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GRADUATE SCHOOL OF HEALTH SCIENCES

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This thesis study has been approved and accepted as a PhD dissertation in "Pharmaceutical Toxicology program" by the assesment, committee, whose members are listed below, on 17.09.2018.

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19 Eylül 2018

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[1] *"Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"*

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** Tez **danışmanının** önerisi ve **enstitü anabilim dalının** uygun görüşü üzerine **enstitü** veya **fakülte yönetim kurulu tarafından karar verilir.***

ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, relate studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor **Assoc. Prof. Dr. Suna Sabuncuoğlu** and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Solmaz Mohammadi Nejad

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ABSTRACT

Mohammadi Nejad S. Evaluation of Developmental Toxicity of Ethephon Using Embryonic Stem Cell Model. Hacettepe University, Graduate School of Health Sciences, Pharmaceutical Toxicology Program, Doctor of Philosophy Thesis, Ankara, 2018. Ethephon, a member of the organophosphorous compounds, is one of the most widely used plant growth regulators for artificial ripening. In recent years, the developmental toxicology studies have increased due to a broad interest in neurodevelopmental problems. Concerning this, new *in vitro* screening models such as embryonic stem cells have been established in predictive toxicology to decrease the expense and time of the experiments number of laboratory animals. The goal of this study was evaluation of the neuro-developmental toxicicity potential of "ethephon". For this purpose, the mES cells were exposed to ethephon and the cytotoxicity, cell cycle, total antioxidant capacity, reactive oxygen species (ROS) and, expression of specific pluripotential genes including Oct-4, SOX2, NANOG, Nrf2, Nestin and neural differentiation genes such as MAP2 and β-Tubulin III were examined. Additionally, effects of ethephon on expression of stemness genes were assessed before and during neural differentiation. Our results from MTT assay with mEs cells, after 24, 48 and 96 h exposure to ethephon demonstrated that at higher doses (1280, 2560 and 5120 μM) ethephon is cytotoxic for mES cells. According to results of oxidative stress assays, it is suggested that the cytotoxic effect of ethephon is not related to the oxidative stress mechanism. In order to genetic evaluation, ethephon treated mES cells were analyzed before and after neuronal-differentiation by Real-time PCR. The expressions of several genes were evaluated. Our observations showed that ethephon is potent to induce a significant difference in expression of these genes in neuronal differentiated cellsFuture studies are necessary to understand the exact mechanism of developmental toxicity of ethephon.

Key Words: Ethephon, cytotoxicity, stemness genes, developmental toxicity, neural differentiation.

ÖZET

Mohammadi Nejad S. Embriyonik Kök Hücre Modeli Kullanılarak Etafonun Gelişimsel Toksisitesinin Değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmasötik Toksikoloji Programı Doktora Tezi, Ankara, 2018. Organfosforlu bileşiklerin bir üyesi olan etafon, yapay olgunlaşma için en yaygın kullanılan bitki büyüme düzenleyicilerinden biridir. Son yıllarda, nörogelişimsel sorunlara olan yoğun ilgi nedeniyle gelişimsel toksikoloji alanındaki çalışmalar artmaktadır. Buna bağlı olarak, deneylerin maliyet ve süreleri ile kullanılan laboratuvar hayvanı sayısını azaltmak için tanımlayıcı toksikolojide embriyonik kök hücreler gibi yeni *in vitro* tarama modelleri geliştirilmektedir. Bu çalışmanın amacı "etafon"un nöro-gelişimsel toksisite potansiyelinin değerlendirilmesidir. Bu amaçla, mES hücreleri etafona maruz bırakılmış ve sitotoksisite, hücre döngüsü, toplam antioksidan kapasite, reaktif oksijen bileşikleri (ROB) ve Oct-4, SOX2, NANOG, Nrf2, Nestinin de dahil olduğu özel pluripotential genler ile nöral farklılaşma genleri olan MAP2 ve β-Tubulin III genlerinin ekspresyonları incelenmiştir. Nöral farklılaşma öncesi ve sonrasında, etafon uygulanmış ve etafonun kök hücre genlerinin ekspresyonu üzerindeki etkileri değerlendirilmiştir. Etfona 24, 48 ve 96 saat süreyle maruz bırakılan mEs hücrelerinde yapılan MTT analizinden elde edilen sonuçlar etefonun yüksek dozlarında (1280, 2560 ve 5120 μM) hücre proliferasyonunda belirgin bir azalma olduğunu göstermiştir. Oksidatif stres yöntemlerinden elde edilen bulgulara göre, etafonun sitotoksik etkisinin oksidatif stres mekanizmasına bağlı olmadığı düşünülmüştür. Genetik değerlendirme sonuçları, etafon uygulanan mES hücreleri, Real-time PCR ile nöronal farklılaşma öncesi ve sonrasında analiz edilmiştir. Kök hücre formu, nöral ektoderm ve dendritik dönemin çeşitli genlerinin ekspresyonu bu yöntemle değerlendirilmiştir. Sonuçlarımız, nöral farklılaşma olan hücrelerde, etafonun bu genlerin ekspresyonunu indükleyebileceğini doğrulamaktadır. Etafonun gelişimsel toksisite mekanizmasının anlaşılabilmesi için gelecekte yapılacak yeni çalışmalara ihtiyaç bulunmaktadır.

Anahtar kelimeler: Etefon, sitotoksisite, stemness genleri, gelişimsel toksisite, nöral farklılaşma.

TABLE OF CONTENTS

SYMBOLS AND ABBREVIATIONS

ACC	Aminocyclopropane carboxylic acid
AchE	Acetylcholine esterase
ALS	Amyotrophic lateral sclerosis
ASCs	Adult Stem Cells
BMP	Bone Morphogenic Proteins
BSA	Bovine Serum Albumin
Buche	Butyrylcholinesterase
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's medium
DPI	Diphenyleneiodonium
EDTA	Ethylenediamine Tetraacetic Acid
EMS	Embryonic Stem cell
ERK	Extracellular signal–Regulated kinase
ES	Embryonic Stem cell
ES- FBS	Embryonic Stem cell
ESCs	Embryonic stem cells
EST	Mouse embryonic stem cell tests
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
FDA	The U.S Food and Drug Administration
FRAP	Ferric Reducing Ability Of Plasma
GABA	Gamma-Amino Butyric Acid
GUP	General used pesticide
HDT	Highest dose tested
ICM	Inner cell mass
IC_{50}	Half Maximal Inhibitory Concentration
IPS	Induced pluripotential stem cells
IUPAC	International Union of Pure and Applied Chemistry

LIF	Leukemia Inhibitory Factor
LOEL	Lowest Observed Adverse Effect Level
MAP2	Microtubule Associated Protein 2
MAPK	Mitogen-activated protein kinases
mES	Mouse Embryonic stem
MTs	Metallothioneins
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEAA	Non Essential Amino Acid
NOEL	No Observed Adverse Effect Level
NSE	Neuron specific enolase
Oct-4	Octamer-binding transcription factor 4
OPs	Organophosphates
OPCs	Organophosphorus compounds
PI	propidium iodide
RA	Retinoic Acid
ROS	Reactive oxygen species
SOX2	Sex determining region Y)-box 2
TAE buffer	Tris Acetic acid EDTA buffer
TGF	Transforming Growth Factor
TUMS	Tehran University of Medical Sciences

FIGURES

TABLES

1. INTRODUCTION

In the 21st century the agricultural systems have been changed rapidly due to the new demands for natural resources. Currently, because of increasing population and consumption, the merger market demands and modifications in an ecosystem the governments require new strategies to provide natural food. Indeed, agriculture has a critical role in feeding policies and must control the growth and development of plants by new methods (1).

Organophosphates are widely used pesticides in agriculture with neurotoxic effects and exposure to high level of these compounds at prenatal duration causes adverse effects in the neural development. In organophosphate toxicity, Acetylcholine Esterase enzyme is a considerable biomarker because Organophosphates inhibit the AChE activity in nervous system (1).

Ethephon is an ethylene releasing compound which has been classified as an organophosphate since 1973. In recent years, ethephon is one of the most widely used plant growth regulators. Scientific reports confirm that ethephon is potent to induce faster ripening in many agricultural crops especially in fruit by overexpression of some of the ripening related genes (2).

As people living in agricultural regions may be exposed to higher levels of these chemicals, the main question is that what the effects of such chemicals on developmental growth are. The most important point is that the potential effects of higher levels are well observed because of clinical symptoms. Thought the effects of low level exposure are still undefined (3).

Stem cells are well equipped models for drug discovery and predictive toxicology because their unique properties such as renewal ability and giving rise to various types of cells. Scientists now are able to use stem cells as an alternative model in developmental toxicity testing (4).

Chemicals and environmental factors in food chain and environment have serious toxic effects on genome. One of the main reasons of such effect is the oxidative stress (5). Many teratogenic and mutagenic damages have no clinical symptoms, so the evaluation of molecular and cellular mechanism of embryonic damage is still required.

The aim of this study is:

- Evaluation of an environmental toxicity of ethephon in embryonic stem cell models.
- Assessment the molecular mechanism of genetic effects of ethephon in embryonic stem cells.

In order to doing this research;

- ✓ The morphology of mES cells before and after neural differentiation;
- ✓ Oxidative stress status in mES cells
- ✓ Neural differentiation of mES cells;
- ✓ Flow cytometric analysis of cell cycle in mES cells;
- ✓ Gene Expression pattern in mES cells
- ✓ Gene expression pattern in neural differentiated mES cells

Were evaluated before and after ethephon treatment.

2. GENERAL INFORMATION

2.1 Organophosphorus Compounds

Organophosphorus compounds (OPCs) are phosphorus containing organic compounds which generally are used as pesticides.(6)

The main subcategories are phosphate esters, phosphate amides and thiol derivatives, phosphonic and phosphinic acids and their esters, phosphine oxides, imides, and chalcogenides, phosphonium salts and phosphoranes. They cause one of the main clinical problems all over the world per year due to their toxicities. They are also used as lethal chemical weapons (nerve gas) which include some of the most deadly substances, including sarin and VX nerve agents. Similar to organophosphates compounds, ethephon has anti-cholinesterase activity in mammals (1).

Table 2.1. Classification of Organophosphprus compounds (1).

Organophosphorus Compounds	
Phosphorus (III)	**Phosphorous (V)**
Phosphine	Phosphine oxide
Phosphinite, Phosphine	Phosphinate, Phosphine (amide)
Phosphonite, Phosphonamidite, Phosphin (diamine)	Phosphonate, Phosphoamidate Phosphonamide
Phosphine (triamin), Phosphite, Phosphoramidite, Phosphorodiamidite	Phosphorodiamidate, Phosphate, Phosphoramidate, Phosphoramidate

Organophosphates compounds (OPs) are one of the essential subclasses of organophosphorus compounds. Most of these chemicals are used as pesticides or insectisides however, some of them have therapeutic effects in cancer therapy (7).

2.1.1 Physicochemical Properties of Organophosphates

OPs normally are found in white powder or colorless liquids forms at room temperature. In fact the physicochemical properties of the Ops such as the volatility, molecular weight and lipid-solubility depend on the chemical structure of the substituents. They are stable in aqueous solutions and are more soluble in polar solvents (6).

2.1.2 Toxicity of Organophosphates

Almost every year nearly 3 million poisonings are reported which about 15% of these cases result in death. Exposure to organophosphates normally can be occurred by breathing of vapors and skin exposure. Some of the occupational groups such as farmers, farmworkers, sheep dippers, people living in agriculture regions and students of urban schools have greater risk of Ops poisoning (8).The annual incidence of organophosphate poisonings among agricultural workers varies from 3-10% per country and (9) the rate of OPs pexposure is higher in the developing countries in Asia and Africa. There are about 8000 Ops exposure cases in the United States per year; however the rate of death is few (8).

The contact with these agents may happen by accidentally eating or drinking of these agents. OPs absorbed via inhalation and skin and poisoning by these compounds is one of the common causes of suicide (10).

The nervous system is target organ of OPs and the mechanism of action of OPs involves the inhibition of acetylcholinesterase by making a covalent bond to the enzyme by these compounds. The AchE is a catalyzing enzyme which hydrolysis the neurotransmitting agent acetylcholine (11). Because of inhibition of the enzyme by OPs, acetylcholine accumulates in neuronal synapses and neuromuscular junctions which lead to prolonged over-stimulation of cholinergic receptors and resulting cholinergic toxicity or cholinergic crisis (12).

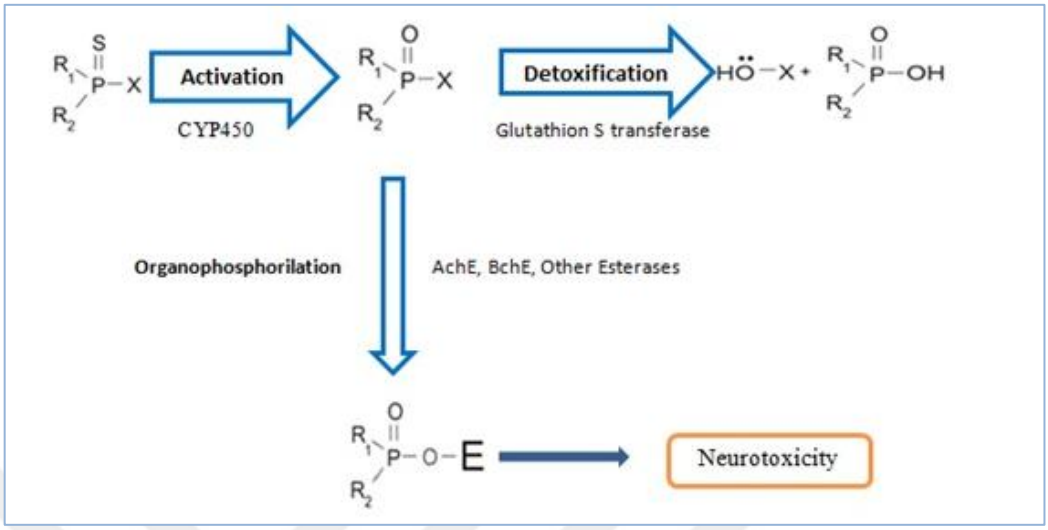


Figure 2.1. Mechanism of Organophosphates (1).

Esterases catalyze the hydrolysis of esters and there are two main types of esterases. The type “choline esterase I”, which is available in nervous system and erythrocytes, and the other type “choline esterase II” or “butyryl choline esterase” which is present in plasma. When inactivation of choline esterase is achieved at 75%, it leads to death seemingly. The organophosphorous compounds have effect on both types of choline esterase enzymes. However; type of toxin, the patient’s reaction and the type of effects on tissues or other enzymes also play important role in toxicity (1, 13).

Table 2.2. Common Organophosphate Insecticides (13).

Organophosphate Insecticides	
Acephate #	Dioxabenzophos
Azamethiphos	Dioxathion
Azinphos ethyl	Disulfoton *#
Azinphos methyl *	Ditalmifos
Bromophos	Edifenphos
Bromophos ethyl	EPBP
Cadusofos	EPN
Carbophenythion	ESP
Chlormephos	Ethion *
Chlorphoxim	Ethopropos *
Chlorpyrifos *#	Ethylparathion *
Chlorpyrifos-methyl	Etrimfos
Chlorthiophos *	Famphur
Chlorvinohos *	Fenamiphos *
Coumaphos *	Fenchlorphos
Crotoxyphos	Fenitrothion *
Crufomate	Fensulfothion
Cyanofenphos	Fenthion
Cyanophos	Fonofos *
Demephron-O and -S	Formothion
Demeton-O and -S	Fosmethilan
Demeton-S-methyl	Heptenophos
Demeton-S-methylsulphon	Isazofos
Dialifos	Isofenphos
Diazinon #	Isothioate
Dichlofenthion	Isoxathion
Dichlorvos	Jodfenphos
Dicrotophons	Leptophos

* Use of these OPs is restricted by EPA

Toxic for mammalians

Table 2.2. Common Organophosphate Insecticides (continue).

Organophosphate Insecticides	
Malathion	Profenofos ***#**
Menazon	Propaphos
Mephosfolan	Propetamphos
Methacrifos	Prothiofos
Methamidophos *#	Prothoate
Methidathion *#	Pyraclofos
Mevinphos	Pyridaphenthion
Monocrotophos	Quinlphos
Naled #	Schradan
Omethoate	Sulfotep *****
Oxydemeton-methyl #	Sulprofos *****
Parathion	Temephos
Parathion-methyl	TEPP
Phenthoate	Terbufos *
Phorate #	Tetrachlorvinphos
Phosalone	Thiometon
Phosmet #	Thionazin
Phosphamidon	Triazophos
Phospholan	Trichlorfon
Phoxim	Vamidothion

* Use of these OPs is restricted by EPA

Toxic for mammalians

These compounds play the role by two following mechanisms (14).

1. The direct inhibition of acetylcholine esterase which toxicity symptoms onset instantly after exposure.
2. The indirect inhibition of acetylcholine esterase (such as parathion) which toxicity symptoms onset 6-24 h after exposure.

The acetylcholine could be found in the central nerve system, at the end of parasympathetic and sympathetic nerves and at the somatic nerves (15). Diagnosis is

based on symptoms and also by measurement of the activity of butyrylcholinesterase of blood. The blood oxygen and intravenous fluids are measured too. The symptoms of this toxicity include salivation, urination, lacrimation, fatigue, hypertension, vomiting, hypoglycemia, sweating and tremor of muscles, diarrhea, and confusion in some cases. These symptoms start within minutes to hours and may last for weeks (10).

The primary medical treatment for organophosphates toxicities are atropine, oximes such as Pralidoxime and benzodiazepines such as Diazepam. Atropine, which is an anticholinergic and antimuscarinic drug, blocks the Ach receptors. It is a frequently used medication for nerve agent or OPs poisoning and is given to patients by muscular injection or intravenously (16).

Pralidoxime is an antidote in Ops intoxications which cleavages the phosphate-ester bond formed in Ops poisoning and reactivates the acetylcholinesterase (16).

Benzodiazepines (Diazepam), decrease the stimulation of neurons and ameliorates the muscle fasciculation via increasing the entrance of GABA. Diazepam in the Treatment of Organophosphorus Ester Pesticide Poisoning (16).

Whether acute or chronic poisoning, it depends on the specialty of chemical agent and the way of exposure, dose and the ability of body in degradation of the compound (12).

2.1.3 Non-Neurotoxic Effects of Organophosphates

Organophosphates have harmful effects on various biologic processes. The carboxylase enzymes are the most important group of enzymes in the aerobic decomposition of organic compounds and are inhibited by organophosphates which ends in blockage of metabolic transformation of some compounds (17 , 18).

Organophosphates also are able to release the active sulfur atom that is produced in the first phase of the dephosphorization stage of metabolism. The release of sulfur

atom inhibits of the CYP enzymes and finally leads to the decrease of the xenobiotics metabolism (19).

In vivo studies also demonstrated those organophosphates compounds are potent to disrupt the following mechanism:

- Inactivation by phosphorylation estrases
- Modification of release of the neurotransmitters such as aminobutyric acid and glutamic acid.
- An Increase in number of the GABA/ Dopamine receptors
- Blockage of the mitochondrial enzymes and ATP production
- Induction of mast cell degranulation and releasing of the histamine (18, 20).

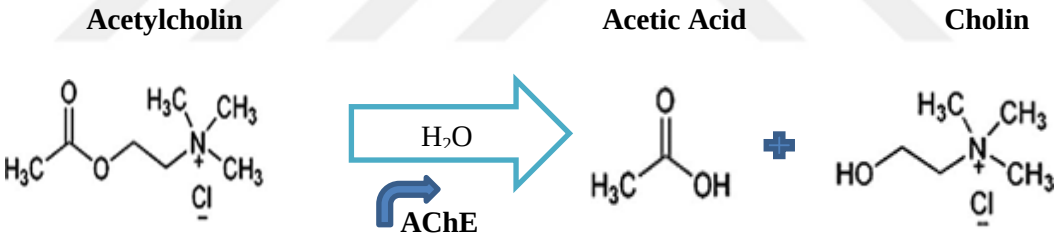


Figure 2.2. Analysis of Acetylcholine by Esterase enzyme (2)

Organophosphates also inhibit the lipase enzymes which are the critical in cellular signaling process. These compounds inactive the DAG-lipase indirectly and by this mechanism activation of signaling pathways and cell growth are affected by organophosphates (21).

Additionally, it has been showed that organophosphates compounds inhibit the androgenic receptors of steroidal hormones and result in deactivation of these hormones (22).

2.1.4 Immunotoxic Effects of Organophosphates

These compounds effect on the immune system both directly and indirectly. Inhibition of serine hydrolase enzyme in complement system and esterases in lymphocytes and monocytes, induction of the oxidative stress effects on immune system and induction of some modifications in growth and developmental pathways of the immune system are some of the direct effects (23).

Some modifications in the expression of some genes in neural cells and changing the metabolism of neural cells are some indirect effects of organophosphates (18).

2.2 Ethephon

Ethephon ($C_2H_6ClO_3P$), a member of the organophosphate compounds, was discovered in 1965. Since 1973 it is a registered pesticide in USA and in 1988 EPA issued a registration standard for this pesticide. Ethephon is one of the most widely used plant growth regulators. It is a phosphonic acid with a chloroethyl substituent attached to the phosphorous atom as shown in figure 2.3. (2).

The IUPAC name is 2-Chloroethylphosphonic acid, however, other trade names such as Bromeflor, Arvest, Ethrel, Florel, Cerone, Prep, and Flordimex are common.

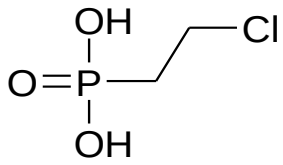


Figure 2.3. Chemical structure of Ethephon (2)

2.2.1 Physicochemical Properties of Ethephon

The pure ethephon is a white waxy solid or white crystalline powder with a light smell which is completely soluble in water, alcohol (especially short chain alcohols) and acetone but is practically insoluble in oil. Ethephon is lightly soluble in aromatic solvents such as benzene and toluene. The melting point is about 74 -75℃ and the boiling point is at 265℃. The molecular weight is 144.5 g/mol and the density is 1.41 g/cm³. Ethephon is a source of ethylene that is stable at pH<5. At higher pH, the hydrolysis process begins and results in the release of free ethylene (24).

Ethephon degrades in soil under aerobic conditions at 20℃. The half-life time is from 2.7 to 37.6 days, depending on the type and pH of the soil. It has two major degrades named ethylene (gaseous) and 2-hydroxyethylphosphonic acid (nonvolatile). The mobility in soil is moderate to low, and in neutral and alkaline environments is hydrolyzed rapidly. Generally, it considered as not contaminant for water and environment (25).

2.2.2 Pharmacokinetics of Ethephon

The absorption, distribution and excretion of ethephon has been evaluated in rats. Ethephon (dissolved in normal saline at the concentrations of 50 and 1000 mg/kg/) was administrated orally for 14 days and the residue of ethephon was determined in various tissues. Findings of *in vivo* studies show that ethephon is rapidly absorbed in the gastrointestinal tract in rats (78-84%) within 2-3 hours. It is widely distributed in body organs such as liver and kidneys and is mainly excreted in urine (50-60%) and faeces. Ethephon showed no potential for accumulation in body tissues (26).

Ethephon mainly degrades to ethylene (96–98%) and a small fraction of it (2–4%) is excreted as carbon dioxide. Also no significant differences was observed in absorption and excretion between sexes (27).

Ethephon degrades in plants very rapidly and at physiological pH releases ethylene, which is absorbed in the plant tissues and intervenes in the growth process.

Ethylene ($H_2C=CH_2$) is a gaseous hydrocarbon which is able to induce ripening and senescence in plant tissues (28). The two major residues found in plants following ethephon application, were ethephon or its metabolite 2-hydroxyethyl phosphonic acid (HEPA), thus some studies suggest that the toxic potency of HEPA is more than ethephon (29).

At alkaline pH, ethephon is converted to a cyclic oxaphosphetane (3%) that is an actual BuChE inhibitor (30). It has been demonstrated that ethephon residues are stable for two years when stored at $\leq -20°C$ if they are in water sources (26). In plants, ethephon swiftly metabolizes to phosphate, ethylene, and chloride. However; ethylene gas is the main metabolites in plants. Remains of monochloroacetic acid may also be discovered in plants under ethephon treatment (31).

2.2.3 Agricultural Applications of Ethephon

Ethephon is a growth regulator which is used for artificial ripening in plants. Ethephon generally is used for stimulation of flowering, coloration, abscission and increasing the shelf life of fruit, vegetables, and cereals to meet marketing requirements (28). Ethephon affects plants by regulation of the growth phases or by application to the various growth sites (29).

It is frequently used on plants such as wheat, rice, tomato, cotton, coffee, pistachio, walnut, tobacco and many of fruit in order to help these plants to reach faster fruit ripeness. However, the application of ethephon changes due to its concentration, the plant species and the time of application For instance in rubber trees, stimulates latex flow (29) and in pineapple induces the flowering. The pineapple growers also widely use ethephon to initiate reproductive development of their products. Ethephon often is used in spray form on green and mature pineapples to degreen them and achieve marketing requirements. While pineapple can be fruited and harvested several times per year by using ethephon, some harmful effects on quality of fruit can be occurred (31).

Ethephon induce fruiting during several weeks and promotes early boll opening in cotton plant. It also enhances flowering in a plant which facilitates and improves the efficiency of scheduled harvesting. The quality of harvested cotton is also ameliorated by using ethephon. The ethephon can be applied onto the plant leaves and foliage by hand sprayer or either ground or aerial equipment (32).

Ethephon is presently registered in the U.S. for use on various fruit and plants. It may also be used in combination with Terpal (another plant growth regulator) and Terpal C (chlormequat-chloride) (33).

In sweet potato, releasing and accumulation of ethylene leads to increasing the concentration of Ca^{2+} ion that mediates ethylene signaling and finally causes a specific physiological response such as leaf senescence and the increase of H_2O_2 amount. Also, ethephon plays a key role in the genetic expression of SPCAT1 mRNA gene, which is relevant to senescence (34).

Studies on the molecular mechanism of flowering indicated that some genes play a significant role in ethylene biosynthesis in pineapple. AcACSs are three isolated genes which synthase the aminocyclopropane carboxylic acid (ACC) which is important in ethylene production (35).

In another experimental study, findings demonstrated that stimulation by ethephon may result in expression of specific genes of flowering such as AP1-like, AG-like, and CAL-like. The expression of such genes in floral organ of the plant lead to artificial ripening. Although other factors such as the concentration of ethephon, the level of plant maturity and climatic factors also play role in flowering. These results show the importance of molecular and genetic studies to discover the action mechanism of ethephon (35).

Results of an investigation on ethephon treated grapevine, suggest that ethephon induces an increase in the copies of genes related to the biosynthesis and accumulation of phytoalexinand stilbenes (antimicrobial substances). In conclusion, ethephon

treatment promotes a protection mechanism in grapevine against some pathogens such as powdery mildew (36).

Application of ethephon usually results in latex stimulation in rubber trees. A research study on the molecular mechanism of ethephon in rubber trees indicated that ethephon can affect the expression of responsible genes which are involved in ROS production, ROS scavenging and biosynthesis of rubber (37).

Plant studies in olive demonstrated that various genes are included in oxidative stress which is activated by the ethephon treatment in fruit. Additionally, it has been discovered that ROS caused abscission of olive leaves in response to ethephon treatment. (38).

In plants, senescence has a developmental function. Free radicals, especially those derived from oxygen, play an essential role in senescence. Disturbance in the balance between the production of ROS and antioxidant defenses induces senescence. It has been shown in a study that reduced glutathione levels were decreased and H_2O_2 levels increased significantly 72 h after ethephon administration in sweet potatoes. These events are correlated with leaf senescence. Diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, causes the delay of leaf senescence and reduces the H_2O_2 elevation. In other words the senescence induced by ethephon signaling van be decreased by DPI.(39, 40)

Studies revealed that ethephon potentially can induce the transcription of some genes (HbM T2) related to metallothioneins (MTs). These cysteine-rich proteins have low molecular weight and also play significant roles in vital intracellular ion homeostasis. They also detoxify heavy metal ions and protect the cellular structure against oxidative damaged (41).

2.2.4 Toxicity of Ethephon

Ethephon is a member of organophosphorous pesticides since 1973. Although the hazards of organophosphorous compounds are well-known but the toxic interactions of mixture of the compounds are still not completely studied. Especially, toxicity of the compounds on growth hormones and reproductive system is one of the most important issues which environmental groups and organizations are concerned. Similarly, ethephon toxicity is literally very limited, because ethephon is metabolized to ethylene quickly in the plants.ahmed gan (13, 27).

However, there are limited certain data related to the adverse effects of this agent or the residues of it on human health. In such situation, the maximum residue levels (MRL) and tolerance limits for ethephon in production of agricultural primary crops is 0.5-30 mg/kg and the ADI is 0.03-0.05 mg/kg by weight per day. Based on a study in female rats the NOAEL was 100 ppm which is equal to 22 mg/kg bw per day and the LOAEL was 3000 ppm (equal to 530 mg/kg bw per day) (42).

Plant studies also show that the concentrations higher that 4 g/L of ethephon are phytotoxic and can damage plant tissues. Ethephon also has an inhibitory role on growth of *Ipomoea cairica* and all leaves fell and the plant was killed totally at the concentration of 7.2 g/L. Ethephon is an alternative herbicide to control the invasive species of *Ipomoea cairica*. Ethephon promotes the production of abscisic acid and the accumulation of H_2O_2 at the concentration of 4.06 g/L. Moreover releasing abundant ethylene causes tissue damage in the *Ipomoea cairica.* In higher doses ethephon can kill the leaves, stems, and roots of this plant (28).

A study data displayed that exposure to the combination of two pesticide, chlorpyrifos (CPF) and ethephon, can cause expanded clinical responses due to the cholinergic effects of these agents. So the use of these compounds is also a risk for individual health (43).

The plant growth regulators cause morphologic and physiologic alterations in plants that lead to qualitative or quantitative changes in final products (29). However, the performed long-term animal studies showed no significant morphologic changes, clinical signs, hematologic and carcinogenic results related to ethephon treatment in both rats and mice (2).

The toxicity level of ethylene is very low and there is no registered risk for human health in exposure to atmospheric levels of this gas. Although, in situations of excessive exposure, symptoms such as headache, dizziness, anesthesia, drowsiness are observable. Also eye and skin contact with aqueous solution may cause frostbite. And there are no reports of adverse effects of ethylene through skin exposure. In rats, function of liver was affected after exposure to low concentrations (44).

2.2.5 Acute Toxicity of Ethephon

Acute toxicity generally happens after exposure to a single dose of toxicant agent after several exposurements during a short period.

Studies on rat show that acute oral LD_{50} is 1.56 g/kg for males and 2.63 g/kg for females. On the other hand, oral LD_{50} value for mice is 2.850 g/kg; 5 g/kg for rabbits and 4.2 g/kg for guinea pigs. Furthermore LC_{50} of ethephon is 4.52 mg/L by acute inhalation when the purity of ethephon is 75% (2, 27).

Results of dermal studies of ethephon on rabbits demonstrated that 1-4 h of exposure with 0.5 mL ethephon (with 70% purity) resulted in erythema (slight to well-defined degree) in both male/ female rabbits (27).

In an *in vivo* study, the various dose levels of ethephon (0, 50, 100, and 200 mg/kg/day) were administered by gavage to 20 rats in both male and female sexes. Then the activity of plasma cholinesterase and brain cholinesterase were measured and findings demonstrated a significant difference between the activity of brain cholinesterase in controls and other groups. However; the red blood cell cholinesterase activity was not different from the controls in any dose group (23).

In most previous studies, obtained results showed no systemic toxic effects of ethephon and no treatment-related effects on body weight, the behavior of the animal and clinical chemistry in short-term treatments (23).

However, some symptoms such as gastroenteritis, respiratory disorders and necrotic hepatitis, pancreatic tumors, nephritis, and brain mineralization were observed after oral administration. Also, some teratogenic effects such as folded retina and absent tail were founded at higher exposure doses in the mouse. Some observations in mice suggest the mutagenic effects and chromosomal aberration induced by ethephon (45, 46)

The inhibitory effect of ethephon on cholinesterase in red blood cells and plasma has been demonstrated. These effects occur at low levels of exposure and even show no clinical symptoms (2).

Ethephon, at the concentration of 2.5μM has an inhibitory effect on human butyrylcholinesterase (BuChE) by producing a covalent adduct on the active site of BuChE serine 198. In other word, ethephon phosphorylates the BChE active site at S198. (33).

Study on Albino rats has been confirmed that after exposure to 200mg/kg of ethephon (neutralized in Na_2Co_3), the mouse liver esterases showed lower sensitivity to ethephon in compare to the plasma BchE both *in vitro* and *in vivo.* Thus, BChE inhibition is the most sensitive marker for diagnosis of ethephon exposure (47).

Both human and animal studies suggest that the potential effect of ethephon is to inhibit the activity of butyrylcholinesterase (BuChE) and acetylcholinesterase (48) in plasma. As BuChE was found more sensitive esterase target for ethephon, it has been used as a sensitive marker in ethephon exposure studies (49).

2.2.6 Chronic Toxicity of Ethephon

Chronic toxicity is the dverse effects of long term exposure to toxicants. Studies of chronical toxicology have important role in discovery of unknown new toxicants.

In a study on both male and female dogs, ethephon was applied to the food of 4 dogs at the concentrations of 0, 5.0, 25.0, or 187.5 mg/kg/day for 13 weeks. Results demonstrated the decrease in the activity of plasma cholinesterase in both sexes at all dose levels (2).

A chronic toxicity study has been performed on 85 Swiss albino mice. The animals were fed by administrated diets including 0, 4.5, 45 and 150 mg/kg/day of ethephon during 78 weeks. The inhibition of plasma cholinesterase at the doses of 45 and 150 mg/kg/day in both males and female animals were measured. The results displayed that activity of cholinesterase is dose-dependent in female animals. A considerable depression of activity of RBC cholinesterase at the doses of 45 and 150 mg/kg/day, was objected. While in females, the same depression was exhibited at the dose of 4.5 mg/kg/day (24).

A conducted carcinogenicity study in CD-1 mice using 70.6-72.1% ethephon purity, with concentrations of 0, 15.5, 156 and 1630 mg/kg/day during 78 weeks showed no indication of carcinogenicity (20).

A recent rat study also indicated significant degenerative changes of hepatocytes in rats after administration of 200 mg/kg/day of ethephon for two weeks. The inflammatory and increase of kupffer cells were observed in the liver. Also, the body weight was found decreased obviously. Results showed that ethephon has hepatotoxic potential (25).

Toxicological studies in reproductive system also showed that ethephon causes a significant decrease in sperm viability and mobility and induces oxidative stress in the reproductive system. However; these gonadal toxic effects were dose and time-dependent (50).

The cytotoxic effect of ethephon in HepG2, Hep2, and Vero cells were also studied and obtained results confirmed the relation between cytotoxicity and increased doses of ethephon (29).

Other chronic toxicology studies show that ethephon, as a corrosive agent, also causes skin irritations (26).

Although some genotoxic studies in rats considered ethephon as a non-genotoxic and non-oncogenic agent in another side, ethephon is a synthetic compound which is potent to react spontaneously to generated phosphoproteins. Such reaction results in an alteration of their biological activity. Indeed, the cellular function is affected by ethephon. However; the teratogenicity of ethephon has been evaluated in rabbits and findings showed no fetal variations or malformations (51).

US EPA (the United States Environmental Protection Agency) has recognized that use of the ethephon in agriculture is not harmful for the population (2).

The Agency also has emphasized that neurotoxicity (both acute and subacute) studies on mammals are required to support the current level of registration of ethephon (2)However; currently some of the ethephon containing products have the signal words such as "Danger," "Warning," or "Caution" dependent on the product (33).

2.3 Developmental Toxicity

The developmental deficiency is an anomaly in the structure or function of the body which is a detectable consequence of a modification in normal developmental process. It has been estimated that approximately 2-3% of all live newborn infants suffer from a major developmental deficiency. The most recent global reports showed that in 2015, about 3.6 million children aged 1–9 years have disorders such as autism; moreover, there are millions of people which are living with idiopathic developmental disabilities. Yet these are just 2 examples of other frequent neurological deficiencies. Still there is no exact global data of prevalence of developmental disabilities (52).

Developmental toxicity is any structural abnormality or functional alteration, reversible or irreversible, which interferes with normal growth, differentiation in development of an organism and results in malformation, deficiency or death. The

environmental agents including drugs, toxic chemicals or physical factors may result in developmental toxicity. In other words, it is the study of adverse effects of toxic agents on the development of a living organism during parental development until maturation. (53).

Developmental toxicants have various effects dependent on their dose and duration of exposure. These substances which cause adverse effects on various stages from early development (embryogenesis) to birth are called teratogens (2, 54). Additionally, it has been proved that such toxicants influence more during early development than in adult age. Developmental disorders such as physical abnormalities, organ deformities, behavioral and learning difficulties may continue through the entire lifetime (55).

Although the main causes of these defects are still controversial (such as malnutrition, radiations, genetic disorders and etc,) but we know that exposure to chemicals and toxins may end in such abnormalities. Generally, nearly 3% of developmental deficiencies are imputable to the exposure of the mother to chemicals and toxic substances, especially environmental compounds (53). The main reason to response this concern is that there are more than 90,000 chemicals in commercial and industrial use which just about 60,000 of them have been evaluated and classified for their potential toxicity (56).

According to the human health consideration and regarded to the environmental pollutants and their influence on developing embryos or fetuses, urgent attempts by scientists are required to discover these dangers. The related organizations must adopt protection actions for risk assessment in this issue and protect human against these hazards (53).

Several environmental agents are proved as developmental toxicants in human such as lead, ionizing radiation, polychlorinated biphenyls, and methylmercury. While there are other suspected developmental toxicants for human on the basis of obtained data from several experimental animal studies such as some pesticides, phthalates, heavy metals and alcohols (2).

This information encourages a strong basis to design animal experiments and use of obtained data in conducting the risk assessment of the human. However; there is no clear evidence of developmental toxicity hazard of many agents which have been found toxicants in animals. And human data are too limited to predict the cause and effects of developmental toxicity (51). The four major indications of developmental toxicity due to FDA guideline are fatality, growth alterations, dysmorphogenesis (structural abnormalities) and functional destructions (57).

Once fertilization occurs, the environmental toxicants are able to pass through the mother to the embryo or fetus through the placental barrier. During the period of formation of major organs, the first 14^{th} to 60^{th} day of pregnancy, the fetus is very sensitive to chemicals and medications and most of malformations or deficiencies happen in this critical duration. However; due to the type and dose of toxicant or time of exposure, the fetus may be damaged at any time of pregnancy (58).

In recent years, researchers have an increasing focus on *in vitro* developmental toxicology models for evaluation of teratogenic agents. The main goal is to find trustworthy and predictable *in vitro* assays which are replaceable with *in vivo* model for evaluation of teratogenicity. The mES tests are the most widely used model for screening of developmental toxicity (59).

This method has been helped the researchers to reduce the use of animals in embryonic experiments and has been validated by European Union Reference Laboratory for alternatives to animal testing (51).

These *in vitro* assays allow us to evaluate the differentiation process and to identify the adverse effects of pollutants and developmental toxicants in this stage of growth (60) .

2.4 Stem Cell

After fertilization in mammals, the fusion of male and female gametes occurs, the zygote forms and the parental development initiate. In the first three days the first cleavage happens and on the fourth day, the young embryo consists of 16-32 cells called blastomere or morula stage which during this step, blastomere separates into embryoblast and trophoblast. The embryoblast is the mass of cells lies inside the blastocyst cavity and forms at the first steps of embryonic development, even before implantation and will ultimately provide the whole structures of the fetus which is completely surrounded by a single layer of cells called trophoblast (61).

Stem cells are the remarkable cells in the body with an exclusive ability to renew them and develop into different specialized cells both in early life and during growth. Furthermore, in many tissues, they have functions such as part of repairing system and dividing without limitation. After the division of stem cells, the new cells are potent either to remain a stem cell or continue their life as a more specialized type of cells with more function such as the muscular cell or blood cell, skin, heart, and brain cells (62).

Both of the stem cells and progenitor cells have important roles in repairing system of tissues in adult organisms. The progenitor cells, like stem cells, are tended to differentiate into specific cell types, however; it is more specific than a stem cell and is able to divide for limited times (63).

All stem cells have three main characteristics:

1. They are potent to renew themselves through cell division, even after a long inactivity period.
2. They are unspecialized cells
3. Under some physiologic or experimental conditions, they are capable to become specific functional cells (64).

In some organs such as gut and bone marrow, damaged or worn out tissues require to be repaired or replaced with the new one. In such organs stem cells are

divided regularly. However, in some organs, such as the heart or stomach, stem cells divide individually under exclusive conditions (65).

In mammals, stem cells are divided into two main categories: the embryonic stem cells (ES cells) and the somatic or adult stem cells (66). The stem cells which are derived from the inner cell mass of blastocysts are named embryonic and the stem cells which are detected in different tissues are called adult stem cells. The essential role of ASCs is to maintain and repair the tissues which they are found in. (67). There are three known reachable sources of ASCs in mammals:

- The adipose tissue, which stem cells are extracted by liposuction.
- The bone marrow, which stem cells are extracted by harvesting typically from the femur and ribs. (mesenchymal or skeletal stem cells)
- The blood, which is require extracting stem cells by apheresis and are also called hematopoietic stem cells. In this method, blood is drawn out from the donor's body and passed completely through a machine which extracts the stem cells. Then after, the other portion of the blood returns to the donor (68).

The adult stem cells continuously increase the different types of cells of the tissue which they are consisting of. Due to the experiments, it is acceptable that the adult stem cells of one main source normally generate the specialized cells of the same tissue. For instance, the adult stem cells of the bone marrow (hematopoietic) generally differentiate into various blood cells such as red and white blood cells. There are several specific factors or conditions which allow stem cells to stay unspecialized or give rise to various specialized cells (69).

The process of generation of the specialized cell from unspecialized stem cell is called differentiation. During this process, the cell passes through different stages which in each step, it becomes more specialized. It has been discovered that there are signals both inside and outside stem cells that provoke each step of the whole differentiation procedure. It has been obvious that the internal signals are under the cell's genes control

and the external signals of differentiation are chemicals either in the body or the environment. The cellular proliferation is the process of increasing the number of cells and as cells differentiate, the rate of cell proliferation usually decreases (70).

2.4.1 Classification of Stem Cells

Stem cells have been classified into four main types by their differential potency.

1. **Totipotent stem cells:** They are potent to differentiate into all cell types. The example is the zygote cell and the first divided cells of the zygote.
2. **Pluripotent stem cells:** They are capable to differentiate into almost all cell forms. Such as embryonic stem cells and derived cells from the mesoderm, endoderm, and ectoderm germ layers.
3. **Multipotent stem cells:** They can differentiate into intimately related cells. An example is the hematopoietic stem cells which differentiated into red or white blood cells.
4. **Oligopotent stem cells:** They are potent to differentiate into a few cells such as lymphoid cells.
5. **Unipotent stem cells:** They can only generate cells of their own type; however; has the potency of self-renewing. Examples include (adult) muscle cells (62).

Classification of stem cells based on their potency has been shown in figure 2.4.

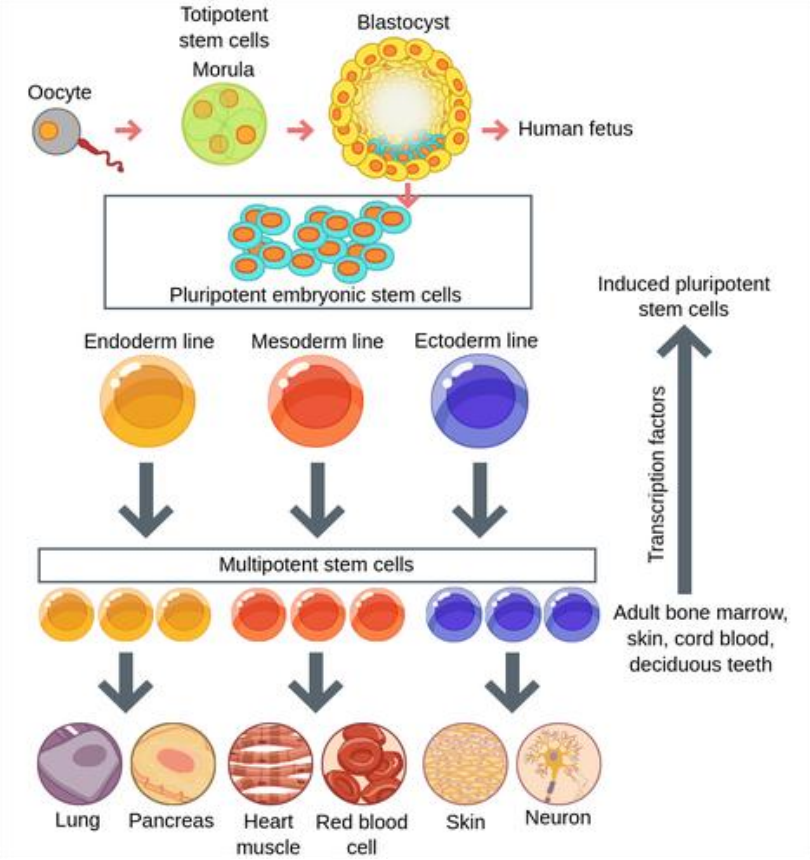


Figure 2.4. Classification of stem cells (71).

There is another type of stem cells which have been engineered in the laboratory. These cells have been produced by conversion of adult tissue-mature cells, into cells that behave like pluripotent embryonic stem cells and called Induced Pluripotential Stem cells (IPS cells) which have characteristics of embryonic stem cells (72).

These types of cells actually are very critical and controversial tools to assist scientific researches in the evaluation of developmental toxicology and new therapies (73).

The mES cell testing is one of the subdivided classes of *in vitro* models in developmental toxicity which have some advantages such as more accessibility, no animal use, and easy performance. Indeed in evaluation of the chemical effects while the

differentiation process; the stem cell testing is one of the best-verified methods to classify the toxic power of substances (74).

2.5 Neural Development

The biological definition of neuronal development is the process of the formation of neurocytes during development and includes the stages of neural proliferation, neural differentiation (from progenitor stem cells), neural migration (migration of immature cells to their final position), synaptogenesis, axon guidance and synthesis of neurotransmitters. Formation of the neural system is one of the earliest stages of development which begins during first days after fertilization and completes after birth. Disturbance of the neurodevelopment process mostly is irreversible and generally results in long-lasting disorders (75).

At the 1990s, scientists showed that stem cells exist in the adult brain and are potent to generate the brain's two main cell types: The non-neuronal cells (astrocytes, oligodendrocytes) and nerve cells (neurons).

Normally this process has two main types of mechanisms which are called activity-independent and activity-dependent.

a) The activity-independent types are mechanisms of differentiation, migration and axon guidance that genetic programs have an important role in them.
b) After axons reach to their final target, the activity-dependent mechanisms start and synapses are generated.

The neural tube stem cells generate two main classes of cells called neurons and glia which both types are potent to differentiate into various types of functional cells (61).

In recent years, the developmental studies with a broad interest in neurodevelopmental problems have increased. The main cause of this enormous concern is the change of the lifestyle and environmental factors which contribute to such

disorders. Due to studies, the developmental neurotoxicity is among the least studied issues and (59) there are very limited data related to neural toxicants which contrast with the increasing amount of environmental toxicants. Additionally, it has been proved that some of the environmental chemicals are potent to influence the nervous system directly (56).

The importance of these studies is because of the immature blood-brain barrier that makes developing brain more sensitive (76).

Epidemiological studies confirmed that chemicals such as methylmercury, lead, arsenic, PCBs, toluene, and ethanol, manganese, fluoride, chlorpyrifos, dichlorodiphenyltrichloroethane, tetrachloroethylene, and the polybrominated diphenyl ethers are the most important neural developmental toxicant (60).

The current guidelines for neurodevelopmental toxicity studies are based on *in vivo* experimentations. These tests are costly, time-consuming and also there is no possibility to test various types of chemicals and agents. Due to these reasons, there are very limited data to identify the exact neurodevelopmental toxicants and their action mechanism (59).

Recognition of the mechanism of brain function is one of the most controversial issues in new medicine. The ES cells, mES, and IPS cells in mouse and human, have appeared as strong tools in discovering the complicated queries in neural differentiation, neurobiology and brain developmental disorders. As stem cells differentiate into various neural subtype cells, the *in vitro* models are pretended to be proper relevant models to study the molecular and cellular mechanism, pathophysiology of neural development disorders and the whole *in vivo* developmental processes (77).

Recent studies have demonstrated that stem cell-derived neural progenitors and neurons help to regenerate the damaged neural cells which results in the wide use of cell therapy (77).

Among *in vitro* studies, neural cells were one of the first cell lines which were differentiated from mES cells in the laboratory. Such studies opened a new window to understanding the pathways of neural differentiation and the related specific markers. (78).

As shown in figure 2.4, observations of studies helped scientist to show the schematic pathways of neural development from ES cells into different types of neural cells (77).

2.5.1 The Pathways of Neural Differentiation

As mentioned before, there are some responsible signals in stem cell models which cause imitation of differentiation and result in proper development. In the adult body, the division of undifferentiated ES cells is regulated by these signaling pathways. Therefore knowing the signaling molecules and related pathways are important in discovering the complete process of differentiation. The first step of this pathway is the self-renewing of pluripotent stem cells that is possible by its renewing factors such as Activin A, BMP-4, FGF basic, LIF, and TGF-β (63).

Transforming Growth Factor beta (TGF-β) is a cytokine, belongs to a multifunctional superfamily of proteins with different isoforms such as TGFβ1, TGFβ2, TGFβ3, and TGFβ4. Other members of this family are Activins and Bone morphogenic proteins (BMP) which are produced by white blood cells and all together make a chain of reactions demanding in the regulation of immune system, the proliferation of ES cells, and transition of epithelial-mesenchymal. TGF-β is critical in the signaling pathway of pluripotent stem cells. Lack of members of this protein family in mES cells showed decreased cell proliferation. The signaling pathway of TGF-β has an essential role in the preservation and growth of neural stem cells and is one of the most interesting issues of basic stem cell research in recent years (79).

Activin A, a member of TGF-β superfamily, is a crucial cytokine, which is crucial for tissue morphogenesis and tissue repair, neural development, the function of

immune system, hematopoiesis and generation of the reproductive system. This protein binds to Activin II receptor, in various cell types and causes activation of other cytoplasmic proteins called Smad proteins by phosphorylation process. The phosphorylated Smad proteins form the Smad complexes accumulate in the nucleus and finally bind to the promoter segment of target genes. The expression of these genes results in initiation signaling pathways related to the developmental process or other series of functional actions (80).

Bone morphogenetic proteins (BMPs) are a class of morphogen proteins that are important for building tissue structure all through the body. The base of their nomination is their first discovery in osteoinductive properties, though recent evidence show that BMP signaling is a critical regulator of stem cells in both the central and peripheral nervous system. BMP signaling has an important role in the maturation of stem cell and neural development (81). BMP-4 signals through Activin and is important in the formation of mesenchyme and tissue repairing but also suppresses the neural formation. It has also other homodimers which can act greatly in osteogenesis (79).

Fibroblast growth factors, FGF 1-22, are signaling protein family which act in various growth processes and any lack or deficiency in their function ends in growth deficiencies (82). FGF basics (also known as FGF-2) are growth factors with an angiogenesis function. However; it also contributes to the wound healing, formation of brain and learning process and the morphogenesis of heart and bone. Generally, at the time of inflammation or in tumors, it has been upregulated. This factor is regulated by other factors such as heparin, FGF –BP, fibrinogen and alpha 2-Macroglobulin which act as cellular receptors of FGF-2 (83).

It has been suggested that FGFs induce the neutralization effect regularly by inhibition of BMP-signaling pathway. They phosphorylate the Smad1 proteins or repress the expression of genes associated in BMP-signaling. Alternately, recent animal studies confirmed that the role of FGF-signaling in neural differentiation into ectoderm is

independent to the BMP pathway. However, human studies suggest that BMP pathway is essential for neural development (82).

LIF (Leukemia Inhibitory Factor) is an IL-6 class multifunctional cytokine which inhibits the growth of cells by inhibition of the differentiation system. This cytokine is secreted by various cell types and is potent to induce differentiation of myeloid leukemia cell into the macrophage. In human, the LIF encoding gene is localized on chromosome 22q12 and in the mouse is located on chromosome 11 (84).

In both mouse and human, LIF is a long chain polypeptide which assists in self-renewal of ES cells, represses ES cells differentiation through the blockage of related differentiation programs. In human and rodents, LIF plays role in embryo implantation in uterine, supports the pluripotency of ES cells, reproduction of progenitor cells and also in T cells maturation. It causes promotion of motor neurons and myelination of oligodendrocyte, LIF also plays a role as an endocrine factor for production of Adrenal hormones (cortisol and aldosterone) and causes differentiation of adipocytes and cardiac smooth muscle cells. Recent experiments indicated the role of LIF in tissue reconstruction following brain injuries (84).

The mesoderm pathway ends in cardiac progenitors, hemangioblasts, and mesenchymal stem cells. The products of endoderm pathways are intestinal stem cells, immature lung cells, hepatocyte progenitors, pancreatic progenitors and beta cells. Neural progenitors have important expressed markers which are known with them. In the ectoderm pathway, the products are neural progenitors and epithelial stem cells (82). Stem cells are distinguished from other cells based on tissues which they are harvested, their ability for differentiation, the developmental stage and their expressed genes. There are specific genes such as Oct-4, SOX2, NANOG, Nrf2, Nestin, NSE, MAP2 and h.

β-Tubulin class III (TUBB3), which are used as biomarkers in evaluation of various stages of neural differentiation.

a) Oct-4

In mouse, Oct-4 is detectable on chromosome 17 and in human, it is on chromosome 6p21.33 and some of its pseudogenes have been discovered on chromosomes 1, 3, 8, 10, and 12. The Oct-4 protein is Oct-4 protein is in the nuclei of elementary glioma cells. The Oct-4 has a key role in both sustaining and re-establishing of the pluripotential specificity of stem cells. Indeed, Oct-4 is a maternal factor in oocytes which is effective in the embryo during the preimplantation stage (85). The overexpression of Oct-4 alone leads to the generation of Induced pluripotential stem cell but does not lead to spontaneous differentiation or loss of pluripotent cell identity (86).

In vitro studies show that low expression of Oct-4 or any deficiency in function of this gene in mES cells causes fail in the formation of ICM and lessening of pluripotency and results in differentiation of mES cells to trophoectoderm. Therefore, the level of Oct-4 demonstrates the potency of renewing in stem cells, however; in differentiated cells, the level of this marker has been decreased. Normally Oct-4 forms a heterodimer with SOX2, which is another stem cell marker, and bind to DNA together (87).

The adult neural stem cells gain the pluripotency specialty only after overexpression of Oct-4. These cells then are allowed to be differentiated into the midbrain neurons. In rat models, neurons are developed from neural stem cells which have been reprogrammed by Oct-4 and improve the behavioral motor deficiencies in Parkinson disease. Further, it has been reported that adult neural stem cells are capable to differentiate into the functional neural cells by Oct-4 induced pluripotency (88). Also observations showed considerably higher expression of the Oct-4 in cervical tumors compared to the normal cervix (89).

b) SOX2

Sex Determining Region Y-Box 2 (SOX2), is associated with a great transcription factor family. It is an intronless gene and based on its critical role in stem cell differentiation, it is crucial for embryonic development. SOX2 plays role in the

provision of required protein structures in the formation of tissues eespecially in eye development and regulation of activity of genes by attachment to the particular regions in DNA. This gene lies within the intron of SOX2OT gene and products of this gene are vital in stem cells which maintain in the central nervous system.(90)

SOX2, Oct-4, and NANOG, are able to control the gene expression in pluripotential stem cells to maintain the pluripotency. Moreover, SOX2 is able to act in the reprogramming of somatic cell, reverse some epigenetic modifications of differentiated cells and direct differentiation of pluripotential stem cells into the neural progenitors(89)

It is remarkable that SOX2 regulates gene expression in eye and stomach, so any mutation of it may result in a drastic deficiency in the structure of optical system such as syndromic microphthalmia that is a kind of eye malformation (91).

c) NANOG

In fact, NANOG is a gene which encodes the NANOG protein (a transcription factor) in ES cells and has data displayed that pluripotency marker genes are Oct-4, SOX2 and, NANOG. They are capable to induce cellular differentiation in the three germ layers and have also a key role in pluripotency of stem cells (90, 91).

The NANOG protein is an activator for the promoter of SOX2 gene, which sustains the expression of SOX2. Alongside the effects of NANOG in the embryonic phases of mammal's life, the ectopic expression of NANOG in the adult stem cells may lead in reestablishing of the proliferation and differentiation ability which is missed due to aging or cellular senescence (92).

d) Nrf2

This gene is also known as the Nfe2l2 nuclear factor, erythroid derived 2 and both in human and mouse, encodes a transcription factor which is called leucine zipper protein. The function of this factor is regulation of the expression of antioxidant proteins that protect cells from oxidative damage (93).

Multiple drugs which are used in the treatment of oxidative stress diseases act by stimulation of this gene. In normal circumstances, Nrf2 is maintained in the cytoplasm and is related to a cluster protein. In oxidative stress conditions, this protein quickly degrades the Nrf2 gene moves to the nucleus and binds to DNA which results in transcription of anti-oxidative genes. Therefor Nrf2 gene has an essential role in anti-inflammatory and detoxification situations in mammals and mutation of this gene cause multisystem disorder with loss of growth, immunodeficiency and neurological symptoms. (94, 95)

e) Nestin

This gene has been identified on the q arm of chromosome 1 in human and primarily is expressed in stem cell of the central nervous system (CNS) and encodes a protein called intermediate filament. However; it cannot be found in mature nerve and *in vitro* cultured cells. Expression of this gene is very critical in neural differentiation pathway especially in renewing of stem cells and proliferation stimulated by mitogens. However; it has been also discovered in other population of stem cells such as hematopoietic progenitors. The brain cancer and Ependymoblastoma are genetic diseases related to the delicacy of expression of this gene (96).

f) NSE

Neuron-specific enolase (NSE) or Gamma-enolase or neuron-specific enolase is an enzyme that in humans is encoded by the NSE gene (ENO2*).* This phosphopyruvate hydratase is one of the three enolase enzymes which have been discovered in both mature neurons and original neuronal cells. Therefore this is a good marker for the detection of neural and peripheral neuroendocrine differentiated from stem cells (97).

Animal studies on rats and primates confirmed that neuron-specific enolase (NSE) exist in two forms, the γγ which is expressed in neurons and the αγ that is expressed in microglia, oligodendrocyte and astrocyte. It has been also indicated that NSE acts in the regulation of neuronal growth and differentiation (97).

g) Map2:

In rat and mouse, Map2 is the gene, which encodes the Microtubule Associated Protein2, a long amino acid chain, which interacts with protein kinase A and tubulin, and involves in assembling of microtubules, that is an indispensable stage of neurogenesis. Many similarities (>80%) have been found between Map2 gene in human, mouse, and rat. These proteins involve in determination and stabilization of dendritic shape of neurons during neural development both in normal and malignant cells. Any deficiency in the expression of this gene may cause neural degenerations. The role of Map2 in some diseases such as Alzheimer, schizophrenia and disorderly mood has been confirmed (98, (98).

h) β-Tubulin Class III (TUBB3)

This gene, also known as the TUBB3 gene, is located on chromosome 16 q24.3 and modified splicing ends in multiple transcript alternatives. This gene encodes the β-tubulin III, a microtubular protein of the tubulin family which is found particularly in neurons, also involves in neurogenesis and axon guidance both in human and mouse. Beta tubulins are one of the essential protein families (α and β) which heterodimer and assemble the muscle formation. Therefore it is a specific marker for separation of neural from glial cells. Disorders such as congenital fibrosis in muscles, developmental abnormalities, and brain malformation are related to mutation of this gene (99).

Research on mutation of β-tubulin identified that any mutation results in disruption of interactions between β-tubulin III with other tubulin monomers. Therefore the formation of protofilaments may be damaged. Also, the mutations may reduce the affinity of this protein for motor proteins, which are required in the regulation and maintenance of axonal transport. (99, 100).

2.6 Stem Cell in Toxicology

It is evident that during the last century, the pattern of disease among the population has been turned from infectious disease to the upper respiratory disease such as asthma , and neurodevelopmental or congenital deficiencies and cancer.

Animal studies have provided evidence that the embryo can be adversely affected by therapeutic and chemical agents. There is an extreme attention to find novel predictive models to assess the risk of the environmental toxicants before they can harm embryo in both pre-implantation and post-implantation. Traditional developmental toxicology is depending on achieved data from animal studies especially the vertebrate species. Concerning this, new *in vitro* screening models have been established in predictive toxicology to decrease both the expense of the experiments and use of animals (101).

During the past two decades, *in vitro* screening tests have been broadly used in drug pre-clinical screening and toxicity testing. The pluripotency of stem cells provide these cells as a good opportunity in the evaluation of the environmental toxicants in contrast to the traditional screening tests which were depended on evidence of animal studies especially the vertebrates. Besides the traditional methods are generally expensive, prolonged and need a large number of animals (102).

In 1981 for the first time, the mES cells were derived from mouse embryo by scientists. After some years, in 1998, researchers isolated the ES cells and could grow these cells in artificial culture in the laboratory (4).

Stem cells have unique characteristics such as the capability to express the inner cell mass markers, unlimited proliferation and potency to differentiate into multiple cell types, which make them proper alternative substitute model for *in vitro* studies in large scale (103).

Also in some diseases or injuries, the adult stem cell therapies have been come up with regenerative medicine and tissue replacement (e.g. bone marrow

transplantation). Recently pluripotent stem cells are suggested to be used potentially in the treatment of some genetic disorders of the immune system, cancer, heart diseases, diabetes and Parkinson (101).

Moreover, stem cells in culture medium go through a comparatively normal developmental process that may help scientists to study the cytotoxicity endpoints (IC_{50}), differentiation endpoint which shows the concentration of agent that causes inhibition of 50% of cell differentiation in (ID_{50}), developmental toxicity, developmental neurotoxicity, reproductive and predictive toxicity testing (104).

Scientists are now capable to grow stem cell artificially in laboratories and transform them (differentiate) into functional cell types of various tissues such as muscles or nerves. Additional research in this field will progress the information about the developmental process of an organism from a single cell and may help us to understand the mechanism of replacement of healthy cells with damaged ones and the regulation of cell proliferation in both normal and abnormal embryonic development. There are also other laboratory tests such as gene expression, flow cytometry, and microarrays which improve the sensitivity of ES cells testing (58).

Various studies have confirmed that somatic adult cells such as fibroblasts are able to be reprogrammed to the pluripotent stem cells by specific transcription factors such as Oct-4, SOX2, NANOG and other stemness genes.

The goal of this study was evaluation of the developmental cytotoxic of a pesticide “ethephon”. The mES cells were exposure to ethephon and the total antioxidant power, and also specific pluripotential gene expressions were examined. Also after the neuronal differentiation, the ethephon treatment was performed on the same cells and effects of ethephon on gene expression during differential period was assesses.

3. MATERIAL AND METHODS

3.1 Chemicals

Chemicals	Country	Company
ES-FBS	England	Sigma
LIF	England	Sigma
mES Cell	England	Sigma
Nuclease-free water	England	Sigma
PD-180970 SB-204741	England	Sigma
Abcam DCFDA cellular ROS detection assay kit	USA	Abcam
Ethephon	Iran	Agroxir
DMEM	France	Biosera
DMEM/F12	France	Biosera
FBS	France	Biosera
Phosphotangestic acid	Germany	Merck
4,5 dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT)	England	Sigma
5-5 –dithiobis-2- nitrobenzoic acid	England	Sigma
Bovine Serum Albomin (BSA)	England	Sigma
Dichlorophenolindophenol (DCPIP)	England	Sigma
K_2HPO_4	England	Sigma
$MgCl_2$	England	Sigma
NADH	England	Sigma
NaHCO3	England	Sigma

Chemicals	Country	Company
PBS	England	Sigma
Phosphate buffer	England	Sigma
Potassium chloride	England	Sigma
Potassium ferricyanide (III)	England	Sigma
Potassium hydroxide	England	Sigma
Propidium Iodide	England	Sigma
Sodium citrate	England	Sigma
Sodium sulfate	England	Sigma
Sulforic acid	England	Sigma
Trichloroacetic acid	England	Sigma
Tris/HCl	England	Sigma
Triton X-100	England	Sigma
Trypsin	England	Sigma
DTNB	Germany	Sigma
TPTZ	Germany	Sigma
Tris-EDTA	Germany	Sigma
Falcon tube	Iran	Soha
Microtube	Iran	Soha
Rat	Iran	TUMS

3.2 Instruments

Instrument	Country	Name	Company
Centrifuge	Germany	universal 320R	Hettich
ELISA Reader	USA	Synergy- BioTek-Microplate Reader	Bio tek
Flow cytometer	China	Bri Cyte E6	Mindrey
Homogenizer	Germany	Ultrasonic processor UI 250v	Hielscher
Nitrogen Tank	England	MVE SC series	Wharton Taylor
PH meter	Switzerland	pH LAB 827	Metohm
Rotator	Germany	2000WB	Memmert
Real-time PCR	Germany	LightCycler® 96 Instrument	Roche
Refrigerator -80	Taiwan	U410-86	Kaltis
Spectrophotometer	Japan	204-05837-01	Shimadzu
Scale	Germany	Pt600	Sartorius

3.3 Preparation of Solutions

3.3.1 Preparation of Cell Culture Medium

Cell culture mediums have various types. Some of them such as Dulbecco's Modified Eagle's medium (DMEM) include the lowest compounds which are required for cell growth such as essential amino acids, vitamins, and salts. Some of them such as RPMI include all amino acids, vitamins, and salts which are completed by addition of minerals and metabolites. Fetal Bovine Serum (FBS) is full of growth factors and dependent on the cell line, different concentrations of this serum are used. In our experiment the medium including DMEM high glucose, L-Glutamine and Sodium pyruvate which was diluted with 10% of FBS and 1% antibiotic

(penicillin/streptomycin) under sterile condition. The prepared medium was restored at 4℃ and was warmed up at 37℃ before use.

3.3.2 Preparation of Gelatin

Gelatin was required to coat the culture flasks and dishes. To prepare the gelatin solution, 0.1 g gelatin was dissolved at 100 ml of sterilized distilled water and was autoclaved for sterilization.

3.3.3 Preparation of FBS

Firstly, FBS was melted for 30 min in 56℃ for deactivation of the complement system. After that, serum was aliquoted under sterile conditions and restored at -20℃. It has been melted at 37℃ each time before use.

3.3.4 Preparation of Trypsin-EDTA

Solution 2.5% trypsin-EDTA 10X was diluted by PBS buffer and was warmed up to the environmental temperature.

3.3.5 Preparation of MTT solution

50 mg of MTT powder was soluted in 10 ml of PBS buffer.

3.3.6 Preparation of Neural Differentiation Culture Medium

1. At first, 19.5 ml of DMEM/F12 medium and 19 ml of neurobasal medium was mixed in a 50 ml falcon tube under sterile conditions.
2. Then, 1 ml of ES-FBS and 7.5 ml of KSR was added to the falcon. 1 ml of B27 and 0.5 ml of N2 was applied to the mixture.
3. Then 0.5 ml of NEAA, 0.5 ml of L-glutamine, 0.5 ml of pen/step and 0.5 ml Mercapto-ethanol were added to the falcon.

3.3.7 Preparation of Retinoic Acid

The Retinoic acidpowder was dissolved in 16.67 ml DMSO. The solution is sensitive to light and should be stored at -20℃.

3.3.8 Preparation of FRAP Assay Solutions

1. Acetate buffer 300 mM

This buffer was prepared with 3.1 g sodium acetate ($C_2H_3Na_2$, $3H_2O$) and 16 ml acetic acid and PH was adjusted at 3.6. This solution is stable for 3 months at 4°C.

2. HCL 40 mM

For preparation of this solution, 3.3 ml HCL was dissolved in 1 lit distilled water.

3. TPTZ 10 mM/L solution in HCL 40 mM (0.031 gr TPTZ in 10 ml HCL 40 mM).

This solution is so unstable and very sensitive to light and the color converts to blue. It is stable for 30 minutes.

4. $FeCl_3$, $6H_2O$ 20 mM which is made by solving 3.24 gr $FeCl_3$ in one liter of distilled H_2O.
5. Ferric sulfate Standard solution ($FeSO_4$,$7H_2O$) 20 mM/L.
   For the preparation, 3.24 gr of the FeSO4 powder was dissolved in 1 liter of distilled H_2O.
6. FRAP reagent was prepared by mixing of following solutions:

1.	Acetate buffer	300 mM	25	ml
2.	TPTZ	10 mM	2.5	ml
3.	$FeCl_3(6H_2O)$	20 mM	2.5	ml
4.	FRAP solution		30	Ml

3.3.9 Preparation of ROS Assay Solutions

i) Preparation of Assay Buffer

To prepare the assay buffer following process was performed.

1. ADP 1.7 mM Reagent

For the preparation, 0.72 gr ADP was dissolved in 1000 ml of distilled water.

2. KCl 130 mM

For the preparation, 9.7 gr KCl was dissolved in 1000 ml of distilled water.

3. $MgCl_2$ 5 mM

For the preparation of this solution, 0.47 gr of $MgCl_2$ was dissolved in 1000 ml of distilled water.

4. NADPH 0.1 mM

For the preparation, 0.048 gr of NADPH was dissolved in 1000 ml of distilled Water.

5. FeCl3 0.1 mM

For the preparation, 0.048 gr of NADPH was dissolved in 1000 ml of distilled Water.

6. NaH_2PO_4 20 mM

For the preparation, 3.12 gr of NaH_2PO_4 was dissolved in 1000 ml of distilled Water.

7. Tris-HCl 20 mM

For the preparation, 3.15 gr of Tris-HCL was dissolved in 1000 ml of distilled Water.

ii) Preparation of Extraction Buffer

For preparation of extraction buffer following process was followed and PH of solution was adjusted at 7.4.

1. EDTA 1 mM

For preparation, 0.2 ml of EDTA 0.5 M was dissolved in 99.8 ml of distilled water.

2. HEPES 5 mM

For preparation, 0.19 gr of HEPES was dissolved in 100 ml of distilled water.

3. KCL 20 mM

For preparation, 1.49 gr KCl was dissolved in 1000 ml distilled water.

4. Sucrose 0.25 mM

For preparation, 85.57 gr of sucrose 1 M powder was dissolved in 1000 ml distilled water.

3.3.10 Preparation of DCFH-Diacetate 5mM

For preparation of this solution, 0.00243 gr of the DCF powder was dissolved in ethanol (purity 99%). The powder should be stored at dark and -20˚C.

3.3.11 Preparation of PI solution

1 mg of PI added to 1 ml of distilled water. This solution stored at 4˚C and be protected from light.

3.3.12 Preparation of RNase

0.2mg of RNase A added to 1 ml of distilled water. The solution was aliquoted and stored at -20˚C freezer.

3.4 Methods

The mESC cell strain C57BL/6 was purchased from Rouyan reproductive biomedicine and stem cell Institute, Tehran, Iran. For culturing the mES cells a previously performed protocol was used (105).

3.4.1 Preparation of Gelatinized Culture Flask

About 2-4 ml (dependent on the size of flask or dish) of prepared gelatin solution was applied to the required flasks and dishes to coat them. Then dishes and flasks were incubated at 37℃ and 5% CO_2 for 45 min. Then the gelatin was discarded. The flasks and dishes were ready to use.

3.4.2 Thawing Frozen Cells

1. Frozen cells were thawed rapidly (< 1 minute) in a 37°C water bath.
2. Then the thawed cells were diluted slowly in a 15 ml sterile falcon, using pre-warmed growth medium to neutralize DMSO.
3. Thawed cells were centrifuged at 1200 rpm for 5 min for preparing cell precipitation without DMSO.
4. The upper liquid was discarded.

5. Then 5 ml of fresh cell culture medium was added to cell precipitant in a 15 ml falcon and cells were suspended.
6. The cell suspension was transferred to a T25 culture flask.
7. The flask was transferred into the incubator at 37℃ and 5% CO_2.
8. Always proper aseptic techniques were used in a laminar flow hood.
9. The flask was observed and the rate of growth and contamination was controlled under the microscope every day and the medium was changed daily.

3.4.3 Changing Culture

When the color of the medium was starting to go to orange rather to pinky, then the medium was changed to keep the correct PH. The medium was warmed up at 37°C in a water bath incubator for at least 30 mins. The old medium was removed carefully and replaced with fresh warmed culture medium immediately. Then the flask was returned to the CO_2 37°C incubator.

3.4.4 Cell Passage

When confluency of cells in each flask was up to 90% ($5*10^5$ cells/ flask), cells were passaged.

1. Cells were washed up by PBS buffer to remove the residue of serum and medium.
2. Then the buffer was discarded and 2.5 ml Trypsin-EDTA was added to the flask and flask was incubated at 37°C, 5% CO_2, and 95% humidity for 2-5 minutes.
3. 5 ml of cell culture medium was applied to the flask to neutralize the trypsin effect.
4. The contents of the flask were transferred to the falcon tube and were centrifuged at 1200 rpm and 25°C for 5 minutes.
5. Then the upper liquid was removed and 2 ml of cell culture medium was added to cell precipitant. Cell counting was done by trypan blue and Neubauer lam.
6. Then the whole cell suspension was added to a new T25 flask containing 5 ml cell culture medium and the flask was incubated at 37°C and 5% CO_2 incubator.

3.4.5 Freezing Cells

Freezing cells at subzero temperatures cause cell membrane damage. Osmotic changes results in irreversible changes in membrane lipoproteins and the permeability of cell membrane alteration. To keep the membrane barrier properties, some compounds are added to the cell medium to protect cells from freezing. One of the most common freezing mediums is 90% FBS/DMSO. By addition of DMSO the formation of ice is prevented. Freezing the cells should be slowly and reducing the temperature must be approximately 1°C per minute.

1. Cells with a concentration of ($1*10^6$) were transferred into the cryotube.
2. 10% of the total volume of cell suspension was DMSO.
3. Then cryotube was transferred to the freezing container including isopropanol.
4. The container then was put at -20°C for one hour and after that the cryotube was transferred into the -70°C for another 24 h.
5. Finally, the cryotubes were put at azote tank -156 °C.

3.4.6 Cell Counting by Trypan Blue Dye Exclusion

1. 20 µL of cell suspension and 20 µL of trypan blue 0.4% were mixed.
2. 20 µL of the mixed suspension was put on the Neubauer lam.
3. The glass cover was put on the central part of the Neubauer lam.
4. The viable cells were counted under the microscope.

As the cell membrane is not damaged in viable cells, color cannot enter the cells. So the viable cells are counted in 5 big squares of the lam (one in the center and 4 in the sides) and finally, the concentration of cell suspension was calculated by this formula.

C=n/v , C= cell concentration

The n= mean of the total number of counted cells in 5 squares

Concentration = (number of cells*10000) / (number of squares *dilution)

3.4.7 Neural Differentiation of mES Cells

For neural differentiation of mES cells we used a previously performed with some modifications (106)

1. The cultured mES Cells were washed up by PBS buffer to remove the residue of serum and medium.
2. Then the buffer was discarded and 2.5 ml Trypsin-EDTA was added to the flask and flask was incubated at 37°C for 2-5 minutes.
3. 5 ml of cell culture medium was applied to the flask to neutralize the trypsin effect.
4. The contents of the flask were transferred to the falcon tube and were centrifuged at 1200 g and 25°C for 5 minutes.
5. Then the upper liquid was removed and 2 ml of neural differentiation culture medium was added to cell precipitant. Cell counting was done by trypan blue and Neubauer lam.
6. As mES cells grow very fast, ($3*10^5$) cells were seeded in two separate 10 cm gelatinized dishes containing 5 ml neural differentiation culture medium and the flask was incubated at 37°C and 5% CO_2 incubator.
7. The first dish contains control cell group (dish A) and the ethephon was added to the medium of the second dish (dish B).
8. After 24 h the medium was changed and 5 ml of new neural differentiation culture medium was added to the dishes. Ethephon at the required concentration was added to dish B.
9. After 2-5 days the Rosset forms were obvious as shown in figure 4.8 (D, E).
10. On the 5^{th} day, the medium was discarded and new neural differentiation medium containing RA was added to the dishes.
11. During 7-10^{th} day the neural cells obviously appeared.

3.4.8 Assessment of the Cytotoxic Effect of Ethephon on mES

Cell-based assays are widely used to determine the effects of test compounds on cell viability or cell proliferation or show the cytotoxic effects which may lead to cell death. The Thiazolyl Blue Tetrazolium Bromide (MTT) test is a reliable colorimetric test which determines the cell metabolic activity. Reduction of tetrazolium dye is dependent on mitochondrial succinate dehydrogenase enzymes which are available in the cytosolic compartment of the cell. Therefore, the cellular metabolic affects the reduction of these dyes. Whenever the little amount of MTT is reduced it means that the metabolic activity of cells is low. In this test, the yellow crystals of tetrazolium are reduced to the insoluble purple formazan crystals.

3.4.9 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Tetrazolium Reduction Assay

For assessment of cytotoxic effect a reviously described protocol was used (107). and the mES cells at third passage stage were selected.

1. Cells were seeded in two 96 well plates (108)/well.
2. Ethephon (purity 90%) solutions were prepared in medium at different concentrations (40, 80, 160, 320, 640, 1280, 2560 and 5120 µM).
3. Then 100 µL of solution of each concentration was added to each well and incubated for 24h.
4. The 50 µL of the medium was discarded and 50 µL MTT solvent was added to the wells after 24 h to the first plate and after 96 h to the second plate. The concentration of MTT was 5mg/ml.
5. Plates were incubated at 37°C and 5% CO_2 incubator for 3 h.
6. The MTT solution was discarded after 3h and 150 µL of DMSO solvent was added to the wells. As Formazan crystals are insoluble, addition of DMSO was required to convert them into the soluble form.
7. The plate was wrapped in foil and was put on an orbital shaker for 15 minutes.

8. The absorbance was read by microplate reader at OD 570 nm and 690 nm within 1 h.
9. Negative control group: Mouse stem cells in normal cell culture medium with no ethephon treatment
10. Groups 2-10: mES cells treated with various concentrations of ethephon: (20, 40, 80, 160, 320, 640, 1280, 2560, 5120) μM prepared from stock solution of ethephon: 100 mg/ml

3.4.10 Total Antioxidant Assay

The ferric reducing ability of plasma (Frap) assay used to measurement of the antioxidant power of ethephon in mES cells. The base of this method is the potency of the antioxidant agent in the reduction of ferric (Fe^{3+}) to the ferrous (Fe^{2+}) ions in presence of (2, 4, 6-tris-(2-pyridyl)-s-triazin) TPTZ agent at low PH. This reaction results in the formation of a blue colored ferrous-tripyridyltriazine complex with the maximum absorbance at 593 nm. In other words, the absorbance of the test reaction and those concentrations which contain ferrous ion are measured and compared at 593 nm by a spectrophotometer. The higher antioxidant power causes more colored solution.

Firstly, cells were seeded in 6 well plates and were treated with various concentrations of ethephon. After 24 h, the upper liquids were transferred to the microtubes and were collected. To performing the test, 1.5 ml of prepared FRAP reagent was added to the sample tubes and incubated at 37 °C for 5 min. Then 50 μL of upper liquid and various standards were transferred to the other tubes and incubated at 37 °C for another 15 min. At the end of incubation time, the absorbance was read at 593 nm against blank.

3.4.11 Reactive Oxygen Species Assay

In this method formation of ROS free radicals in cells are measured by 2', 7'–dichlorofluorescin diacetate (DCFDA). This fluorescent substance penetrates into the cell and is converted to a non-fluorescent compound (DCFH) by cellular esterase. Then DCFH reacts with oxygen and converts to 2', 7'–dichlorofluorescin (DCF). Therefore,

conversion of DCFH to the DCF is a marker for measurement of reactive oxygen species. The advantage of this test in comparison to the FRAP assay is the possibility of measuring the total produced ROS in all pathways. At the final step, absorbance is read at 488 nm and 525 nm by flourometric ELISA, respectively.

For this test solutions such as assay buffer, extraction buffer and dithiothreitol (DTT) were required and test was performed base on a previously described (109).

Firstly cells were seeded in a six-well plate.

1. After treating cells with various concentrations of ethephon, they were washed up with PBS.
2. Then Trypsin-EDTA was added to each well to detach cells from wells
3. The contents were transferred to other vials to be centrifuged for 5 min at 5000 rpm and the purred extraction was collected.
4. After that 70 µL of extraction buffer and 10 µL of DTT were added to the cell precipitant.
5. The mixtures were homogenized with the ultrasonic homogenizer.
6. Then 25 µL of each sample was transferred to the 96 well Elisa plates and 81 µL of reaction buffer and 5 µL of DCFH_DA5 µM solution were added to each plate
7. Plates were incubated at 37℃ for 15 min.
8. Finally, the rate of oxidation of DCFH to DCF was measured by flourometric ELISA reader at Excitation: 485 and emission: 528 every 5 minutes for the whole 1h.

3.4.12 Flow Cytometry Technique

Flow cytometry is a laser-based technology which is used for quantitative and qualitative assessment of physical and chemical characteristics of cells and other biologic particles. In this method, sample fluid (cell suspension) passed through detection apparatus under laser radiation. This instrument allows multi-parametric analysis of the cell characteristics. The laser light after incidence to the cells is dispersed

in different directions which part of these rays are collected by sensitive detectors and analyzed.

Flow cytometry is consistently used in the investigation of diseases, particularly blood cancers; however, it has multifold functions in fundamental research and clinical experiments. A typical variation includes uniting the analytic capability of the flow-cytometer to a sorting device, to physically isolate and clarify particles of the mixture based on their optical characters. Such procedure is cell sorting and the instrument is a cell sorter.

3.4.13 Cell Cycle Analysis by Flow Cytometry

Flow cytometry quantitates the DNA content of cells, therefor is able to assess the various phases of cell cycle. At the G0 and G1 stages of cell cycle, cells are diploid (2n) and contain two sets of chromosomes. Cells at G2 and M stages are tetraploid (4n) which means the DNA content is twice. As cells are permeable to various dyes, in this method cells were treated with propidium iodide (PI), a fluorescent dye which makes a complex with DNA and DNA is stained. The intensity of evoked fluorescence by UV radiant in stained cells equates the DNA amount that they contain. Technically cells with higher DNA content are brighter which means more fluorescent intensity. To prepare PI solution, 2 ul of PI Stock Solution and 20 ul RNase Stock Solution was added to 2 ml of Triton/ PBS 0.01 %.

3.4.14 Preparation of Cells for Flow Cytometry

The cell cycle analysis was performed as set up and described previously (109).

1. Cells were seeded in a 24 well plate (1.5*104) cells /well.
2. Then cells were treated with various concentrations of ethephon for 24 h and incubated at37 °C with 5% CO2 and 95% humidity.
3. After 24 h, wells were washed up with PBS.
4. Then Trypsin/EDTA solution was added to each well to detach the cells.
5. The fresh cell culture medium was added to the plate to stop the effect of trypsin.

6. Cell counting was performed and about 104 cells were transferred to the 1.5 ml tubes. Then cells were centrifuged for 5 min at 2500 g at 25 °C. The supernatant was discarded and the cell precipitant was washed up two times with cold PBS.
7. The 70% ethanol was added to the cell precipitant and the tube was stored at
8. 2-4°C for 24h.
9. Tubes were centrifuged for 5 min at 2500 g at 25 °C and the supernatant was discarded.
10. The PI solution, which should prepare freshly, was prepared.
11. 1 ml of the PI solution was added to the cell precipitant and a cell suspension was made.
12. The cell suspension was incubated for 15 min at 37 °C and darkness.
13. Analysis of each cell suspension was performed and the histograms were drawn to understand of the percent of cells in each cell cycle phase.

3.4.15 RNA Extraction from mES cells

RNA extraction was performed by Bioera total RNA extraction kit.

1. Cells were seeded at six-well plate (5*105 cell per well) and after 24 h incubation at 37 °C with 5% CO_2 and 95% humidity, the cell culture medium was discarded and cells were treated with various concentrations of ethephon for 24 h.
2. After 24 h, the contents of each well were transferred to the separate microtubes by trypsinization.
3. 1 ml of total RNA extraction reagent Biozol was added to each microtube and microtubes were incubated at room temperature for 15 min.
4. 250 µL of cold chloroform was added to all microtubes
5. Microtubes were vortexed for 15 seconds. Then microtubes were incubated at 4℃ for 15 min.
6. Then samples were centrifuged at 4℃ and 12000 rpm for 20 min. The centrifuged sample has three phases in which RNA is the upper phase. The

lower phase is a phenol-chloroform phase which contains protein. The middle phase is a white color and contains DNA.

7. The upper blue colored phase was transferred to a new microtube.
8. Then the same volume of cold isopropanol was added to the microtube.
9. Then samples were incubated at -20℃ for 30 min.
10. Then after microtubes were centrifuged for 10 min at 12000 rpm and 4℃.
11. The supernatant was discarded and 750 µL of ethanol 75% (prepared of absolute ethanol and nuclease-free water)
12. 12. Then microtubes were centrifuged for 7 min at 4℃ and 12000 g.
13. The supernatant was discarded and cells were washed up with 1 ml of ethanol 75%.
14. Then samples were centrifuged at for at 4℃ and 12000 rpm for 5 min.
15. The RNA precipitant was soluted in 50 µL of nuclease-free water and then the concentration of RNA was determined by spectrophotometer.
16. The extracted RNA was stored at -20℃ for RT_PCR test.
17. For measurement the concentration of total RNA, extracted RNA was quantified by Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer.
18. Then 2 µL of total extracted RNA was added to 2 µL of nuclease-free water and the absorbance was read at 260nm and 280 nm. The purity of the extracted RNA is calculated by the ratio of absorbance at 260 and 280 nm. The ratio higher than 1.6 is acceptable as pure for RNA.

3.4.16 RNA Qualification Assay on Gel Electrophoresis

Qualification assay of RNA was performed using agarose gel electrophoresis. In molecular biology, gel electrophoresis method is used for separation of macromolecules such as DNA, RNA, and proteins in agarose gel.

Firstly the EDTA 0.5 M (PH=8) and TAE buffer stock solution (50X) were prepared

A) Steps of Gel Electrophoresis

1. For the first step, the agarose gel 1% in TAE buffer (1X) was dissolved and the gel was prepared. Then prepared gel was heated in the microwave until agarose was dissolved. Then it was left to be cool.

2. Then the comb was posed 1 ml above the plate to let the wells to be formed completely.

3. The agarose gel was poured into the mold for the thickness of about 5 mm.

4. After 30 minutes the comb was removed gently and the gel was posed into the electrophoresis tank without bubbles.

5. Then enough TAE buffer (stained with ethidium bromide) was added to cover the gel.

6. The RNA samples were mixed with gel loading buffer with pipettes.

7. The mixture was loaded very slowly into the slots.

8. The electric leads were attached and the RNA started to move toward the anode. The applied voltage was 300 and then when the gel loading buffer migrated the appropriate distance, about 40 minutes.

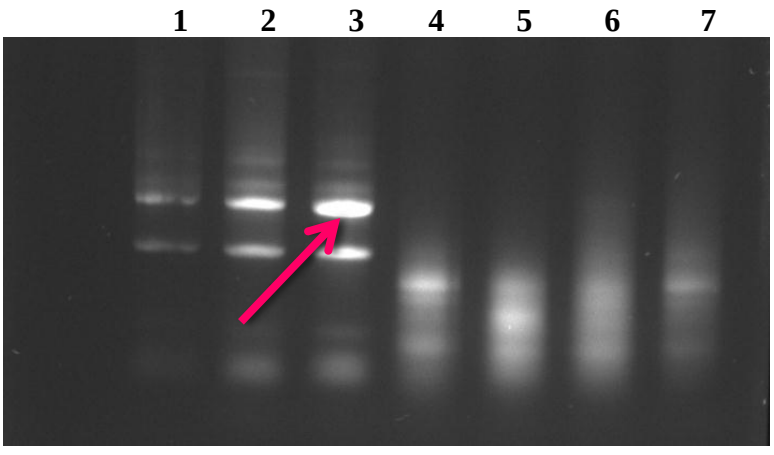


Figure 3.1. RNA qualification assay.

The 28s rRNA and 18 sRNA bonds are detected. Line 3 shows RNA with high quality, while other lines are degraded RNA.

3.4.17 First Strand cDNA Synthesis

For cDNA synthesis, the Iscript cDNA synthesis Kit made by Thermo Scientist Company was used and all steps of the process were performed on ice, under sterile conditions under a laminar flow hood.

1. Firstly, after thawing, the contents and reagents of the kit was mixed and centrifuged briefly, and then all were stored on ice.
2. Then 0.2 µg (1 µL) of extracted total RNA was added to the 10 µL of nuclease-free water.
3. Then 1 µL of random hexamer primer was applied to the mixture and was centrifuged briefly.
4. 1 µL of RiboLock RNase inhibitor and 2 µL of dNTP Mix 10mM were added to the PCR strip. Then 1 µL of Revert Aid M-Mul VRT (200 u/ µL) was added.
5. Finally, 4 µL of 5X Reaction buffer was applied, then was mixed gently and centrifuged.
6. The strips were incubated at 5℃ for 5 min.
7. The reaction was terminated by heating at 70℃ for 5 min.
8. The product of this process was stored at -20℃ for less than one week and then used in the Real Time PCR process. However, for longer times samples could be stored at -70℃.

3.4.18 Real Time-Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To investigate the molecular effects of ethephon on neural differentiation in mES cells, the expression levels of Oct-4, SOX2, NANOG, NSE, Nestin, Map2 and β-tubulin III genes were investigated by quantitative RT-PCR.

List of Primers

1) Oct-4 (Pou5f1)

	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	99	20	61.02	60.00	1.00	0.00	
	GGAGAAGTGGGTGGAGGAAG						
RIGHT PRIMER	284	20	60.08	45.00	3.00	2.00	
	TGATTGGCGATGTGAGTGAT						
PRODUCT SIZE: 186,							

2) SOX2

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	37	24	59.48	37.50	2.00	0.00	
	GCAAAAAGAGGAGAGTAGGAAAAA						
RIGHT PRIMER	208	20	59.42	50.00	2.00	1.00	
	AGTCCCCCAAAAAGAAGTCC						
PRODUCT SIZE: 172,							

3) NANOG

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	862	20	57.35	55.00	2.00	1.00	
	CGAGGATGAGACAGAAGGAC						
RIGHT PRIMER	1031	18	57.88	55.56	2.00	0.00	
	CCAAGGACAAGCAAGCAC						
PRODUCT SIZE: 170,							

4) Nrf2

OLIGO	start	len	tm	gc%	any	3'	seq

LEFT PRIMER	372	22	59.98	45.45	2.00	0.00
GAAAACGACAGAAACCTCCATC						
RIGHT PRIMER	573	18	60.00	61.11	2.00	0.00
CTCCATCCTCCCGAACCT						

PRODUCT SIZE: 202,

5) NSE

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	186	21	59.48	57.14	2.00	0.00	
GCTCTCTCTGCTCTTCCTCCT							
RIGHT PRIMER	358	20	59.99	45.00	2.00	2.00	
AAACACCCCAACACACCAAT							

PRODUCT SIZE: 173,

6) Map2

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	1007	22	57.47	45.45	3.00	0.00	
AAGTCCCTCTGTAGGAAAAGGT							
RIGHT PRIMER	1233	20	58.20	40.00	3.00	0.00	
ACAACCAGCCATTGAAGAAA							

PRODUCT SIZE: 227,

7) β-tubulin III

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	9	18	59.74	61.11	3.00	0.00	
GGGGACCTCAACCACCTT							
RIGHT PRIMER	225	18	60.75	55.56	3.00	1.00	
CGAACATCTGCTGCGTGA							

PRODUCT SIZE: 217,

1. For the first step, the required primers were designed.
2. Then left and right primers, the synthesized cDNA samples, which had been synthesized from extracted RNA from mES cells and ethephon treated and differentiated mES cells, $MgCl_2$, the syber green solution and nuclease-free water were centrifuged gently.

3. Later, 13 µL of nuclease-free water was added to all PCR strips.
4. Soon after, 1 µL of cDNA, 1 µL of left primer and 1 µL of right primer were added to the strips carefully.
5. And finally, the 4 µL of syber green was applied to all strips.
6. The mixtures were blended softly.
7. At the final step, samples were placed in RT-PCR instrument and the thermal settings were performed consequently.
8. The whole process takes 45 cycles.
9. The numbers of cycles were adjusted from the amplification curve to conclude the relative gene expression by usage of the comparative cycle threshold method (110).
10. 2-ΔΔCt was used for relative gene expression analysis.
11. All determined values from the target genes were adjusted using those from the untreated mES cells as the control group.

3.4.19 Statistical Analysis

In this study, all tests were performed three times and the obtained data were presented as mean ± SEM. The Windows SPSS version 15 was used with one-way analysis of variance (ANOVA) followed by Tukey's multi-comparison tests to calculate the statistical difference ($p<0.05$) between treated and control group.

4. RESULTS

4.1 Results of The Cell Cycle Assay

The distribution of the cell cycle in both normal and treated mES cells with Ethephon were assessed (figures 4.1 and 4.2).

Results showed that 44.7 % of the control group were in G0/G1, 41.5% in S phase and 12.8 % were in G2/M phase.

As compared to the control group, at the concentration of 80 μM, 44.2% of the control group were in G0/G1, 44.9% in S phase, and 9.98 % were in G2/M phase.

At the concentration of 160 μM, 50.9 % of the treated cells were in G0/G1, 41.8 % in S phase and 6.71 % were in G2/M phase.

At the concentration of 320 μM, 52.3 % of the treated cells were in G0/G1, 40.4 % in S phase and 6.56 % were in G2/M phase.

As displayed in figures 4.1 and 4.2 distribution of mES cells in G0/G1 phase have been increased at higher doses. However, during dose excision, the distributions of these cells in G2/M phase have been decreased.

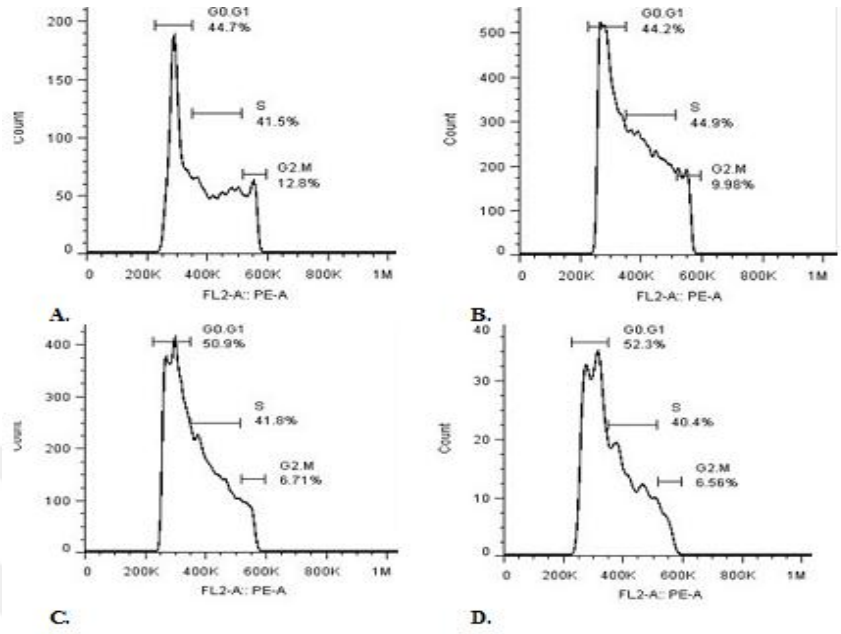


Figure 4.1. Effect of ethephon on cell cycle analysis

A. Control group. Non-treated with ethephonm

B. mES cells treated with 80 µM of ethephon

C. mES cells treated with 160 µM of ethephon

D. mES cells treated with 320 µM of ethephon

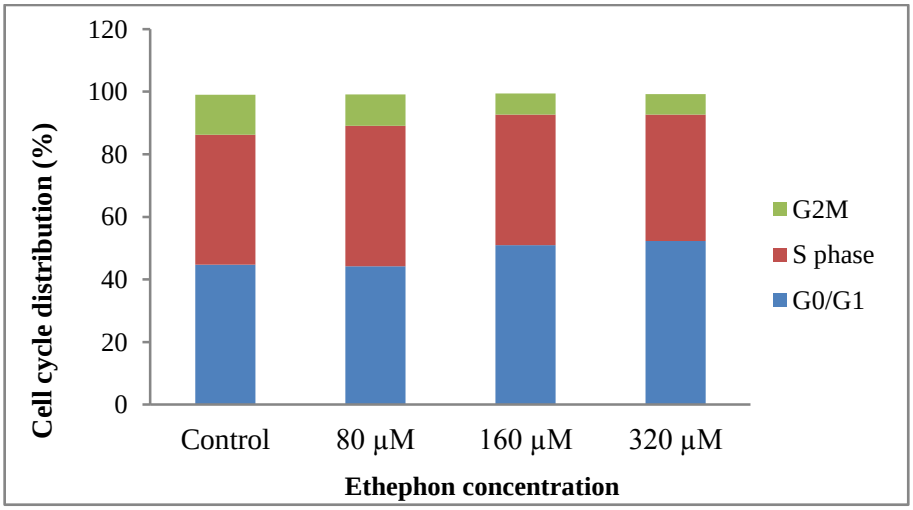
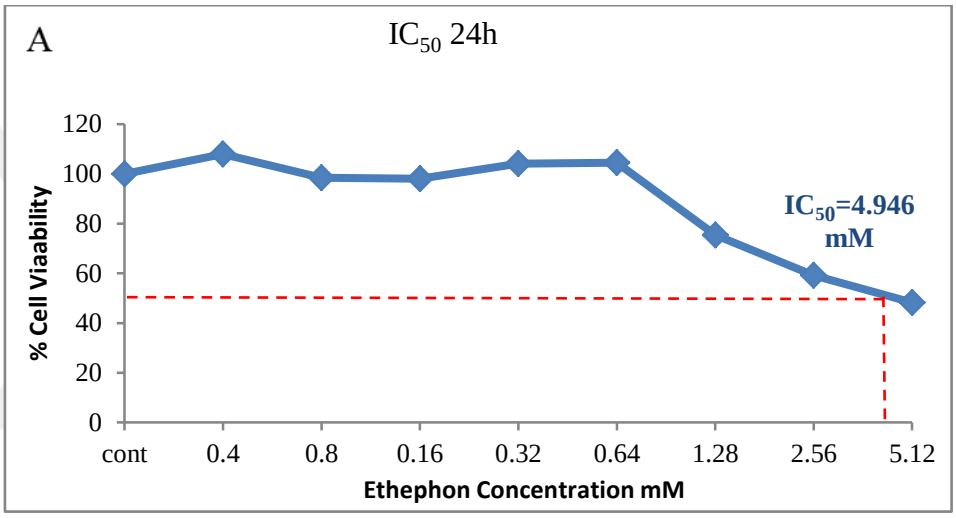


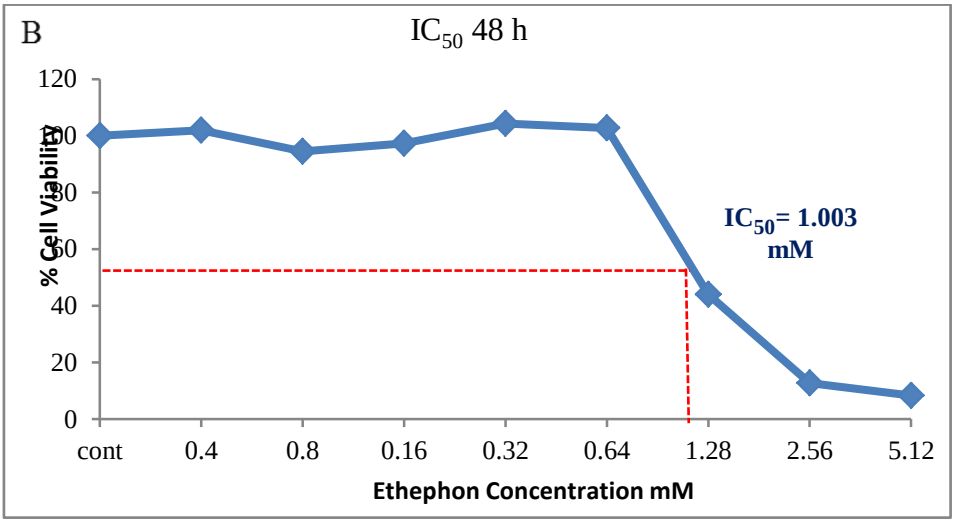
Figure 4.2. Graph of phases of cell cycle.

No significant differences were found between the concentrations.

4.2 Results of Cytotoxicity Assay (MTT)

As shown in figure 4.3 and figure 4.4, the effect of different concentrations of ethephon on mES cells has been treated with various concentrations (40, 80,160,320,640, 1280, 2560 and 5120 µM) of ethephon at different times. The IC_{50} was 4.946 mM. Results show that the viability of mES cells at the concentrations of 1280, 2560 and 5120 µM have been decreased significantly in compare to the control group.





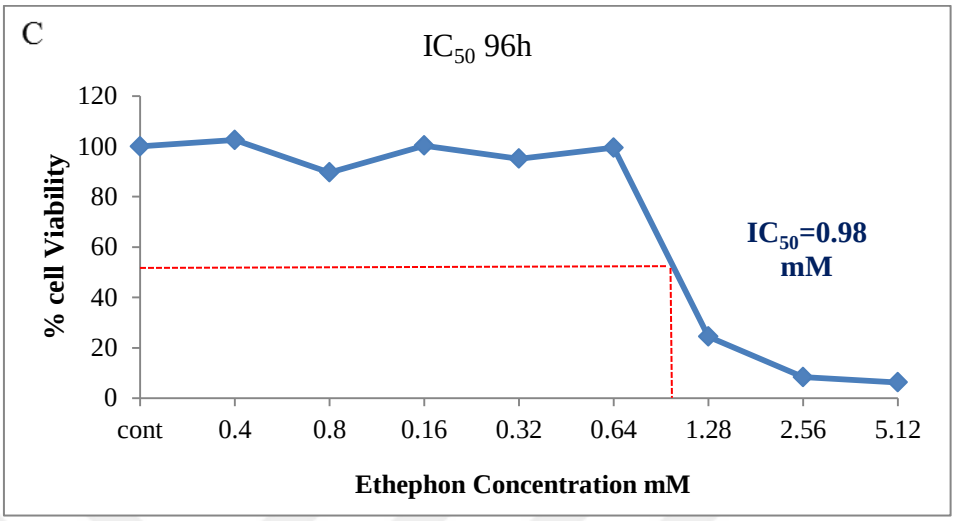


Fig 4. 3. Determination of ethephon IC_{50} .

A. 24h ethephon treatment. IC_{50}= 4.946 mM

B. 48h ethephon treatment. IC_{50}=1.003 mM

C. 96h ethephon treatment. IC_{50}= 0.98 mM

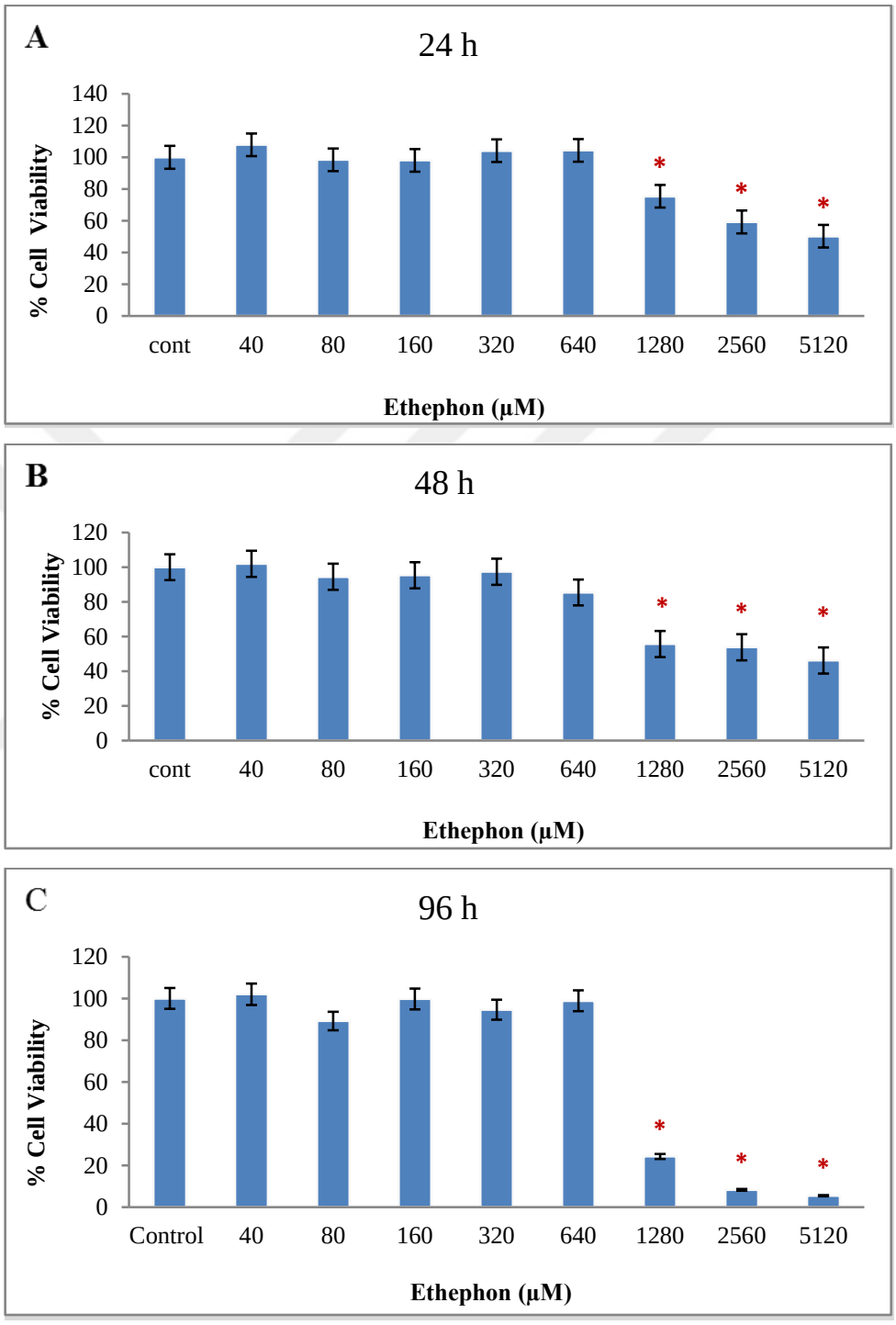


Figure 4.4. Evaluation of Ethephon cytotoxicity by MTT assay for 24h (A), 48h (B) and 96h (C) exposures. The results are shown with Mean ±SEM.

* Significantly different from control (p<0.001)

4.3 Evaluation of Antioxidant Status by FRAP

The effect of ethephon on FRAP is presented in Figure 4.5. The concurrent exposure of mES cells to ethephon demonstrated the highest antioxidant potential (p <0.001) than non -treated mES cells at 160 µM.

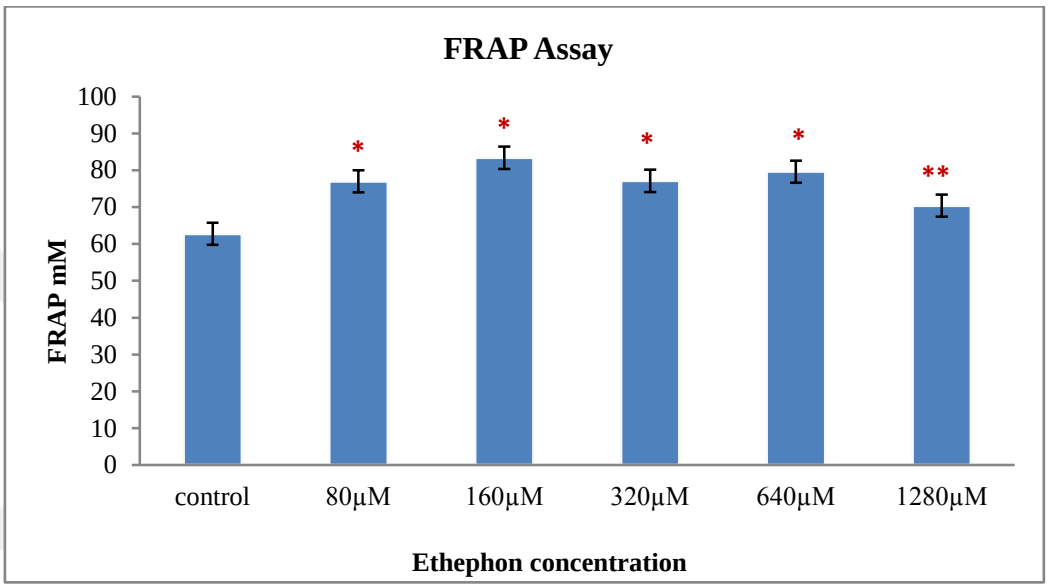


Figure 4.5. Evaluation of Antioxidant Status by FRAP Assay at different concentrations of ethephon.

Results are shown with Mean ±SEM

* Significantly different from control (p<0.001)

** Significantly different from control (p<0.05)

4.4 Effects of Ethephon ROS Level

Evaluation of the effect of ethephon on generation of ROS in mES cells showed that in comparison to control group, ethephon at the concentrations of 640 and 1280 µM significantly decreased the ROS level in compare to control group. Results have been shown in figure 4.6.

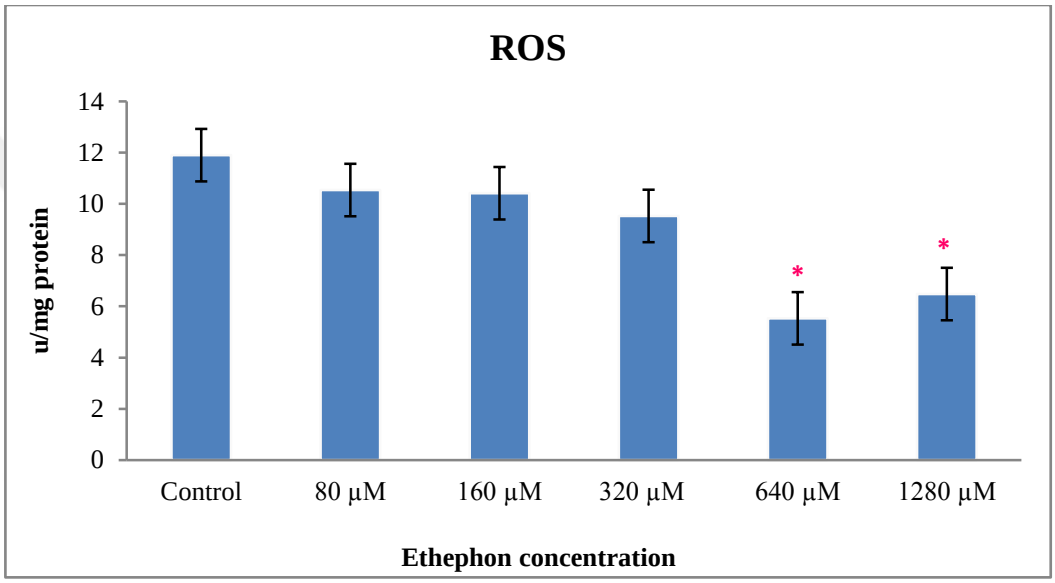


Figure 4.6. Effects of Ethephon on oxidative stress by ROS assay

Results are displayed with Mean ±SEM.

*****Significant difference with the control group (p<0.001)

Summary of the results for oxidative stress and antioxidant status are given in table 4.1.

Table 4. 1. Effect of ethephon on stress oxidative markers in mES cells.

Parameter/ unit	**ROS**	**FRAP**
Mean ±SEM	**(u/mg protein)**	**mM**
Control	12.79 ± 0.80	63.54 ± 0.50
80 µM	10.54 ± 1.05	82.29 ± 3.45 *
160 µM	10.41 ± 0.68	82.95 ± 2.02 *
320 µM	9.52 ± 1.09	78.95 ± 1.16 *
640 µM	5.53 ± 0.59 *	81.54 ± 1.51 *
1280 µM	6.48 ± 0.37 *	72.87 ± 1.59 **

Results are displayed with Mean ±SEM.

Results are displayed with Mean ±SEM.

* Significantly different from the control, p <0.001

** Significantly different from the control, p <0.05

4.5 Evaluation of Morphology of mES Cells

The mES cells were morphologically evaluated before and after neural differentiation as shown in figures 4.7 and 4.8.

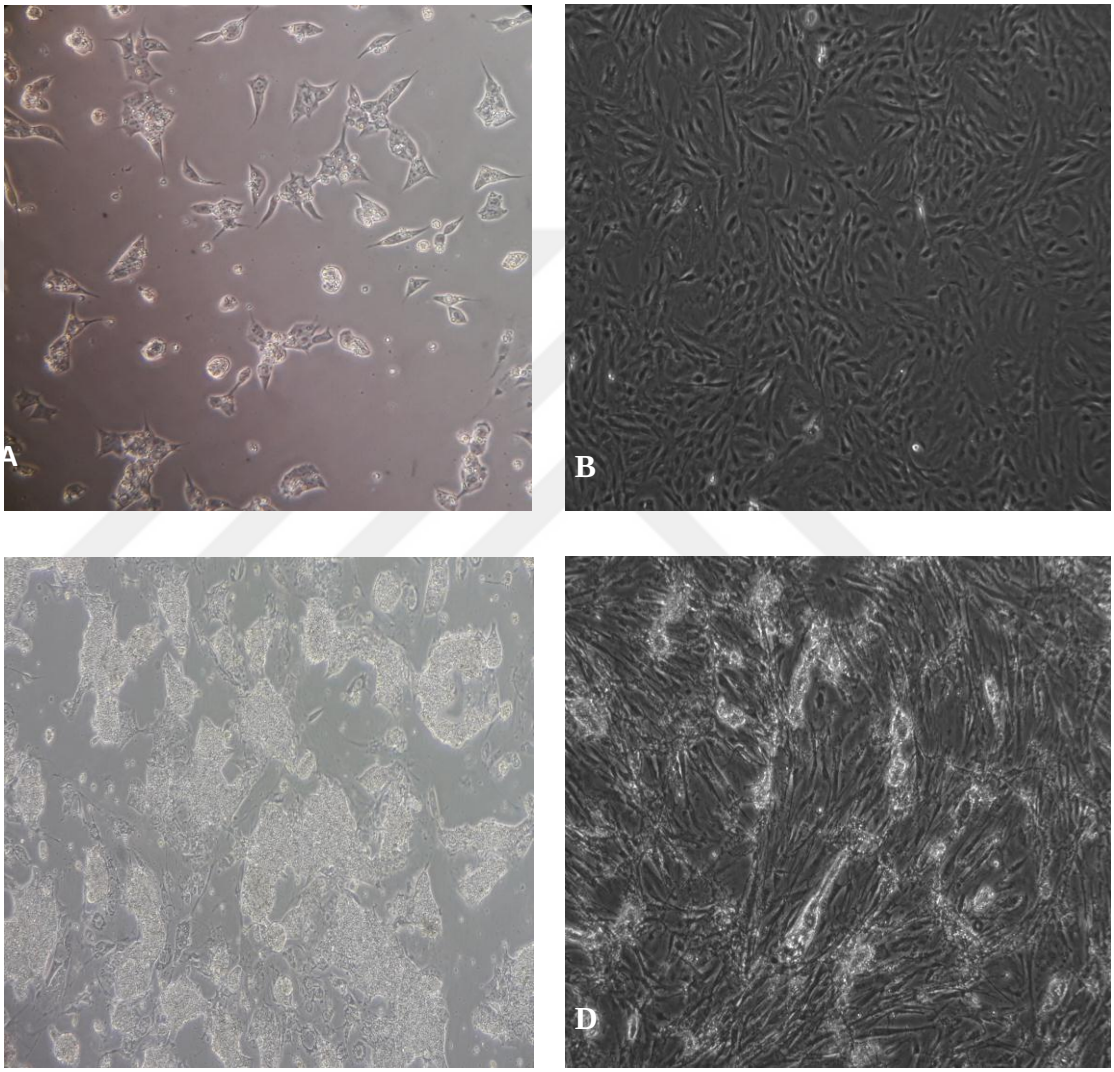


Figure 4. 7. Diagram of undifferentiated mES cell morphology

A. Cultured mES cell. Day 2
B. Cultured mES cell. Day 5
C. Cultured mES cell. Day 6 with 70-80 % frequency
D. Cultured mES cell. Day 8 with 90% confluency

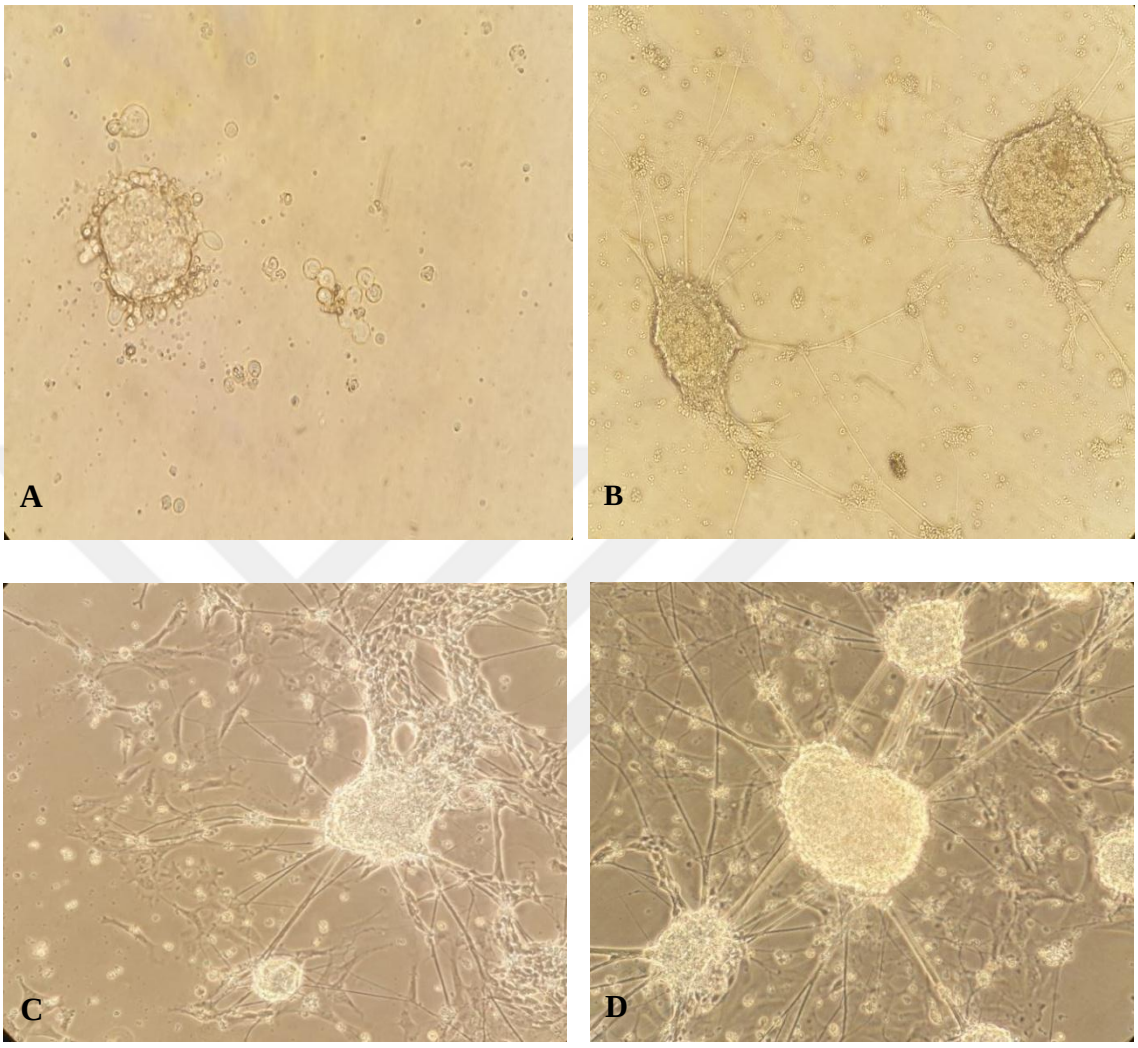


Figure 4.8. Diagram of mES cells morphology after neural differentiation

A. Differentiated mES cell. Day 6 the Rosset formation.

B. Differentiated mES cell. Day 7-12

C. Differentiated mES cell. Day 13-17 neuron formation

D. Differentiated mES cell. Day 18-22 neurons and dendrites.

4.6 Results of Gene Expression Assay in mES Cell by Real Time-PCR

Results of evaluated genes Oct-4, NANOG, Nestin, SOX2, MAP2, β-Tubulin III, Nrf2 and NSE have been provided in figure 4.9-13 and table 4.2. The investigation of statistical data illustrated that ethephon disrupt and/or mutate gene expression significantly different as compared to control ($p<0,001$).

As presented in table 4.2 the Oct-4 (housekeeping gene), SOX2 and NSE were downregulated in mES cell at the concentration of 320µM in compare to the control group.

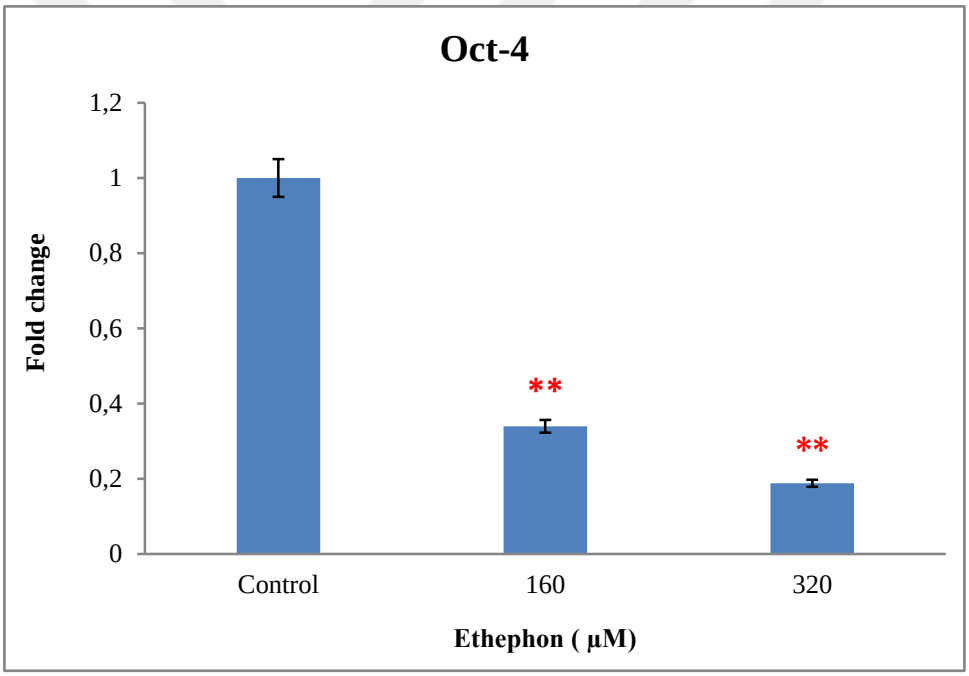


Figure 4.9. Expression of Oct-4in mES cells

** Significantly different at significantly different from control, $p< 0.001$.

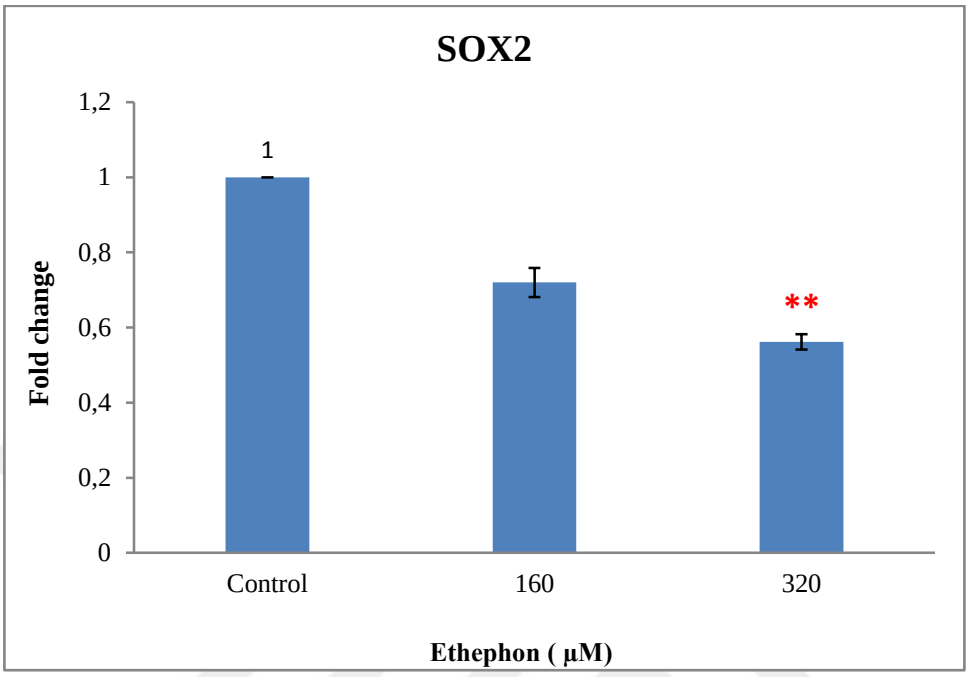


Figure 4.10. Expression of SOX2 in mES cells

** Significantly different at significantly different from control, $p < 0.001$

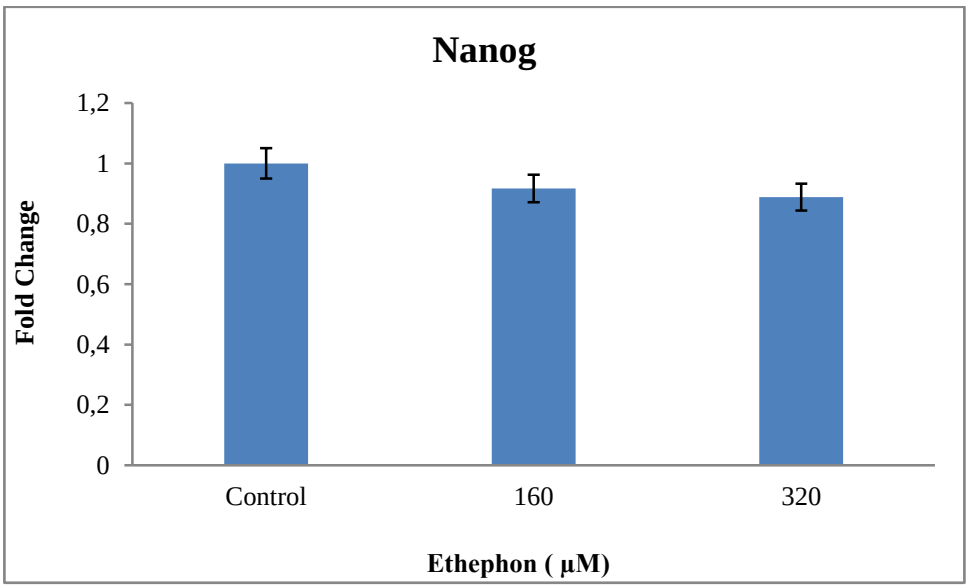


Figure 4.11. Expression of NANOG gene in mES cells.

No significant differences were found between the concentrations.

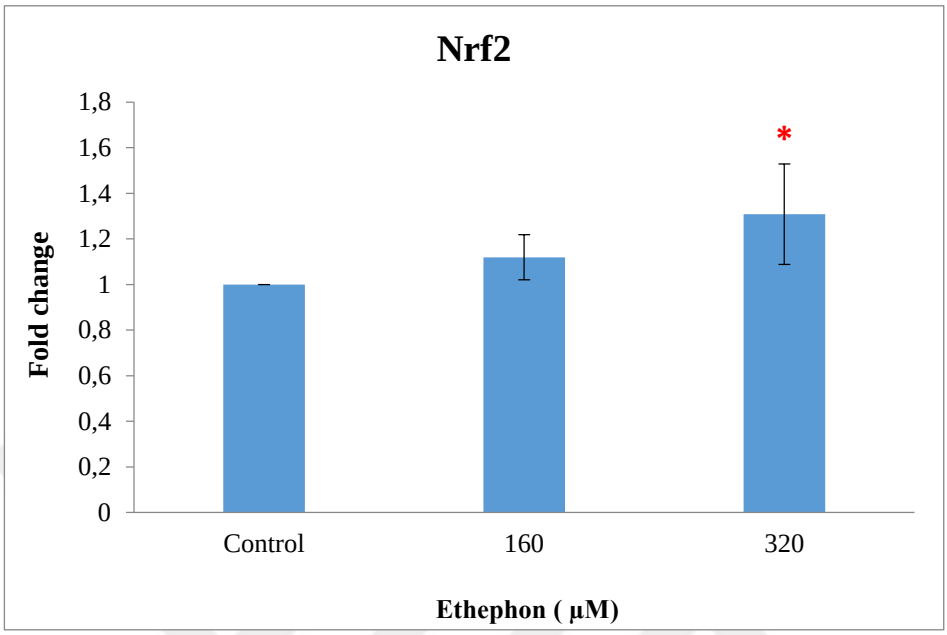


Figure 4.12. Expression of Nrf2 gene in mES cells

****** Significantly different from control, $p < 0.05$

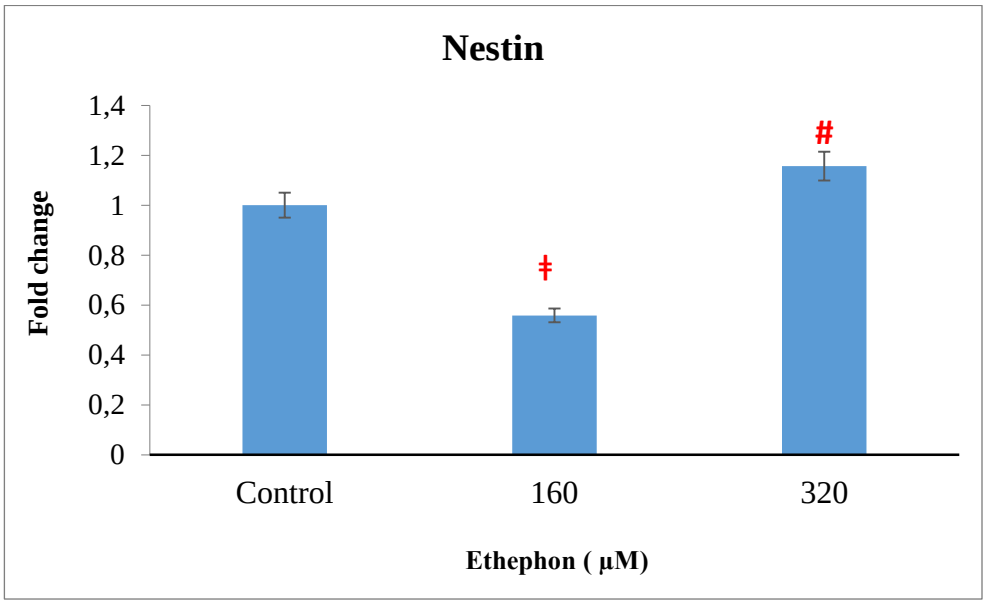


Figure 4.13. Expression of Nestin gene in mES cells.

‡ Significantly different from control, $p < 0.01$

Significantly different from 160 µM, $p < 0.001$

Table 4.2. Description of relative fold changes of stemness genes in mES cells.

	Experimental groups (relative fold change ± SEM)	
Gene symbol	**160 µM**	**320 µM**
Oct-4	1.12 ± 0.06 **	1.30 ± 0.06 **
SOX2	0.71 ± 0.03	0.56 ± 0.02 **
NANOG	0.91 ± 0.04	0.88 ± 0.04
Nrf2	1.12 ± 0.05	1.30 ± 0.06 *
Nestin	0.59 ± 0.02 ǂ	1.15 ± 0.05 #

Values are expressed as mean ± SEM in each group.

* Significantly different from control, $p < 0.05$

** Significantly different from control, $p < 0.001$

ǂ Significantly different from control, $P < 0.01$

Significantly different from 160 µM, $p < 0.001$

4.7 Results of Gene Expression in Neuronal Differentiated mES Cells

As shown in figure 4.14-18 and table 4.3, the expression of Oct-4, SOX2, NSE, Map2 and β-Tubulin III were evaluated after neural differentiation of mES cells by Real-time PCR at the concentration of 160 µM of ethephon. The data demonstrated that the expression of NSE, Map2 and β-Tubulin III in the differentiated cells have been significantly increased in compare to the un-differentiated mES cells ($p<0.001$). Although in ethephon treated cells during the differentiation process, the expression of NSE, Map2 and β-Tubulin III, have been decreased significantly in comparison with control neurons ($p<0.001$).

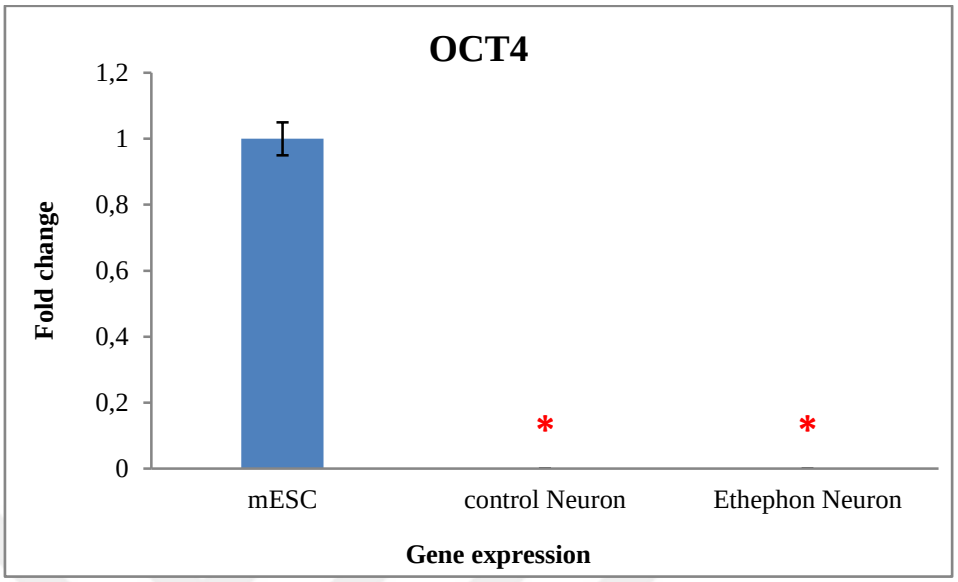


Figure 4.3. Expression of Oct-4 gene in the neural cell

* Significantly different from control (mES cell), $p < 0.05$.

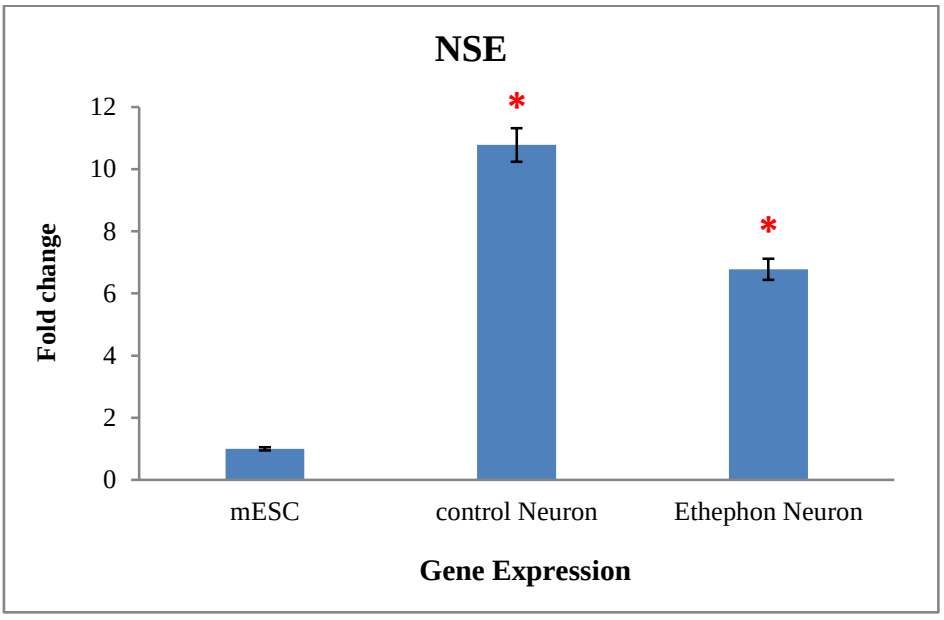


Figure 4.45. Expression of NSE gene in the neural cell

* Significantly different from control (mES cell), $p < 0.05$.

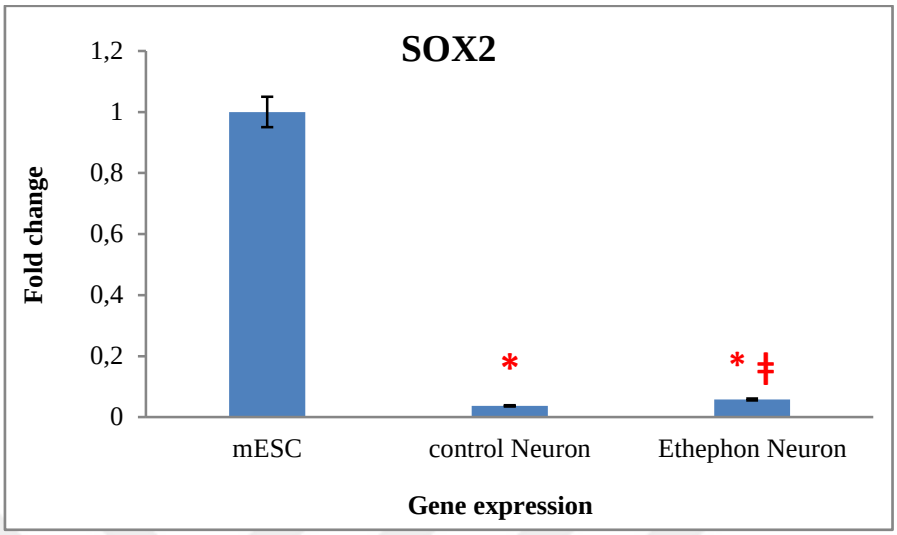


Figure 4.5. Expression of SOX2 gene in the neural cell

***** Significantly different from control, $p < 0.05$

ǂ Significantly different from control, $p < 0.01$

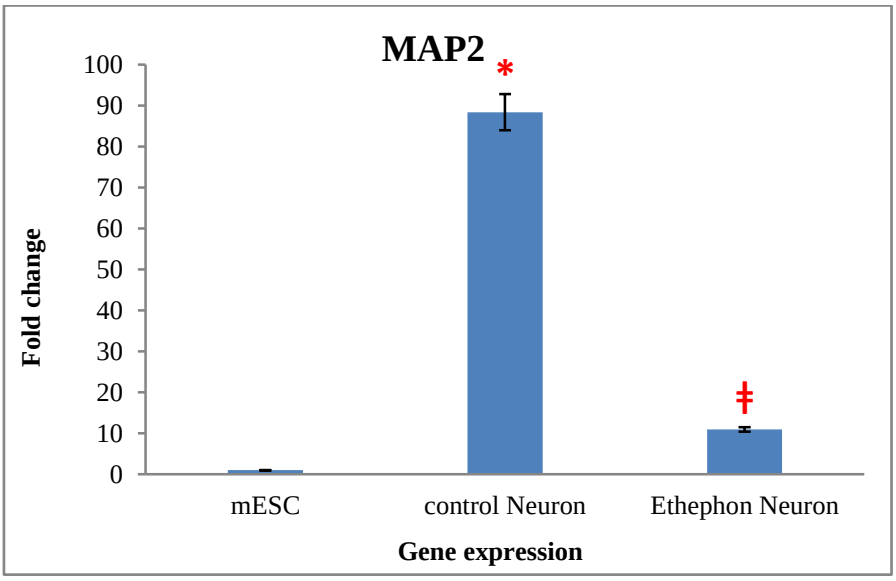


Figure 4.6. Expression of MAP2 gene in the neural cell.

* Significantly different from control, $p < 0.05$.

ǂ Significantly different from control, $p < 0.01$.

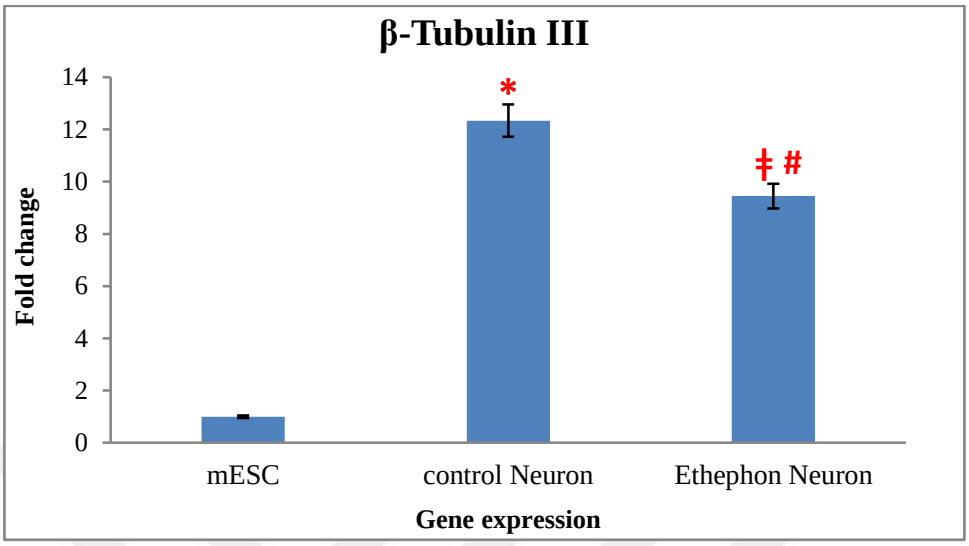


Figure 4.18. Expression of β-Tubulin III gene in the neural cell.

* Significantly different from mES cells, $p < 0.05$.

ǂ Significantly different from mES, $p < 0.01$.

Significantly different from Control neuron, p <0.01.

Table 4.3. Description of relative fold changes of stemness and neural specific genes after neural differentiation of mES cells

Gene symbol	Untreated neuron	Treated neuron
OCT-4	0.0003 ± 0.000016 *	0.0005 ± 0.000026 *
SOX2	0.036 ± 0.001 *	0.057± 0.002 *
NSE	10.777± 0.53*	6.773 ± 0.338 * ǂ
Map2	88.340 ± 4.41*	10.966 ± 0.548 ǂ
βTubulin III	12.337 ± 0.61*	9.447 ± 0.472 # ǂ

** Significantly different from mES control at $p < 0.001$.

ǂ Significantly different from neuron control group, $p < 0.001$.

Significantly different from neuron control group, $p < 0.01$.

Expressions of all genes before and after differentiation of mES cells were compared in figure 4.19 and 20.

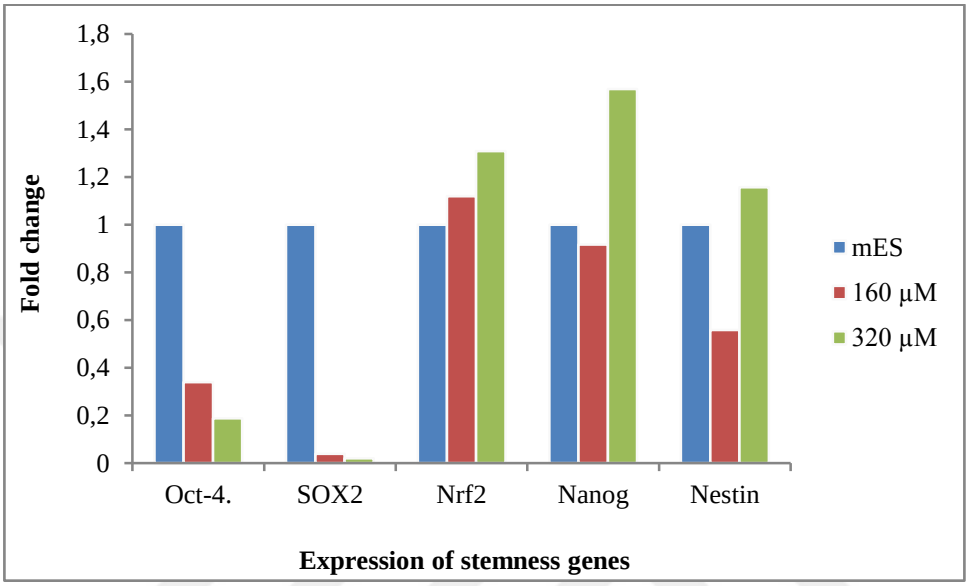


Figure 4.19. Effects of ethephon on gene expression in undifferentiated mES cells.

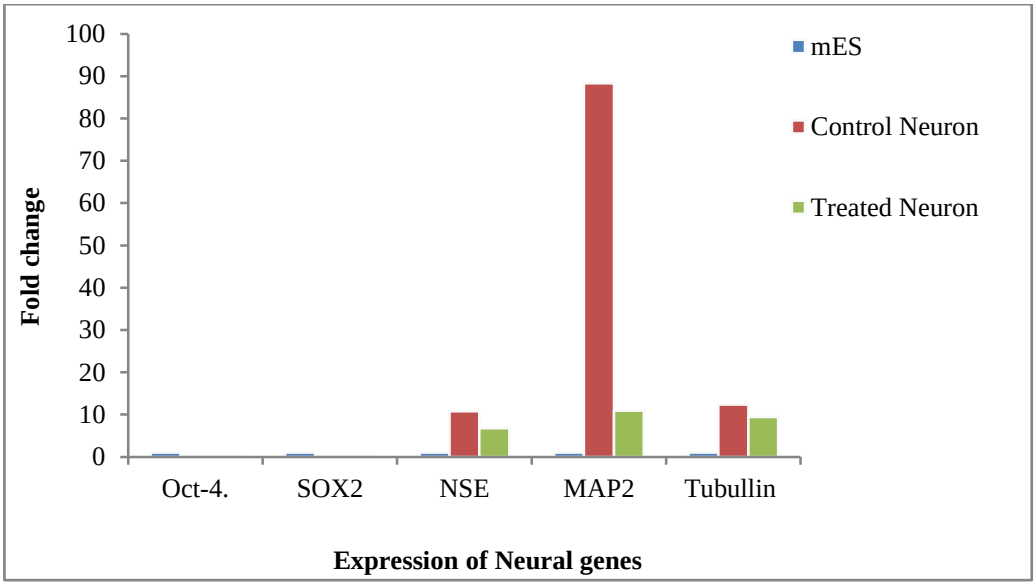


Figure 4.20. Effects of ethephon on gene expression in neural differentiated mES

4.4 Results of the correlation

The evaluation of the correlation between measured parameteres has been shown in table 4.4

Table4.4. Correlation Data

		MTT	ROS	FRAP	Oct-4	SOX2	NSE	Nrf2
MTT	Pearson Correlation	1	-0.547	-0.098	-0.369	-0.216	-0.216	0.754
	Sig. (2-tailed)		0.631	0.937	0.759	0.862	0.861	0.456
	N	3	3	3	3	3	3	3
ROS	Pearson Correlation	-0.547	1	-0.779	0.98	0.935	-0.699	-0.962
	Sig. (2-tailed)	0.631		0.432	0.128	0.23	0.508	0.175
	N	3	3	3	3	3	3	3
FRAP	Pearson Correlation	-0.098	-0.779	1	-0.889	-0.95	0.993	0.579
	Sig. (2-tailed)	0.937	0.432		0.303	0.201	0.076	0.607
	N	3	3	3	3	3	3	3
Oct-4	Pearson Correlation	-0.369	0.98	-0.889	1	0.987	-0.828	-0.889
	Sig. (2-tailed)	0.759	0.128	0.303		0.102	0.379	0.303
	N	3	3	3	3	3	3	3
SOX2	Pearson Correlation	-0.216	0.935	-0.95	0.987	1	-0.907	-0.804
	Sig. (2-tailed)	0.862	0.23	0.201	0.102		0.277	0.405
	N	3	3	3	3	3	3	3
NSE	Pearson Correlation	-0.216	-0.699	0.993	-0.828	-0.907	1	0.478
	Sig. (2-tailed)	0.861	0.508	0.076	0.379	0.277		0.683
	N	3	3	3	3	3	3	3
Nrf2	Pearson Correlation	0.754	-0.962	0.579	-0.889	-0.804	0.478	1
	Sig. (2-tailed)	0.456	0.175	0.607	0.303	0.405	0.683	
	N	3	3	3	3	3	3	3
Nanog	Pearson Correlation	0.979	-0.706	0.106	-0.55	-0.41	-0.013	0.872
	Sig. (2-tailed)	0.131	0.501	0.932	0.629	0.731	0.992	0.326

No correlation was found between cytotoxicity, antioxidant power and expression of stemness genes under effect of ethephon.

5. DISCUSSION

Plant growth regulators or plant hormones are chemical agents that have effect on the growth rate and cell or tissue differentiation of plants. Among plant hormones, ethephon is extensively used for its role in different features of plant development and ripening. Ethephon has been categorized as a non-harmful substance. However, there are limited certain data related to the adverse effects of this agent or the residues of it on human health. In such situation, the maximum residue levels (MRL) and tolerance limits for ethephon in production of agricultural primary crops is 0.05-20 mg/kg and the ADI is 0.03-0.05 mg/kg by weight per day (42).

It has been indicated that use of 1.5-2.2 mg/kg bw/day of ethephon for 28 days causes gastrointestinal effects in both male and female human volunteers. Use of 0.5 mg/kg bw/day for 16 days resulted in inhibition of plasma AchE activity (111).

Recently, the ES cells are encouraging tools for study of the developmental process and cell origin specialties. Currently, the lack of knowledge about pathways of neuronal function and differentiation is partially due to the absence of accessible model systems which allow scientist to evaluate the process of mammalian development directly.

The flow cytometric assays in this study indicated a decrease of G2/M and S phase of cell cycle in compare with control group and this reduction is dose-dependent. Also the distribution of the mES cells in G0/G1 phase has been increased at higher doses. The data showed that there was an increased growth of cells at high concentrations. Based on flow cytometric data, ethephon has the similar to the other organophosphates such as Diazoxon and Clorpyrifosoxon could induce an increase in number of cells at the G0/G1 phase (112). In this case ethephon shows a different pattern of cell viability percent in G2/M phase when compared to other organophosphates such as Dichlorvos (113).

In our study, the cytotoxicity and effects of ethephon with commercial formulation on gene expression have been examined in mES cells. The effect of

ethephon on mES viability was quantified by MTT assay and cell counting. Obtained results from 24 h exposure to ethephon demonstrated a normal cell proliferation at low doses. However, at higher doses (1280, 2560 and 5120 µM) considerable decrease of cell proliferation was detected in compare to the control. The results of 96 h exposure time also indicated more significantly decrease in cell viability at the same doses with 24 and 48 h which confirms that longer exposure time of higher doses of ethephon is toxic for mES cells.

In a previous study on mammalian cell lines of mouse NIH3T3, it has been proved that such cells react significantly to 0.3 mM ethephon and the proliferative activity has been increased in ethephon treated cells However, human Hela cells are more resistant and ethephon induced proliferative activity at the concentration of 2 mM of ethephon (114).

In an *in vitro* study, various mammalian cells were treated with different concentrations of ethephon (2.81-180 µM). MTT assay was performed and low cytotoxic effect was observed in Vero (monkey kidney cells), HepG2 (human hepatocellular carcinoma cells) and Hep2 (human epidermoid cancer cells) after 24 h of ethephon treatment. The highest IC_{50} value was measured by MTT assay and results showed the highest value in vero cells (126 µg/ml) and then followed by HepG2 (108/7 µg/ml) and Hep2 cells (137.29µg/ml) (29).

In all, comparing of our findings and the observations of previous studies show that the cell response to ethephon and viability of cellular population varies due to the examined cell line.

The oxidative stress has an important role in mechanism of various neurodevelopmental diseases such as dyslexia, autism, attention deficiency or hyperactivity disorder. Also increasing evidence confirmed the relation between fatty acid abnormalities or altered membrane phospholipids with these pathologic conditions. Organophosphates induced oxidative stress may generate the neuronal injury (116,117). Despite the fact that neurodevelopmental disorders are regularly correlated with

exposure with toxicants in fetal duration, ROS related conversions seem to be related to embryopathies and birth defects (118,119).

In another *in vitro* study showed that ethephon was significantly induce oxidative stress in rat fibroblasts at the concentration of 160 μM. However, at this dose, there was no significant difference in antioxidant status (115).

To determine the action mechanism of ethephon, we explored the ROS level and total antioxidant capacity. Our results demonstrated a meaningful decrease of ROS level between control and ethephon treated mES cells at the concentrations of 640 and 1280 μM. However, there was no significant difference at lower concentrations. Achieved data of FRAP assay as a measurement of total antioxidant status indicated a significant difference between treated cells and control just at the concentration of 1280 μM. Given all our observations, it might be suggested that the cytotoxic effect of ethephon is not related to the oxidative stress mechanism.

The nervous system includes of a large multitude of cell types which are derived from the initial neural plate. In short, at early embryogenesis, the neural induction happens and this plate begins to expand, self-renew and /or differentiate into various cell types. During this modification process of neurogenesis, oligo genesis occurs and oligodendrocytes and astrocytes (major nerve cell types) form (90).

The Oct-4 protein is the most well-known member of Oct proteins which are crucial for regulation of pluripotency, and is a certain element of the self-renewal process in ES cell and their distinction into trophoblast. Oct-4 is expressed in the early mammalian embryo and in the germline (116).

It has been indicated that pluripotent cells are connected to each other via a strong network of transcription factors. The Oct-4 is one of them and has a critical role in continuity and controlling of initial pluripotency in this network. In the early embryo stage, Oct-4 is expressed in the inner cell mass of blastocyst and epiblast, whichever conclusively will induce all cells of the embryo conclusively. Any deficiency in the structure or expression of Oct-4 in embryos results in the decline of the pluripotency. In

this situation after implication, the formation of ICM fails and the whole embryo cells differentiate into the trophoblast which means fail of development (116, 117).The Oct-4 is also crucial for lineage specification, regulation of processes related to the identity of cells. According to the some theories, environmental factors may influence the function of Oct-4 during embryogenesis (86).

Similar to Oct-4, SOX2 has a key role in the maintenance of pluripotency of stem cells as a transcription factor. The interaction of these two factors ends in the formation of a binary complex. Then after the other nuclear factors bind to this initial complex and activate the expression of pluripotent genes and inhibit the differentiation genes. Besides SOX2 is also an essential transcription factor for initialization of initiating the neural induction which maintenance the properties of neural progenitors (118).

The data from mechanistic studies of SOX2 also shows that this gene and NANOG promote and maintain expression of Oct-4. Interestingly, low or overexpression of SOX2 leads to lack of pluripotential property in embryonic stem cells. In fact, both increased and decreased level of SOX2 will reduce the activity of SOX2-Oct-4 complex. Depletion of SOX2 gene may result in morphological changes of both human and mES cells, lack of other pluripotent markers such as NANOG and difficulties in differentiation (122,123).

Similar to Oct-4, SOX2 also is an important regulator which makes it a reliable marker to identify neural progenitors. The SOX2 particularly play important role in neurogenesis and gliogenesis (90)

Mutation in SOX2 gene causes severe eye disabilities such as anophthalmia or severe microphthalmia (small eye) in human. This is associated with the reduced expression level of SOX2 in progenitor neural cells of in retina. As well, molecular evidence have provided that SOX2 essentially acts in the regulation of other signaling pathways to maintain the progenitor cells in the retina (119).

Another transcription factor which is associated with self-renewing in embryonic stem cells is NANOG which can maintain the undifferentiated form. Both in human and

mES cells, Oct-4, SOX2 and NANOG genes make an essence transcriptional structure which keeps the self-renewal ability. This gene also plays an important role in apoptosis, regulation of angiogenesis, disturbing the exposure of tumor cells to cytotoxic T lymphocytes (120). *In vitro* studies confirmed that any inhibition in the expression of NANOG ends in the decrease of cellular migration and cellular proliferation. Lack of this gene causes failure of development in embryonic cells (121). On the other hand, some experiments demonstrated the relation between overexpression of NANOG in cancer stem cells. This makes NANOG as a diagnostic biomarker in the prediction of cancer (122).

Nrf2 gene is another essential regulatory factor in mammals which maintains the characteristics of stem cells. Nrf2 also acts in maintenance of the normal physiologic condition of cells by induction of various antioxidant enzymes. However, epigenetic studies showed that Nrf2 expression could be controlled by epigenetic changes such as modification of chromatin structure or DNA methylation. Nrf2 also influences cell proliferation and differentiation especially by prevention of apoptosis in oxidative stress. This gene is able to regulate the promoter of many antioxidant genes in cell signaling pathway. Overexpression of Nrf2 causes enhancement of mesenchymal stem cells differentiation into osteoblasts. However, deficiencies of Nrf2 or genetic disruptions result in neuronal death or decrease of its function in oxidative stress (93, 94).

The NSE gene primarily is expressed at the final steps of neural differentiation in mature neurons (glial cells), especially in brain and neuroendocrine cells. This gene has been upregulated when cells are under stress conditions. Loss of NSE expression may end in neural damage, because of its neuroprotective role, and is a valuable marker in malignancy. At the situation of silencing of NSE, the proliferation of neural cells will be suppressed and because of the inhibitory function of this gene in apoptosis, the number of apoptotic cells will increase. Moreover, increased level of NSE gene in plasma is an indicator of neuronal and neuroendocrine damage and neuroendocrine cells (123). Royds et al investigated level of NSE enzyme in cerebrospinal fluid. The results showed that a

raised level of encoded isozymes of this gene is a sensitive marker in diagnosis of pathological anomalies in brain damages (124).

NSE plays a dual role in the neural system. The overexpression of NSE causes an elevation of the neuron specific enolase enzyme that promotes degradation of neural cells. In contrast, the inhibition or silencing of NSE gene causes decrease of inflammatory cytokines and correspondingly the numbers of glia cells. NSE levels increase during neuronal differentiation, while the level of NSE is low during the cell development (97).

Nestin is another genetic marker in neuroectodermal progenitor stem cells, glia precursor, and endothelial cells. It has a functional role in self-renewing, proliferation, differentiation and migration of neural stem cells. In fact, any overexpression or silencing of Nestin gene affects the function of Nestin protein as an intermediate filament in various tissues in brain, muscles, bone marrow, kidney, lungs, and skin. During the differentiation process of stem cells, modification of cellular shape is based on the remodeling of intermediate filaments (125). Furthermore, overexpression of Nestin has been observed in different malignancies such as neuroblastoma and melanoma and some of the epithelial tumors. The DNA methylation and histone acetylation affect Nestin expression in the neural differentiation process (126).

In an adult brain, the cells with Nestin marker construct neutrosphere and engender differentiated astrocytes. Moreover, it has been proved that downregulation of this gene results in a significant rise of cellular invasion which shows other effects of Nestin in malignancies. In this manner, Nestin is an essential marker to evaluate both the stem cell functions and tumor cells (127).

MAP2 gene is a marker of mature neurons and its encoded protein has a key role in the formation of microtubular network. Studies showed the overexpression of this gene in some malignancies such as melanoma. In neural differentiation, Map2 is crucially required for dendritic elongation development and function. It has been confirmed by Harada et al, that any lack or silencing of this gene both *in vitro* and *in vivo* may result in a reduction of density in microtubular proteins which ends in

structural deficiencies and anomalies in the morphology of dendritic cells Map2 is also able to control the signaling pathways of axonal growth (132,133,134).

In an *in vivo* study, it has been shown that, suppression of MAP2 expression caused deficiencies in neurite formation and blocked the neural differentiation two weeks after birth. Additionally, suppression of this gene changed the neural polarity in culture medium. In contrast, the elongation of non-neural cells was detectable when the gene is overexpressed (128).

β-tubulin III, a class member of β-tubulins is a structural protein which forms the microtubule network in vertebrates. Generally, tubulins are essential for important processes (such as mitosis) in cells and β-tubulin III is located exactly in neurons. Expression of this gene specifically correlates with the initial state of neural differentiation. Thus, β-tubulin III has a key role in neurogenesis and axon instruction (129).

Downregulation of β-tubulin III in neural cells makes cells more sensitive to some chemotherapy drugs and chemicals. In neurons, this gene encodes tubular proteins which are important in neuronal morphogenesis. It may depend on the binding mechanism of cells which are related to the protein encoded by β-tubulin III. Conversely, the increase of β-tubulin III expression caused cellular resistance to anti-microtubule compounds (130).

The results of mutations in this gene are some difficulties in cortex construction. The relation between mutations of β-tubulin III gene and some of the brain anomalies (such as microcephaly) has been discovered. Depletion of this gene causes alteration of the cell cycle, disruption of the division of progenitor cells, deformity of basal ganglia(108).

In mouse, the Oct-4 and NANOG genes are expressed at pluripotent stem cells and the SOX2 and Nestin genes are expressed especially in the neural ectoderm cells. Nrf2 gene encodes proteins which are responsible for cellular resistance to oxidation. In order to genetic evaluation, we tested the mES cells and differentiated mES cells by

Real-time PCR method to discover the expression of several genes before and after ethephon treatment. We investigated the expression of Oct-4, SOX2, NANOG, Nrf2, and Nestin in mES cells before differentiated at the concentrations of 160 and 320 μM of ethephon. At these concentrations we did not observe any cytotoxic effect. However, previous study on mouse embryonic fibroblast cells showed the cytotxic effects of ethephon at the concentration of 160μM (115).Our data demonstrated a significant decreased expression of Oct-4, SOX2 and NANOG genes, dose dependently. These findings show that ethephon can affect expression of stemness genes at these doses. Although the genotoxic effects of ethephon and the DNA damage at the same concentrations has been confirmed, ut more molecular studies are required to identify the mechanism of these effects (119).

Conversely to the other examined genes, the Nrf2 was overexpressed with the increased concentration of ethephon. Due to a previous study, ethephon induces the oxidative stress in mouse embryonic fibroblasts at the concentration of 160 μM of ethephon (119). Although our observations did not show any similar effect of ethephon in oxidative stress assays.

The effects of ethephon after mES differentiation process were also examined by evaluation of Oct-4, SOX2, NSE, MAP2 and β-Tubulin III. The Oct-4 gene was selected and used as a progenitor stem cell marker. Nestin and SOX2 were used as common markers in the detection of predominant stem cells of the central nervous system. When neural cells in the central nervous system are nearly matured, the expression of Nestin gene is suppressed automatically. While NSE, MAP2 and β-Tubulin III were selected and used as a mature neuronal lineage and dendritic markers (96).

Our observations confirmed that ethephon is potent to induce a significant decrease in the expression of NSE, MAP2, and β-Tubulin III when treated neurons were compared to the control group in neural differentiated mES cells. In other words this study demonstrated that ethephon is potent to decrease the expression of these genes in mature neurons.

We suggest that more *in vivo* and *in vitro* studies are required to determine the exact mechanism of developmental toxicity and effects of ethephon on cellular signaling pathways during long term exposurements.

6. CONCLUSION AND SUGGESTIONS

In conclusion, our study confirmed that developmental toxicity of ethephon in both undifferentiated mES cells and neuronal differentiated mES cells. Ethephon has been known as a non-harmful plant regulator hormonem and this is the first study provides data for neural developmental toxicity of ethephon.

- ✓ Ethephon is cytotoxic for mES cells at higher doses.
- ✓ Ethephon induces developmental toxicity in mES cells at non-cytotoxic concentrations. However the mechanism of action is still unknown.
- ✓ Our findings showed no relation between cytotoxic effect and induction of oxidative stress in ethephon treated mES cells.
- ✓ The impact of ethephon during neuronal differentiation process has been proved by overexpression of stemness genes (Oct-44, SOX2, NANOG and Nestin).
- ✓ Ethephon is potent todecrease the expression of specific genes such as MAP2 and β-Tubulin III in mature neurons. This effect is important in the neural lineage during developmental process.

As ethephon is a globally used agent in today's agriculture, the importance of this research is the evaluation of the cytotoxic and other effects of a widely used compound in crops. Moreover, research on the action mechanism of ethephon will facilitate the diagnosis of various developmental deficiencies and on the other hand may help scientists to produce chemicals with no or lower toxic effects.

The most important issue about this substance is the chronical exposure of human and other organisms to ethephon. According to the lack of studies in the field of cytotoxicity and genotoxicity of ethephon in mammals, we suggest further *in vitro* and *in vivo* experimental researches to assess the influence of ethephon and its residues in

human health to understand the molecular mechanism of ethephon in developmental pathways.

The measurement of acetylcholine esterase activity to find the toxic dose would be helpful for determination of study doses in future *in vitro* study models. Use of more developed technologies such as Next Generation sequencing in IPS cells will help researchers to achieve analytical data of ethephon and the effectiveness of this substance in various signaling pathways during development.

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8. ANNEXES

8.1 Digital Reciept

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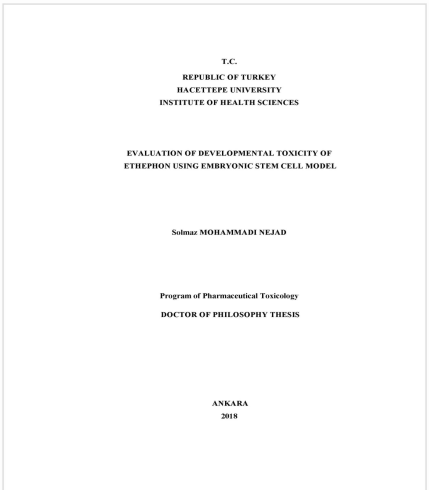

T.C.
REPUBLIC OF TURKEY
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INSTITUTE OF HEALTH SCIENCES

EVALUATION OF DEVELOPMENTAL TOXICITY OF
ETHEPHON USING EMBRYONIC STEM CELL MODEL

Solmaz MOHAMMADI NEJAD

Program of Pharmaceutical Toxicology
DOCTOR OF PHILOSOPHY THESIS

ANKARA
2018

8.2 Thesis Originality Report

Evaluation of Developmental Toxicity Of Ethephon Using Embryonic Stem Cell Model

Solmaz Mohammadi Nejad

Total 116 pages

9. CURRICULUM VITAE

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Education

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BSc: Medical laboratory sciences, Shahid Beheshti University of Medical Science and Health services, Tehran, Iran Feb 2006.

Publications:

- Molecular and biochemical evidence on the protective role of ellagic acid and silybin against oxidative stress-induced cellular aging. Molecular and Cellular Biochemistry. (Sep 2017) https://www.ncbi.nlm.nih.gov/pubmed/28887692
- Pharmacological and Toxicological Properties of Eugenol. Turkish journal of pharmaceutical sciences. (Aug 2017)

http://turkjps.org/article_15167/Pharmacological-And-Toxicological-Properties-Of-Eugenol

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- Work in "DNV GL. safeguarding life, property and the environment". Department of Risk assessment. 2009 -2011.
- Work in "Karestan Kala" company, Active in trading medical diagnostic kits as Quality control expert. Tehran. 2006- 2009.
- Work in Iran pathobiology & clinical diagnosis lab. Department of Biochemistry. 2005-2006.
- Work in Taleghani hospital as a summer student in clinical Biochemistry department. Summer student. June-Sep 2005.
- Work in laboratories National Research Institute for Tuberculosis and Lung Diseases. medical technologist. Department of Biochemistry and immunology. Shahid Beheshti University of Medical Science and Health services 2001-2006.

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- Student member of Society of Toxicology (SOT)
- Member of Iranian Society of Toxicolog