ketamine) (*P<0.05 compared with positive control)

3.9. FATTY ACID PROFILING

In order to determine the effect of CM combined Pluronics on membrane structure, fatty acid profiling assay was done. After incubation of SH-SY5Y cells with the different types of solutions for 24 hours, they were observed under light microscope. Figure 3.18 shows the light microscope images of cells exposed Ketamine for 24 hours. As it is seen in the Figure 3.18 cell viability of Ketamine treated cells (positive control) (Figure 3.18.i) decreased remarkably when compared to negative control (Figure 3.18.h) which contains only growth medium (DMEM). Growth medium with CM (Figure 3.18.a), CM combined Pluronic F68 (Figure 3.18.b) and F127 (Figure 3.18.c) improved the cell viability after Ketamine induced stress but growth medium with Pluronic F68 (Figure 3.18.d) and F127 (Figure 3.18.e) without combined with CM are not as successful as growth medium with CM (Figure 3.18.a), CM combined Pluronics F68 (Figure 3.18.b) and F127 (Figure 3.18.c)

are in increasing cell viability. Moreover in Pluronic F68 (Figure 3.18.f) and F127 (Figure 3.18.g) treated cells enhanced the cell viability was observed.

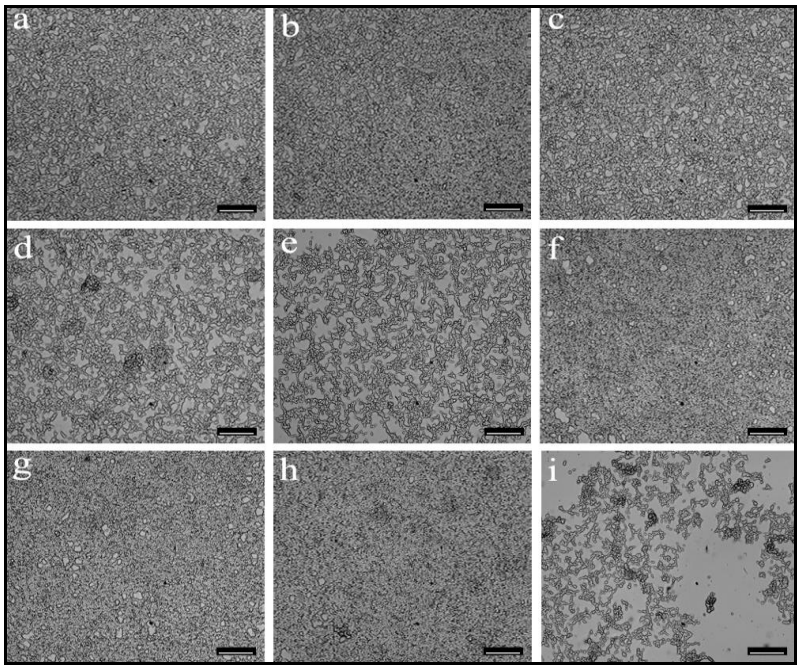


Figure 3.18. Light microscope images of cells (20x) prepared for fatty acid profiling experiment. Growth medium with Ketamine and CM (a); Ketamine, CM and Pluronic F68 (b); Ketamine, CM and Pluronic F127 (c); Ketamine and Pluronic F68 (d); Ketamine and Pluronic F127 (e); Pluronic F68 (f); Pluronic F127 (g); and Negative control (h); Positive control (i) (scale bar, 100 μm)

Fatty acid analysis showed that Ketamine had a slight effect on fatty acid profile of the cells when they were compared with the control group. According to our results in all Ketamine exposed groups (Growth medium with Ketamine and CM; Ketamine, CM and Pluronic F68; Ketamine, CM and Pluronic F127; Ketamine and Pluronic F68; Ketamine and Pluronic F127; and Positive control) levels of Palmitic acid (saturated, 16:0), stearic acid (saturated, 18:0) and Oleic acid (monounsaturated, 18:1 w9c) were increased when it was compared with the negative control. On the other hand the levels of these fatty acids for growth medium with Pluronic F68 and F127 treated group were nearly close to the negative control suggesting that pluronics do not alter the fatty acid profile negatively. Moreover, Palmitoleic acid (monounsaturated, 16:1 w7c) and Oleic acid (monounsaturated, 18:1 w9t) could not be found in Ketamine exposed groups. They

existed only in the growth medium with Pluronic F68, F127 and negative control. Taken together, this data suggests that Ketamine destroyed Palmitoleic and Oleic acids and pluronics included in the growth media could not help to preserve these two monounsaturated fatty acids. Results were shown in Table 3.1.

Table 3.1. Fatty acid content of CM combined Pluronic F68 and F127

Type	Isomer	Name	Group	%
Saturated	16:0	Palmitic Acid	K+C	36,36
Saturated	16:0	Palmitic Acid	K+C+F68	32,92
Saturated	16:0	Palmitic Acid	K+C+F127	35,68
Saturated	16:0	Palmitic Acid	K+F68	34,05
Saturated	16:0	Palmitic Acid	K+F127	35,15
Saturated	16:0	Palmitic Acid	F68	26,12
Saturated	16:0	Palmitic Acid	F127	30,68
Saturated	16:0	Palmitic Acid	PC	34,84
Saturated	16:0	Palmitic Acid	NC	30,59
Saturated	18:0	Stearic Acid	K+C	23,83
Saturated	18:0	Stearic Acid	K+C+F68	23,5
Saturated	18:0	Stearic Acid	K+C+F127	21,94
Saturated	18:0	Stearic Acid	K+F68	22,03
Saturated	18:0	Stearic Acid	K+F127	21,6
Saturated	18:0	Stearic Acid	F68	14,97
Saturated	18:0	Stearic Acid	F127	15,7
Saturated	18:0	Stearic Acid	PC	23,04
Saturated	18:0	Stearic Acid	NC	16,66
Monounsaturated	18:1 w9c	Oleic Acid	K+C	39,82
Monounsaturated	18:1 w9c	Oleic Acid	K+C+F68	43,58
Monounsaturated	18:1 w9c	Oleic Acid	K+C+F127	42,38
Monounsaturated	18:1 w9c	Oleic Acid	K+F68	37,69
Monounsaturated	18:1 w9c	Oleic Acid	K+F127	43,26
Monounsaturated	18:1 w9c	Oleic Acid	F68	32,16
Monounsaturated	18:1 w9c	Oleic Acid	F127	35,46
Monounsaturated	18:1 w9c	Oleic Acid	PC	38,94
Monounsaturated	18:1 w9c	Oleic Acid	NC	34
Monounsaturated	16:1w7c	Palmitoleic Acid	F68	8,65
Monounsaturated	16:1w7c	Palmitoleic Acid	F127	9,08
Monounsaturated	16:1w7c	Palmitoleic Acid	NC	10,48
Monounsaturated	18:1 w9t	Oleic Acid	F68	8,05
Monounsaturated	18:1 w9t	Oleic Acid	F127	9,08
Monounsaturated	18:1 w9t	Oleic Acid	NC	8,27

3.10. CO-CULTURE OF SH-SY5Y CELL LINE AND hTGSCs

In order to determine the effect of hTGSCs on the viability of SH-SY5Y cells against Ketamine induced neurotoxicity, co-culture system was established by SH-SY5Y cells and hTGSCs were plated together at different ratios (1:1 and 1:3) on tissue culture well plates and cells exposed to Ketamine for 24 hours. After 24 hours cells were observed under flourescent microscope. Figure 3.19 and 3.20 show flourescent images of cells.

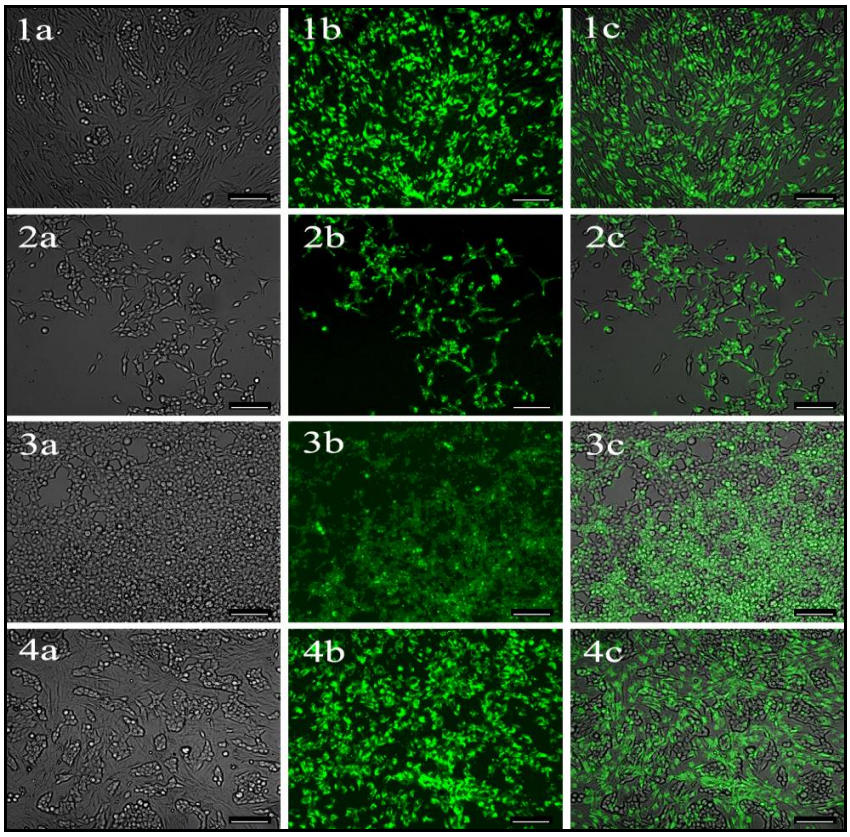


Figure 3.19. Co-culture of SH-SY5Y and hTGSCs at 1:1 ratio (20x) (scale bar, 100 µm) 1.Ketamine treated SH-SY5Y/SH-SY5Y cells (positive control). SH-SY5Y (1a-bright field image)/SH-SY5Y (1b-green fluorescence image); 2. Ketamine treated SH-SY5Y/hTGSCs. SH-SY5Y (2a-bright field image)/hTGSCs (2b-green fluorescence image); 3.Untreated SH-SY5Y/SH-SY5Y cells (negative control). SH-SY5Y (3a-bright field image)/SH-SY5Y (3b-green fluorescence image); 4. Untreated SH-SY5Y/hTGSCs. SH-SY5Y (4a-bright field image)/hTGSCs (4b-green fluorescence image); (c) Merged images of panels a and b

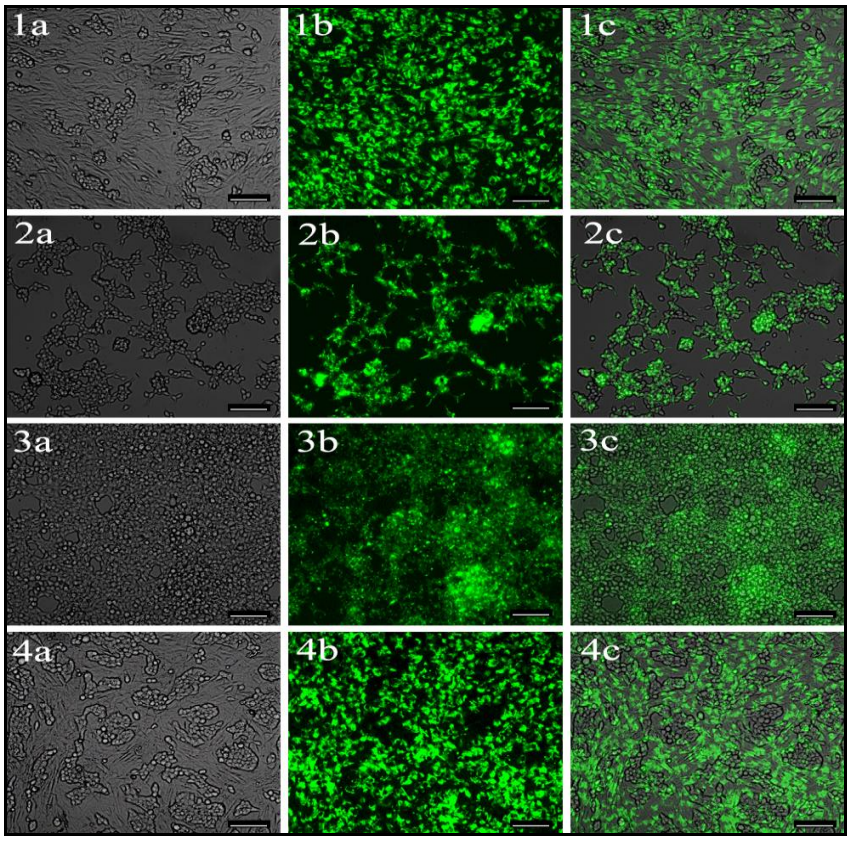


Figure 3.20. Co-culture of SH-SY5Yand hTGSCs at 1:3 ratio (20x) (scale bar, 100 μm) 1.Ketamine treated SH-SY5Y/SH-SY5Y cells (positive control). SH-SY5Y (1a-bright field image)/SH-SY5Y (1b-green fluorescence image); 2. Ketamine treated SH-SY5Y/hTGSCs. SH-SY5Y (2a-bright field image)/hTGSCs (2b-green fluorescence image); 3.Untreated SH-SY5Y/SH-SY5Y cells (negative control). SH-SY5Y (3a-bright field image)/SH-SY5Y (3b-green fluorescence image); 4. Untreated SH-SY5Y/hTGSCs. SH-SY5Y (4a-bright field image)/hTGSCs (4b-green fluorescence image); (c) Merged images of panels a and b

Cells were stained with PKH67. Untreated (without Ketamine), unlabeled and labeled SH-SY5Y cells were accepted as a negative control and cell viability of SH-SY5Y cells in co-culture with hTGSCs were analysed by flow cytometry. This allowing the monitorization of two different cell populations according to their green fluorescence intensity. Our results showed that both co-culture cell ratios at 1:1 and 1:3 increased the cell viability. The viability of SH-SY5Y cells in co-culture with hTGSCs at 1:1 and 1:3 ratio under Ketamine exposure improved remarkably by 51% and 45%, respectively when compared to positive control. In addition to this, the viability of SH-SY5Y cells in

co-culture with hTGSCs at 1:1 and 1:3 ratio (without any treatment with Ketamine) increased by 53% and 130%, respectively when compared to negative control. Results were shown in Figure 3.21 and Figure 3.22.

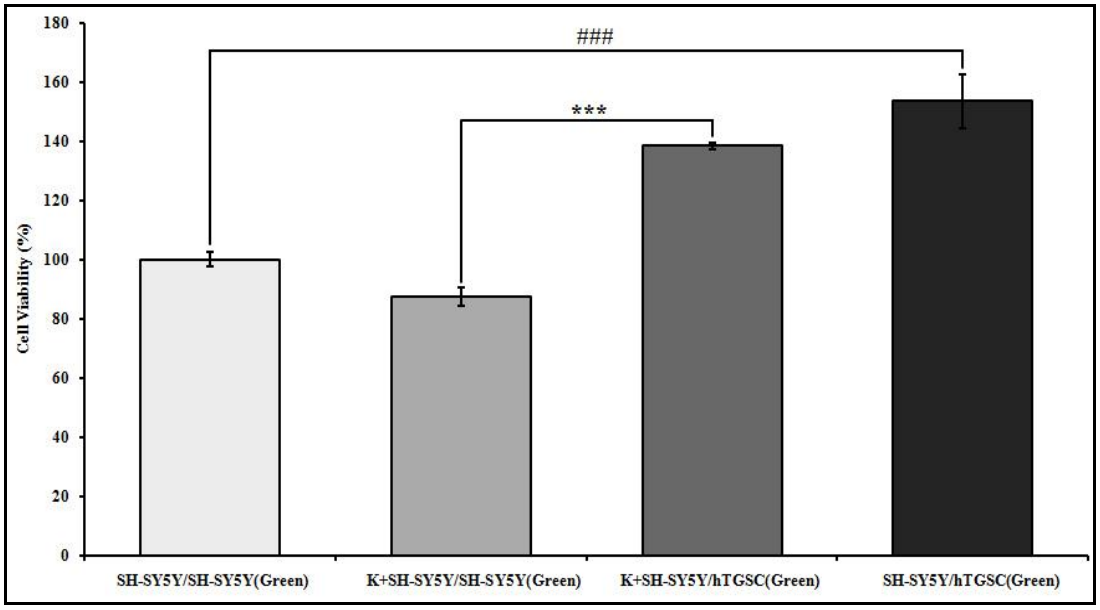


Figure 3.21. Cell viability of SH-SY5Y cells in co-culture with hTGSCs at 1:1 ratio (K: Ketamine, SH-SY5Y/SH-SY5Y (Green): Untreated SH-SY5Y/SH-SY5Y(PKH67 labeled) cells (negative control), K+SH-SY5Y/SH-SY5Y (Green): Ketamine treated SH-SY5Y/SH-SY5Y (PKH67 labeled) cells (positive control), K+SH-SY5Y/hTGSCs (Green): Ketamine treated SH-SY5Y/hTGSCs (PKH67 labeled), SH-SY5Y/hTGSCs (Green): Untreated SH-SY5Y/hTGSCs (PKH67 labeled)) (***P<0.001 compared with positive control, $^{###}P$<0.001 compared with negative control)

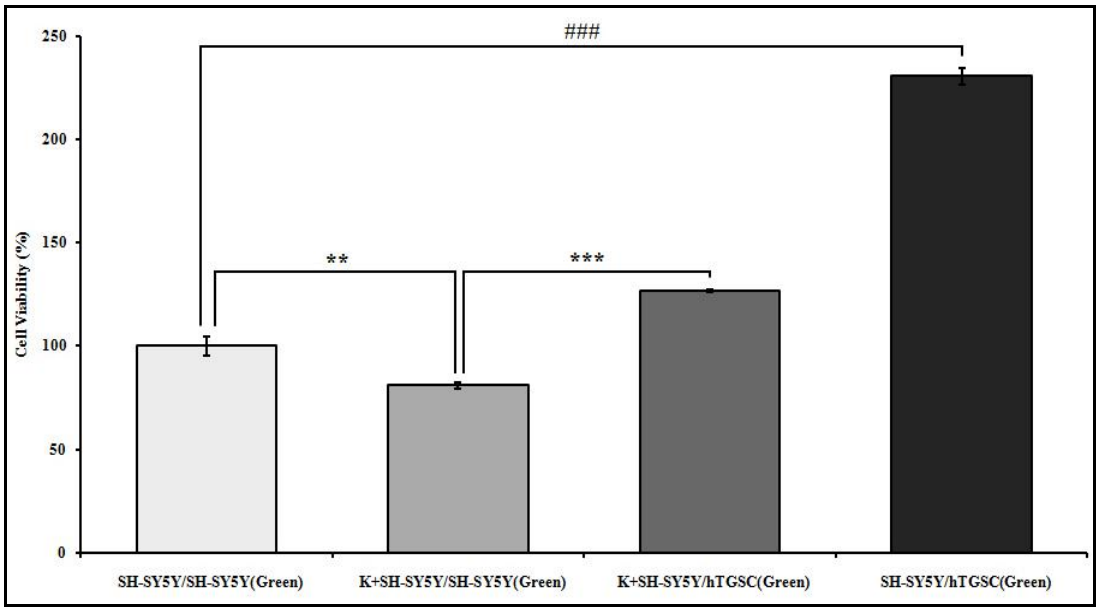


Figure 3.22. Cell viability of SH-SY5Y cells in co-culture with hTGSCs at 1:3 ratio (K: Ketamine, SH-SY5Y/SH-SY5Y (Green): Untreated SH-SY5Y/SH-SY5Y(PKH67 labeled) cells (negative control), K+SH-SY5Y/SH-SY5Y (Green): Ketamine treated SH-SY5Y/SH-SY5Y (PKH67 labeled) cells (positive control), K+SH-SY5Y/hTGSCs (Green): Ketamine treated SH-SY5Y/hTGSCs (PKH67 labeled), SH-SY5Y/hTGSCs (Green): Untreated SH-SY5Y/hTGSCs (PKH67 labeled)) (**P<0.01 compared with positive control, ***P<0.001 compared with positive control, $^{###}P$<0.001 compared with negative control)

3.11. DETERMINATION OF EXISTENCE OF NMDA RECEPTOR NR-1 SUBUNIT BY USING IMMUNOCHEMICAL ANALYSIS ON SH-SY5Y AND PRIMARY MIX BRAIN CULTURE CELLS

The immunofluorescent staining was performed in order to show the presence of NMDA receptor NR-1 subunit in the SH-SY5Y cell line. Figure 3.23 and Figure 3.24 show the existence of NR-1 subunit in SH-SY5Y and primary mix brain culture cells.

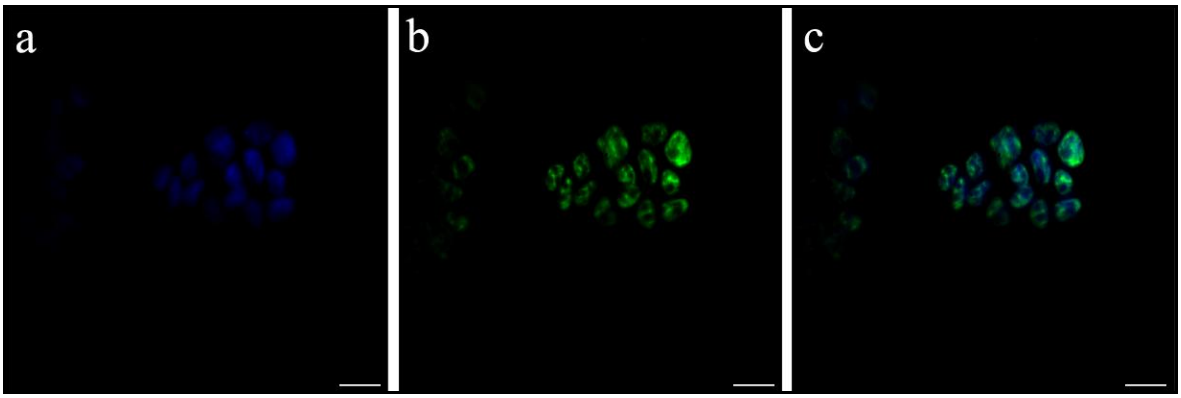


Figure 3.23. Immunocytochemistry of SH-SY5Y cells expressing NMDA receptor NR-1 subunits (40x) (scale bar, 50 μm)
Cells labeled with (a) DAPI and (b) NR-1 antibody, (c) merged image of a and b

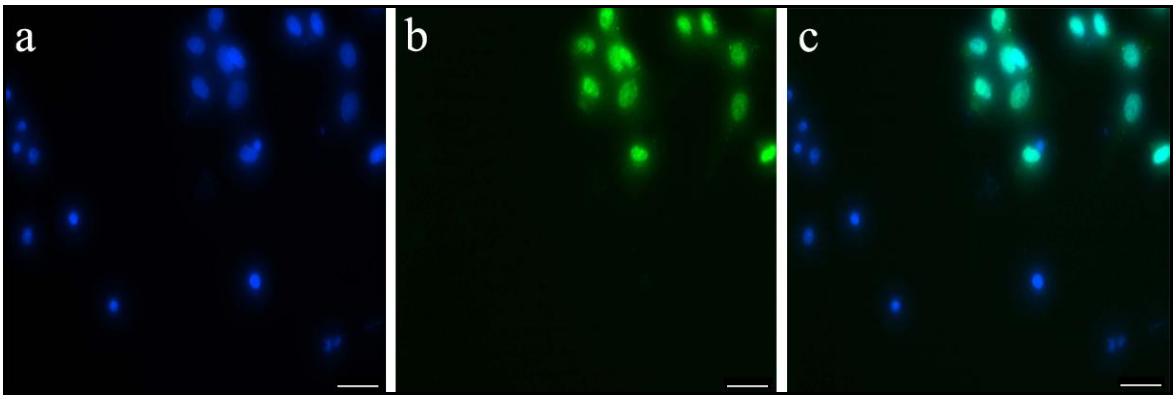


Figure 3.24. Immunocytochemistry of primary mix brain culture cells expressing NMDA receptor NR-1 subunits (40x) (scale bar, 100 μm)
Cells labeled with (a) DAPI and (b) NR-1 antibody, (c) merged image of a and b

3.12. REAL TIME PCR ANALYSIS OF CASPASE-3 AND NR-1 SUBUNIT mRNA LEVELS

Expression levels of Caspase-3, apoptosis marker, and NMDA receptor NR-1 subunit, related to Ketamine induced neurotoxicity, were detected by using Real time PCR. β-actin was used as the house keeping gene to normalize the results. In the literature, it is pointed that Ketamine induced neurotoxicity is related to calcium overload caused by the glutamatergic sitimulation due to the overactivated NMDA receptor especially NR-1

subunit receptor [111]. The upregulation of NMDA receptor leads to necrosis [119] and apoptosis [120]. Results showed that CM combined Pluronics reduced the expression levels of NR-1 and Caspase-3. Moreover, according to cell viability assays VEGF and FGF-2 combined Pluronics were the most effective GFs in reducing the neurotoxicity. The results revealed that they also reduce Caspase-3 expression. The results were summarized in Figure 3.25, Figure 3.26 and Figure 3.27.

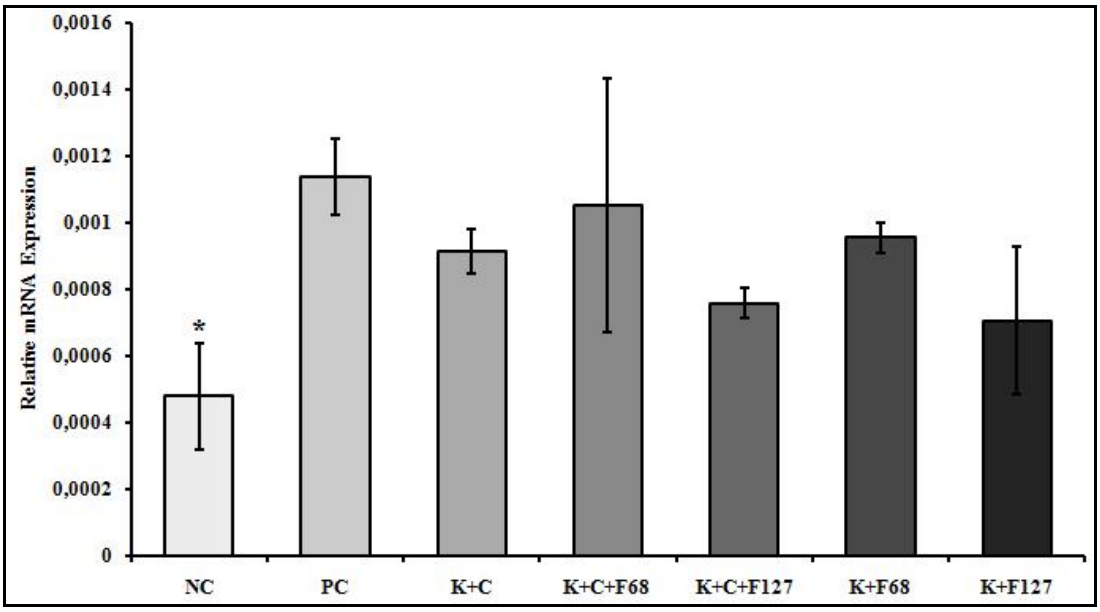


Figure 3.25. Real-time PCR analysis of NMDA receptor NR-1 subunit messenger RNA (mRNA) expression levels in SH-SY5Y cell line. Y axis represents the NR-1 mRNA expression normalized against the house keeping gene β-actin expression. (K: Ketamine, CM: Conditioned Medium, F68: Pluronic F68, F127: Pluronic F127, NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, K+CM: Growth medium with CM and Ketamine, K+CM+F68: Growth medium with CM, Pluronic F68 and Ketamine, K+CM+F127: Growth medium with CM, Pluronic F127 and Ketamine, K+F68: Growth medium with Pluronic F68 and Ketamine, K+F127: Growth medium with Pluronic F127 and Ketamine) (*P<0.05 compared with positive control)

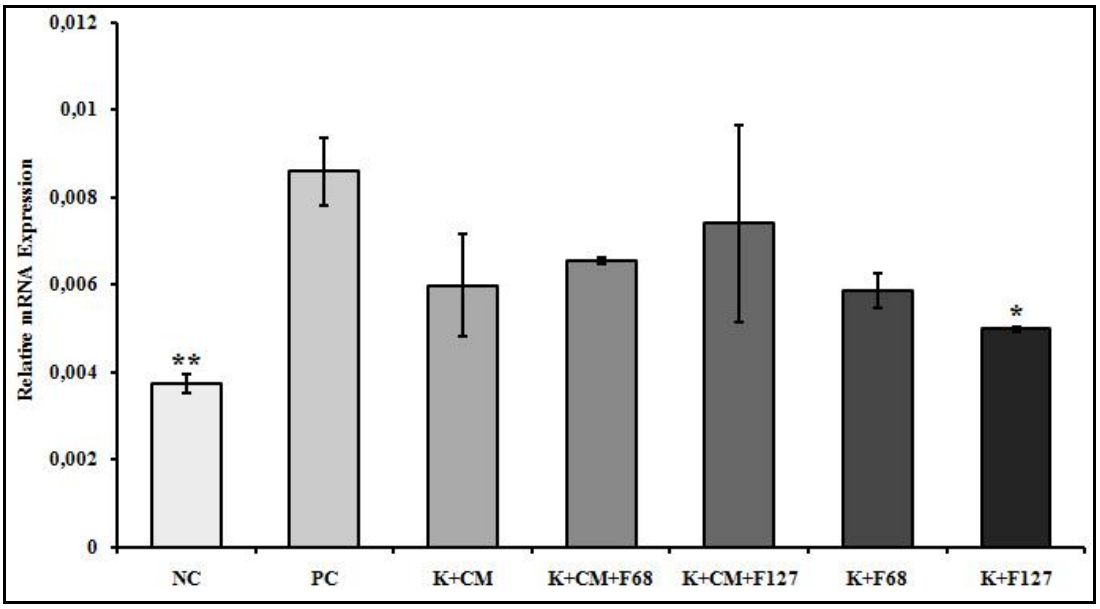


Figure 3.26. Real-time PCR analysis of Caspase-3 messenger RNA (mRNA) expression levels in SH-SY5Y cell line. Y axis indicates the Caspase-3 mRNA expression normalized against the house keeping gene β-actin expression (K: Ketamine, CM: Conditioned Medium, F68: Pluronic F68, F127: Pluronic F127, NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, K+CM: Growth medium with CM and Ketamine, K+CM+F68: Growth medium with CM, Pluronic F68 and Ketamine, K+CM+F127: Growth medium with CM, Pluronic F127 and Ketamine, K+F68: Growth medium with Pluronic F68 and Ketamine, K+F127: Growth medium with Pluronic F127 and Ketamine) (*P<0.05/**P<0.01 compared with positive control)

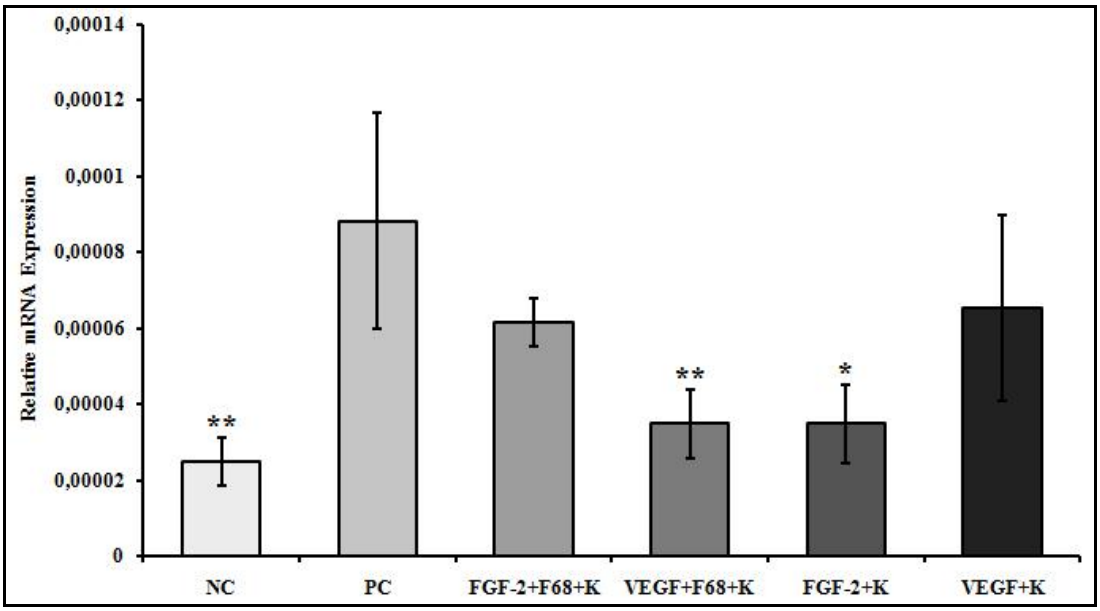


Figure 3.27. Real-time PCR analysis of Caspase-3 messenger RNA (mRNA) expression levels in SH-SY5Y cell line. Y axis shows the Caspase-3 mRNA expression normalized against the house keeping gene β-actin expression (K: Ketamine, F68: Pluronic 68, F127: Pluronic 127, Fibroblast Growth Factor-2 [FGF-2], Vascular Endothelial Growth Factor [VEGF], NC: Negative Control/Growth Medium, PC: Positive Control/Growth Medium with Ketamine, FGF/VEGF+F127+K: Growth medium with FGF/VEGF, F127 and Ketamine, FGF/VEGF+K: Growth medium with FGF/VEGF and Ketamine) (*P<0.05/**P<0.01 compared with positive control)

4. DISCUSSION

Number of people who suffer from neurodegenerative disorders stemming from sporadic, and familial reasons or using some narcotics have increased dramatically in recent years [140]. Deliviring medicines to the central nervous system is not an easy task since the brain have some barriers such as blood brain barrier and blood-cerebrospinal fluid barrier and mechanisms such as drug efflux transport systems [51]. Today great efforts have been made to develop strategies for delivering drugs efficiently to the central nervous system (CNS) in order to treat diseases. In this point synthetic polymers have received a great deal of interest for several reasons. First of all, polymers can be designed for multifunctionality, solubility (combining drugs covalently or non-covalently), stability and permeability etc. [49]. Secondly, polymers can be used as targeted delivery agents [49]. Thirdly, controlled or sustained release of the drug achived by using drug combined polymers [49]. Lastly, they can interact and alter the endogenous drug transport systems because of their biological activity and this property is significant for drug delivery [49]. One significant and promising drug delivery agent which has amphiphilic property is called Pluronic triblock copolymers. Pluronic triblock copolymers have the ability to self assemble into micelles and drug molecules can be incorporated into them. They can be transported into body. They also possess a great potential for biological modifying activity [49].

Over the years, the usage of stem cells in order to treat neurodegenerative diseases such as Alzheimer, Amyotrophic Lateral Sclerosis (ALS) etc. has taken tremendous interest because of their proliferation, self renewal ability and differentiation capacity. Among stem cells dental tissues including dental pulp and follicle are promising stem cells source. Stem cell isolation does not have any ethical problem like embryonic stem cells because dental tissues are waste materials from the surgical operations. Dental stem cells have capacity for transdifferentiation into neurogenic cells and neuroprotection [37].

Within stem cells Human Tooth Germ Stem Cells (hTGSCs), which have many kind of advantages in application, have a part in regenerative medicine as a new alternative

source. According the our characterization results it appears that hTGSCs carries MSCs properties [31].

Ketamine is an uncompetitive NMDA receptor antagonist, that is used as a general anesthetic [141]. It dissociates rapidly after intravenous administration. Ketamine levels are the highest at the first 5 minutes in the plasma and brain. 4 hours after the last administration, no significant apoptotic situation is determined [142]. Although the beginning concentration of Ketamine in brain is lower than that in plasma, brain is exposed to Ketamine more than plasma due to slower elimination from this tissue [142- 144]. Furthermore from the first 6 hours through 18 hours after last injection when Ketamine level is detected as almost zero in plasma and brain, increased apoptosis become apparent (it is nearly 2 fold). Thus, it can be understood from this information that, increased apoptosis in brain is not directly linked to acute level of Ketamine in blood or in brain, but it might be linked to Ketamin related receptors (NMDA receptors) in the brain [142].

In this study, 200µg/ml Ketamine was tested on SH-SY5Y and primary mix brain culture cells. Ketamine causes neurodegeneration and increased cell death detected by MTS test which also is confirmed by literature [141, 145,146]. Wang *et al* in 2006 showed that Ketamine produces loss of cultured frontal cortical neurons from the postnatal day 3 monkey [141]. Mak *et al* in 2010 also showed the toxic effect of Ketamine on SH-SY5Y cells [145]. The areas of the damage in brain induced by Ketamine have been identified mostly subependymal [146], existing at the lateral walls of the lateral ventricles of the brain containing adult neural stem cells [147], around the central canal [146] and the narrow passageway in the spinal cord [148]. The morphological properties are most frequently characterized as the demyelination and necrosis. In addition, it was reported that after intrathecal administration of the Ketamine chromatolysis, which is a late, but not pathognomonic, the morphological sign of apoptosis, was taken place [114]. Moreover there are other regions in brain which get damaged because of Ketamine. As a result of multiple Ketamine injections apoptotic neurodegeneration were detected in layers II and III of the frontal cortex, the striatum, hippocampus, thalamus, and amygdala in rats treated with six injections of 20 mg/kg Ketamine [142]. Furthermore, it was reported that Ketamine affects the glial cells including microglia and astrocytes in response to inflammatory insults so Ketamine may show an indirect effect on neurons but further

studies is necassary because there is no definite information about whether is toxic to the glial cells [145].

Studies pointed that there is a relationship between Ketamine, NMDA receptor ion channel blocker, and NMDA receptor subunits particularly NR-1 which is widely dispersed all around the brain and is the essential subunit required for the NMDA channel function [149]. In our study, we showed NMDA receptor NR-1 subunit expression in the SH-SY5Y and primary mix brain culture cells using immunocytochemistry and Real Time PCR analysis. Upregulation in the level of NR-1 protein expression was demonstrated in Ketamine exposed forebrain cultures and this result confirmed that the treatment with Ketamine gives rise to the upregulation of the NMDA receptor complex [150]. As a result of this upregulation increased Ca^{2+} influx is triggered. The buffering capacity of the mitochondria is exceeded by the increased Ca^{2+} and the excessive intracellular Ca^{2+} loading by mitochondria reduces the membrane potential and disrupts the electron transport and leading to excess generation of reactive oxygen species (ROS) [142, 149]. Ca^{2+} dependent activation of neuronal nitric oxide (nNOS) causes increased nitricaoxide (NO) production and the formation of toxic peroxynitrite ($ONOO^-$) [151]. Oliveria *et al* in 2009 showed that Ketamine increase the oxidative stress in the brain of rats. It was proved that Ketamine increases the lipid peroxidation, decreases the activity of super oxide dismutase, catalase and antioxidant enzymes [152]. Furthermore, Ca^{2+} overload of mitochondria causes the activation of caspases [146].

In order to reduce Ketamine induced neurotoxicity CM and GFs combined Pluronic F68 and F127, which are known drug delivery agent, were used. Although Pluronic F127 was not commonly used in neurological studies, Pluronic F68 was shown to be protective as it increased the cell growth, survival and the attachment to the culture dish surfaces [153, 154]. It stabilizes also lipid blayer [155]. Pluronic F68 reseals the damaged cell membrane and increases the functional recovery of the cells injured by various reasons such as the electric shock, heat (thermal) shock, and excitotoxic and oxidative agents. It protects both neuronal and nonneuronal cells from cell death. Serbest et al in 2005 demonstrated that Pluronic F68 reduces the rate of apoptosis and necrosis significantly after mechanical trauma in the neural cell culture. It was suggested that Pluronic F68 inserts into the cell membrane and promotes the resealing. In addition to this, same group

also proved that Pluronic F68 restores the membrane integrity and the viability of mechanically injured neuronal cells. Moreover, another study showed that it provides an acute recovery axonal function in the spinal cord injury models [156]. Pluronic F68 treatment also presented a good effect in NMDA exposed embryonic rat hippocampal pyramidal neurons. It was shown that after the Pluronic F68 application Ca^{2+} mechanism is healthy, voltage-gated Ca^{2+} channels and NMDA receptor-coupled Ca^{+2} channels work normally; and free intracellular Ca^{2+} concentration was properly diminishes to the baseline values after an intracellular Ca^{2+} load. It was proved that lipid peroxidation was induced in brains of rats exposed to Ketamine [152]. It was demonstrated that Pluronic F68, which inserts into lipid membrane, reduces the lipid peroxidation by scavenging reactive oxygen species and directly inhibits it [157]. Pluronic F68 inserts into the damaged monolayers, bilayers, and cell membranes whose lipid surface pressure diminished and increases the lipid paking density in order to repair the injured membrane where the lipid packing density reduced. Pluronic F68 can also prevent the uncontrolled flux of ions and cellular contents through the puncture sites. Furthermore, Mina *et al* in 2009 showed that Pluronic F68 reduces the toxicity of amyloid oligomers in SH-SY5Y cell line and primary neurons [158]. Pluronic F127 is a good drug delivery agent because of its thermoreversible properties in the aqueous solutions. In addition, they enhance the stabilization of incorporated drugs especially proteins and decreases the tendency for the peptide unfolding [159]. Pluronic F127 enhances the transport of proteins, it is also used for the wound healing [160, 161].

In the literature, it was demonstrated that dental stem cells are able to secrete many growth factors such as vascular endothelial growth factor (VEGF) [162], insulin-like growth factor-1 (IGF-1), insulin-like growth factor-2 (IGF-2) [163-165], stem cell factor (SCF), granulocytecolony stimulation factor (GCSF) [166, 167], nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF) and fibroblast growth factor-2 (basic) (FGF-2, bFGF) [34]. Studies proved that BDNF, NGF, IGF-1, FGF-2 and VEGF used for this study show neuroprotective properties on various neurogenerative diseases [168, 169]. Moreover, hTGSCs might secrete these GFs into its medium because it is derived from dental tissues and dental stem cells can express many GFs into their medium. GFs carry neuroprotective features as mentioned above. When CM and GFs combined Pluronic F68 and F127 were applied on SH-SY5Y

and primary mix brain culture cells, it was observed that cell death diminished and cell viability increased because Pluronics increase the efficiency of CM and GFs.

In co-culture experiments, it was shown that hTGSCs protects SH-SY5Y cells from the Ketamine induced stress. This effects might stem from the fact that stem cells can express many growth factors which help the restoration of the cell viability as well as stimulation of the plastic responses (for triggering plasticity), thereby improve the survival and function of SH-SY5Y cells.

It is known that upregulation of NMDA receptors gives rise to apoptosis but it also leads necrosis defined by excessive sodium and calcium intake together with chloride and water entry which causes cell swelling and death [119]. This type of toxicity may depend on concentration of glutamate agonist, duration time, the receptor subtype activated, cell type and its stage of the development or maturity [142]. In addition While Slikker *et al* in 2007 showed Ketamine induced apoptosis in the perinatal rhesus Monkey [149], Wang *et al* in 2006 demonstrated that Ketamine induced neurotoxicity eventuated both apoptosis and necrosis in the postnatal day 3 monkey frontal cortical neurons [141]. It was demonsrated that when Ketamine exposure time is 3 hours which resembles to a typical general pediatric anesthetic application period, there is no significant neurotoxic effects. However, 24 hours (long term) duration generates a significant increase in the neuronal cell death in the frontal cortex [142].

In this study SH-SY5Y and primary mix brain culture cells were exposed to Ketamine for 24 hours and cell death was observed clearly. The results showed that pluronics combined with both CM and GFs reduced the cell death and diminished the expression of Caspase-3 hence reducing the Ketamine induced stress. It was found that, NR-1 expression was reduced by pluronic combined CM which directly alters Ketamine's effect on cells.

We tested the effects of Ketamine on the fatty acid profiles of SH-SY5Y cells in the presence of Pluronics and CM. Studies proved that Ketamine causes lipid peroxidation and as a consequence of decrease in membrane fluidity gives rise to structural and functional changes in the membrane. These might play a crucial role in neurotoxicity [139]. Our

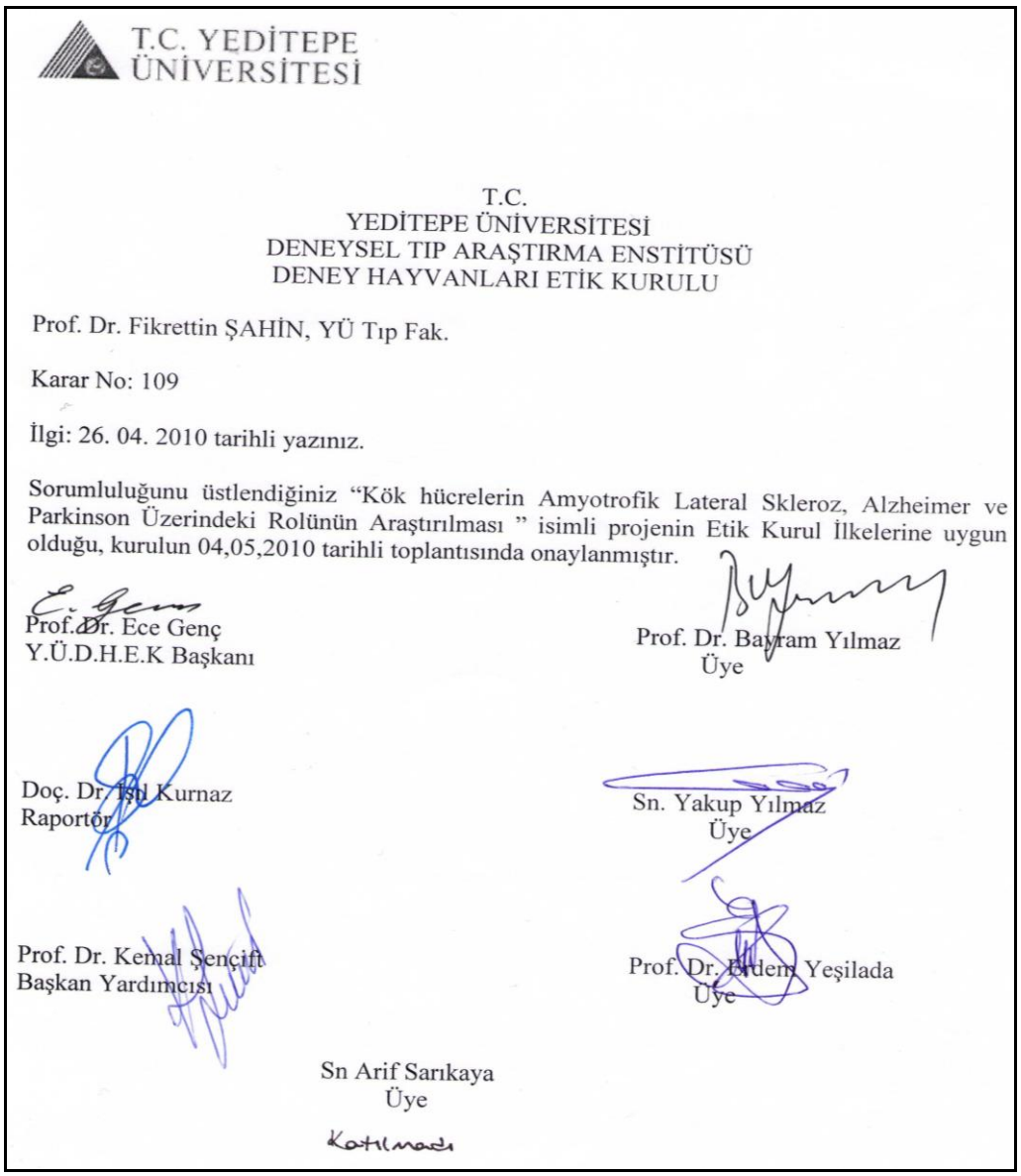
results revealed that Ketamine which has altered the fatty acid profile of cells abolishes the presence of two monounsaturated fatty acids named Palmitoleic and Oleic acids. CM combined Pluronics can not restore the levels of these two fatty acids back to their basal levels.

5. CONCLUSION

In conclusion, Ketamine gives rise to neurotoxicity in a time and dose dependent manner. hTGSCs are new, alternative, promising stem cell sources containing neuroprotective properties. Pluronic F68 and F127 are perfect drug delivery agents. When they were combined with CM and GFs they enhance effects of CM and GFs, increase cell viability and reduce Ketamine induced stress in cells. Our results suggest that MSCs might play an important role in reducing Ketamine induced neurotoxicity, it also suggests that pluronic combined growth factors might treat Ketamine induced neurotoxicity which might lead to develop new treatment strategies for patients suffered from Ketamine use. Lastly, CM and GFs combined Pluronics might also provide an alternate approach to treatment of other neurodenerative diseases.

APPENDIX A: ETHICAL APPROVAL

Ethical approval was obtained for animal use in the experimental research from Research Ethics Committee, Yeditepe University, Istanbul, Turkey in 2010. It was shown in Figure A.1.



T.C. YEDİTEPE ÜNİVERSİTESİ

T.C.
YEDİTEPE ÜNİVERSİTESİ
DENEYSEL TIP ARAŞTIRMA ENSTİTÜSÜ
DENEY HAYVANLARI ETİK KURULU

Prof. Dr. Fikrettin ŞAHİN, YÜ Tıp Fak.

Karar No: 109

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Sorumluluğunu üstlendiğiniz "Kök hücrelerin Amyotrofik Lateral Skleroz, Alzheimer ve Parkinson Üzerindeki Rolünün Araştırılması " isimli projenin Etik Kurul İlkelerine uygun olduğu, kurulun 04,05,2010 tarihli toplantısında onaylanmıştır.

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Katılmadı

Figure A.1. Ethic approval for animal use in the experimental research

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