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# EFFECTS OF ENVIRONMENTAL DIESEL EXHAUST PARTICLES ON GASTRIC STEM CELLS

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Heba Ahmed Mazen Al Sadik

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sciences

Under the Supervision of Professor Sherif M. Karam

April 2017

# Declaration of Original Work

I, Heba Ahmed Mazen Al Sadik, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled *"Effects of Environmental Diesel Exhaust Particles on Gastric Stem Cells"*, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Sherif M. Karam, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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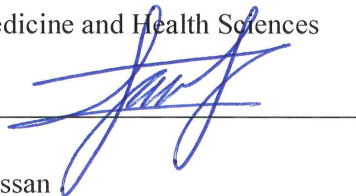   Signature _____    Date *April 19, 2017*

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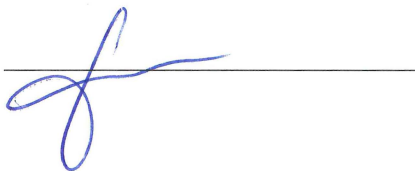
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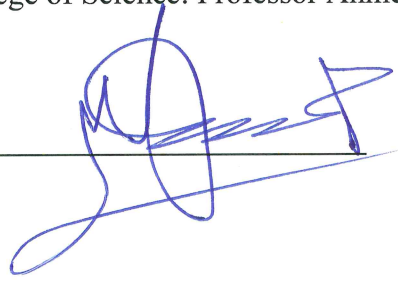
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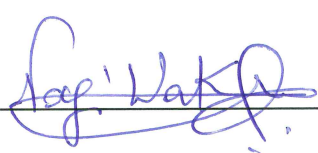
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# Abstract

Even though the stem cells have attracted many scientists because of their unique properties and therapeutic applications, it is not known how the environmental toxic factors could affect their features and functions. This study is focusing on the interaction between environmental diesel exhaust particles (DEP) and the stomach stem cells. In the stomach, the stem cells are responsible for generating and maintaining different types of cells which are organized to form numerous tubular glands. The aim of this study was to examine the effects of DEP on the mouse gastric stem cells. The hypothesis is that DEP could have deleterious effects on the properties of mouse gastric stem cells such as their viability and migration. An immortalized mouse gastric stem cell line was used to develop a simple *in vitro* model to test the effects of exposure to various concentrations of DEP. Stem cells were cultured using routine tissue culture protocols. The DEP were added to the culture media at different concentrations: 1, 10, and 100 µg/ml for different time points up to 72 hours. Then, stem cells were analyzed using: cell viability assay, wound healing or migration assay, oxidative stress analysis by measurement of reduced glutathione, lipid peroxidation, and nitric oxide, expression analysis of genes specific for cell proliferation, cell death and oxidative stress using quantitative real-time polymerase chain reaction, and expression of stem cell specific proteins using western blotting. While DEP induced a reduction in the growth rate of gastric stem cells only at high concentration, no significant effects were found on cell migration, cell death related genes/proteins. Only minor changes were observed in oxidative stress parameters. However, these findings were interestingly associated with down-regulation of Notch 1, 2 and 3 proteins. Since Notch signaling pathways play an important role in development and differentiation of stem cells, it will be interesting to determine which mechanisms and target genes are involved in an animal model of DEP exposure. In conclusion, this study establishes an *in vitro* model system to investigate the biological feature of gastric stem cells when exposed to environmental pollutants. In addition, demonstrating the effects of DEP on adult stem cells will help in raising public awareness about environmental hazardous agents.

# Title and Abstract (in Arabic)

## التأثيرات البيئية لجزيئات الديزل لعوادم السيارات على الخلايا الجذعية المِعَدِيَة

### الملخص

بالرغم من ان الخلايا الجذعية قد جذبت العديد من العلماء بسبب ما تتميز به من خواص متميزة وتطبيقات علاجية واسعة، إلا أنه من غير المعروف إلى الآن كيف تؤثر العوامل البيئية السمية على خواصها وعملها. في هذه الدراسة سوف نركز على تأثيرات جزيئات الديزل البيئية لعوادم السيارات على الخلايا الجذعية الخاصة بالمعدة.

تعتبر الخلايا الجذعية في المعدة هي المسؤولة عن عملية التجديد والحفاظ على بطانة الطبقة النسيجية الظهارية للمعدة التي تنتظم لتشكل العديد من الغدد الأنبوبية التي يسكنها أنواع مختلفة من خلايا إفراز المخاط، الأحامض، مولد الببسين، ومختلف الهرمونات / الببتيدات. خطة هذه الدراسة هي إستخدام جزيئات الديزل لعوادم السيارات لإختبار تأثيراتها على الخلايا الجذعية لمعدة الفئران. الإفتراض هنا يكمن في إمكانية وجود تأثيرات ضارة لجزيئات عوادم السيارات على الخواص الحيوية لهذا النوع من الخلايا مثل خاصية تكاثرها وإنتشارها وقدرتها على البقاء. سوف يتم إستخدام الخلايا الجذعية للمعدة لتطوير طراز مخبري بسيط لإختبار تأثيرات التعرض لجزيئات السابقة بمختلف التراكيز الموجودة. الخلايا الجذعية سوف يتم زراعتها بإستخدام بروتوكول زراعة الانسجة الروتيني. جزيئات الديزل لعوادم السيارات سوف يتم إضافتها بمختلف تركيزاتها إلى البيئة الزراعية. ضمن الدراسة سوف يتم تحليل العديد من المعايير والعوامل. أولا قابيلة الخلايا الجذعية للحياة والناتجة من عن طريق استخدام (فحص قابلية الحياة) التي تعتمد على تحديد كمية جزيئات الطاقة الناتجة بعد تعرضها للتركيزات المختلفة من جزيئات عوادم السيارات وإستخدام (فحص قابلية هجرة الخلايا) التي تعتمد بدورها على قياس حجم الشقوق التي تم تشكيلها ضمن طبقة من الخلايا الجذعية. ثانيا تحديد مؤشرات الإجهاد التأكسدي للخلايا الجذعية مثل الاكسدة الليبيدية، الغلوتاثيون المخفف و النيتريك المؤكسد عن طريق استخدام طريقة (فحص الإجهاد التأكسدي) الناتجة من الخلايا المختلفة. ثالثا التعرف على تعبير جينات محددة داخل الخلايا الجذعية عن طريق استخدام الطريقة المشهورة (تفاعلات السلسلة البوليميرازية في الوقت الحقيقي لها) و طريقة (هجرة الجزيئات الصبغية). إستنتاج التأثيرات السمية لجزيئات الديزل لعوادم السيارات على الخلايا الجذعية الناضجة سوف يساعد في تطوير الوعي للمجتمع بشأن المخاطر البيئية والمساعدة في اتخاذ تدابير وقائية من

قبل السلطة ضد الآثار الضارة للجزيئات عادم الديزل. بالاضافة الى المساهمة في تطوير مجال العلاجات المتجددة.

**مفاهيم البحث الرئيسية**: الخلايا الجذعية، الجزيئات البيئية لعادم الديزل، فحص قابلية الحياة، فحص قابلية هجرة الخلايا، فحص الإجهاد التأكسدي، بروتينات النوتش.

## Acknowledgements

I would like to thank my committee for their guidance, support, and assistance throughout my preparation of this thesis, especially my advisor Dr. Sherif Karam. I would like to thank the chair and all members of the Department of biology, Science College and graduate study College at the United Arab Emirates University for assisting me all over my studies and research.

# Dedication

*To my beloved the most beautiful place in the world my country SYRIA, Parents, brother, sisters and family (my husband and my little baby…)*

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# List of Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BCA | Bicinchoninic acid |
| BMl | B cell-specific Moloney murine leukemia virus integration site 1 |
| DEP | Diesel exhaust particles |
| DTNB | Dithionitrobenzoic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ESC | Embryonic stem cells |
| GSH | Reduced Glutathione |
| GSTP1 | Glutathione S-transferase |
| HPRT | Hypoxanthine-guanine phosphoribosyltransferase |
| iPS | Induced pluripotent stem cells |
| mGS | Mouse gastric stem cells |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NO | Nitric oxide |
| Oct4 | Octamer-binding transcription factor 4 |
| PBS | Phosphate buffered saline |
| PM | Particulate matter |
| pSTAT3 | phosphorylated signal transducer and activator of transcription 3 |
| RIPA buffer | Radioimmunoprecipitation assay buffer |
| RPMI | Roswell Park Memorial Institute medium |
| RT-PCR | Real time polymerase chain reaction |
| SDS | Sodium dodecyl sulfate |
| STAT3 | Signal transducer and activator of transcription 3 |
| TBARS | Thiobarbituric acid reactive substances |
| TBA | Thiobarbituric acid |

TBS         Tris-buffered saline

TBST        Tris-buffered saline with Tween

TNB         5-thio-2-nitrobenzoic acid

# Chapter 1: Introduction

## 1.1 Overview

The human body is made of hundreds of types of cells which can be classified into two main categories: somatic and gonadal cells. A small fraction or a subpopulation of the somatic cells is similar to many embryonic cells. Members of this subpopulation can divide by mitosis to maintain themselves and to ensure continuous production of mature cells that perform the functions of the body organs. These mitotic cells are referred to as the "stem cells". While there are many studies on the effects of environmental hazardous particles on body organs, little is known about their effects on stem cells.

## 1.2 Stem cells

The stem cells are undifferentiated cells that have the ability to renew themselves and to give rise to various cell types (Verfaillie, 2002). Morphologically, the stem cells are characterized by small size with high nucleus-to-cytoplasm ratio. They also have undifferentiated embryonic cell like features: few small organelles, many free ribosomes and prominent nucleoli (Karam, 2012). Functionally, the stem cells are capable of division to renew themselves and to differentiate throughout the life of the organism. Therefore, they are responsible for the homeostasis of body organs. The stem cells are not only responsible for renewing but also repairing different tissues and organs in the body (Brown, 2013).

In humans, there are two main types of stem cells. The first type is the embryonic stem cells which form the inner cell mass of the blastocyst (Passier & Mummery, 2003). This mass of cells forms after three to five days of fertilization

and gives rise to the more specialized cells which then form the whole body. These stem cells are pluripotent, which means they can develop into all types of body cells to form the whole human body. Therefore, embryonic stem cells are of great value because they are considered a renewable source for the body tissues and can be used to study the development of the human body in addition to testing new drugs on human development (Jun & Park, 2009).

Scientists have been able to use adult somatic cells to generate induced pluripotent stem (iPS) cells similar to those of embryonic stem cells. The development of this technology and the generation of this new type of stem cells led to awarding Noble Prize to Shinya Yamanaka in 2006 (Takahashi & Yamanaka, 2006). These cells have been engineered in the lab by converting adult somatic cells, like the cells of the dermis of the skin (fibroblasts), into cells that behave like embryonic stem cells and possess their features. For the generation of iPS cells, some retroviruses were used to transfect the fibroblasts of the mouse skin with four key pluripotency genes: Oct4, SOX2, c-Myc, and Klf4. The embryonic stem cells share many properties with iPS cells, involving their ability to develop into all different types of body cells (Yu et al., 2007) However, there are some negative features for iPS cells. They showed some DNA methylation errors compared to the original patterns in embryonic stem cells (Ooi et al., 2010). Also, they failed to produce a viable chimer if injected into developing embryos (Singh et al., 2015).

The second type of stem cells is the adult or tissue-specific stem cells. They are more specialized than embryonic stem cells and located in the various organs of the body, such as the skin, the brain, and the liver. The main function of these cells is to generate different specialized types of the cells for the specific organs.

Hematopoietic stem cells residing in the bone marrow represent another example of tissue-specific stem cells. They are responsible for the production of white blood cells, red blood cells, and platelets (Korbling et al., 1995). In a tissue section, the stem cells can be identified by *in situ* hybridization or by immunohistochemistry using specific markers which include genes and their protein products such as Oct4 (Amini et al., 2014; Schöler et al., 1990).

In the stomach there are also tissue specific adult stem cells located among the epithelial cells lining the luminal surface. During development these epithelial cells form a monolayer sheet which gradually invaginates to form millions of tubular glands. In the adult stomach, the gland is divided into four regions: pit, isthmus, neck, and base (Figure 1). Along the wall of these glands there are different types of cells: pit (foveolar) cells, parietal (oxyntic) cells, zymogenic (chief) cells, and enteroendocrine cells. These mature cells are respectively responsible for the production of mucus, hydrochloric acid, pepsinogen, and several types of peptides or hormones throughout the life of the organism. Electron microscopic studies and tritium thymidine cell labeling analysis revealed that all these cells originate from the adult stem cells located in the isthmus region of each gland. These cells are undifferentiated and located in the mid-isthmic region of the epithelial gland (Karam & Leblond, 1992).

The stem cells give rise to poorly differentiated epithelial progenitors. Detailed radioautographic and ultrastructural analyses showed that these progenitor cells are responsible for the continuous production of all cell types that populate the gastric glands (Karam & Leblond, 1993). Little information is known about the gastric progenitor cells. Some evidences have indicated that alterations in their

proliferation and differentiation programs play a key role in the pathogenesis of gastric dysplasia and neoplasia (Giannakis et al., 2008; Farook et al. 2008). Moreover, some studies provided evidences that these progenitor cells express receptors for binding of *Helicobacter pylori*, a major factor involved in the pathogenesis of peptic ulcer, gastritis, and even adenocarcinoma (Stein et al., 2013). Thus, further studies on these epithelial progenitors of the gastric glands are very important to provide some basic information for the better understanding of the pathogenesis of several stomach diseases including gastric cancer which are relatively common worldwide and also in the United Arab Emirates.

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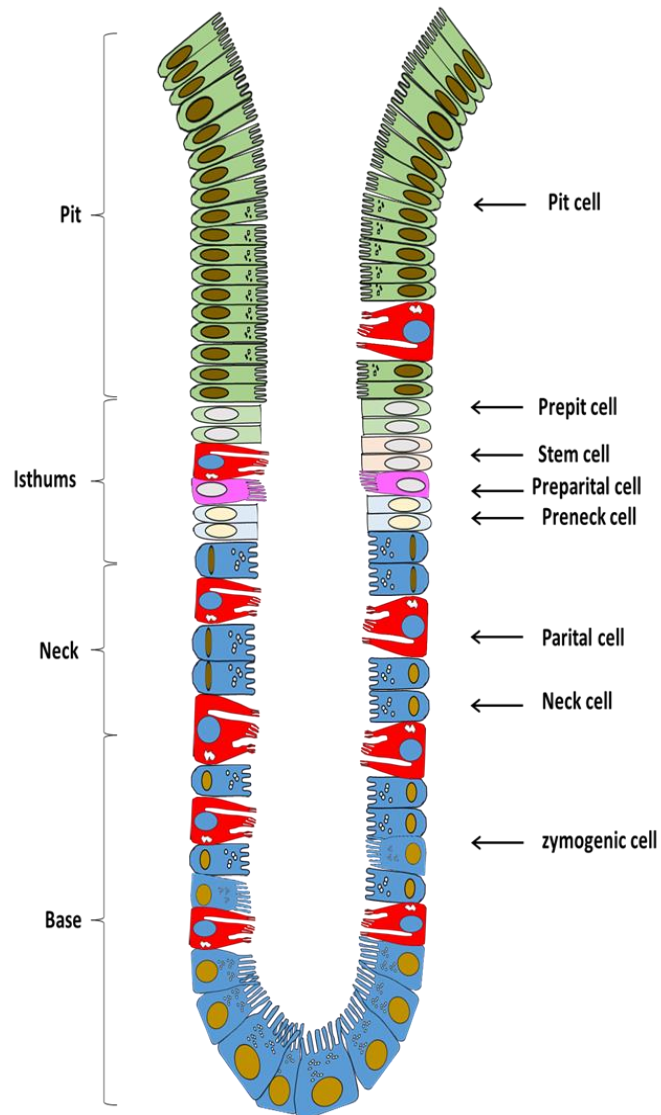
Figure 1: Diagramtic representation of the gastric gland showing the different cell types and their locations along the four regions: pit, isthmus, neck and base. The stem cells are located in the isthmus region. They divide and give rise to prepit, preparietal and preneck cells which undergo differentiation and become pit, parietal and neck cells. The latter continue to differentiate and become zymogenic cells

Gastric cancer has long been considered as one of the major global human-killing disease. It accounts for around 10% of newly diagnosed cancer in some areas of the world (Roder, 2002). More recently, the world health organization and National Cancer Institute of USA indicated that every year the total number of gastric cancer cases reported throughout the world averages about 670,000 cases and more than 24,000 cases are diagnosed in the United States each year (Bafna et al., 2008). Despite a gradual decline in the incidence and mortality rates of gastric cancer, it remains the fourth most common cancer and the second leading cause of cancer related death worldwide (Fox & Wang, 2007). The high frequency and mortality rates of gastric cancer are warning signals to improve our understanding on the proliferative stem cells of the gastric glands and predisposing factors that may affect their behavior. In the United Arab Emirates, data kindly provided by National Cancer Registry in Tawam Hospital shows an increased number of gastric cancer cases in 2007 with higher prevalence among males than females.

**1.2.1 Medical applications of stem cells**

Long time ago, the value of the medical applications of stem cells was explored. The use of stem cells in medicine continues to evolve gradually to the present day. For example, studies have shown that the stem cells have potential applications in various cardiac diseases, such as acute myocardial infarction, chronic heart failure, and advanced coronary artery disease. Experimental studies have shown that transplantation of stem cells could have a positive effect on the contractile performance and tissue perfusion of the injured heart (Abdelwahid et al., 2011).

Stem cell transplantation also has potential application in diabetic patients who are in a continuous need for insulin injections due to lack of beta cells. This transplantation procedure will minimize the need of insulin injections on a regular basis. However, the use of embryonic stem cells for treatment of diabetes has faced some ethical problems along with high potential for the rejection (Lumelsky et al., 2001). In order to solve these implications, the embryonic stem cells have been replaced by autologous stem cells (Palumbo et al., 2014). Therefore, adult stem cells have become a good alternative because they resolved the problem of rejection and the ethical stigma of embryonic stem cells.

The use of the regenerative power of stem cells has been demonstrated in many diseases and cancers of blood that used to destroy the immune system leading to death. Nowadays, patients with leukemia, lymphoma, anemia and multiple myeloma have more hope with the development of bone marrow stem cell transplantation. Every year, thousands of such transplants are done in many centers worldwide using autologous stem cells. To improve efficiency of such procedures in pediatrics and elderly patients, scientists have even developed new protocols for personalized bone marrow transplantation (ten Brink et al., 2014; Hassan & Abedi-Valugerdi, 2014).

With the advancement of knowledge about stem cells and their medical applications, very little is known about the effects of environmental toxins and/or particles that may affect their features during health, disease, and therapy.

**1.3 Diesel exhaust particles (DEP)**

The emissions of pollutants from diesel engines include a complex mixture of hundreds of constituents in either gas or solid forms. The diesel solid emissions include particles which consist of a central core of elemental carbon with organic compounds including polyaromatic hydrocarbons and nitro-polyaromatic hydrocarbons, heavy metals, gaseous materials, and other trace elements. Therefore, the diesel exhaust includes particles which are ultrafine with a large surface area and are considered highly respirable and can cross the air-blood barrier. So, many studies are conducted on the effects of these particles on the respiratory and cardiovascular systems (Nemmar et al., 2015; Yoshizaki et al., 2015). In addition, to the possibility of inhaling these particles, they can easily reach the oral cavity, mix with the saliva and swallowed into the esophagus and the stomach. Surprisingly, nothing is known about the effects of these particles on the gastrointestinal tract or any of the gastrointestinal stem cells.

**1.3.1 Effects of DEP**

Studies have shown that exposure to the DEP leads to many diseases affecting different body parts including the respiratory (Yoshizaki et al., 2015), cardiovascular (Nemmar et al., 2015), nervous (Hougaard, 2009) and immune (Müller, 2013) systems. In addition, the risk of cancer was found to increase with the exposure to the exhaust of diesel engines (Birch & Cary, 1996). Moreover, the ambient fine and ultrafine particles, which are an important component of DEP, are found to be involved in the pathogenesis of cardiopulmonary disorders and lung cancer (Wichmann, 2007) .

The emissions of the heavy-duty diesel engines have a 30–100 times as many particles as gasoline engines with variable diameter, 0.02 to 0.2 μm. These particles can adsorb over 450 various organic compounds such as mutagenic and carcinogenic polycyclic aromatic hydrocarbons. The DEP include fine to ultrafine sizes of particles which can readily deposit in the alveoli of the lungs. For these serious reasons, the exposure of truckers, engine mechanics, railroad and construction workers to DEPs is considered a serious health problem worthy of attention (Castranova et al., 2001).

The DEP include smaller particulate matters that are considered more dangerous than the larger ones because of their higher potential to cause health problems in humans. Only these particles with diameters less than 10 micrometers can enter the human airways cause health problems even in very small amounts. Ultra-fine particles with diameter less than 0.1 micrometer are considered the most dangerous for human health due to their ability to penetrate deep into the lungs, to cross the air-blood barrier, and to reach the bloodstream (Lekkas, 2008). The particles of 20-40 nm in diameter are considered problematic for humans. These particles are able to easily become stuck to the alveoli and cause serious effects on respiration.

One of the most serious effects of DEP in the lungs and heart is to cause oxidative stress and inflammation which lead to a decrease in the respiratory function, an increase of coagulation of the blood, and a high susceptibility to coronary thrombosis. Studies showed that within 24 hours, the acute single-dose of intra-tracheal instillation of DEP (15–30 μg/mouse), a one type of particulate matter like PM2.5, can cause influx of inflammatory cells, which lead to the oxidative

stress. The characteristics of DEP, such as size, shape, quantity, chemical composition, and solubility, play an important role in their effects on the organism. The particles which dissolve rapidly in aqueous solutions are diluted in the body while the insoluble particles retain their toxicity and thus their impact becomes chronic (Mayer et al., 2009).

Because of the exceptional thin membrane, the wall of the alveoli allows the oxygen to cross and enter the bloodstream. Thus, the fat-soluble and other compounds on the surface of bigger particles which are so small can pass through this thin membrane and via the bloodstream reach to any organ (Kgabi et al., 2015). The body's reaction to these particles varies depending on the size of the particles. Generally, the body can dispose the larger particles through exhalation, during a matter of hours. But the smaller size of particles may remain inside the body for several months or years. Normally, these smaller particles are carried to lymph nodes and phagocytosed by immune system macrophages. Also, there is a possibility that these particles remain in the organs as long as the body lives (Dobrovolskaia et al., 2008; Sydbom et al., 2001) .

The most prevalent and common health effects of DEP are the respiratory and cardiovascular diseases. The common symptoms of respiratory problems, like coughing, difficulty breathing and irritation of the airways are immediately detected on a dusty street overcrowded with transportation vehicles.  Alterations in brain blood circulation may also appear in a few patients. Children or old individuals are the most likely to be affected by particulate matters. In addition, studies showed that the worst negative effect as a result of exposure to these DEP is the premature death, especially in patients with asthma and respiratory diseases. The increasing amount of

DEP content in the air can deteriorate the working orders of the lungs and increase the rates of diseases and hospital admissions (Anderson et al., 2012) . Eventually, the presence of these particles significantly in atmosphere surrounding human beings inevitably leads to a high rate of mortality (Xu et al., 2012) .

**1.3.2 Defense mechanism against DEP**

The human body has various mechanisms for the disposal of the accumulated DEP. During the process of breathing, the body inhales the dust, noxious gases and other components of air. Most of these particles are disposed in the respiratory system by coughing, and others are attached to the luminal layer of mucus and then disposed with the sputum. The water-soluble contents of DEP can probably reach the bloodstream through small capillaries of the respiratory tract, and then reach the kidneys and finally excreted with the urine without causing any harm. However, the other compounds like lipid-soluble compounds can reach the phospholipid membrane inside the cells and thus disposing them will be more difficult and slower. The body cannot eliminate all DEP; it depends on their chemical compositions and size. Thus, the immune cells in some cases in the lungs may not be able to fight against them. The ultrafine particles can reach and pass into the bloodstream and their path is very unpredictable. There are different possibilities, for example, they can attach to endothelial cells lining the walls of blood vessels, the heart, and sinusoids of the bone marrow or the liver. Also, in their path they can induce inflammation, oxidative stress, DNA dysfunctions, and finally cell death. One of the most important concerns in this issue is the increased risk of cancer due to the DNA alterations. The oxidative stress can also lead to inflammation in the bronchial walls and alveoli which are considered one of the main causes of chronic asthma and

chronic obstructive pulmonary disease (Repine et al., 1997). Patients suffering from these diseases are more vulnerable to particles.

The DEP can also cause a decrease in the efficiency of gas exchange in the lungs and enhancement of blood coagulation. However, it should be noted that because of individual variations, it is difficult to predict and determine the effects of DEP.

**1.4 Aims of the study**

While much is known about the effects of diesel exhaust particles on the functions of different body organs and their specialized cells, there is no information available regarding their effects on epithelial stem cells. Therefore, the overall aim of this study was to obtain new information about the effects of environmental DEP on epithelial stem cells. Immortalized adult mouse gastric stem (mGS) cells were used as an *in vitro* model system for the following specific objectives:

1. To identify the effects of DEP on the growth and proliferation of mGS cells by using CellTiter-Glo viability assay.

2. To detect the effects of DEP on the migration of mGS cells by using the wound-healing assay.

3. To determine whether mGS cells' exposure to DEP causes oxidative stress by using different biochemical assays for the measurement of the released levels of the antioxidant glutathione (GSH) and oxidative stress markers such as lipid peroxidation (Thiobarbituric acid reactive substances or TBARS) and nitric oxide (NO).

4. To obtain some clues about how DEP incubation with mGS cells would affect the expression levels of proteins and genes specific for stem cells and cell death using western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR).

## Chapter 2: Methods

### 2.1 Preparation of DEP

The DEP (SRM 2975) were obtained from the National Institute of Standards and Technology (Gaithersburg MD, USA) and were suspended (1000µg of DEP) in 1 ml sterile saline (0.9% NaCl) containing 0.01% Tween 80. To minimize aggregation of particles, the DEP suspensions were sonicated for 15 minutes and vortexed immediately before preparing dilutions for use. For control, saline containing 0.01% Tween 80 was used. These particles were previously analyzed by transmission electron microscope and shown to have a substantial amount of ultrafine (nano) sized particles aggregates and larger particles aggregates (Nemmar et al., 2007; Nemmar et al., 2012)

### 2.2 Culture of mGS cells

Frozen aliquots of the mGS cells of passages 20-25 were cultured in T25 or T75 flasks using RPMI media containing 10% fetal bovine serum and a 37˚C incubator adjusted to 5% $CO_2$ and 95% $O_2$. The culture media was changed every other day. When semi-confluent, the cells were passaged twice to stabilize their morphology and growth rate before used in this study (Farook et al., 2008).

### 2.3 Cell viability (CellTiter-Glo) assay

The mGS cells of passage 17 were plated in petri dish, then trypsinized and counted by handheld automated cell counter (Merck-Millipore, Billerica, MA, USA). In the 96 well plates, the cells were seeded at 5000 cells/100 µl media per well. After 24 hours of incubation the cells were incubated with different concentrations of DEP (0, 1, 10, 100 µg/ml) prepared in saline containing 0.01% Tween 80. For control,

cells were incubated with media containing only vehicle. After 1, 6, 24, 48, 72 hours, the cells with different concentrations of DEP were incubated with 100 µL of CellTiter-Glo® 2.0 reagent for 10 min at room temperature. The amount of ATP released from the cells was quantified in Eppendorf tubes by using GloMax-Integrated luciferase technologies (Promega). The measured amount of released ATP reflects the amount of viable cells in each well. This experiment was repeated three times. The data were analyzed by using the one-way ANOVA with Dunnett's multiple comparison test model. This method of analysis was used to create confidence intervals for differences between the mean of each factor level and the mean of a control group. P value of less than 0.05 was taken to indicate a significant difference. Graphical representation of the data (mean ± SEM) was performed using GraphPad Prism software (La Jolla, CA, USA).

## 2.4 Migration (or Wound-Healing) assay

The mGS cells were cultured in Petri dish, trypsinized and then counted by handheld automated cell counter. In the 6 well plates the cells were seeded at 1.5x10^6 cells/well/2ml. After 24 hours of incubation, when the cells formed a monolayer, a lesion (scrape or wound) was made along the middle of each well with a sterile pipette tip. Then, the media was removed and the cells were washed with phosphate buffered saline (PBS). Media containing different concentrations of DEP (1, 10 µg/ml) prepared as described above were added. For control, vehicle was only added without any DEP. After 1, 6 and 24 hours, the cells with the lesions in the center of wells were photographed using inverted Olympus microscope and DP70 digital camera. For each well, the width of the wound was measured at three different places at each time point. The difference between the measurements at 1 hour and the

measurements at 6 and 24 hours was taken as the migration distance and expressed as mean ± SEM. The percentage of wound healing was also calculated and blotted against time. This assay was repeated three times. The difference between the data of control and experimental groups were analyzed by using the one-way ANOVA with Dunnett's multiple comparison test.

## 2.5 Measurements of oxidative stress markers

The mGS cells of passage 20, were cultured in a Petri dish using RPMI containing 10% serum. When confluent, the cells were trypsinized and counted by handheld automated cell counter. In the 12 well plates the cells were seeded at 35,000 cells/well/1ml. After 24 hours of incubation, the media was removed and the cells were incubated with fresh media containing different concentrations of DEP (1, 10, 100 µg/ml) prepared in saline containing 1% Tween 80. For control, cells were cultured in media containing only vehicle. After 24 hours of incubation, the media (supernatant) of each well (1 ml) were removed and divided equally into three Eppendorf tubes and processed for different assays. These following three assays were repeated three times.

## 2.5.1 Glutathione measurement

The samples were deproteinized with 5% sulfosalicylic acid, centrifuged to remove the precipitated protein, then assayed for glutathione using dithionitrobenzoic acid (DTNB) method (Banerjee et al., 2002). The assay was carried out in a 96 well plate as follows. The first 2 wells were loaded with only 10 µl of the 5% 5-Sulfosalicylic acid as a blank. Then, 10 µl glutathione standard solutions were added in duplicate into separate wells. The unknown samples were

added also in duplicate into separate wells in various amounts (up to 10 µl). Then 150 µl of the assay buffer containing potassium phosphate, EDTA, glutathione reductase, NADPH, DTNB were added to each well with a multichannel pipette and mixed. After 5 min incubation at room temperature, 50 µl of the diluted NADPH solution was added. The presence of glutathione in the samples causes reduction of DTNB to a yellow product, 5-thio-2-nitrobenzoic acid (TNB) which was measured spectrophotometrically using a plate reader at a wave length of 412 nm. The reaction rate was proportional to the concentration of glutathione. A standard curve of reduced glutathione was used to determine the amount of glutathione in the samples. The experiment The data were presented as mean ± SEM and graphically presented using GraphPad Prism software (La Jolla, CA, USA). For statistical analysis, the one-way ANOVA with Dunnett's multiple comparison test was used.

### 2.5.2 TBARS measurement

In labeled 5 ml tubes, 100 µl of different samples were obtained from the culture media of mGS cells incubated with 0, 1, 10, 100 DEP. Equal volumes of standard solutions were added in separate tubes. Then, 100 µl of sodium dodecyl sulfate (SDS) solution were added to the samples and mixed well. The color reagent (4 ml) containing thiobarbituric acid, acetic acid, and sodium hydroxide was added forcefully in each Eppendorf tube. The samples-containing tubes were caped and heated in a water bath at $95^0$C. After one hour, the samples were placed on ice for 10 minutes to stop the reaction and then centrifuged for 10 minutes at 3,000 rpm. Then, 150 µl of each sample were loaded in duplicate to 96-well plate to measure the absorbance by spectrophotometer at a wave length of 490 nm. The data were analyzed statistically by using the one-way ANOVA with Dunnett's multiple

comparison test. Graphical representation of the data (mean ± SEM) was performed using GraphPad Prism software (La Jolla, CA, USA).

### 2.5.3 NO measurement

The nitrite/nitrate assay kit (Sigma, St Louis, MO, U.S.A.) was used for measurement of NO according to the instructions of the manufacturer. Briefly, all samples and standards were run in duplicate using a 96 well plate. Equal volumes (100 µl) of standard solutions (NaNO2 in H2O) and the unknown samples of the culture media of mGS cells incubated for 24 hours with DEP at different concentrations were transferred into separate wells. Then, 100 µl of the Griese reagent was added to the standards solutions and unknown samples. All samples were incubated for 5-10 minutes at room temperature for the color reaction to develop. The intensity of the colored product was measured by Biotek reader (ELx 800) at 492/650 - 562/570 nm. The readings were proportional to the NO metabolite present in the samples. The data were analyzed statistically by using the one-way ANOVA with Dunnett's multiple comparison test. Graphical representation of the data (mean ± SEM) was performed using GraphPad Prism software (La Jolla, CA, USA).

### 2.6 SDS-PAGE and Western blot analysis

The mGS cells of passage 20 were cultured in T75 flask using same conditions mentioned above. Near confluence, the cells were trypsinized and counted by handheld automated cell counter (Merck-Millipore, Billerica, MA, USA). In the petri dishes the cells were cultured at 2-million cells/dish containing 7 ml media. After 24 hours of incubation, cells were incubated with different concentrations of

DEP (1, 10, 100 µg/ml) prepared in saline containing 1% Tween 80. For control, some dishes were incubated vehicle without DEP (0 µg/ml). After 24 hours of incubation, the culture media were collected and the cells were double washed with PBS followed by trypsinization. The cells were collected and centrifuged for 5 minutes. The media were discarded and replaced by 1 ml PBS for washing. Cells were collected with PBS, transferred to new Eppendorf tubes, centrifuged, and then stored at -80°C. Lysis buffer was added to the samples, mixed well and kept on ice. After 15 minutes samples were centrifuged at 14,000 rbm for 20 minutes. The supernatants of samples were collected to new Eppendorf tubes.

For measurement of protein concentration, the bicinchoninic acid (BCA) assay was used. The BCA reagent (Pierce, Rockford, IL, USA) was added in Eppendorf tubes for samples set and standards solutions set. The bovine serum albumin (BSA) standards were prepared in 20 µl of reagent. For the set of unknown samples, 1 µl of each sample was added to 200 µl of reagent. All tubes were incubated for 30 minutes at 37°C and protein concentrations were measured by NanoDrop (ThermoFisher, USA) at 562 nm.

For protein separation, the RIPA buffer wad added to protein samples in new Eppendorf tubes. The loading buffer was added and samples were kept in the heating block at 99°C for five minutes. The resolving and stacking gels were prepared and the running buffer was added inside the tank (Bio Rad, Mini-protean tetra system GE health care life Sciences Whatman TM) for 90 minutes at 100v. Proteins separated in the gel were transferred onto nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany) using transferring buffer for 80 minutes at 100 v. The membranes were incubated with the blocking buffer (1xTBS in fat-free milk) for 1

hour. The primary antibodies for cleaved caspase 3 (Cell Signaling Technology) was prepared (1:500) in TBST, the nitrocellulose membrane sealed with the antibody solution and kept in cold room at 4ºC for 24 hours. Then the membrane was washed with TBST five times for five minutes each. The secondary antibody horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, USA) was

diluted at 1:1000 in blocking buffer, added to the probed membrane and incubated for 1 hour at room temperature (Table 1). The membrane was washed with TBST 3 times, 5 minutes each. To control equal loading of proteins in the different lanes, the blots were probed with mouse monoclonal anti-β-actin antibody. Immunoreactive proteins in the blots were detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Barrington, IL, USA). Densitometric analyses of the bands were conducted using C-DiGit blot scanner (LI-COR Biotechnology, Cambridge, UK). The analysis for anti-Notch 2, Notch 3, Oct4 and Bmi-1 antibodies was repeated using proteins extracted from three different experiments. For the other antibodies, the blots were done using proteins extracted from only one or two different experiments. Representative blots were presented.

Table 1: List of primary antibodies used in the Western blot analysis of mGS cells after incubation with 0, 1, 10 µg/ml DEP for 1 day

|  | Antibody | | Dilution | Company |
|---|---|---|---|---|
| 1 | Notch 1 | Rabbit polyclonal | 1:2000 | Cell Signaling Technology |
| 2 | Notch 2 | Rabbit polyclonal | 1:1000 | Cell Signaling Technology |
| 3 | Notch 3 | Rabbit polyclonal | 1: 300 | Cell Signaling Technology |
| 4 | Notch 4 | Mouse monoclonal | 1:2000 | Cell Signaling Technology |
| 5 | Oct 4 | Rabbit polyclonal | 1:200 | Cell Signaling Technology |
| 6 | Bmi-1 | Rabbit polyclonal | 1:500 | Cell Signaling Technology |
| 7 | Cleaved caspase 3 | Rabbit polyclonal | 1:500 | Cell Signaling Technology |
| 8 | Caspase 3 (H-277) | Rabbit polyclonal | 1:150 | Santa Cruz Biotechnology |
| 9 | Cleaved PARP | Mouse monoclonal | 1:150 | Cell Signaling Technology |
| 10 | Bcl2 | Rabbit polyclonal | 1:300 | Cell Signaling Technology |
| 11 | STAT3 | Mouse monoclonal | 1:500 | Cell Signaling Technology |
| 12 | pSTAT3 | Rabbit polyclonal | 1:500 | Cell Signaling Technology |
| 13 | b-actin | Mouse monoclonal | 1:1000 | Santa Cruz Biotechnology |

## 2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

The mGS cells of passage 24 were cultured in T25 flask, then trypsinized and counted using hemocytometer. In the 12-well plates the cells were cultured in 500 µl RPMI containing 10% serum at 25,000 or 30,000 cells per well. After 24 hours of incubation, the media was removed and the cells were incubated with different concentrations of DEP (1, 10, 100 µg/ml) prepared in saline containing 1% Tween 80. Control cells were incubated with vehicle without DEP (0 µg/ml). After 6, 24, 48 hours of treatment cells were washed with PBS buffer and then processed for RNA extraction using RNeasy kit according to manufacturer instruction (Qiagen, Hilden, Germany). The RNA lysis buffer (600 µl) was added into each well. The lysates were collected into 1.5 ml microcentrifuge tubes. Equal volume of 70% ethanol was added to each well. The mixture was transferred into an RNeasy spin column and centrifuged at 10,000 rpm for 15 sec. The flow-through and collection tube were discarded. Spin column membrane was washed and total RNA was eluted by using 30 µl of nuclease free water and spinning at 10,000 rpm for 1 min. The RNA was quantified using NanoDrop spectrophotometer and stored at -80ºC.

The cDNA first strand synthesis was carried using GoScript reverse transcription kit (Promega, Madison, WI, USA) and Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). So, 10 µl of RNA (1µg) were added to the master mix containing random primers (0.5µg/reaction) and the volume was made up to 10 µl with nuclease free water in 0.2 ml PCR tube. The tubes were heated at 70ºC for 5 min and then immediately chilled on ice. Then,10 µl of the reverse transcription reaction mix was added to each tube. The reaction was carried out for annealing at 25ºC for 5 min and extension at 42ºC for 1 hr followed by the

inactivation of reverse transcriptase enzyme at 70ºC for 15 min in thermal cycler. Samples of the synthesized cDNA were stored at -20ºC.

For real-time PCR, the TaqMan method and the QuantStudio 7 Flex real-time PCR system (Applied Biosystems, California, USA) with the primers obtained from ThermoFisher were used. In new Eppendorf tubes, cDNA and the PCR mix were added and centrifuged. The samples were loaded to PCR 96 well plate. Reaction mixture (25 μl) containing 2 μl of cDNA template, 1.5 μl each of primer and probe mix, and TaqMan Universal PCR master mix (Applied Biosystems) amplified as follows: denaturation at 95°C for 10 min and 40 cycles at 95°C for 10 s, 60°C for 20 s. Direct detection of PCR products monitored by measuring the fluorescence produced by the result of TaqMan probe hydrolysis after every cycle. The expressions of different genes were analyzed using ΔΔCT method and the fold differences were calculated using 2-ΔΔCT. The expression levels of the genes examined were determined in dupicate and normalized using hypoxanthine phosphoribosyltransferase 1 (HPRT1). The experiment was repeated three times and data were analyzed statistically by using the one-way ANOVA with Dunnett's multiple comparison test. Graphical representation of the data (mean ± SD) was performed using GraphPad Prism software (La Jolla, CA, USA). This assay was done for three times.

## Chapter 3: Results

The effects of different concentrations of DEP on the morphological, biochemical, and molecular features of cultured mGS cells are summarized below.

### 3.1 Effect of DEP on the morphology of mGS cells

When the mGS cells were incubated with various concentrations of DEP and compared with control group after different time points, several findings were observed regarding cellular density, size, and shape. First, at DEP concentrations of 1 µg/ml, there were no differences noted between the cells incubated for 24, 48 and 72 hours when compared with control cells incubated with the vehicle (Figure 2a,b,c). However, at 10 µg/ml DEP concentration, there were some spaces observed in the culture plates between the small groups of attached cells after 24 hours incubation with DEP indicating a reduction in the density or amount of attached cells as compared to those in control wells (Figure 2d). After 48 and 72 hours of incubation, there were no differences observed when DEP-treated cells were compared with those of control wells (Figure 2e,f). When mGS cells were incubated with DEP at the high concentration of 100 µg/ml, only a small amount of attached cells were observed in all culture wells for the different time points (Figure 2j,k,l).

Second, there was no difference between the size and shape of cells after incubation with DEP for different periods except when mGS cells were incubated with 100 µg/ml concentration of DEP for different time points. Therefore, only at this high concentration of DEP, the cultured mGS cells appeared smaller than those of control wells (Figure 2j,k,l). The shape of the cells also appeared round as compared to the polyhedral or stellate cells of the control wells.
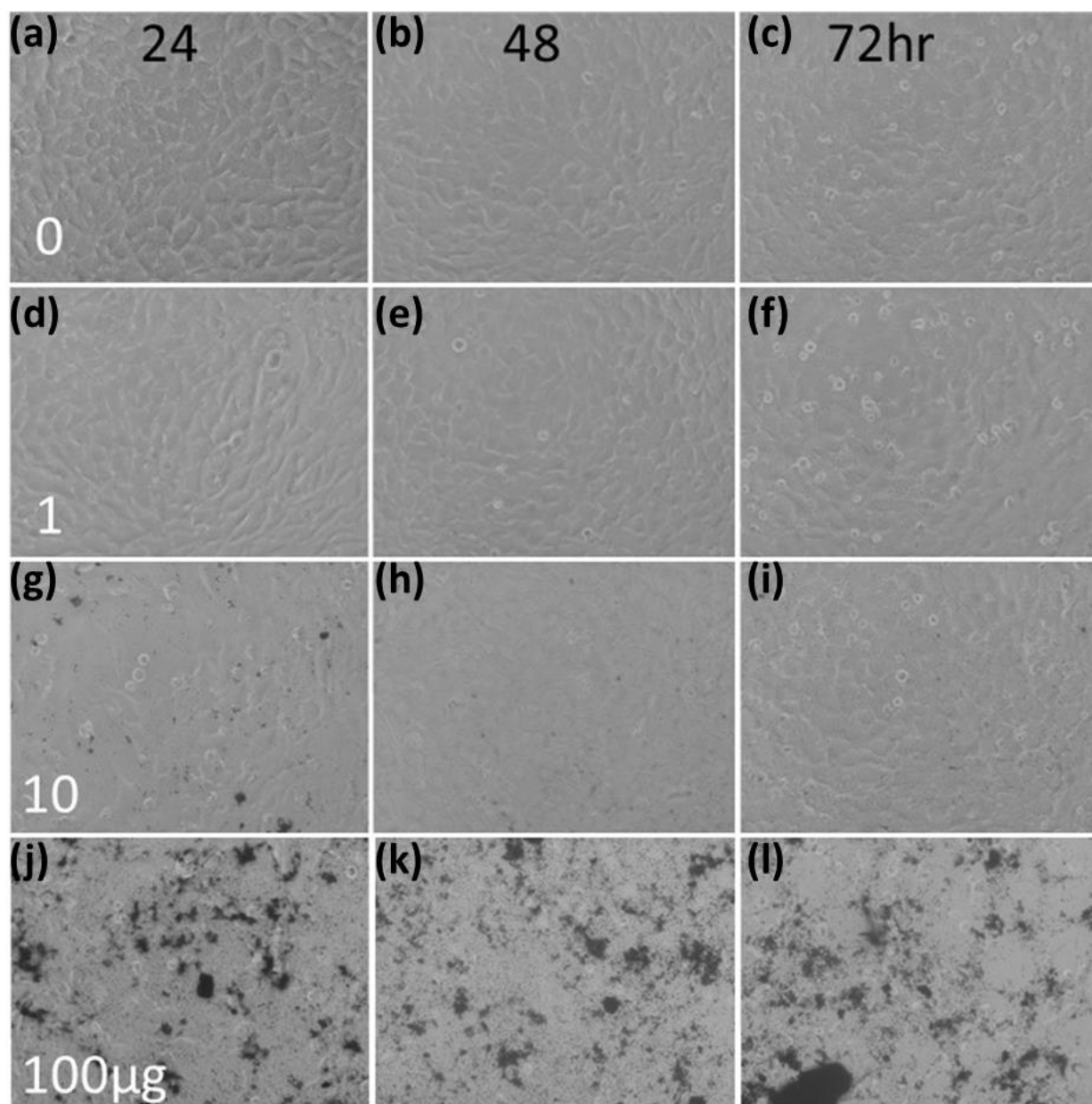
Figure 2: Light micrographs showing mGS cells after 24, 48 and 72 hours of incubation with different concentrations of DEP (0, 1, 10, 100 μg/ml) as shown with 20x objective lens. Note that control cells (a,b,c) do not show apparent difference when compared with cells incubated with 1 μg/ml of DEP after 24, 48 and 72 hours (d, e, f, respectively). Incubation with 100 μg (j,k,l) of DEP shows a decrease in amount of cells attached. DEP appear as dark small particles which tend to form clumps in high concentration

**3.2 Effect of DEP on the viability of mGS cells**

After 1 and 6 hours of incubating mGS cells with different concentrations of 1, 10, 100 µg/ml of DEP, the cell viability assay showed a slight reduction in the amount of ATP in the cultured cells which reflects the percentage of viable cells. Statistical analysis showed that this decrease was not significant (Figure 3a,b). However, the percentages of viable mGS cells after 24, 48 and 72 hours of incubation with high concentration (100 µg/ml) of DEP showed a significant decrease in the percentage of viable cells ($P < 0.05$, 0.001, 0.001 respectively) when compared with control cells (Figure 3c,d,e). But at the lower concentrations of DEP (1 and 10 µg/ml), there were slight reductions in the percentages of viable cells which were not statistically significant when compared with control cells incubated only with the vehicle (Figure 3c,d,e). Therefore, mGS cells respond to high concentration of DEP after 24 and more hours of incubation by reducing the percentage of their viable attached cells due possibly to the inhibition of cell proliferation and increase of cell death.

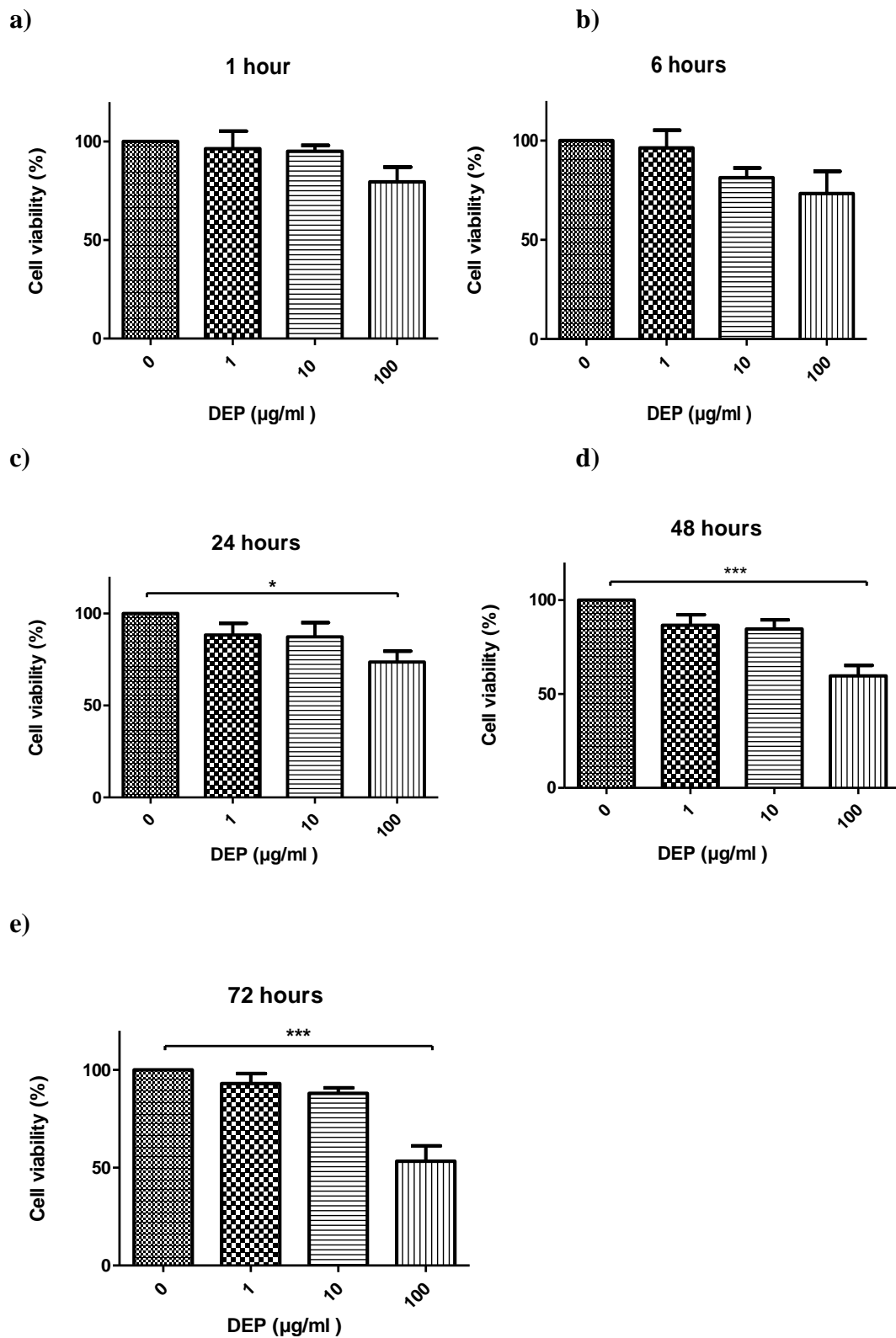Figure 3: Cell viability assay for mGS cells after 1 (a), 6 (b), 24 (c), 48 (d), and 72 (e) hours incubation with DEP at different concentrations (0, 1, 10, 100 µg/ml)

**3.3 Effect of DEP on the migration of the mGS cells**

After inducing wounds in the monolayers of cultured mGS cells, low concentrations (1, 10 µg/ml) of DEP were added for different time points of 1, 6 and 24 hours. Measurements of the widths of the wounds at each time point were recorded and showed no changes (Figure 4). These cell migration data were expressed in two different ways (Figure 5a,b). First, the migration distance in µm of the mGS cells was blotted against time following 1, 6 and 24 hr incubation with 0, 1, 10 µg/ml DEP (Figure 5a). In control wells, the distance of cell migration gradually increased to reach about 750 µm at 24 hours. There was a slight difference when the cells were incubated with either concentration of DEP. Second, the percent of wound healing for each condition was calculated and blotted against time. Again, control cells showed a gradual increase in the percentage of wound healing reaching about 80% after 24 hours (Figure 5b). Statistical analysis demonstrated no significant difference between control cells and those incubated with DEP regarding either the migration distance or the percent healing. Therefore, at the concentrations and time points examined, DEP does not seem to affect the migration of mGS cells.
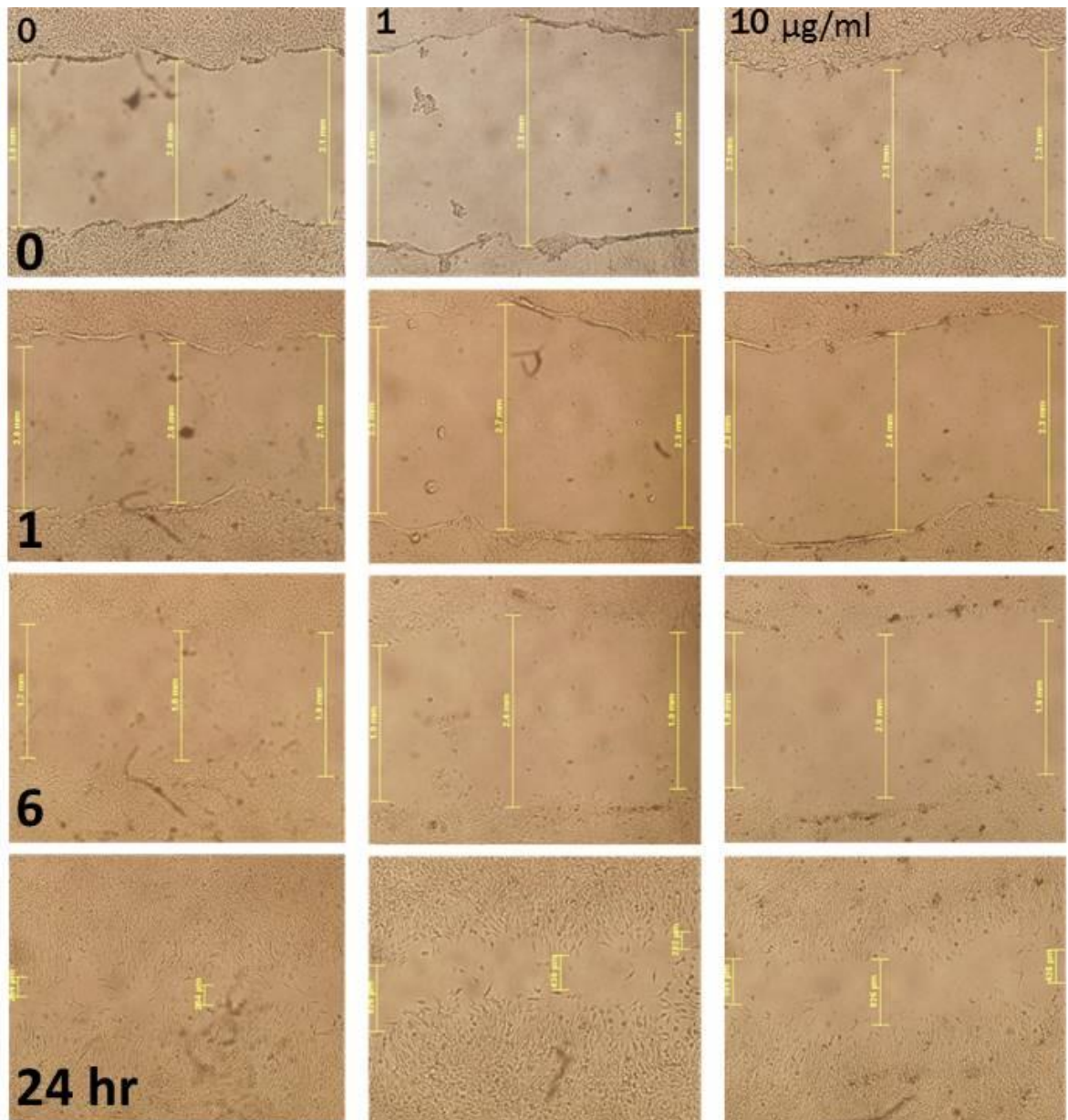
Figure 4: Light micrographs showing the effect of DEP (0, 1, and 10 µg/ml) on the migration of mGS cells after 0, 1, 6, and 24 hours incubation. The widths of the wounds at each time point show little or no differences when compared with control cells (0 µg/ml)
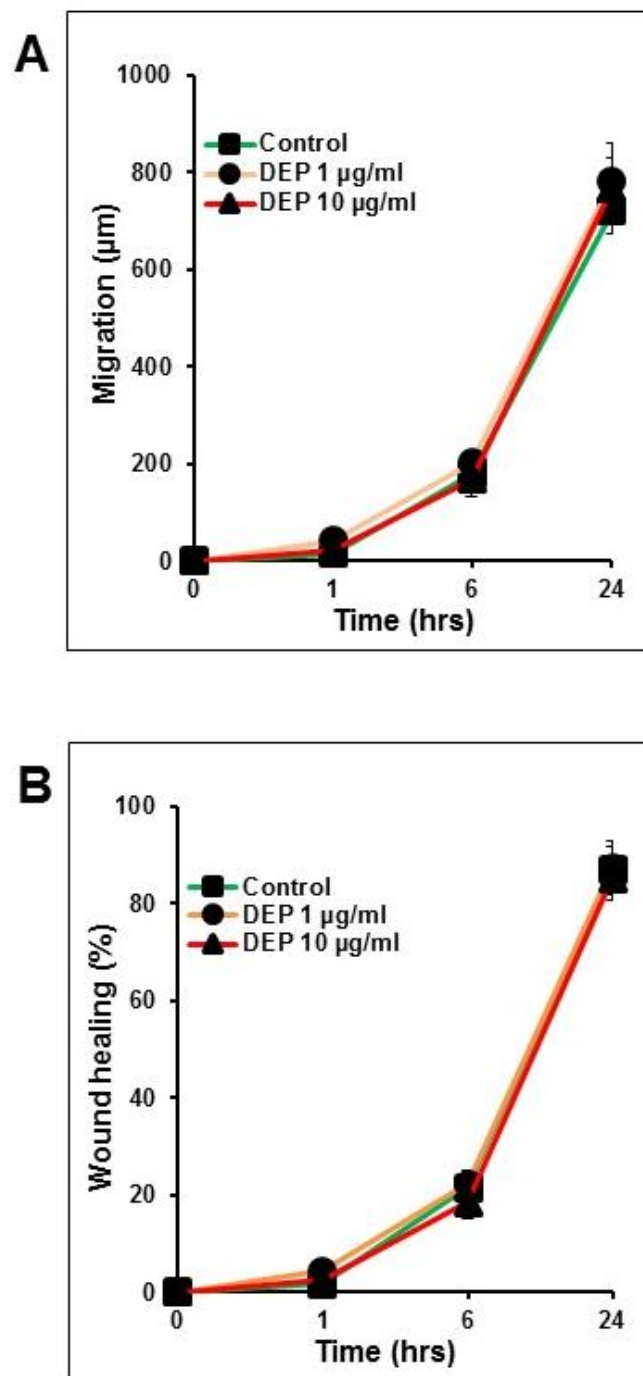
Figure 5: Graphs showing the effect of DEP on the migration and wound healing of mGS cells. The migration data from three different experiments (means ± SEM) in the previous figure are presented in graphs showing migration distance in micrometer (A) and percent of wound healing (B). In both graphs, the data shows correlation between the values of control and cells incubated with DEP at 1 and 10 µg/ml for 1, 6 and 24 hrs

**3.4 Effect of DEP on the production of GSH by mGS cells**

Measurements of GSH levels released in the media of cultured mGS cells incubated with different concentrations of DEP revealed some changes after 24 and 48 hours of incubation (Figures 6 and 7). After 24 hours, the data obtained from three different experiments were considered together. The average level of GSH in control mGS cells was 13.8 nmol/µl, while the average values for cells exposed to different DEP concentrations (1, 10, 100 µg/ml) were 11.89, 11.34, and 14.57  respectively. Thus, there was a gradual decrease in the GSH values in cells incubated with 1 and 10 µg/ml concentrations of DEP compared with control cells. But with the cells incubated with 100 µg/ml of DEP there was a slight increase in GSH values compared with those of control cells. Statistically, these differences were not significant when compared with control cells (Figure 6).



Figure 6: Average levels of GSH released in the culture media of mGS cells incubated with different concentrations of DEP: 0 (control), 1, 10, and 100 µg/ml for 24 hours

After 48 hours of incubation, the data of three different experiments were considered together. The average level of released GSH in the culture media of control mGS cells was 1.36 nmol/µl, while the average values for cells exposed to different DEP concentrations (1, 10, 100 µg/ml) were 2.69, 2.44 and 2.38 respectively (Figure 7). Thus, there was an increase in the GSH values in cells incubated with 1,10 and 100 µg/ml concentrations of DEP compared with control cells. However, statistically these differences were not significant when compared with control cells except the cells incubated with 1 µg/ml of DEP, there was a significant increase and the P value was 0.047 (Figure 7).
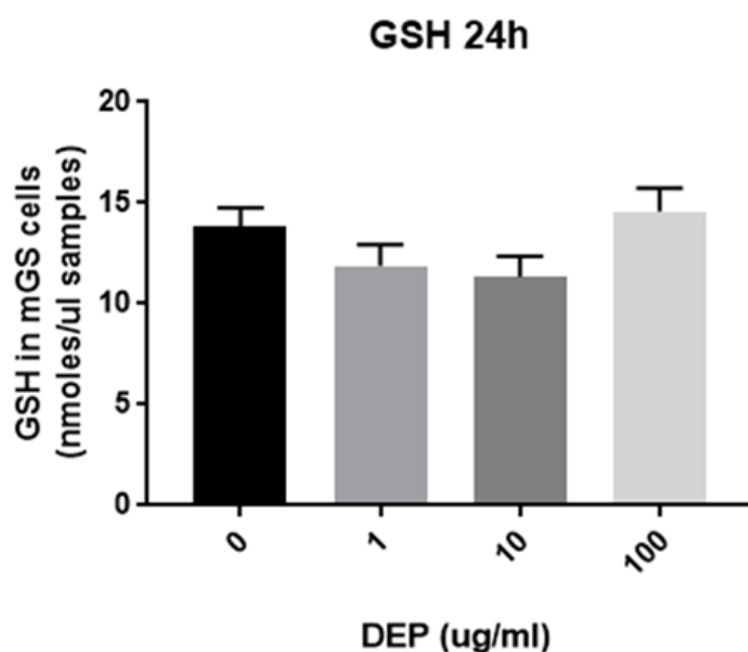


Figure 7: Average levels of GSH released in the media of mGS cells incubated with different concentrations of DEP: 0 (control), 1, 10, and 100 µg/ml for 48 hours

## 3.5 Effect of DEP on the production of TBARS by mGS cells

Measurements of the levels of TBARS released in the media of cultured mGS cells incubated with different concentrations of DEP revealed some changes as compared to control values (Figures 8 and 9). After 24 hours of incubation, the data of three different experiments were considered together. The average levels of TBARS in control mGS cells was 19.8 nmol/µl, while the average values for cells exposed to different DEP concentrations (1, 10, 100 µg/ml) were 13.5, 13.3, and 15.4 respectively. So, there was a decrease in the level of TBARS in mGS cells incubated with DEP after 24 hours when compared with control cells. Statistical analyses showed that this decrease was significant in case of cells incubated with 1 and 10 µg/ml of DEP and the P values were 0.011, 0.0036, respectively.
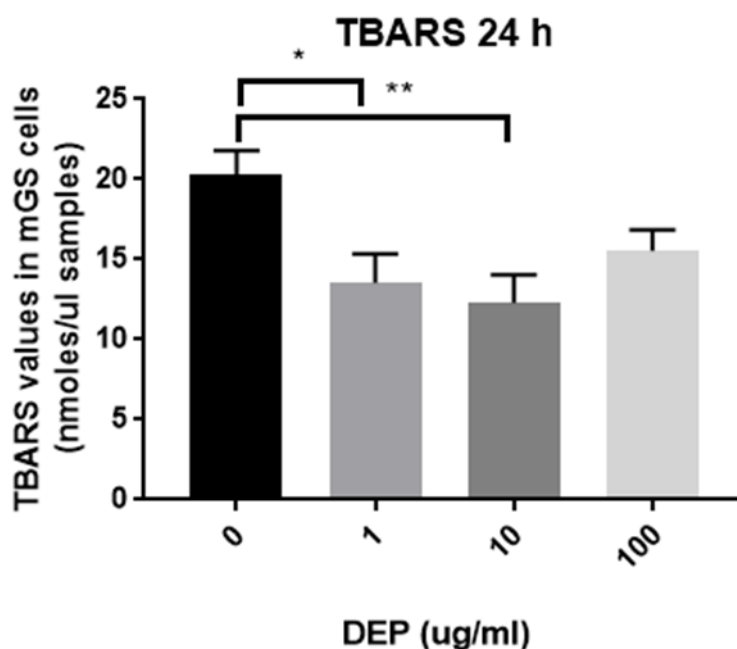


Figure 8: The levels of TBARS in mGS cells incubated with different DEP concentrations: 0 (control), 1, 10, and 100 µg/ml for 24 hours

After 48 hours of incubation with DEP, the data of three different experiments were considered together. The average level of released TBARS in the culture media of control mGS cells was 28.57 nmol/µl, while the average values for cells incubated with different DEP concentrations (1, 10, 100 µg/ml) were 40.1, 34.5, 32.8, respectively. However, statistical analyses showed no significance when any of these values were compared with those of control cells (Figure 9).
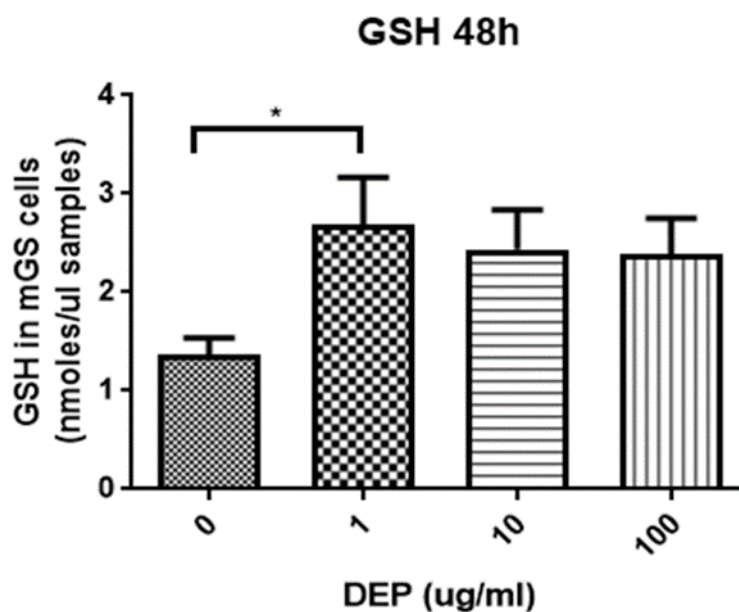


Figure 9: The levels of released TBARS in culture media of mGS cells incubated with different DEP concentrations: 0 (control), 1, 10, and 100 µg/ml for 48 hours

### 3.6 Effect of DEP on the production of NO by mGS cells

When the data of four different experiments were considered together, the average level of released NO in culture media of control mGS cells was 16.2 nmol/μl. However, the average values for cells exposed to different DEP concentrations (1, 10, 100 μg/ml) were 17, 19.1, and 15.6 respectively. As shown in figure 10, there was a slight increase in the levels of NO in mGS cells incubated with 1 and 10 μg/ml of DEP concentrations for 24h when compared with control cells. But with 100 μg/ml concentrations of DEP, there was a slight decrease in the values of NO compared with control cells. Statistical analyses showed that only the increase in NO following incubation with 10 μg/ml of DEP was significant (Figure 10).



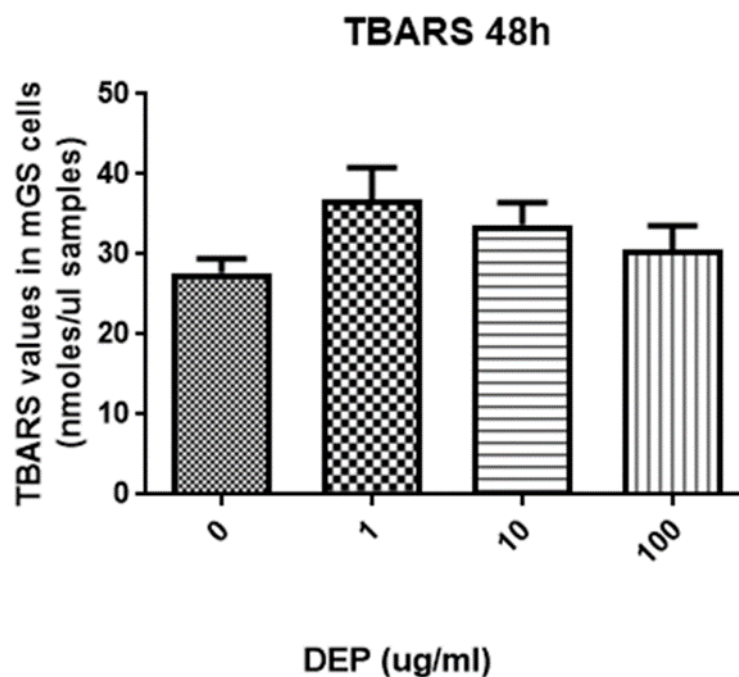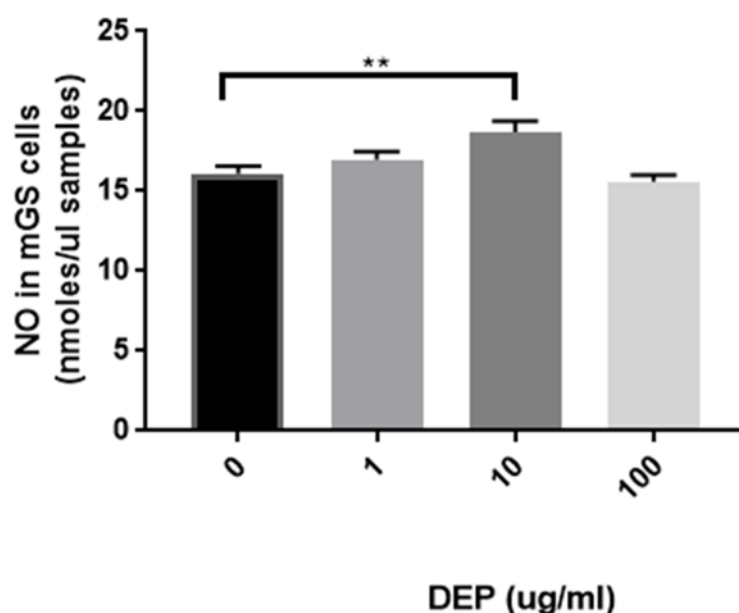Figure 10: The levels of NO released in culture media of mGS cells incubated with different DEP concentrations: 0 (control), 1, 10, and 100 μg/ml for 24 hours

**3.7 Effects of DEP on the expression of stem cell-related proteins in mGS cells**

To test whether the phenotype of mGS cells is affected following their incubation with DEP at 1 and 10 µg/ml for 24 hours, total cellular proteins were extracted. Then, equal amounts of proteins (30 µg) were processed for SDS gel electrophoresis and western blot analysis using different antibodies specific for stem cells. When membranes were probed with Notch antibodies, it was interesting to reveal a change in their expression patterns. This was apparent with Notch 2 and 3 where a gradual decrease in the band density was observed with increased concentration of DEP (Figures 11 and 12). Notch 4 was not detected and Notch 1 showed relatively higher density than control at 1 and 10 µg/ml concentrations and a very low level at 100 µg/ml DEP. When probing was carried out using antibodies specific for Oct4 and BMI-1, there was an apparent down-regulation at the concentration of 100 µg/ml DEP (Figure 11).

Figure 11: Protein expression analysis of stem cell-related genes following incubation of mGS cells with DEP at 0, 1, 10, and 100 μg/ml for 24 hours. Proteins were extracted and 30 μg from each sample were processed for SDS-PAGE and western blot analysis using antibodies specific for Notch 1, 2, 3 and 4, Oct4, and BMI-1
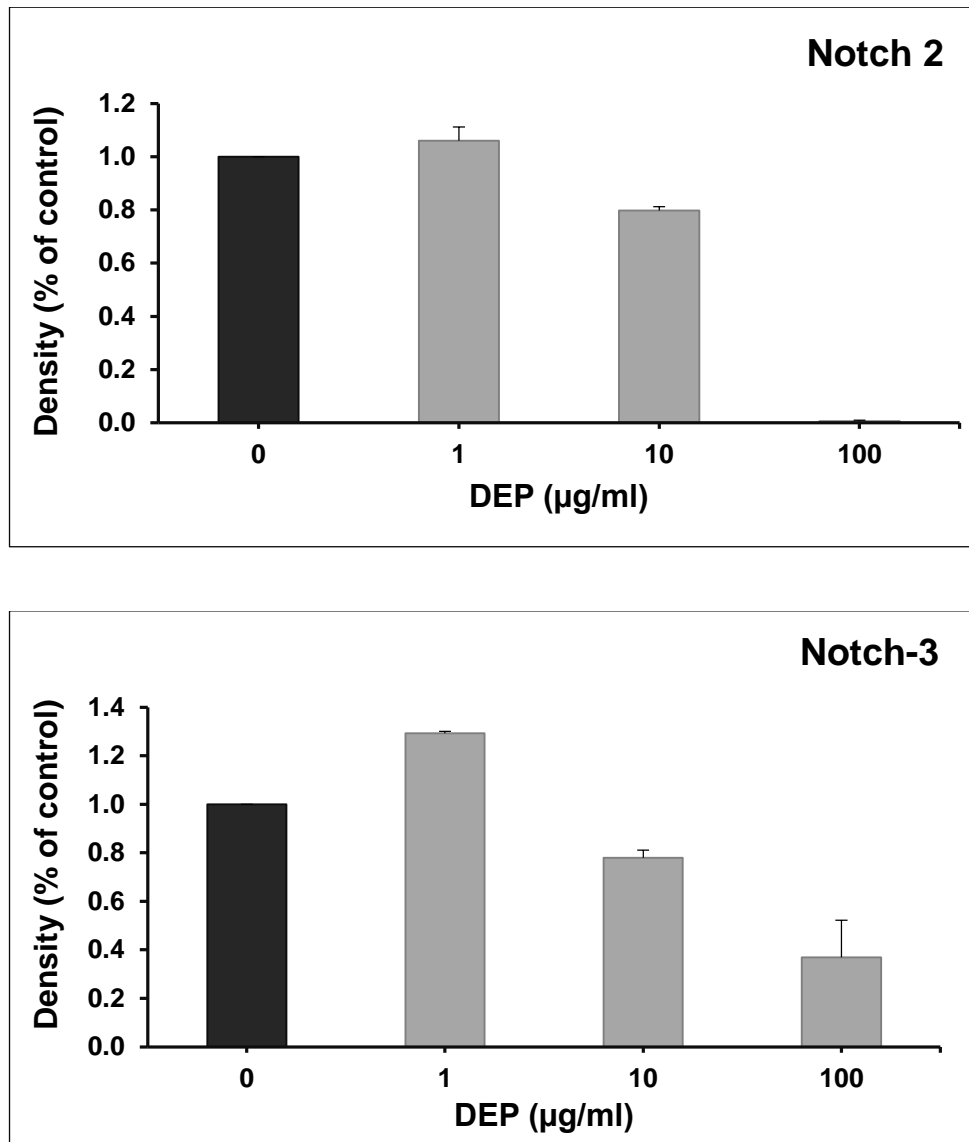
Figure 12: Densitometric analysis of the expressions of Notch 2 and Notch 3 proteins averaged from three different experiments. Cultured mGS cells were incubated with 1, 10, and 100 µg/ml DEP and then, after 24 hours, processed for western blot analysis and measurement of optical density. The values of control cells were averaged and the percent differences of the three experimental conditions were also averaged (± SEM)

**3.8 Effects of DEP on the expression of apoptosis-related proteins in mGS cells**

Since morphological analysis and cell viability assay showed a decrease in the number of cultured mGS cells when incubated with DEP, it was necessary to test whether DEP alters the expression of some apoptosis-related proteins. Therefore, mGS cells were incubated with DEP at 1, 10, and 100 µg/ml for 24 hours. Then, proteins were extracted and processed for western blot analysis using antibodies specific for intact and cleaved caspase-3, cleaved PARP, and Bcl2. The results showed that there was no change in the levels of caspase-3 and the cleaved form of this protein was not detected (Figure 13). Similarly, Bcl2 and the cleaved form of PARP were not detected in mGS cells following any of the DEP concentrations tested (Figure 13).
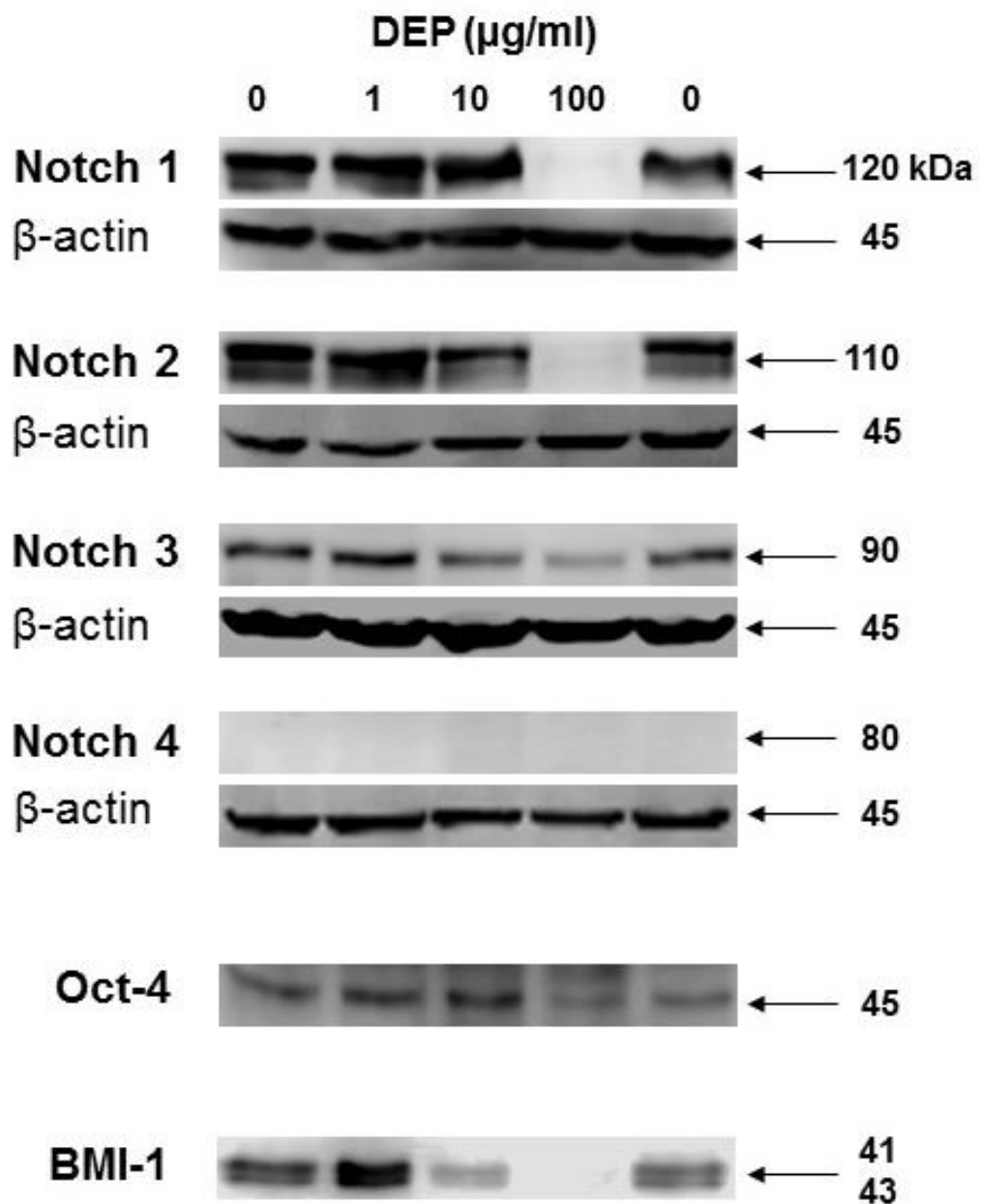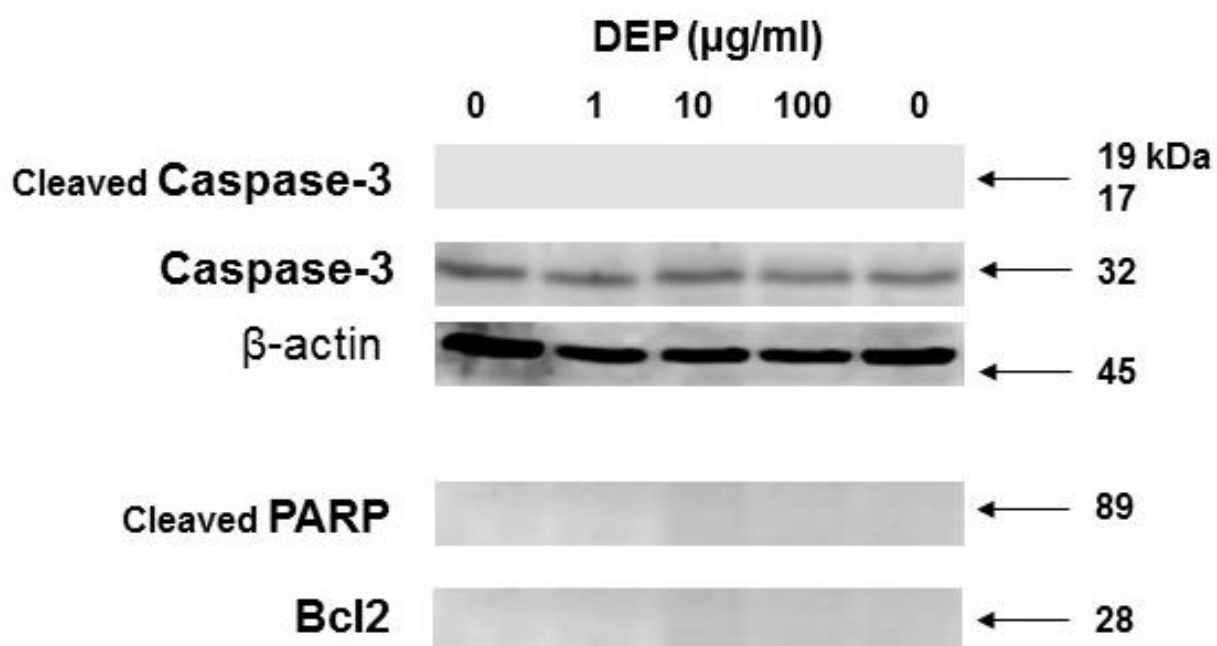
Figure 13: Protein expression analysis of apoptosis-related genes following incubation of mGS cells with DEP at 0, 1, 10, and 100 µg/ml for 24 hours

**3.9 Effects of DEP on the expression of the onco-protein STAT3 in mGS cells**

Because of their role in stem cell fate and differentiation, and because of the down-regulation of the growth rate of mGS cells following DEP incubation, the expression of STAT3 was tested in mGS cells to determine whether it is altered with DEP incubation. Western blot analysis was carried out using two different antibodies specific for STAT3 and its phosphorylated form. The results showed an increased expression of both STAT3 and its phosphorylated form following treatment with DEP in a dose dependent manner (Figure 14).
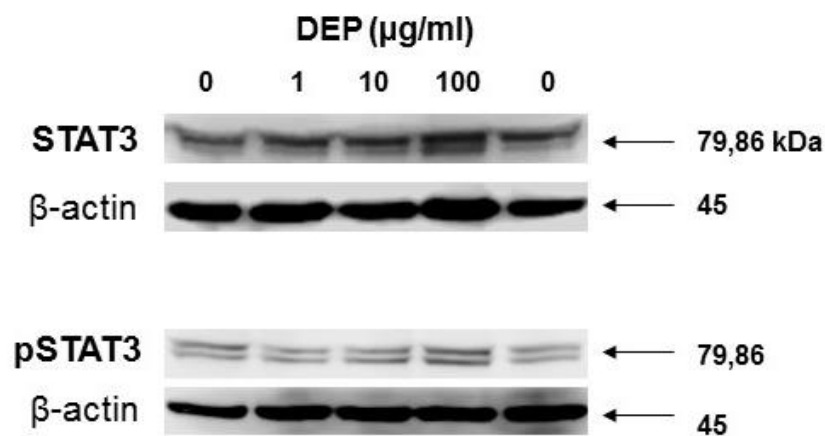


Figure 14: Protein expression analysis of STAT3 and its phosphorylated form following incubation of mGS cells with DEP at 0, 1, 10, and 100 µg/ml for 24 hours

**3.10 Gene expression analysis using qRT-PCR**

The RNA samples extracted from mGS cells cultured in the presence of different concentrations of DEP for 24 hours, were subjected to cDNA synthesis and quantitative real time PCR analysis using primers specific for GSTP1, Ki67 and Bcl2 genes. The experiment was repeated three times. In each experiment, the gene expression levels were determined in duplicates for all three genes. The expression of the house-keeping gene HPRT showed consistent levels and, therefore, was used to normalize the expression levels of the three genes examined.

Incubation of mGS cells with 1 µg/ml of DEP was associated with down-regulation in mRNA levels of GSTP1. But with increased concentrations of DEP (10 and 100 µg/ml), there was a gradual up-regulation in mRNA levels of GSTP1. Statistical analysis showed that only with 100 µg/ml concentration of DEP there was a significant up-regulation in the expression of GSTP1 (Figure 15).

As for the Ki67, 24h-incubations of mGS cells with 1, 10 and 100 µg/ml of DEP were associated with slight changes in mRNA levels.  However, statistical analysis showed that these changes were not significant (Figure 16).

For Bcl2, the incubation with different concentrations of DEP showed little changes in its expression levels after 24 h. Also statistical analysis showed that there was no significant up or down-regulation in the expression of Bcl2 (Figure 17).

Figure 15: Quantitative RT-PCR showing changes of mRNA expression of GSTP1 gene in mGS cells incubated with DEP (1, 10, 100 µg/ml) for 24 hours as compared with control (0 µg/ml). At 1 µg/ml of DEP, there was no significant change in the GSTP1 expression, but incubation with 10 and 100 µg/ml DEP induced a gradual up-regulation in mRNA levels which become statistically significant at 100 µg/ml concentration

Figure 16: Quantitative RT-PCR showing changes of mRNA expression of Ki67 gene in mGS cells incubated with DEP (1, 10, 100 µg/ml) for 24 hours as compared with control (0 µg/ml). There is a slight down- (1 and 100 µg/ml of DEP) and up- (10 µg/ml of DEP) regulation in mRNA expression levels which were not statistically significant
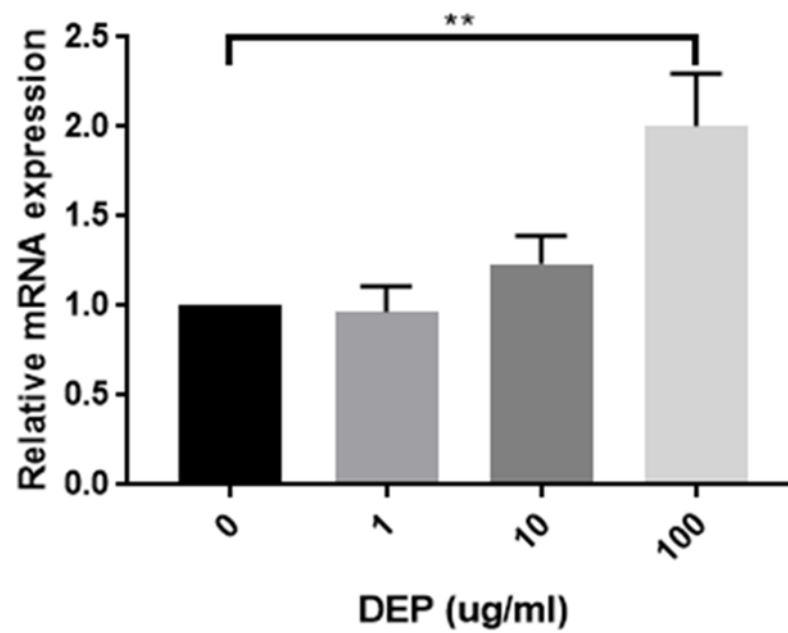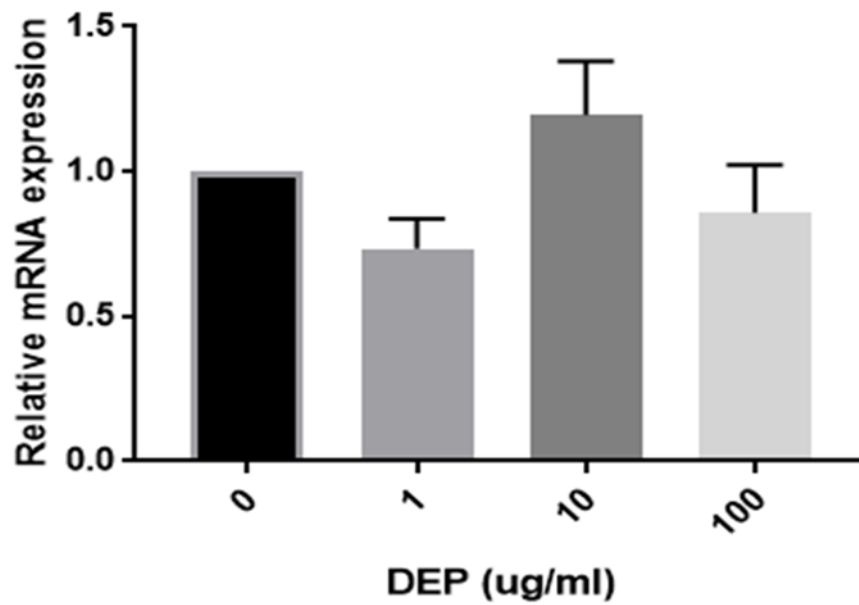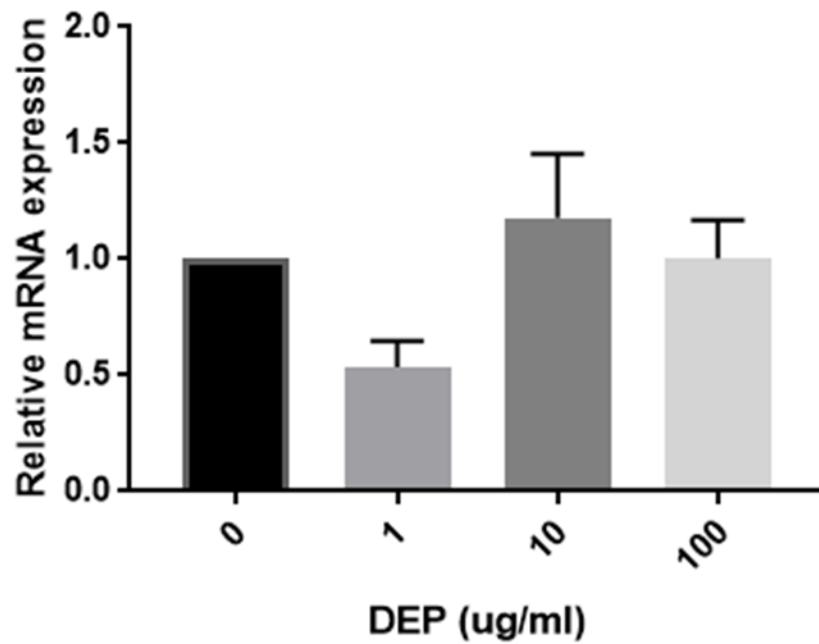
Figure 17: Quantitative RT-PCR showing changes of mRNA expression of Bcl2 gene in mGS cells incubated with DEP (1, 10, 100 µg/ml) for 24 hours as compared with control (0 µg/ml). There is some down- (1 and 100 µg/ml of DEP) and up- (10 µg/ml of DEP) regulation in mRNA expression levels which were not statistically significant

## Chapter 4: Discussion

It is well established in many studies that exposure to DEP is an important risk factor for some common diseases affecting the cardiovascular and respiratory systems (Nemmar et al., 2015; Yoshizaki et al., 2015). Therefore, the DEP play a significant role in increasing rates of both morbidity and mortality around the world (Nemmar et al., 2003). Analysis of the toxic effects of crude and fractionated extracts of DEP on liver cells in the rat revealed involvement of several mechanisms: increased levels of aryl hydrocarbon receptor-mediated activity, modulation of cell proliferation, formation of DNA adducts, oxidative DNA damage, induction of DNA damage responses, and phosphorylation of p53 tumor suppressor and checkpoint kinases (Pálková et al., 2015).

Very little is known on the effects of DEP on stem/progenitor cells. In a recent study, the endothelial progenitor cells in the human lung and the stem cells in bone marrow were examined after DEP exposure (Pöss et al., 2013). The potential effects were formation of reactive oxygen species leading to some clinical implications. The antioxidant enzyme and antioxidant supplementation have been examined for their impact on cardio-respiratory effects of the particulate pollution (PM2.5) exposure. Animal studies have shown an increase in the expression levels of antioxidant gene in epithelial cells after exposure to DEP (Cui et al., 2016). It was reported that omega-3 polyunsaturated fatty acid could attenuate the adverse effect of PM2.5 on heart rate variability (Cui et al., 2016). Antioxidant supplementations such as vitamins C and E were shown to have beneficial effects against human lung damage by air pollution. The lipid-lowering drug called probucol could reduce

cigarette smoke-induced impairment of neovascularization along with improved function of endothelial progenitor cells (Cui et al., 2016).

The aim of this study was to test whether DEP can affect gastric epithelial progenitors or stem cells. So, this study demonstrates an *in vitro* model for analyzing the effects of the environmental DEP on mGS cells with some emphasis on cell viability, migration, oxidative stress, and gene expression. This study will shed some light on the biological features of gastric stem cells when exposed to environmental pollutants, such as DEP. This will also provide a wide scope of various future applications for the understanding of the pathogenesis of some chronic stomach diseases which are common in the United Arab Emirates.

## 4.1 DEP affect mGS cell morphology only at a high concentration

In this study, the light microscopic features of mGS cells incubated for various time points with DEP at low concentrations of 1 and 10 µg/ml had no effect on cell morphology.  However, at the concentration of 100 µg/ml there was no increase in the number of attached cells with time, from 1 hr up to 72 hrs. Also the shape of attached cells appeared more round. Surprisingly, very little floating cells were observed in any of the wells treated with different concentrations for any time point. This finding may indicate that the mGS cells are resistant to damage by DEP even at high concentrations.  Some resistance to the toxic effects of DEP was also observed in lung cells. Electron microscopic analysis showed that incubation of lung epithelial cell with particulate matter for 72 hours is associated with particles interaction with the surface and then internalization into the cytoplasm. However, the cells appeared healthy without any apoptotic changes. The main effect was the increase of multilamellar bodies, formation of vacuoles containing particles and

development of atypical nuclei (Esposito et al., 2012). Even though electron microscopic studies were not carried out in the present study, nuclear atypia were not observed with light microscopic examinations.

## 4.2 DEP reduce the growth rate of mGS cells only at a high concentration

The CellTiter-Glo luminescent assay used in the present study is a good indicator for the change in cell number of viable cells (or cell proliferation) based on quantitation of the amount of ATP. Incubation of mGS cells with different concentrations (1, 10, 100 μg/ml) of DEP for 1 and 6 hours did not reveal any significant effect on their number. Similarly, with increasing the incubation time to 24, 48 and 72 hours for mGS cells with 1 and 10 μg/ml of DEP, there was no significant effect on the cell viability or proliferation. But at high concentration of 100 μg/ml DEP, the number of viable mGS cells after 24, 48 and 72 hours was significantly decreased when compared with control cells incubated only with vehicle. Thus, the number of mGS cells is negatively affected by DEP, but only at a high concentration. These findings correlate with the changes observed with the light microscopy where the amount of attached mGS cells in the wells incubated with high concentration of DEP was reduced. However, in a previous study using the immortalized human bronchial epithelial cells (16HBE14o) exposed to aerosolized DEP (0, 0.5, 1, 7.5, 10, and 50 μg/ml of particles) at the air–liquid interface and to suspensions of collected particles, there was a reduction in the viability of all treated cells compared to unexposed control cells (Esposito et al., 2012).

In a more recent study, the exposure to DEP not only reduces the number of circulating endothelial progenitor cells but also impairs their function in both C57Bl/6 wild-type and ApoE-knockout mice. The detrimental effect of DEP on the

functional capacity of endothelial progenitors was confirmed in an *in vitro* model system in a concentration-dependent manner. In ApoE-knockout mice, the reduction in endothelial cell numbers and function was associated with a reduction in neoangiogenesis and a significant increase in atherosclerotic lesion formation (Pöss et al., 2013).

## 4.3 The mGS cells incubated with DEP are resistant to oxidative stress as revealed by measurements of GSH, TBARS and NO

After 24-hour incubation of mGS cells with different concentrations of DEP, measurements of GSH, TBARS and NO revealed different changes in their levels. The levels of released GSH did not significantly change. But the levels of released TBARS were decreased when cells were incubated with different concentrations of DEP. This effect was statistically significant with 1 and 10 µg/ml of DEP. Also with the measurements of NO levels, there was a significant increase in case of cells incubated with 10 µg/ml of DEP. So, these results could be due to some oxidative stress which was not detectable in cells incubated with 100 µg/ml of DEP due to the low number of attached cells as indicated from the microscopic analysis and viability assay.

The measured values of NO were also correlated and standardized with the values obtained from the cell viability assays because of two reasons: 1) the cell viability assay showed some decrease in the number of viable cells with increasing the concentration and time of incubation with DEP, and 2) the oxidative stress markers including NO were measured in equal volumes of the culture media of cells incubated with DEP. Therefore, after considering the slight gradual decrease in attached cell numbers with treatment of DEP, the values of NO were recalculated

and found to increase from 17.0 to 19.1 μM/μL in case of 1 μg/ml of DEP and from 19.1 to 22.4 μM/μL (10 μg/ml of DEP) and from 15.6 to 22.0 μM/μL (100 μg/ml of DEP).

After 48 hours of incubations with DEP, the levels of released GSH and TBARS into the culture media of mGS cells revealed minor changes except for GSH level at the concentration of 10 μg/ml. Therefore, insignificant changes in antioxidant levels indicate that the cells tend to acquire resistance to damage by these environmental particles.

Other body cells, tissues and organs might be more vulnerable to environmental particles/toxins than mGS cells. Exposure of mice to the hazard of smoking was associated with elevation in the levels of TBARS in the heart tissue, suggesting an increase in oxidative stress. Also, this smoking exposure caused a significant decrease of GSH concentrations as compared to control mice, suggesting depletion of the antioxidant GSH (Nemmar et al., 2013). In another study, proteomic analysis was used to demonstrate a hierarchical oxidative stress response to DEP in macrophages. Reduction in the level of GSH was observed and suggested to play an important role in scavenging of reactive oxygen species and maintenance of cellular redox equilibrium (Xiao et al., 2003). When the effects of ultrafine DEP on oxidative stress generation and dopamine metabolism in a dopaminergic cell model system were studied, a significant increase in the cellular nitrate level and the generation of reactive oxygen species were observed in a dose-dependent manner. Thus, incubation with the ultrafine DEP leads to cytoplasmic dopamine accumulation, possibly contributing to the formation of reactive oxygen species (Kim et al., 2014). Therefore, the oxidative stress plays a role in DEP toxicity. This may be also the case

for mGS cells, but only after 24-hour exposure to DEP, then the cells acquire resistance.

## 4.4 Down-regulations of Notch proteins in mGS cells following DEP incubation

Notch proteins represent a family of transmembrane proteins which are involved in direct cell-cell communication, thereby controlling cell proliferation and differentiation (Previs et al., 2015). Notch 3 expression was previously demonstrated in mGS cells (Giannakis et al., 2008). In the present study, Notch 1 and Notch 2 were also found to be expressed in mGS cells. When they were exposed to DEP, there was a gradual decrease in the levels of Notch 2 and Notch 3 proteins (Figures 11 and 12). Notch 4 was not detected and Notch 1 level was relatively higher than control at 1 and 10 µg/ml concentrations and it was in very low level at 100 µg/ml DEP (Figure 11). When the expressions of Oct4 and BMI-1 proteins were evaluated, there was down-regulation only when mGS cells were incubated with high concentration (100 µg/ml) of DEP (Figure 11).

Alterations in Notch signaling was previously reported in neoplastic lesions of the human cervix in the form of several lower mass polypeptides recognized by the antibody, presumably reflecting breakdown of Notch polypeptide (Zagouras et al., 1995). The decrease in the band density of Notch 2 and Notch 3 came as a result of DEP exposure and may reflect changes in the phenotype of mGS cells. Similarly, the down-regulation of Oct4 and BMI-1 also came as a result of exposure to DEP. The changes in these stem cell-related genes may reflect changes in the stemness of mGS cells. These alterations may also indicate a change in their fate as suggested by the decrease of their viability. Therefore, these changes may affect either the capacity of mGS cells to differentiate or the initiation of an apoptotic pathway.

Apoptosis is a highly regulated form of cell death which is necessary for homeostasis in renewing tissues. Caspase 3 is a member of the apoptotic family of genes that plays a central role in the execution phase of cellular apoptosis (Cory, Huang, & Adams, 2003). In the present study, while there was no change in the levels of caspase-3, the cleaved form of this protein was not detected. Bcl-2 is a family member of the regulatory genes that control apoptosis. It has an anti-apoptotic function and, thus, classified as an oncogene. Both Bcl-2 and the cleaved form of PARP were not detected in mGS cells incubated with DEP at any of the concentrations tested. These results may suggest resistance of stem cells to the toxic effects of DEP which correlate with the slight or no changes observed with the oxidative stress markers as previously discussed.

STAT3 is a signal transducer and activator of transcription and a family member of latent cytoplasmic proteins that are activated to participate in gene control (Darnell, 1997). This protein has been implicated in stem cell fate. In the present study, mGS cells showed an increase in the expression of STAT3 protein and its phosphorylated form following treatment with DEP in a dose dependent manner (Figure 14). While these data are interesting, there is a need further analysis and confirmation. A similar activation of STAT3 expression was previously reported when airway epithelial cells were incubated with DEP, but it was associated with oxidative stress (Cao et al., 2007). Therefore, in the present study the data suggest that mGS cells have some resistance to the toxic effects of DEP and instead of up-regulating genes involved in oxidative stress and apoptosis, they down-regulate stem cell-specific genes suggesting a change in the phenotype of these stem cells with a possible change in their fate. These cell fate changes and possible initiation of differentiation remains to be investigated and proved.

## 4.5 Gene expression analysis of mGS cells revealed some molecular explanations to their resistance to DEP damaging effects

The quantitative real-time PCR technique was used to analyze changes in the expression pattern of some genes specific for the dynamics of mGS cells after exposure to DEP. Genes responsible for the detoxification, proliferation and death of stem cells were explored. Glutathione S-transferases (GST) represent a family of enzymes that plays an important role in detoxification of noxious agents by catalyzing their conjugation with reduced glutathione. In animals, enhanced expression of GST is mediated by products of gut fermentation (Ebert et al., 2003). In the present study, incubating mGS cells with DEP has a tendency to upregulate the expression of GSTP1 gene. This may explain the resistance of stem cells to the damaging effects of DEP. But, the slight down-regulation of GSTP1 following incubation with 10µg/ml of DEP may indicate some initiation for the effect of DEP to the cells.

The Ki67 gene is associated with cell proliferation. It is tightly linked to the cell cycle and expressed during all the phases: G1, S, G2, and mitosis (Scholzen & Gerdes, 2000). Therefore, the expression of Ki67 is a good indicator of cell proliferation. In the present study, the quantitative PCR analysis shows some alteration in the levels of Ki67 expression after incubation of mGS cells with different concentrations of DEP for 24 hours. The decreased expression of Ki67 with 100 µg/ml of DEP correlates with the decreased amount of attached mGS cells as observed microscopically and quantitatively with the viability assay (Figures 2 and 3). However, these changes in gene expression were minor and not statistically significant (Figure 16).

Bcl-2 is a family member of the regulatory genes that control apoptosis. It has an anti-apoptotic function and, thus, classified as an oncogene (Kroemer, 1997). Incubation of mGS cells with DEP at 1, 10 and 100 µg/ml for 24 hours have no significant change in the expression of Bcl2. It seems that the normal levels of Bcl2 following DEP incubation is due to the unique resistance of mGS cells to toxicity and their ability to defend their survival by keeping away from the signals for cell death and maintaining the normal anti-apoptotic signals. Previous studies have shown that  Bcl2 gene expression in specific cells is associated with the regulation of caspase3 (Seriani et al., 2016).

Some studies show a correlation between Ki67 expression with transcriptional regulation of genes related to cell death, such as Bcl2. It was noted to be down-regulated in tumors with high levels of Ki67 immunostaining. Differential regulation of these genes, especially Bcl2, may contribute to the biological nature of clinically more aggressive and highly proliferative cancer tissue (Tan et al., 2005). In the present study, the slight changes in the expression of Ki67 and Bcl2 may indicate resistance of mGS cells to DEP toxic effects.

# Chapter 5: Conclusion

While it is well established that environmental DEP has many toxic effects on cells and tissues of the cardio-pulmonary system, this study demonstrates that the stem cells lining the gastric glands have tendency to resist DEP toxicity. An *in vitro* model of mGS cells exposure to DEP was established. The cells were analyzed using different morphological, biochemical and molecular techniques. The cellular viability, migration, release of oxidative stress markers and expression of some proteins and mRNAs were systematically analyzed. While the viability assay showed a significant effect only when a high concentration (100 µg/ml) of DEP was used, the wound-healing assay did not reveal any significant effects on the migration of mGS cells. This was associated with little, or temporary or no change in the levels of oxidative stress markers: GSH, TBARS, and NO. To detect any early molecular changes in mGS cells that might affect their fate or nature some proteins and mRNAs related to cell proliferation, apoptosis and the stemness of stem cells were analyzed. While the data revealed no change in the expression levels of proteins or mRNAs related to apoptosis or cell proliferation, markers of the stemness of stem cells were altered. Not only Notch 1, 2 and 3, but also Oct4 and Bmi1 proteins were down-regulated. Therefore, the overall data indicates that DEP affects mainly the phenotype of gastric stem cells with only a little effect on their viability at high concentration. These findings will be useful for the preventive medicine department and health authorities in the United Arab Emirates and also may have some clinical implications during the use of stem cells in regenerative therapy.

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