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Lung injury by D-galactosamine
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Aims and Scope

IUFS Journal of Biology is a semi-annual, international, peer-reviewed journal in English and publishes original research articles, reviews and short communications in the field of biological sciences. The journal therefore welcomes papers on biology ranging from molecular and cell biology, biochemistry and physiology to ecology and environment, also systematics, microbiology, toxicology, hydrobiology, radiobiology and biotechnology.

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Research Article

The influence of vitamin U supplementation on liver injury of amiodarone-administered rats

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Abstract

Amiodarone is a currently used drug with high efficacy for the treatment acute life-threatening arrhythmias. Although the use of this drug is widespread, it is associated with unwanted systemic effects. S-methylmethionine sulphonium, which is a derivative of the methionine amino acid, defined mostly as vitamin U and various beneficial effects of Vit U have been demonstrated. The present study was planned to determine whether the vitamin U exhibits preventive effects on amiodarone-induced liver toxicity. Male Sprague-Dawley rats were randomly divided into four groups. Group I; control animals receiving corn oil. Group II; control animals receiving Vit U (50 mg/kg) for 7 days orally. Group III; animals receiving 100 mg/kg amiodarone for 7 days orally. Group IV; animals receiving Vit U orally for 7 days (in the same dose and time) 1 h prior to the administration of amiodarone. Pretreatment with vitamin U particularly decreased degenerative morphological changes such as picnotic nucleus in hepatocytes, sinusoidal dilatation, hyperemia seen in amiodarone treated individuals. On the other hand liver aspartate transaminase, alanine transaminase and alkaline phosphatase activities were increased, catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase activities were decreased in amiodarone group. Administration of vitamin U reversed these effects in amiodarone group. In conclusion, it might be suggested that pretreatment with vitamin U have protective effects on liver injury induced with amiodarone through decreasing oxidative stress.

Keywords: Arrhythmia, amiodarone, liver injury, vitamin U.
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Amiodaron verilen sıçanların karaciğer hasarı üzerine vitamin U desteğinin etkisi

Özet

Amiodaron, yaşamı tehdit eden akut aritmilerin tedavisinde günümüzde kullanılmakta olan yüksek etkinliğe sahip bir ilaçtır. Bu ilaç kullanımı yaygın olmasına rağmen, istenmeyen sistemik etkiler ile ilişkilidir. Çoğunlukla U vitamini olarak tanımlanan ve metiyonin amino asidinin bir türevi olan S-metilmetiyonin sulfonyum'un çeşitli yararlı etkileri ortaya konmuştur. Bu çalışma, U vitaminin amiodaron ile indüklenen karaciğer toksisitesi üzerinde önleyici etki gösterip göstermediğini belirlemek amacı ile planlanmıştır. Erkek Sprague-Dawley sıçanlar rastgele dört gruba ayrılmıştır: 1. Grup; mısır yağı verilen kontrol hayvanlar. 2. Grup; 7 gün boyunca oral olarak U vitamini (50 mg/kg) verilen hayvanlar. 3. Grup; 7 gün boyunca oral olarak 100 mg/kg amiodaron verilen hayvanlar. 4. Grup; amiodaron uygulamasından 1 saat önce 7 gün boyunca oral olarak U vitamini (aynı doz ve zamanda) verilen hayvanlar. U vitaminin amiodaron uygulamasından önce verilmesi, amiodaron uygulanan hayvanlarda gözlenen hepatositlerdeki piknotik nukleus, sinüzoidal dilatasyon, hiperemi gibi dejeneratif morfolojik değişiklikleri kısmen azaltmıştır. Diğer taraftan, amiodaron grubunda karaciğer aspartat transaminaz, alanine transaminaz ve alkalin fosfataz aktiviteleri artmış, katalaz, süperoksit dismutaz, glutatyon peroksidaz, glutatyon-S- transferaz aktiviteleri ise azalmıştır. U vitaminin uygulanması amiodaron grubundaki bu etkileri tersine çevirmiştir. Sonuç olarak, U vitamininin önceden uygulanmasının, amiodaron ile indüklenen karaciğer hasarı üzerinde oksidatif stresi azaltarak koruyucu etkilere sahip olduğu öne sürülebilir.

Anahtar Kelimeler: Aritmi, amiodaron, karaciğer hasarı, U vitamini.

Introduction

Amiodarone (AMD; 2-butyl-3-[3',5'-diiodo-4'α-diethylaminoethoxybenzoyl]-benzofuran) is an iodinated amphiphilic and a class III anti-arrhythmic drug and has been widely used for treatment supraventricular and ventricular tachyarrhythmias and atrial fibrillation (Waldhauser et al. 2006; Goldschlager et al. 2007). It has been well documented that AMD causes side effects in the skin, thyroid, lung, liver, cornea, peripheral nervous system and muscle during long-term therapy (Harris, 1983; Vassallo and Trohman 2007) and AMD and its metabolite desethylamiodarone accumulate in several tissues such as liver, lung, pancreas, thyroid gland, kidney, brain, heart (Brien et al. 1987).

The liver is the central organ for drug metabolism and removal (Pandit et al. 2012) and any imbalance in the activity of drug metabolizing enzymes leads to a free radical generation which is harmful to macromolecules and causes liver toxicity (Singh et al. 2016). AMD causes idiosyncratic, drug-induced liver injury in human especially after long-term oral intake and acute intravenous administration (Rotmensch et al. 1984; Lewis et al. 1989; Rätz Bravo et al. 2005). It has been reported that AMD and its major metabolite desethylamiodarone accumulate in the highly-perfused liver due to their lipophilic characters (Lewis et al. 1989) and the relationship between amiodarone and its hepatic toxicity based on AMD elimination by CYP3A4, one of the isoforms of cytochrome P450 (Shayeganpour et al. 2006; Waldhauser et al. 2006; Zahno et al. 2011).

S-Methylmethionine sulfonium is a derivative of the amino acid methionine and referred as vitamin U (Vit U) due to its effects in the treatment gastrointestinal ulcers (Salim et al. 1993a,b; Kopinski et al. 2007). The raw cabbage has anti-ulcer properties and is rich in Vit U (Roche-Vitec, 1990). In addition to anti-ulcer properties, Vit U has anti-inflammatory, antidepressant, wound-healing, reduction of blood lipid, cytoprotective, and adipocyte differentiation effects (Urazaeva 1976; Stoliarov and Mys'ko 1981; Watanabe et

al. 1996; Kim et al. 2010; Lee et al. 2012). It has also been shown that Vit U has protective effects on liver and renal injury induced by valproic acid, an anti-epileptic drug (Sokmen et al. 2012; Gezginci-Oktayoglu et al. 2016).

The studies related to possible protective effects of vitamins and flavonoid-type antioxidants against AMD-induced toxicity remained limited with vitamin E, silibinin and silymarin (Kachel et al. 1990; Kannan et al. 1990; Vereckei et al. 1993; Honegger et al. 1995; Agoston et al. 2003). According to our knowledge, this is the first study that shows protective effects of Vit U on liver injury induced by amiodarone (AMD) in rats.

Materials and methods

Animals

The experimental procedures were approved by the local Animal Care and Use Committee of Istanbul University, with the certification on the Application for the Use of Animals dated September 27, 2012 (approval ID: 2012 / 127). In this study, 3.5-4 months aged male Sprague-Dawley rats (Istanbul University Experimental Medical Research and Application Institute, DETAE) were used. Their diet consisted of standard animal pellet food and tap water *ad libitum*. Application of AMD dose and time were determined as Reasor et al. (1996). Vit U dose were administered according to Sokmen et al. (2012).

Experimental design

A total of twenty nine rats were divided into 4 groups as follows. The groups include: Group I, control animals receiving corn oil for 7 days (n=6); Group II, animals receiving Vit U (50 mg/kg) for 7 days (n=7); Group III; animals receiving AMD (100 mg/kg) for 7 days (n=8); and Group IV, animals receiving Vit U (50 mg/kg) for 7 days 1 h prior to the administration of AMD (100 mg/kg) (n=8). AMD and Vit U were administered to rats by gavage. On the 8th day, all the animals fasted overnight were sacrificed.

Histological evaluation of liver tissues

The liver tissues were fixed in Bouin's solution for 24 h and washed with 70% ethanol to remove picric acid. The tissues were dehydrated in a series of graded ethanol, cleared in xylene and embedded in paraffin. Sections at 5 µm thickness were taken at rotary microtome (Leica, RT) and stained with Hematoxylin-Eosin and Masson's trichrome. Sections were evaluated histologically using a light microscope (Olympus, CX23) and photomicrographs were taken using a photomicroscope (Olympus, CX41) at a magnification of x200.

Biochemical assays

For biochemical assays, the liver tissues were taken from animals under anesthesia. Tissue samples were washed with physiological saline (0.9 % NaCl) and kept frozen until the day of the experiments. On the day of the experiments, liver samples were homogenized in cold saline with a glass homogenizer to make up to a 10 % (w/v) homogenate. The homogenates were centrifuged, and the clear supernatant fraction was removed for biochemical analysis. Liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assessed according to Reitman and Frankel (1957), alkaline phosphatase (ALP) activity was determined by Two Point Method (Walter and Schutt 1974). The catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities were assessed according to the following methods, respectively: Aebi (1984), Mylorie et al. (1986), Wendel (1981), Habig and Jacoby (1981). Protein levels of liver homogenizates were determined according to Lowry method (Lowry et al. 1951).

Statistical analysis

The biochemical results were evaluated using an unpaired t-test and ANOVA variance analysis using the NCSS statistical computer software. Data were expressed as mean±standard deviation (SD). $p<0.05$ was considered as significant.

Results

Histopathological results

The control group and Vit U given control group have the normal liver microstructure (Fig.1). AMD caused mild degenerative

morphological changes such as picnotic nucleus in hepatocytes, sinusoidal dilatation, hyperemia, rupturings in endothelium of central vein seen in amiodarone treated rats (Fig.1). Pretreatment with Vit U particularly regressed these degenerative changes (Fig.1).

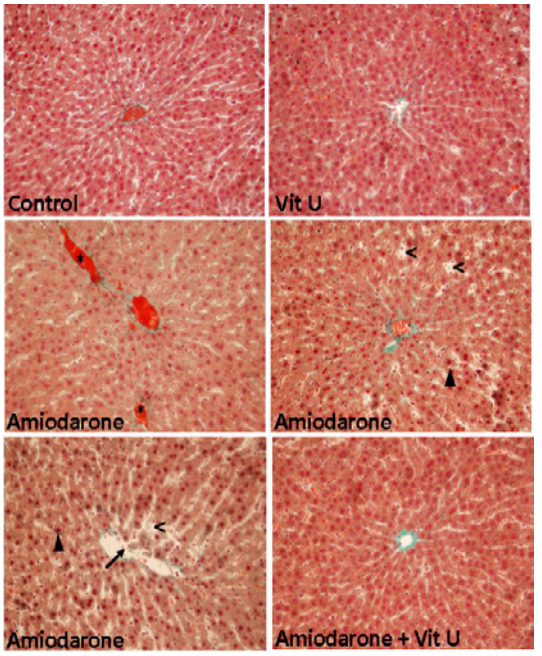

Figure 1. Endothelial rupture in central vein (→), sinusoidal dilatation (<), hyperemia (*) and picnotic nucleus ▲) were observed in amiodarone treated group. Vit U prevented these degenerative changes.

Biochemical results

Liver AST, ALT and ALP activities of all groups are shown in Table 1. According to the results, all of the enzyme activities were significantly increased in AMD group as compared to control group, respectively ($p<0.05$, $p<0.01$, $p<0.0001$). Administration of Vit U reversed these enzyme activities of AMD group in a significant manner, respectively ($p<0.0001$, $p<0.01$, $p<0.05$).

In Table 2, liver CAT and SOD activities are given. Both the enzyme activities were found to be significantly decreased in AMD group when compared to control group, respectively ($p<0.05$, $p< 0.01$). In Vit U given AMD group, CAT and SOD activities were increased significantly ($p<0.05$).

Liver GPx and GST activities are shown in Table 3. GST activity in Vit U group showed a significant decrease when compared to control group ($p<0.0001$). In AMD group, both GPx and GST activities were found to be decreased significantly as compared to control group, respectively ($p<0.0001$, $p<0.01$). Administration of Vit U reversed these activities significantly in AMD group ($p < 0.05$, $p< 0.01$).

Table 1. Liver aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities of control and experimental groups.

Groups	AST (U.g protein^{-1})*	ALT (U.g protein^{-1})*	ALP (U.g protein^{-1})*
Control	6.89 ± 1.90	6.87 ± 1.15	0.92 ± 0.01
Vit U	8.45 ± 2.07	5.59 ± 2.11	0.94 ± 0.11
AMD	10.78 ± 1.85[a]	12.53 ± 2.89[c]	1.44 ± 0.23[e]
AMD+Vit U	1.86 ± 1.18[b]	5.44 ± 1.87[d]	0.81 ± 0.24[f]
P_{ANOVA}	0.001	0.0001	0.007

*Mean ± SD

[a]$p<0.05$ versus control group, [b]$p<0.0001$ versus AMD group, [c]$p<0.01$ versus control group, [d]$p< 0.01$ versus AMD group, [e]$p< 0.0001$ versus control group, [f]$p< 0.05$ versus AMD group

Table 2. Liver catalase (CAT) and superoxide dismutase (SOD) activites of control and experimental groups.

Groups	CAT (U.mg protein^{-1})*	SOD (U.g protein^{-1})*
Control	81.75 ± 18.35	7.50 ± 0.98
Vit U	87.94 ± 22.53	8.70 ± 0.74
AMD	57.09 ± 17.67[a]	5.03 ± 0.25[c]
AMD+Vit U	80.50 ± 11.32[b]	5.69 ± 0.32[b]
P_{ANOVA}	0.045	0.0001

*Mean ± SD

[a]p<0.05 versus control group, [b]p< 0.05 versus AMD group, [c]p< 0.01 versus control group

Table 3. Liver glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities of control and experimental groups.

Groups	GPx (U.g protein^{-1})*	GST (mU.mg protein^{-1})*
Control	3.31 ± 0.68	501.01 ± 66.79
Vit U	3.31 ± 1.06	352.14 ± 19.22[a]
AMD	1.87 ± 0.13[a]	350.99 ± 39.23[c]
AMD+Vit U	3.79 ± 0.89[b]	469.77 ± 25.70[d]
P_{ANOVA}	0.071	0.010

*Mean± SD

[a]p<0.0001 versus control group, [b]p< 0.05 versus AMD group, [c]p< 0.01 versus control group, [d]p< 0.01 versus AMD group

Discussion

Drug-induced liver toxicity is one of the most common adverse effects of medicines, and the protective compounds gain attention to mitigate the drug-induced liver injury. AMD is a commonly used drug for treatment cardiac arrhythmias, but the use of AMD is often restricted by its side effects. Although the most toxic effect of AMD is seen in lungs, it has also been reported that the level of serum aminotransferases, which show liver damage, of more than 20% of patients was increased (Lewis et al. 1989).

AMD has an amphipathic nature and a weak solubility in water. This situation gives a high affinity for membranes to AMD. Besides, the drug has a very long half-life and is known to be stored in the liver (Harris et al. 1983). AMD induces hepatitis through its toxic effects to mitochondria, uncoupling of oxidative phosphorylation, inhibiting of the electron transport chain and b-oxidation of fatty acids (Fromenty et al. 1990a,b; Berson et al. 1998; Spaniol et al. 2001). Moreover, it has been shown by *in vitro* studies, that AMD increases reactive oxygen species concentration and lipid peroxidation (Waldhauser et al. 2006; Ouazzani-Chahdi et al. 2007). Furthermore, AMD interacts with membrane phospholipids and leads to modifications in membrane phospholipid composition of both tissues and organelles, such as lysosomes and mitochondria (Rabkin 2006; Serviddio et al. 2011). The interactions between AMD and lysosomal membrane produce drug-lipid complexes which are seen as lamellar lysosomal bodies (Poucell et al. 1984). In respect of studies with isolated mitochondria, AMD inhibited complex I activity and uncoupled oxidative phosphorylation, which leads to a decrease in hepatic ATP production (Serviddio et al. 2011).

Numerous studies have shown that long-term or acute AMD use causes steatosis, fibrosis in portal and sinusoidal area, neutrophil infiltration, centrilobular necrosis and ballonning degeneration and Mallory bodies in hepatocytes of liver in humans and animals models (Poucellet al. 1984; Lewis et al. 1989; Chang et al. 1999; Gluck et al. 2011; Vitins et al. 2014). In addition, treatment of cultured mouse hepatocytes with AMD results in swollen hepatocytes and accumulation of lipids which is seen as clear cytoplasmic droplets (Ouazzani-Chahdi et al. 2007). Furthermore, it has been shown by ultrastructural studies that AMD induces the formation of lysosomal lamellar phospholipid inclusions and lipid droplets (Chang et al. 1999; Agoston et al. 2003). The degenerative changes in our animal models were moderate, and we have observed hyperemia, sinusoidal dilatation, rupturings in the endothelium of central vein, the picnotic nucleus in hepatocytes. This discrepancy could be related to dose and duration of the treatment with AMD. On the other hand, we could not observe any lysosomal lamellar bodies due to the fact that our histopathological examination was at light microscopic level. However, Vit U decreased the mild degenerative changes formed by AMD administration, and the tissue morphology was similar to the control groups.

Serum transaminases AST and ALT are the most common used parameters to indicate the liver injury because of their relatively long half-life (Sakka 2007). They are mostly found in the liver as well as in other tissues. When cellular membrane damage occurs during the liver injury, AST and ALT activities rise (Borlak et al. 2014). We have got elevated AST and ALT activities in liver tissue of AMD group as compared to control group. In parallel to our results, Merz and Fuller (2007) and Ng et al. (2012) have found elevated AST and ALT activities in patients who were treated with AMD. Administration of Vit U decreased these enzyme levels *via* its cellular repair function (Racz et al. 2008). In addition to this, phosphatases are also good marker enzymes for determining liver injuries. They hydrolyze different phosphate esters at specific pH levels and liberate phosphate groups of the stored substrates of hepatocytes in various conditions like hepatic injuries (Vijayavel et al. 2013). In our study, AMD caused an elevation in ALP activities. Administration of Vit U reversed this activity in AMD group.

The free radical-induced oxidative stress results from the excess production of reactive oxygen species or diminished antioxidant levels of cells. This situation is accepted as a former mechanism for AMD-induced cytotoxicity. AMD has a tendency for producing free radicals. Because of its cationic amphiphilic nature, AMD accumulates in cells. Meanwhile it inhibits electron transport chain which is related to free radical production. In addition to that, either AMD or a metabolite can start this harmful production. Therefore, generation of ROS becomes at higher amounts. In this point, cellular antioxidants play an important role for detoxifying these substances. CAT becomes important when H_2O_2 levels are low and when H_2O_2 levels are at higher concentrations, GPx plays a role for quenching this substance. SOD is also important because of the very short life of superoxide anion. GST is a marker enzyme as being phase II detoxifying enzyme for liver (Kaufmann et al. 2005; Ozer et al. 2008; Durukan et al. 2012; Kalyanaraman 2013). In the current study, CAT, SOD, GPx and GST activities are found to be decreased in AMD group as compared to control group. These diminished activities may be explained by the excess free radical production caused by AMD. Administration of Vit U increased these activities in AMD group. This protective effect of Vit U may be related its antioxidative effects which is reported by Sokmen et al. (2012).

In conclusion, our morphological and biochemical results indicate that Vit U prevents liver toxicity due to the usage of amiodarone through anti-oxidative mechanisms.

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Research Article

Efficacy of antioxidant vitamins (vitamin C, vitamin E, beta-carotene) and selenium supplement on D-galactosamine-induced lung injury

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Abstract

Effects of vitamin combination with selenium on acute lung injury in rats were examined in this study. Four experimental groups of rats were used as follows: group 1, animals administered intraperitoneally physiological saline solution; group 2, rats fed with vitamin C, vitamin E, beta-carotene and sodium selenate for three days; group 3, a single intraperitoneally injection of D-galactosamine (D-GaIN; 500 mg kg^{-1}) into rats; group 4, animals fed with the antioxidant vitamin combination with selenium for three days, and administered D-GaIN. Lung tissues were examined using light microscope, and the following biochemical parameters were measured glutathione (GSH), lipid peroxidation (LPO) levels and tissue factor (TF), lactate dehydrogenase (LDH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), paraoxonase (PON), myeloperoxidase (MPO), xanthine oxidase (XO) and sodium potassium ATPase (Na^+/K^+-ATPase) activities in lung tissues. Extensive edema in peripheral areas, mononuclear cell infiltrations around venules and locally a honeycomb-like structure were observed in the lung of group 3 rats. GSH, GPx and PON activities were decreased, whereas LPO level, TF, LDH, CAT, SOD, MPO and XO activities were increased in rats treated with D-GalN. Administration of the antioxidant combination protected lung tissue against damage by enhancing biochemical chances and pulmonary edema in group 3 animals, while no significant effect on protection of pulmonary inflammation was observed. In conclusion, the antioxidant vitamin supplementation with selenium can be used in the prevention of acute lung injury.

Keywords: Antioxidant, ascorbic acid, β-carotene, D-galactosamine, selenium, vitamin E, lung injury, oxidative stress
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D-galaktozamin ile oluşan akciğer hasarında antioksidant vitaminlerin (vitamin C, vitamin E, beta-karoten) ve selenyumun etkisi

Özet

Bu çalışmada, akut akciğer hasarı üzerine selenyum ihtiva eden vitamin kombinasyonunun etkileri araştırılmıştır. Çalışmada sıçanlardan dört farklı deney grubu oluşturulmuştur; bu gruplar, grup 1, intraperitonal fizyolojik tuzlu su enjekte edilen sıçanlar; grup 2, üç gün süre ile vitamin C, vitamin E, beta-karoten ve sodyum selenat ile beslenen hayvanlar; grup 3, tek doz intraperitonal D-galaktozamin (D-GaIN; 500 mg kg^{-1}) enjekte edilen sıçanlar; grup 4, üç gün süre ile selenyum ve antioksidan kombinasyonu ile beslenen ve tek doz D-GaIN enjekte edilen sıçanlardan oluşturulmuştur. Akciğer dokuları ışık mikroskobu ile araştırılmış ve şu biyokimyasal parametreler ölçülmüştür; glutatyon (GSH), lipid peroksidasyonu (LPO) düzeyleri ve doku faktörü (TF), laktat dehidrojenaz (LDH), katalaz (CAT), superoksit dismutaz (SOD), glutatyon peroksidaz (GPx), paraoksonaz (PON), miyeloperoksidaz (MPO), ksantin oksidaz (XO) ve sodyum potasyum ATPaz (Na^+/K^+-ATPaz) aktiviteleri. Grup 3 sıçanların akciğer dokularında periferal alanlarda yaygın ödem, venüller etrafında mononuklear hücre infiltrasyonları ve bölgesel peteksi yapılar gözlenmiştir. D-GaIN injekte edilen sıçanlarda GSH, GPx ve PON aktiviteleri azalmasına rağmen, LPO düzeylerinin, TF, LDH, CAT, SOD, MPO ve XO aktivitelerinin arttığı saptanmıştır. Antioksidan kombinasyonunun verilmesinin, grup 3 hayvanlardaki biyokimyasal değişiklikleri ve pulmoner ödem bulgularını iyileştirerek, hasara karşı akciğer dokularını koruduğu, ancak pulmoner inflamasyona karşı önemli bir etkisinin olmadığı saptanmıştır. Sonuç olarak, selenyum ihtiva eden antioksidan vitamin uygulamasının akut akciğer hasarını önlemede kullanılabileceği kanısına varılmıştır.

Anahtar Kelimeler: Antioksidan, askorbik asid, β-karoten, D-galaktozamin, selenyum, vitamin E, akciğer hasarı, oksidatif stres

Introduction

Acute lung injury can be induced by inhalation of some toxins, remote organ failure, and mechanic ventilatory, and local and systemic inflammations. It is characterized by epithelial and endothelial cell damage, inflammation and edema. Moreover, oxidative stress is the first sign of tissue damage in acute lung injury. Previously, various lung injury models in experimental animals were reported. For example, aspiration pneumonia induced acute lung injury (Puig et al. 2016). Lipopolysaccharide and carbon tetrachloride were used for acute lung injury model using rats (Kurt et al. 2016; Lin et al. 2016). Lung injury can also be modeled using hyperoxia and radiation treatment (Kayalar and Oztay 2014; Calik et al. 2016). Experimental animal injury models using chemical agents may lead to secondary organ dysfunctions. It was reported that ischemic-reperfusion model in the kidney causes acute lung injury (Karimi et al. 2016; Oztay et al. 2016). D-Galactosamine (D-GaIN) is a common agent used to sensitize mice and other animals to the lethal effects of tumor necrosis factor-alpha (TNF-α). Previously, Catal et al. (2010) reported D-GaIN-induced liver injury accompanied with the elevated oxidative stress and liver injury also causes kidney injury in rats. In this study, protective effects of antioxidant supplementation together with selenium against D-GalN-induced acute lung injury in rats were investigated. The present study reported lung damage caused by D-GaIN-treatment, and positive effects of an antioxidant combination containing selenium on an injury.

Materials and methods

Experiment

In this study, 2-2.5 months female Sprague-Dawley female rats were used. They were randomly divided into four groups as follows: Group I: rats injected physiological saline solution, intraperitoneally (IP). Group II: animals treated with the combination of vitamin C (100 mg $kg^{-1}.day^{-1}$), vitamin E (100 mg $kg^{-1}.day^{-1}$), beta-carotene (15 mg $kg^{-1}.day^{-1}$), and sodium selenate (0.2 mg $kg^{-1}.day^{-1}$) for three days via

gavage. Group III: rats injected D-GaIN (500 mg kg^{-1}; IP) as a single dose. Group IV: rats given the antioxidant combination for three days, then injected D-GaIN. Rats were sacrificed 6 hours after the injection in groups I and III, 7 hours after the last administration in groups II and IV.

Light microscopy

Samples from the lung were fixed in Bouin's solution for 24 h and embedded in paraffin. The sections 5 µm in thickness were stained with haematoxylin-eosin and examined under light microscope.

Biochemical analysis

Right lung samples were analyzed for biochemical studies. Tissue samples from right lung were washed with physiological saline and stored at –20 °C before the experiments. Right lung samples were homogenized in cold saline using a glass homogenizer in order to make 10 % (w/v) homogenate for spectrophotometric analyses. After centrifugation, the supernatant fraction was removed for biochemical determinations. Supernatants were used to determine reduced glutathione (GSH), lipid peroxidation (LPO), thromboplastic activity (TF) and total protein levels as well as for enzymatic analyses. GSH levels were determined by the method developed by Beutler (1975) by using Ellman's reagent. LPO levels in lung homogenates were estimated according to Ledwozyw et al. (1986). TF activities in the homogenates were performed according to Quick's one-stage method described by Ingram and Hill (1976). Lactate dehydrogenase (LDH) activity was assayed by the method proposed by Wroblewski (1957). Catalase (CAT) activity of the lung tissue was carried out by the method of Aebi (1984). Superoxide dismutase (SOD) activity was done according to Mylroie et al. (1986). Glutathione peroxidase (GPx) activity in the lung tissue samples was determined using the method described by Paglia and Valentine (1967) and modified by Wendel (1981). Determination of paraoxonase (PON) activity in the lung tissues was carried out by the method described by Furlong et al. (1988). Myeloperoxidase (MPO) activity of the lung tissue was assayed according to the method of Wei and Frenkel (1993). Xanthine oxidase (XO) activity was evaluated as uric acid production according to Corte and Stirpe (1968) with a few modifications. Sodium/potassium-ATPase (Na^+/K^+-ATPase) in the lung tissue homogenates was determined by the method developed by Ridderstap and Bonting (1969). The protein content in the supernatants was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

Biochemical test results were evaluated using one-way ANOVA and unpaired Student's t-test using the NCSS statistical computer package (NCSS 2001, Kaysville, UT, USA). Significant differences were considered when $p<0.05$. Data were expressed as the mean ± standard deviation (SD).

Results

Light microscopical results

Control rats exhibited healthy lung structure. In rats treated with D-GalN, lungs were characterized by extensive edema in peripheral areas, mononuclear cell infiltrations around venules and locally a honeycomb-like structure in the alveolar area. Pretreatments of the antioxidant vitamins combined with selenium preserved lung against injury by improving pulmonary edema in D-GalN-treated rats, whereas they had not an effect on prevention of pulmonary inflammation in these rats (Fig. 1).

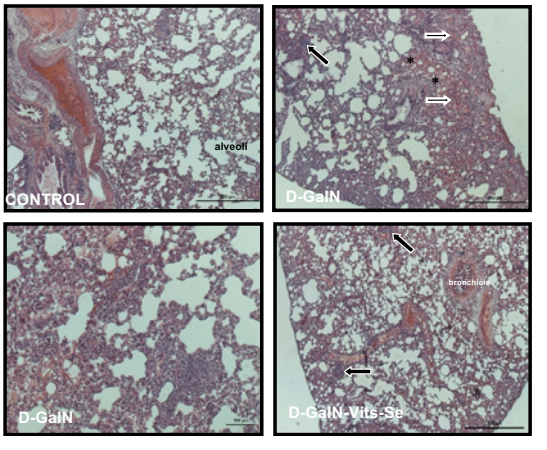

Figure 1. It shows general structure of lungs in experimental rats: Edema (Thin arrow), infiltration areas (Thick arrow) and honeycomb strucure in alveolar areas (*).

Biochemical results

Lung tissue GSH, LPO and TF levels are presented in Table 1. The GSH levels were significantly lower in the lung of treated control+antioxidant and D-GalN group than that of the control group ($p<0.05$). The TF levels were significantly decreased in D-GalN group as compared with control group ($p<0.0001$). In LPO levels, antioxidant treatment to control group and D-GalN administered group were significantly increased when compared to control group, respectively ($p<0.05$; $p< 0.0001$). Administration of antioxidant to D-GalN group reversed these effects ($p<0.05$; $p<0.0001$) (Table 1).

Table 1. Lung tissue glutathione (GSH), lipid peroxidation (LPO), and tissue factor (TF) levels of all groups.

Groups	GSH (nmol GSH/mg protein)*	LPO (nmol MDA/mg protein)*	TF (sec)*
Control	30.86 ± 1.82	3.68 ± 0.52	204.25 ± 17.63
Control + Antioxidant	24.65 ± 3.70[a]	4.43 ± 0.32[a]	194.12 ± 24.22
D-GalN	25.62 ± 1.87[a]	7.18 ± 1.39[c]	151.25 ± 13.66[c]
D-GalN + Antioxidant	38.24 ± 6.58[b]	3.22 ± 1.01[d]	227.69 ± 14.47[d]
P_{ANOVA}	0.002	0. 0001	0.0001

*Mean ± SD
[a]$p<0.05$ versus control group [c]$p<0.0001$ versus control group
[b]$p<0.05$ versus D-GalN group [d]$p<0.0001$ versus D-GalN group

Table 2. Lung tissue lactate dehydrogenase (LDH), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GP_x) of all groups.

Groups	LDH (U/g protein)*	CAT (U/g protein)*	SOD (U/g protein)*	GP_x (U/g protein)*
Control	17.82 ± 6.88	1.54 ± 0.42	4.77 ± 1.49	35.67 ± 4.27
Control + Antioxidant	11.95 ± 2.64	3.75 ± 0.72[c]	5.64 ± 2.48	39.30 ± 3.32
D-GalN	31.48 ± 2.30[a]	4.68 ± 0.88[c]	9.12 ± 2.10[a]	26.59 ± 4.07[a]
D-GalN + Antioxidant	12.39 ± 4.56[b]	1.42 ± 0.64[d]	4.07 ± 1.43[e]	48.63 ± 6.65[e]
P_{ANOVA}	0.0001	0.0001	0.002	0.001

*Mean ± SD
[a]$p<0.05$ versus control group [d]$p<0.001$ versus D-GalN group
[b]$p<0.0001$ versus D-GalN group [e]$p<0.05$ versus D-GalN group
[c]$p<0.001$ versus control group

Lung tissue LDH, CAT, SOD and GPx, activities of all groups are shown in Table 2. According to the table 2, LDH, CAT, and SOD activities were remarkably increased in D-GalN group as compared with the control group, respectively ($p<0.05$; $p<0.001$; $p<0.05$). However, treatment with an antioxidant to D-GalN group gave rise to in a remarkable decrease in the activities of these enzymes according to D-GalN group, respectively ($p<0.0001$; $p<0.001$; $p<0.05$). The GPx activity was notably lower in the lung tissue of treated D-GalN when compared to the control group ($p<0.05$). However, administration of antioxidant caused a significant increase in lung GPx activity in D-GalN group ($p<0.05$) (Table 2).

PON, MPO, XO, and Na^+/K^+-ATPase activities of tissue homogenates are presented in Table 3. There were a significant decrease in PON ($p<0.05$) and an insignificant decrease in Na^+/K^+-ATPase activity in D-GalN treated group when compared with control group.

Administration with an antioxidant to D-GalN group led to both notable increase in activities of PON ($p<0.0001$) and Na^+/K^+-ATPase ($p<0.05$). The MPO activities of tissue homogenates were remarkably higher in the lung of treated control+antioxidant and D-GalN group than that of the control group, respectively ($p<0.001$; $p<0.0001$). Treatment with antioxidant to D-GalN group resulted in a significant decrease in the activity of MPO ($p<0.0001$). A significant increase in the activity of XO was observed in D-GalN given the group as compared to control group ($p<0.001$). Supplementation of antioxidant to D-GalN group gave rise to in an unremarkable decline in the lung XO activity.

Table 3. Lung tissue paraoxonase (PON), myeloperoxidase (MPO), xanthine oxidase (XO) and sodium potassium ATPase (Na^+/K^+-ATPase) activities of all groups

Groups	PON (U/mg protein)*	MPO (U/g tissue)*	XO (U/mg protein)*	Na^+/K^+-ATPase (µmol P_i/mg protein/h)*
Control	13.70 ± 4.31	1.34 ± 0.62	8.89 ± 1.30	1.46 ± 0.17
Control + Antioxidant	17.21 ± 1.99	2.52 ± 0.38[c]	12.11 ± 2.99	2.58 ± 0.99
D-GalN	6.91 ± 1.22[a]	5.34 ± 0.75[d]	16.60 ± 2.04[c]	1.06 ± 0.39
D-GalN + Antioxidant	17.32 ± 1.40[b]	1.83 ± 1.14[b]	14.64 ± 2.20	3.20 ± 0.81[e]
P_{ANOVA}	0.0001	0.0001	0.001	0. 026

*Mean ± SD
[a]$p<0.05$ versus control group [d]$p<0.0001$ versus control group
[b]$p<0.0001$ versus D-GalN group [e]$p<0.05$ versus D-GalN group
[c]$p<0.001$ versus control group

Discussion

Several protective compounds against lung injury models have been reported using experimental animal models. For example, infliximab was reported as a protective compound against carbon tetrachloride-induced lung damage (Kurt et al. 2016). Dexamethasone, nitric oxide synthase inhibitors prevent lung damage (Kozan et al. 2016). Epigallocatechin gallate protects lung damage against fluoride-induced oxidative stress (Shanmugam et al. 2016). Imatinib reduced lung injury in ischemia/reperfusion injury in rats (Tanaka et al. 2016). Protective effects of emodin on lung damage were also reported (Xu et al. 2016). Sivelestat shows beneficial effects on sepsis-related lung damage (Li et al. 2016). Dexmedetomidine protects lung ischemia-reperfusion damage in rats (Zhang et al. 2016). Administration of dexamethasone treatment before lung injury induced by ventilation shows beneficial effects in rats (Reis et al. 2016). Ghorbel et al. (2016) suggested that extra virgin olive oil may be a novel strategy to protect lung tissue injury. Magnolol was reported as a protective agent against lung injury inhibiting nitric oxide and TNF-α (Tsai et al. 2016). Hyperoxia-induced lung injury can be prevented by etanercept and retinoic acid treatments in rats and mice (Kayalar and Oztay 2014; Kaya et al. 2016). It was reported that isoflurane post-conditioning attenuates lipopolysaccharide-induced lung injury induced by reactive oxygen species (ROS) (Yin et al. 2016).

Some protective agents against the liver may also show beneficial effects on the lung. For example, silymarin which is used to protect the liver can also inhibit activation of enzymes such as caspases in the lung in rats (Jin et al. 2016). Previously, a vitamin E-derivative, ETS-GS, was reported as a ROS scavenger improving the lung in crush injury in rats (Nakagawa et al. 2016). Catal and Bolkent (2008) and Catal et al. (2010) reported protective effects of vitamin E, beta-carotene and selenium on liver and kidney injury through inactivation of caspase-3 and ROS scavenging. Various biochemical parameters such as elevated LPO levels, increased activities of MPO, LDH, CAT, SOD and GPx,

and reduced GSH levels were reported in lung and kidney injury models (Catal et al. 2010; Arda-Pirincci et al. 2012; Oztay et al. 2016). Also, it is well known the excessive oxidative stress-induced tissue damage in the acute lung injury. Pulmonary endothelial and epithelial cells and activated alveolar macrophages produce ROS in response to inflammatory. The generated ROS cause pulmonary endothelial/epithelial damage, endothelial/epithelial barrier disruption and pulmonary edema (Arda-Pirincci et al. 2012). In the present study, antioxidant enzymes as well as compounds, such as XO and MPO were shown as an important indicator of D-GaIN-mediated oxidative stress accompanied with inflammation and the damage of alveolar structure in lung tissues. Additionally, it is known that decreased TF activity in tissue samples contributes to high thromboplastin level and cellular damage. In the present study, D-GaIN-mediated biochemical alterations mentioned above resulted in structural damage, inflammation, and pulmonary edema. Because oxidative stress is effective on acute lung injury, antioxidant therapy is useful in the regression of damage. For example, the therapeutic administration of *N-acetylcysteine* to rats after induction of acute lung injury partially attenuated oxidative stress and defects in lung structure (Choi et al. 2012). On the other hand, patients with acute respiratory stress syndrome have a significant decrease at concentrations of GSH, ascorbic acid, α-tocopherol, β-carotene and selenium (Richard et al. 1990; Bowler et al. 2003). In the present study, the antioxidant vitamin supplementation containing vitamin C, vitamin E, beta-carotene and selenium regressed pulmonary edema and structural damage, by inducing of antioxidant enzymes, PON, TF and Na^+/K^+-ATPase activities in D-GalN-treated rats, whereas it had not an effect on prevention of pulmonary inflammation in these rats. In conclusion, the antioxidant vitamins combined with selenium can be used to protect lung tissues against acute lung injury. However, this therapy needs of using of additive anti-inflammatory reagents.

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Interactive effect of static magnetic field and abiotic stressors on growth and biochemical parameters of germinating wheat cultivars

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Abstract

In this study, the effects of the individual and combined application of the static magnetic field and abiotic stressors (60 gL^{-1} PEG6000 (as a drought stress inducer) or 100 mM NaCI (as a salinity stress inducer)) on the cultivars of germinating wheat were investigated by assessing growth, biochemical and antioxidant defence parameters. The seeds of Nina and Flamura-85 species of bread wheat were exposed to 2.9-4.7 mT static magnetic flow density for a period of 0, 2.2 and 19.8 seconds on a band turning with the speed of 1 ms^{-1}. Moreover, this magnetic field application was performed together with abiotic stressors and separately. The growth parameters of both wheat cultivars increased under the static magnetic field applications, while they decreased when subject to the abiotic stress application than the control. The application of the static magnetic field together with abiotic stressors statistically increased the amount of • OH and H_2O_2 in root samples, and the total Chl, Chl a and Chl b amounts in leaf samples. The application of the static magnetic field together with abiotic stressors or separately significantly increased antioxidant enzyme activities (SOD, POD, CAT, APX and GR), and GSH and GSSG content in all experimental groups when compared to the control. Consequently, that the application of the magnetic field triggers the antioxidant defence parameters reduced the negative effects of drought and salinity stresses on the growth parameters in both species.

Key words: Antioxidant defence parameters, biochemical parameters, growth parameters, static magnetic field, salinity, drought, wheat.
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Çimlenmekte olan buğday (*Triticum aestivum* L.) çeşitlerinde büyüme ve biyokimyasal parametreler üzerine statik manyetik alanın ve abiyotik stresörlerin etkileşimleri

Özet

Bu çalışmada, yapay statik manyetik alan ile abiyotik stresörlerin (kuraklık stresi indükleyici (60 gL^{-1} PEG6000) ve tuzluluk stresi indükleyici (100 mM NaCI) tek başına ve beraber uygulanmasının çimlenmekte olan buğday çeşitleri üzerine etkileri büyüme, biyokimyasal ve antioksidan savunma parametreleri değerlendirilerek araştırılmıştır. Nina ve Flamura-85 ekmeklik buğday çeşitlerine ait tohumlar 2.9-4.7 mT statik manyetik akı yoğunluğuna, 1 ms^{-1} hızla dönen bir bant üzerinde 0, 2.2 ve 19.8 saniye süre ile tabi tutulmuşlardır. Ayrıca bu manyetik alan uygulaması abiyotik stresörler ile birlikte ve ayrı ayrı yapılmıştır. Manyetik alan uygulanan her iki buğday çeşidinin büyüme parametreleri kontrol grubuna göre artış gösterirken, abiyotik stres uygulanan deney gruplarında ise kontrole göre düşüş görülmüştür. Manyetik alanın stresörlerle birlikte uygulaması sonucu ise kök örneklerinde • OH ve H_2O_2 miktarlarını, yaprak örneklerinde ise total Chl, Chl a ve Chl b miktarlarını istatistiksel olarak arttırmıştır. Statik manyetik alanın tek başına ve abiyotik stresörler ile kombine uygulaması antioksidan enzim(SOD, POD, CAT, APX ve GR) aktivitelerini ve GSH ve GSSG içeriğini tüm deney gruplarında kontrole göre anlamlı olarak arttırmıştır. Sonuç olarak; manyetik alan uygulaması, antioksidan savunma parametrelerini uyararak her iki buğday çeşidinde, kuraklık ve tuzluluk streslerinin büyüme parametreleri üzerindeki olumsuz etkilerini azaltmıştır.

Anahtar kelimeler: Antioksidan savunma sistemi, biyokimyasal parametreler, büyüme parametreleri, statik manyetik alan, kuraklık, tuzluluk, buğday

Introduction

Increasing the germination performance of seeds is an important aim for plant breeders, especially in cases of low germination percentages due to harsh environmental conditions, dormancy or seed dryness. In recent literature, it has been reported that exposing seeds to magnetic fields (MFs) may accelerate or stimulate seed vigor, growth, and yield (Podleśny et al. 2005; De Souza et al. 2006; Shine et al. 2012; Bilalis et al. 2013). MFs treatments influence photosynthetic pigment contents (Shine et al. 2012) and mineral uptake (Esitken and Turan 2004; Radhakrishnan and Kumari 2012; Bilalis et al. 2013) by altering biochemical processes that involve free radicals and by stimulating the activity of enzymes (Radhakrishnan and Kumari 2012). However, there is not yet a sufficient explanation on exactly how MF affects biological systems via biochemical, biophysical and molecular mechanisms (Harris et al. 2009). Several theories have been proposed, associated with biochemical changes due to the radical-pair mechanism, ion cyclotron resonance mechanisms and ferrimagnetism or enzyme activity (Galland and Pazur 2005).

Wheat (*Triticum aestivum* L.) is a widely cultivated cereal crop worldwide. Abiotic stresses, such as drought, salinity, and low and high temperatures greatly reduce wheat productivity and quality, which is a result of accelerating reactive oxygen species (ROS) accumulations such as hydroxyl radicals ($^{\bullet}OH$), superoxide radicals ($O_2^{\bullet-}$), alkoxyl radicals ($RO^{\bullet}$), hydroperoxide radicals ($HO_2^{\bullet}$) and hydrogen peroxide (H_2O_2). These metabolites are normal products of natural redox reactions in mitochondrial respiratory and photosynthetic electron transport chains, peroxisomes and glyoxysomes. They degrade large macromolecules such as nucleic acid,

proteins, lipids and chloroplast pigments. To avoid the deleterious effects of ROS, plants have evolved antioxidant defence systems. One of these protective mechanisms is the enzymatic antioxidant system, which operates with a sequential and simultaneous action of many enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1. 11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (POX, EC 1. 11.1.7) and glutathione reductase (GR, EC 1.8.1.7). There are also non-enzymatic antioxidant defences such as glutathione, ascorbate, α-tocopherol and carotenoid (Gill and Tuteja 2010). Eliminating salinization and drought stress worldwide is not realistic. For this reason, improving environmental-friendly strategies to avoid the negative effects of drought and salinity is important. In light of these facts, this study focused on interacting effect of artificial static magnetic field (SMF) pre-treatments on abiotic stressors (salinity and drought) in the initial growth stage of wheat cultivars based on growth, biochemical and antioxidant defence parameters under individual and combine applications of SMF and abiotic stressors.

Materials and methods

Plant material

Mature wheat (*Triticum aestivum* L.) seeds from Nina and Flamura-85, which are widely cultivated varieties in Thrace Region, were provided by Directorate of Trakya Agricultural Research Institute in Edirne, Turkey. The seeds were harvested during the 2007–2008 season and their water content was measured to be 9.18% (Nina) and 9.86% (Flamura-85).

Experimental design

The experiment included control, two different abiotic stressors (salinity and drought) and artificial SMF pre-treatment (2.2 s and 19.8 s). Each group was designed to test individual and combined effects of abiotic stressors and SMF pre-treatment on growth and several biochemical parameters measured on the 3rd and 5th germination day. Thirty seeds were used for each combination with three repetitions. For SMF pre-treatment, seed surfaces were sterilized with 70% (v/v) ethanol for 5 min and 20% commercial NaOCl bleach (5% active chlorine) solution for 20 min and then thoroughly rinsed four times in sterile distilled water. Then the seeds were placed in petri dishes under aseptic conditions, which contained sterilized agar 10% (w/v). Various SMF flux densities of 2.9–4.7 mT were applied at 0 (as a control), 2.2 and 19.8 s on a moveable belt at a speed of $1ms^{-1}$ under 23±2°C. Afterward, cultures were transferred to clean petri dishes with fresh agar 10% (w/v) with and without 60 gL^{-1} PEG 6000 (as a drought proxy) or 100 mM NaCl (as a salinity inducer). For germinating, all petri dishes were placed in a growth chamber at 25±2°C with a photoperiod of 16 hours light and 8 hours dark for five days.

Measurement of germination percentage, seedling vigour index and growth parameters

Germination percentage was calculated by counting the number of germinated seeds on the 3rd and 5th day. Seedling vigour index was calculated on the 5th day using the following equation: germination percentage ×seedling length (cm), where seedling length= root length + shoot length (Dhanda et al. 2004). Additionally, average weights and lengths for plants and roots were measured on the 5th day.

Spectrophotometric assay of free radicals (•OH and H_2O_2) contents

The formation of H_2O_2 and •OH in the mixture were measured at 550 nm and 412 nm as described by Puntarulo and Cederbaum (1988) and Holland and Story (1981), respectively. The production of •OH or compounds with the oxidizing power of •OH was assayed by the generation of formaldehyde from dimethyl sulfoxide (DMSO). The basic reaction system consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 0.4 mM NAD(P)H, 30 mM DMSO and 200 µl extracted samples in a final volume of 1 mL. The production of formaldehyde was determined at 412 nm. For spectrophotometric assay of H_2O_2, about 500 µl of homogenate was added to the tubes containing 1.5 mM ferricytochrome. The formation of H_2O_2 in the mixture was measured

at 550 nm by estimating the oxidation product of ferrocytochrome.

Spectrophotometric assay of ferric reducing antioxidant power content

Ferric reducing antioxidant power (FRAP) was measured using Benzie and Strain's (1996) method. An amount of 200 µl extracted samples were mixed with 3 ml FRAP reagent in test tubes and were then incubated in a water bath for 30 minutes at 30ºC. The absorbance of the samples was determined against blank at 593 nm. $FeSO_4.7H_2O$ were used for drawing a standard curve. The values obtained were expressed as µM of ferrous equivalent Fe (II) per gram of freezed sample.

Spectrophotometric assay of pigment contents

Chlorophyll (Chl) *a+b, a, b* and carotenoids (CAR) content was calculated following Litchtenthaler and Wellburn (1985). 100% acetone was used as a homogenizing solution. Extraction ratio was 1:10. Homogenized mixture is separated by centrifugation at 3000 rpm, for 10 minutes. The analytical determination was performed UV/Visible spectrophotometer at the following wavelengths: 645, 662 and 664 nm, for chlorophyll a and b (according to each extraction solvent) and 470 nm for carotene. Equations used for calculation are presented below.

Chlorophyll a = 11.75 A662 – 2.350 A645

Chlorophyll b = 18.61 A645 – 3.960 A662

Carotene = 1000 A470 – 2.270 Chl a – 81.4 Chl b/227

Enzyme extraction and assay

200 mg frozen leaves and roots samples were homogenized in an ice bath with a 2 ml 100 mM phosphate buffer. Afterward, they were centrifuged at 11.000 *x g* for 25 minutes at 4°C and the supernatant fraction was used to prepare the enzyme assays. All of the procedures in the preparation of enzyme extracts were performed at 4°C. Protein content was determined using Bradford's (1976) method. Superoxide dismutase was assayed by monitoring the superoxide radical-induced NBT reduction at 560 nm (Dhinsa et al. 1981). In order to determine different type of SOD isozymes, either 5 mM KCN (inhibitor of Cu/Zn-SOD) or 5 mM H_2O_2 (inhibitor of Cu/Zn-SOD and Fe-SOD) was added to the spectrophotometric assay buffer (Rao et al. 1996). One unit of SOD activity was defined as the amount of enzyme, which causes a 50% inhibition of the photochemical reduction of nitro blue tetrazolium chloride. Guaiacol peroxidase activity was measured at 470 nm by using H_2O_2 and guaiacol as substrates (Panda et al. 2003). Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm according to Aebi (1984) method. Ascorbate peroxidase activity was determined by measuring the consumption of ascorbate by tracking absorbance at 290 nm (Nakano and Asada 1981). Glutathione reductase activity was determined by measuring the enzyme-dependent oxidation of NADPH by the following absorbance at 340 nm (Foyer and Haliwell 1976).

GSH and GSSG contents

Total and oxidized glutathione contents were measured using the methods of Tietze (1969) and Griffith (1980) with minor modifications. Briefly, 100 mg leaf samples were ground with a mortar and pestle in the presence of 1 ml of 5% (w/v) meta-phosphoric acid. Homogenates were centrifuged at 10,000 × *g* for 20 min at 4°C. Glutathione (as GSH + GSSG) was measured using 1 ml of assay mixture containing 100 mM sodium phosphate buffer (pH 7.5) plus 6.3 mM EDTA, 300 µM NADPH, 6 mM DTNB, 25 µl of homogenate and 0.5 U GR. The change in absorbance at 412 nm was recorded for 2 min. Glutathione concentrations were calculated from a standard curve constructed using commercial GSH over the range 0–0.1 mM. To determine the content of GSSG, 100 µl of the samples were incubated with 2 µl of pure 2-vinylpyridine and 6 µl of 1.5 M triethanolamine for 1 h at 25°C. GSSG concentrations were calculated from a standard curve constructed using commercial GSSG over the range 0–0.01 mM.

Statistical analysis

The significance of the interactions of the three factors (SMFs, abiotic stressors, and cultivars) was analyzed with three-way-

ANOVA in the program SPSS Statistics 20.0 (IBM Corp, Chicago, IL, USA). Prior to analysis, data were checked for normality and homogeneity of variance. Comparisons of mean values in each group between (*SMF x abiotic stressor*, *SMF x cultivar* and *abiotic stressor x cultivar*) and within (*SMFs* (Control, 2.2 s and 19.8 s), *abiotic stressors* (Control, Drought and Salinity) and *cultivars* (Nina and Flamura-85) groups were made using a least significant difference (LSD) post-hoc test at the 95% significance level.

Results

Effects of SMF pre-treatments with and without the combinations of PEG 6000 or NaCl on germination percentage, seedling vigour index and growth parameters

Table 1 showed that separate application of SMFs positively affected seed germination ratios and seedling vigour indexes than the groups of control and combined-applications of SMFs with drought and saline conditions than separate applications of either drought or saline conditions. Additionally, SMF applications increased growth of germinated seeds up to 42% and 46% in Nina and Flamura-85 wheat cultivars, respectively, whereas applications of 60 gL^{-1} PEG 6000 or 100 mM NaCl decreased growth 27.5–46% in Nina and 36–51% in Flamura-85 as compared to the control. Artificial SMF pre-treatment alleviated 60 gL^{-1} PEG6000 or 100 mM NaCl effects on seed growth of both cultivars (Fig. 1). The highest increases in growth parameters were measured to be up to 37% in Nina (as an average plant height on 19.8 s+60 gL^{-1} PEG6000 experimental group) and up to 54% in Flamura-85 (as an average root height on 19.8 s+100 mMNaCl) under SMF pre-treatment with either 60 gL^{-1} PEG6000 or NaCl application as compared to the group without SMF pre-treatment (Table 2).

Table 3 showed three-way ANOVA results of individual and combined applications of SMF (2.2 s and 19.8 s) and abiotic stressors (NaCl and PEG 6000) on Nina and Flamura-85 cultivars. Average plant height (r^2=0.939, all p<0.0001) statistically significant affected between the groups, namely *SMF x stress application*, *SMF x cultivar* and stress *application x cultivar*, but within the groups, namely *2.2s and 19.8 s SMF treatments*, *100 mM NaCl and 60 grL^{-1} PEG 6000 applications,* and *Nina and Flamura-85 cultivars* there was no significant differences in average plant height (all p>0.05). The same effect was observed on average root length (r^2=0.796), average plant fresh weight (r^2=0.969) and average root fresh weight (r^2=0.926) (for most groups, p<0.0001); there were no statistical differences within groups (all p>0.05).

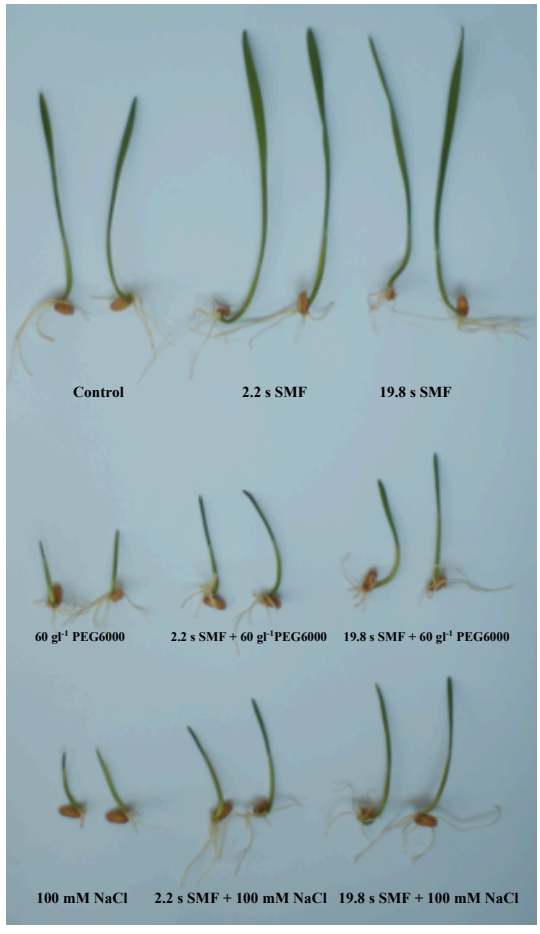

Figure 1. The Flamura-85 cultivar on the 5th day after alone and combined pre-treatments of SMF, 60 gL^{-1} PEG 6000 and 100 mM NaCl.

Table 1. Germination percentage on the 3rd and 5th days and seedling vigour index (SVI) on the 3rd and 5th day.

Experimental Groups	Nina			Flamura-85		
	3rd day (%)	5th day (%)	SVI	3rd day (%)	5th day (%)	SVI
Control	30.00	66.67	371.35	30.00	70.00	413.70
2.2 s	50.00	83.33	594.14	53.33	80.00	646.40
19.8 s	53.33	86.67	630.96	53.33	86.67	747.10
60 gL^{-1} PEG6000	26.67	50.00	202.00	26.67	46.67	175.48
2.2 s + 60 gL^{-1} PEG6000	33.33	70.00	379.40	30.00	66.67	336.68
19.8 s + 60 gL^{-1} PEG6000	36.67	76.67	425.52	33.33	63.33	328.68
100 mMNaCl	30.00	43.33	157.72	30.00	50.00	182.50
2.2 s + 100 mMNaCl	36.67	60.00	283.80	33.33	66.67	335.35
19.8 s + 100 mMNaCl	40.00	63.33	309.68	36.67	70.00	362.60

Table 2. Growth parameters of *in vitro* germinated wheat after 5 days (*T. aestivum* L. cvs. Nina and Flamura-85) pre-treated with SMF and with and without 60 gL^{-1} PEG6000 or 100 mM NaCl.

Cultivars	Experimental Groups	Average Plant Height (cm)	Average Root Height (cm)	Average Plant Fresh Weight (mg)	Average Root Fresh Weight (mg)
Nina	**Control**	5.57^{a*}	2.63^{a}	95.50^{a}	33.20^{a}
	2.2 s	7.13^{b}	3.16^{b}	125.50^{b}	46.45^{b}
	19.8 s	7.28^{b}	3.23^{b}	128.50^{b}	47.20^{b}
	60 gL^{-1}PEG6000	4.04^{c}	1.77^{c}	51.55^{c}	22.75^{c}
	2.2 s+60 gL^{-1} PEG6000	5.42^{ac}	2.12^{ac}	63.60^{ac}	27.95^{ac}
	19.8 s+60 gL^{-1} PEG6000	5.55^{a}	2.15^{ac}	65.50^{ac}	27.15^{ac}
	100 mMNaCl	3.64^{c}	1.67^{c}	53.83^{c}	21.15^{c}
	2.2 s+100 mMNaCl	4.73^{ac}	2.02^{ac}	62.65^{ac}	26.45^{ac}
	19.8 s+100 mMNaCl	4.89^{ac}	2.09^{ac}	62.83^{ac}	26.75^{ac}
Flamura-85	**Control**	5.91^{a*}	2.78^{a}	93.50^{a}	37.20^{a}
	2.2 s	8.08^{b}	3.55^{b}	132.30^{b}	52.45^{b}
	19.8 s	8.62^{b}	3.73^{b}	137.00^{b}	52.85^{b}
	60 gL^{-1}PEG6000	3.76^{c}	1.44^{c}	51.05^{c}	22.55^{c}
	2.2 s+60 gL^{-1} PEG6000	5.05^{ac}	2.02^{ac}	69.60^{ac}	30.95^{ac}
	19.8 s+60 gL^{-1} PEG6000	5.19^{ac}	2.09^{ac}	71.00^{ac}	31.25^{ac}
	100 mMNaCl	3.65^{c}	1.37^{c}	53.50^{c}	21.35^{c}
	2.2 s+100 mMNaCl	5.03^{ac}	2.06^{bc}	70.60^{ac}	29.08^{ac}
	19.8 s+100 mMNaCl	5.18^{ac}	2.12^{bc}	71.15^{ac}	30.45^{ac}

***** Letters indicate significant differences ($p<0.05$) among experimental groups according to one-way ANOVA ($n=5$).

Effects of SMF pre-treatments with and without the combinations of PEG 6000 or NaCl on free radicals (•OH and H_2O_2) and ferric reducing antioxidant power contents

Tables 4, 5 and 7 showed that the effects of •OH, H_2O_2 and FRAP contents both leaf and root samples. Between groups in table 7, SMF pre-treatment and abiotic stressors were correlated strongly to •OH contents in leaves and roots (r^2=0.910, all p<0.0001). Individual applications of abiotic stressors and SMF significantly increased •OH content both in leaf (up to 31% and 182% under SMF and abiotic stress, respectively) and root (up to 64% and 126% under SMF and abiotic stress, respectively) compared to control samples (all p<0.0001). There were no statistical significant within the groups (all p>0.005). •OH content increased by up to 83% in leaves and 80% in roots under combined applications of SMFs and abiotic stressors. H_2O_2 content increased in leafs by up to 434% and 104% under abiotic stress and SMF treatments, respectively (r^2=0.848, all p<0.0001). H_2O_2 increases in roots were also significant (r^2=0.898, all p<0.0001). As in other effects, there were no significant within-group differences (all p>0.005). FRAP contents were statistically affected by SMF and abiotic stressors in leaves (r^2=0.373) and roots in (r^2=0.534, p<0.0001).

Table 3. Results of a 3-wayANOVA of growth parameters of *in vitro* germinated wheat after 5 days, showing significant effects of individual and combined effects of static magnetic fields, abiotic stressors and cultivars.

Parameters[a]	**Factor**	**df**	**MS**	**F**
Average Plant Heights (cm)	Abiotic Stressors	2	181.888	1401.955***
	SMFs	2	73.554	566.931***
	Cultivars	1	3.936	30.340***
	Abiotic Stressors x SMFs	4	1.414	10.898***
	Abiotic Stressors x Cultivars	2	8.150	62.824***
	SMFs x Cultivars	2	0.879	6.773**
	Abiotic Stressors x SMFs x Cultivars	4	0.737	5.680***
	Error	504	0.130	
Average Root Heights (cm)	Abiotic Stressors	2	47.821	419.601***
	SMFs	2	10.337	90.700***
	Cultivars	1	0.033	0.292[ns]
	Abiotic Stressors x SMFs	4	0.767	4.461*
	Abiotic Stressors x Cultivars	2	1.744	15.305***
	SMFs x Cultivars	2	0.666	5.847**
	Abiotic Stressors x SMFs x Cultivars	4	0.012	0.104[ns]
	Error	504	0.114	
Average Plant Fresh Weights (mg)	Abiotic Stressors	2	95536.675	3545.758***
	SMFs	2	14675.161	544.655***
	Cultivars	1	1284.195	47.662***
	Abiotic Stressors x SMFs	4	1555.515	57.732***
	Abiotic Stressors x Cultivars	2	14.796	0.549[ns]
	SMFs x Cultivars	2	469.241	17.415***
	Abiotic Stressors x SMFs x Cultivars	4	11.931	0.443[ns]
	Error	504	26.944	
Average Root Fresh Weights (mg)	Abiotic Stressors	2	10149.668	1259.069***
	SMFs	2	2688.700	333.534***
	Cultivars	1	715.473	88.755***
	Abiotic Stressors x SMFs	4	199.699	24.773***
	Abiotic Stressors x Cultivars	2	60.370	7.489**
	SMFs x Cultivars	2	68.317	8.475***
	Abiotic Stressors x SMFs x Cultivars	4	4.604	0.571[ns]
	Error	504	8.061	

[ns]Not Significant;* p<0.05; ** p<0.001; *** p<0.0001; [a]n=30 for growth parameters.

Table 4. ·OH [nmol g^{-1}(fw)], H_2O_2 [µmol g^{-1}(fw)] and FRAP [µmolFe(II) g^{-1}(fw)] content of leaves samples with and without SMF pre-treatment and abiotic stressors.

Experimental Groups	Nina			Flamura-85		
	·OH	H_2O_2	FRAP	·OH	H_2O_2	FRAP
Control	12.84^{a*}	1.93^{a}	21.38^{a}	14.62^{a}	3.42^{a}	19.24^{a}
2.2 s	14.93^{a}	3.22^{b}	28.62^{b}	17.85^{b}	5.12^{b}	25.76^{b}
19.8 s	15.69^{b}	3.66^{b}	29.34^{b}	18.31^{b}	5.84^{b}	26.62^{b}
60 gL^{-1} PEG6000	31.13^{b}	10.07^{c}	28.66^{b}	32.69^{c}	14.06^{c}	29.38^{c}
2.2 s+60 gL^{-1} PEG6000	23.33^{c}	7.98^{c}	25.5^{ab}	24.49^{bc}	9.40^{bc}	23.36^{b}
19.8 s+60 gL^{-1} PEG6000	25.01^{c}	8.62^{c}	25.7^{ab}	25.22^{bc}	9.98^{bc}	23.96^{b}
100 mMNaCl	26.67^{c}	8.54^{c}	30.36^{b}	29.73^{c}	12.90^{c}	29.18^{c}
2.2 s+100 mMNaCl	19.65^{b}	5.20^{bc}	27.22^{b}	22.06^{bc}	8.98^{bc}	23.56^{b}
19.8 s+100 mMNaCl	21.01^{b}	5.68^{bc}	27.66^{b}	22.11^{bc}	8.55^{bc}	23.69^{b}

* Letters indicate significant differences ($p<0.05$) among experimental groups according to one-way ANOVA ($n=5$).

Table 5. ·OH [nmol g^{-1}(fw)], H_2O_2 [µmol g^{-1}(fw)] and FRAP [µmolFe(II) g^{-1}(fw)] content of root samples with and without SMF pre-treatment and abiotic stressors.

Experimental Groups	Nina			Flamura-85		
	·OH	H_2O_2	FRAP	·OH	H_2O_2	FRAP
Control	16.15^{a*}	2.82^{a}	23.38^{a}	17.28^{a}	4.92^{a}	22.88^{a}
2.2 s	22.31^{b}	4.28^{b}	30.26^{b}	20.16^{a}	6.52^{b}	27.36^{b}
19.8 s	20.67^{b}	4.59^{b}	30.82^{b}	20.95^{b}	6.72^{b}	27.58^{b}
60 gL^{-1}PEG6000	34.97^{c}	10.58^{c}	30.08^{a}	34.03^{c}	17.71^{c}	31.58^{c}
2.2 s+60 gL^{-1} PEG6000	25.27^{b}	8.36^{c}	27.10^{ab}	26.17^{bc}	10.88^{bd}	25.64^{a}
19.8 s+60 gL^{-1} PEG6000	26.65^{b}	8.10^{c}	27.88^{ab}	26.46^{bc}	11.12^{d}	25.28^{a}
100 mMNaCl	29.79^{c}	9.71^{c}	31.08^{b}	29.99^{c}	15.98^{c}	30.92^{c}
2.2 s+100 mMNaCl	23.27^{b}	6.88^{bc}	26.84^{a}	23.72^{bc}	9.14^{ad}	25.68^{a}
19.8 s+100 mMNaCl	24.85^{b}	6.94^{bc}	26.72^{a}	23.43^{bc}	9.90^{d}	25.78^{a}

* Letters indicate significant differences ($p<0.05$) among experimental groups according to one-way ANOVA ($n=5$).

Table 6. Pigment content of 5-old-day *in vitro* germinated wheat (*T. aestivum* L. cvs. Nina and Flamura-85) with and without SMF pre-treatment and abiotic stressors.

Varieties	Experimental Groups	Chl*a+b*	Chl*a*	Chl*b*	CAR
		[mg(gfw)$^{-1}$]			
Nina	**Control**	0.097^{a*}	0.063^{a}	0.034^{a}	0.022^{a}
	2.2 s	0.127^{b}	0.083^{b}	0.044^{b}	0.029^{b}
	19.8 s	0.123^{b}	0.083^{b}	0.040^{a}	0.028^{b}
	60 gL^{-1}PEG6000	0.068^{c}	0.041^{c}	0.027^{c}	0.025^{a}
	2.2 s+60 gL^{-1} PEG6000	0.092^{a}	0.061^{a}	0.031^{a}	0.029^{b}
	19.8 s+60 gL^{-1} PEG6000	0.087^{ac}	0.059^{a}	0.028^{c}	0.030^{b}
	100 mMNaCl	0.063^{c}	0.035^{c}	0.028^{c}	0.026^{a}
	2.2 s+100 mMNaCl	0.082^{ac}	0.053^{ac}	0.029^{c}	0.030^{b}
	19.8 s+100 mMNaCl	0.079^{ac}	0.051^{ac}	0.028^{ac}	0.030^{b}
Flamura-85	**Control**	0.107^{a*}	0.071^{a}	0.036^{a}	0.025^{a}
	2.2 s	0.131^{b}	0.088^{b}	0.043^{b}	0.031^{b}
	19.8 s	0.128^{b}	0.085^{b}	0.043^{b}	0.033^{b}
	60 gL^{-1}PEG6000	0.073^{c}	0.045^{c}	0.028^{c}	0.027^{a}
	2.2 s+60 gL^{-1} PEG6000	0.097^{a}	0.063^{a}	0.034^{a}	0.033^{b}
	19.8 s+60 gL^{-1} PEG6000	0.095^{ac}	0.060^{a}	0.035^{a}	0.032^{b}
	100 mMNaCl	0.069^{c}	0.045^{c}	0.024^{c}	0.025^{a}
	2.2 s+100 mMNaCl	0.092^{a}	0.059^{a}	0.033^{a}	0.032^{b}
	19.8 s+100 mMNaCl	0.088^{ac}	0.057^{a}	0.031^{a}	0.033^{b}

***** Letters indicate significant differences ($p<0.05$) among experimental groups according to one-way ANOVA ($n=5$).

Effects of SMF pre-treatments with and without the combinations of PEG 6000 or NaCl on pigment contents

In our study, Chl*a*+*b*, Chl*a* and Chl*b* content increased significantly in leaf samples under individual and combined interactions between abiotic stress and SMF (r^2=0.693 in Chl*a*+*b*,p<0.001; r^2=0.701in Chl*a*,p<0.0001; r^2=0.399 in Chl*b*, p<0.005). With SMF pre-treatment, there increases were significant compared to the control (p<0.0001 in Chl*a*+*b*andChl*a*, p<0.005 in Chl*b*). Under abiotic stress, these effects decreased significantly (all p<0.0001). The combined effects of SMFs and abiotic stressors statistically increased Chl*a*+*b*, Chl*a* and Chl*b* content (all p<0.0001) compared to individual applications of salinity and drought. Again, there were no statistically significant differences within groups (all p>0.005). Abiotic stress and combinations of abiotic stress treatments with SMFs statistically increased leaf CAR content compared to the control (p<0.005; Tables 6 and 7).

Table 7. Statistical significance of F value (three-way ANOVA) for individual and combined effects of SMF pre-treatments and abiotic stressors on biochemical parameters in leaves and roots.

Parameters[a]	Abiotic Stressors	SMFs	Cultivars	Abiotic Stressors x SMFs	Abiotic Stressors x Cultivars	Cultivars x SMFs	Abiotic Stressors x SMF x Cultivars
Leaf Samples							
•OH	269.384***	76.557***	8.127^{ns}	52.878***	2.796^{ns}	0.723^{ns}	0.758^{ns}
H_2O_2	162.147***	25.605***	20.262***	28.576***	0.504^{ns}	0.184^{ns}	0.330^{ns}
FRAP	2.317^{ns}	2.697^{ns}	2.078^{ns}	11.232***	1.858^{ns}	2.285^{ns}	1.144^{ns}
Chl*a+b*	80.930***	21.335***	1.061^{ns}	3.785*	0.416^{ns}	0.103^{ns}	0.043^{ns}
Chl*a*	83.471***	24.804***	1.406^{ns}	3.505*	0.704^{ns}	0.084^{ns}	0.063^{ns}
Chl*b*	26.620***	4.972**	0.213^{ns}	3.634*	0.137^{ns}	0.531^{ns}	0.206^{ns}
CAR	6.972**	0.080^{ns}	0.217^{ns}	4.535**	0.750^{ns}	0.534^{ns}	0.491^{ns}
Root Samples							
•OH	181.663***	41.456***	0.037^{ns}	83.892***	0.102^{ns}	2.102^{ns}	2.265^{ns}
H_2O_2	212.429***	46.441***	70.635***	35.048***	10.257***	9.892***	8.387***
FRAP	2.397^{ns}	1.762^{ns}	0.813^{ns}	23.577***	4.739^{ns}	2.491^{ns}	0.290^{ns}

^{ns}Not Significant;* $p<0.05$; ** $p<0.001$; *** $p<0.0001$.

Table 8. Antioxidant enzyme activities of 5-old-day *in vitro* germinated wheat leaf samples (*T. aestivum* L. cv. Nina) pre-treated to non-uniform SMF in various times with and without 60g L^{-1}PEG6000 or 100 mMNaCl.

Experimental Groups	Total-SOD (U mg^{-1} protein)	Fe-SOD (U mg^{-1} protein)	Mn-SOD (U mg^{-1} protein)	Cu/Zn-SOD(U mg^{-1} protein)	POX (ΔA_{470} mg^{-1} protein)	CAT (ΔA_{240} mg^{-1} protein)	APX (ΔA_{290} mg^{-1} protein)	GR (U mg^{-1} protein)
Control	164.33^{a*}	32.58^{a}	54.84^{a}	76.91^{a}	104.78^{a}	3.37^{a}	30.71^{a}	156.85^{a}
2.2 s	206.16^{b}	55.59^{b}	55.91^{a}	94.66^{b}	195.25^{b}	5.54^{b}	52.88^{b}	225.30^{b}
19.8 s	207.37^{b}	57.26^{b}	54.86^{a}	95.25^{b}	205.89^{b}	5.68^{b}	53.91^{b}	235.14^{b}
60 gL^{-1} PEG6000	283.16^{b}	87.46^{c}	74.59^{b}	121.11^{c}	362.76^{c}	8.44^{c}	65.94^{c}	575.64^{c}
2.2 s + 60 gL^{-1} PEG6000	213.50^{b}	65.46^{bc}	52.68^{a}	95.36^{b}	252.29^{bc}	5.93^{b}	49.94^{b}	355.49^{bc}
19.8 s +60 gL^{-1} PEG6000	219.58^{b}	67.03^{bc}	55.98^{a}	96.57^{b}	260.60^{bc}	5.31^{b}	51.67^{b}	390.90^{bc}
100 mMNaCl	305.58^{c}	91.22^{c}	83.98^{b}	130.38^{c}	256.77^{c}	9.02^{c}	67.23^{c}	606.06^{c}
2.2 s + 100 mMNaCl	234.12^{bc}	71.29^{bc}	61.09^{a}	101.74^{b}	205.60^{b}	6.74^{bc}	52.58^{b}	364.94^{bc}
19.8 s + 100 mMNaCl	240.14^{bc}	69.60^{bc}	61.22^{a}	109.32^{bc}	222.68^{b}	6.79^{bc}	55.59^{b}	374.79^{bc}

* Different letters indicate significant differences ($p<0.05$) among the experimental groups according to one-way ANOVA, post hoc LSD tests.

Effects of SMF pre-treatments with and without the combinations of PEG 6000 or NaCl on antioxidant enzymes

Activities of antioxidant enzymes in leaf and root samples of both wheat cultivars under the pre-treatments with and without the combination of PEG6000 or NaCl are presented in Table 8-11. Antioxidant enzyme activities increases under SMF pre-treatments were statistically significant ($p<0.005$). For Nina, SMF pre-treatment resulted in maximum increases in total-SOD, POX, APX, CAT and GR activities were observed in the leaf samples of 26.2, 96.5%, 75.6%, 68.6% and 49.9% relative to the control; in root samples they also increased by 34.2%, 41.7%, 40.5%, 67.7% and 70.5%, respectively. Similar increases were observed in Flamura-85. Among the three SOD isozymes (Fe-SOD, Mn-SOD and Zn/Cu-SOD), Fe-SOD was affected more than the others by SMF pre-treatment.

Cultivars exposed to 60 gL^{-1}PEG6000 or 100 mMNaCl were statistically different from the control (at $p<0.05$ level). As for the combined applications of SMF and 60 gL^{-1} PEG6000 or 100 mMNaCl, there were statistical differences in stimulating antioxidant enzyme activity in leaf and root samples of both wheat cultivars except Mn-SOD ($p<0.05$). SMF pre-treatment of Nina with 60 gL^{-1} PEG6000 had maximum increases in total-SOD, POX, APX, CAT and GR activities in leaf samples of 33.6%, 148.7%, 68.3%, 76% and 149.2%; in the root samples increases were 161.5%, 41.4%, 54.7%, 86.8% and 146.8%, respectively. Similar increases were observed under the combination of SMF pre-treatment with 100 mMNaCl. Exposed to SMF and 60 gL^{-1} PEG6000, antioxidant enzyme activity increased in Flamura-85 leaf and root samples. Compared to the control, maximum increases for total-SOD, POX, APX, CAT and GR were 36.8%, 39.5%, 43.4%, 51.5% and 86.30% in leaf samples and 28.98%, 72.15%, 16.08%, 98.15% and 60.34% in root samples, respectively. Similar increases were observed under the combination of pre-treatment and 100 mMNaCl in both wheat cultivars.

A three-way ANOVA showed statistically significant effects on antioxidant enzyme activities in leaf samples for individual effects of SMF pre-treatment and abiotic stress, except for Mn-SOD activity, and under individual and combined interaction effects SMF pre-treatments, abiotic stresses, and cultivars (Table 14). As for root samples, a three-way ANOVA indicated statistically significant effects on the enzyme activities of SOD isozymes and POX under individual SMF pre-treatment, on the all antioxidant enzyme activities under individual effects of abiotic stress, on the enzyme activities of SOD isozymes, POX, CAT and APX under interactions of SMF and abiotic stresses and on the activity of APX under combined effects of abiotic stresses and cultivars and SMF, abiotic stress and cultivars (Table 15). There was no statistically significant differences between the two cultivars, in both leaf and root samples, except for APX activity in root samples.

Effects of SMF pre-treatments with and without the combinations of PEG 6000 or NaCl on the contents of GSH and GSSG and the ratios of GSH/GSSG

The effects of pre-treatment with and without 60 gL^{-1} PEG6000 or 100 mM NaCl on GSH, GSSG contents and GSH/GSSG ratios are presented in Tables 12 and 13. According to these results, GSH and GSSG were stimulated in all groups under SMF pre-treatment alone and combined with NaCl or PEG6000. These increases were remarkable with 60 gL^{-1} PEG6000 or 100 mM NaCl. The applications of SMF with NaCl or PEG 6000 also changed GSH and GSSG content. GSH/GSSG ratios decreased under the combined application of SMF with and without 60 gL^{-1} PEG6000 or 100 mMNaCl. These reductions ratios were greater under PEG6000 or NaCl treatment more than individual SMF pre-treatment or combined with PEG6000 or NaCl.

A three-way analysis of variance showed that statistical significance ($p<0.005$) on the contents of GSH, GSSG and GSH/GSSG ratios in leaves and roots under individual effects of SMF and abiotic stresses. There was no statistically significant difference between the two cultivars (Tables 14-15).

Table 9. Antioxidant enzyme activities of 5-old-day in vitro germinated wheat root samples (T. aestivum L. cv. Nina) pre-treated to non-uniform SMF in various times with and without 60 gL^{-1}PEG6000 or 100 mMNaCl.

Experimental Groups	Total-SOD (U mg^{-1} protein)	Fe-SOD (U mg^{-1} protein)	Mn-SOD (U mg^{-1} protein)	Cu/Zn-SOD (U mg^{-1} protein)	POX (ΔA_{470} mg^{-1}protein)	CAT (ΔA_{240} mg^{-1}protein)	APX (ΔA_{290} mg^{-1}protein)	GR (U mg^{-1} protein)
Control	224.93^{a*}	59.12^{a}	74.16^{a}	91.65^{a}	203.63^{a}	8.11^{a}	18.04^{a}	200.58^{a}
2.2 s	293.33^{b}	91.04^{b}	76.22^{a}	126.07^{b}	277.14^{b}	13.51^{b}	25.03^{b}	321.21^{b}
19.8 s	301.81^{b}	95.93^{b}	75.30^{a}	130.58^{b}	288.45^{b}	13.60^{b}	25.35^{b}	341.96^{b}
60 gL^{-1} PEG6000	464.77^{c}	164.99^{c}	97.49^{b}	202.29^{c}	445.19^{b}	17.28^{c}	32.43^{c}	736.70^{c}
2.2 s +60 gL^{-1} PEG6000	347.32^{bc}	118.51^{bc}	81.58^{a}	147.23^{bc}	278.98^{ab}	15.15^{bc}	27.61^{bc}	491.19^{bc}
19.8 s + 60 gL^{-1} PEG6000	363.05^{bc}	128.36^{bc}	84.28^{a}	150.41^{bc}	287.96^{b}	15.02^{bc}	27.91^{bc}	495.02^{bc}
100 mMNaCl	518.57^{c}	188.75^{c}	111.20^{b}	218.62^{c}	463.43^{ab}	18.93^{c}	34.95^{c}	739.85^{c}
2.2 s + 100 mMNaCl	377.03^{bc}	129.72^{bc}	88.25^{ab}	159.06^{bc}	235.29^{a}	16.80^{bc}	27.65^{bc}	471.70^{bc}
19.8 s + 100 mMNaCl	390.80^{bc}	133.93^{bc}	91.73^{ab}	165.14^{bc}	231.51^{a}	16.39^{bc}	27.25^{bc}	474.78^{bc}

* Different letters indicate significant differences ($p<0.05$) among the experimental groups according to one-way ANOVA, post hoc LSD tests.

Table 10. Antioxidant enzyme activities of 5-old-day *in vitro* germinated wheat leaf samples (*T. aestivum* L. cv. Flamura-85) pre-treated to non-uniform SMF in various times with and without 60 gL^{-1} PEG6000 or 100 mM NaCl.

Experimental Groups	Total-SOD (U mg^{-1} protein)	Fe-SOD (U mg^{-1} protein)	Mn-SOD (U mg^{-1} protein)	Cu/Zn-SOD (Umg^{-1} protein)	POX (ΔA_{470} mg^{-1}protein)	CAT (ΔA_{240} mg^{-1}protein)	APX (ΔA_{290} mg^{-1}protein)	GR (U mg^{-1} protein)
Control	140.59^{a*}	33.28^{a}	47.69^{a}	59.62^{a}	147.72^{a}	3.90^{a}	22.37^{a}	146.57^{a}
2.2 s	180.05^{b}	55.12^{b}	49.37^{a}	75.56^{b}	190.82^{b}	7.51^{b}	33.07^{b}	166.04^{a}
19.8 s	183.06^{b}	58.30^{b}	47.87^{a}	76.89^{b}	190.74^{b}	7.58^{b}	34.91^{b}	165.56^{a}
60 gL^{-1} PEG6000	242.84^{c}	78.27^{c}	73.03^{b}	91.54^{c}	238.57^{c}	6.91^{c}	37.79^{c}	482.71^{b}
2.2 s + 60 gL^{-1} PEG6000	192.33^{ab}	69.92^{bc}	44.54^{a}	77.87^{a}	204.98^{bc}	5.69^{bc}	31.87^{b}	270.04^{c}
19.8 s + 60 gL^{-1} PEG6000	171.37^{b}	68.55^{bc}	41.03^{b}	81.79^{bc}	206.07^{bc}	5.91^{bc}	32.07^{b}	273.06^{c}
100 mMNaCl	270.95^{c}	85.72^{c}	82.92^{c}	102.31^{c}	253.92^{c}	8.29^{b}	38.45^{c}	547.29^{d}
2.2 s + 100 mM NaCl	201.06^{bc}	74.16^{c}	45.46^{a}	81.44^{bc}	204.14^{bc}	6.54^{c}	30.46^{b}	267.17^{c}
19.8 s + 100 mMNaCl	202.27^{bc}	71.72^{bc}	48.43^{a}	82.12^{bc}	208.54^{bc}	6.14^{c}	31.29^{b}	254.94^{c}

* Different letters indicate significant differences ($p<0.05$) among the experimental groups according to one-way ANOVA, post hoc LSD tests.

Table 11. Antioxidant enzyme activities of 5-old-day *in vitro* germinated wheat root samples (*T. aestivum* L. cv. Flamura-85) pre-treated to non-uniform SMF in various times with and without 60 gL^{-1} PEG6000 or 100 mM NaCl.

Experimental Groups	Total-SOD (Umg^{-1} protein)	Fe-SOD (Umg^{-1} protein)	Mn-SOD (Umg^{-1} protein)	Cu/Zn-SOD (Umg^{-1} protein)	POX (ΔA_{470} mg^{-1}protein)	CAT (ΔA_{240} mg^{-1}protein)	APX (ΔA_{290} mg^{-1}protein)	GR (Umg^{-1} protein)
Control	176.57^{a*}	49.55^{a}	57.71^{a}	69.31^{a}	246.11^{a}	6.48^{a}	7.90^{a}	168.49^{a}
2.2 s	251.65^{b}	97.55^{b}	65.32^{a}	88.78^{b}	314.85^{b}	10.01^{b}	10.59^{b}	215.23^{b}
19.8 s	251.92^{b}	93.33^{b}	68.66^{a}	89.93^{b}	319.64^{b}	10.09^{b}	10.95^{b}	245.43^{b}
60 gL^{-1} PEG6000	271.42^{c}	91.10^{b}	78.22^{a}	102.10^{c}	533.09^{c}	15.58^{c}	13.03^{c}	559.56^{c}
2.2 s + 60 gL^{-1} PEG6000	227.73^{ab}	80.72^{b}	60.03^{a}	86.98^{b}	418.12^{bc}	12.31^{bc}	9.04^{a}	270.15^{b}
19.8 s + 60 gL^{-1} PEG6000	225.82^{ab}	79.35^{b}	59.25^{a}	87.22^{a}	423.67^{bc}	12.84^{bc}	9.17^{b}	268.27^{b}
100 mMNaCl	277.55^{c}	87.54^{b}	71.73^{a}	118.28^{c}	570.94^{c}	16.07^{c}	14.59^{c}	555.24^{c}
2.2 s + 100 mMNaCl	210.11^{ab}	69.27^{b}	55.97^{a}	84.87^{b}	435.50^{bc}	13.26^{bc}	10.19^{b}	251.00^{b}
19.8 s + 100 mMNaCl	212.05^{ab}	70.64^{b}	55.84^{a}	85.57^{b}	446.80^{bc}	13.03^{bc}	10.24^{b}	251.93^{b}

* Different letters indicate significant differences ($p<0.05$) among the experimental groups according to one-way ANOVA, post hoc LSD tests.

Table 12. GSH and GSSG content and GSH/GSSG ratios in leaf and root samples of 5-old-day *in vitro* germinated Nina wheat, pre-treated to non-uniform SMF in various times with and without 60 gL^{-1} PEG6000 or 100 mMNaCl

The Part of Organs	Experimental Groups	GSH nmolmg^{-1}protein	GSSG nmolmg^{-1}protein	GSH/GSSG
Leaves	**Control**	131.57^{a*}	35.57^{a}	3.70^{a}
	2.2 s	150.53^{a}	47.88^{b}	3.14^{a}
	19.8 s	153.33^{a}	49.76^{b}	3.08^{a}
	60 gL^{-1}PEG6000	289.22^{b}	114.97^{c}	2.52^{b}
	2.2 s+60 gL^{-1} PEG6000	207.65^{c}	75.88^{bc}	2.73^{b}
	19.8 s+60 gL^{-1} PEG6000	204.06^{c}	75.55^{bc}	2.70^{b}
	100 mMNaCl	272.75^{b}	109.12^{c}	2.49^{b}
	2.2 s+100 mMNaCl	199.74^{c}	75.15^{bc}	2.66^{b}
	19.8 s+100 mMNaCl	195.75^{c}	75.43^{bc}	2.60^{b}
Roots	**Control**	163.77^{a*}	42.22^{a}	3.88^{a}
	2.2 s	211.09^{b}	66.38^{b}	3.18^{a}
	19.8 s	219.65^{b}	69.53^{b}	3.16^{a}
	60 gL^{-1}PEG6000	354.99^{c}	174.23^{c}	2.04^{b}
	2.2 s+60 gL^{-1} PEG6000	284.43^{bc}	132.69^{d}	2.14^{b}
	19.8 s+60 gL^{-1} PEG6000	293.94^{bc}	135.68^{d}	2.17^{b}
	100 mMNaCl	387.67^{c}	195.77^{c}	1.98^{b}
	2.2 s+100 mMNaCl	286.75^{bc}	129.71^{d}	2.21^{b}
	19.8 s+100 mMNaCl	289.77^{bc}	127.99^{d}	2.26^{b}

* Different letters indicate significant differences ($p<0.05$) among the experimental groups according to one-way ANOVA, post hoc LSD tests

Table 13. GSH and GSSG content and GSH/GSSG ratios in leaf and root samples of 5-old-day *in vitro* germinated Flamura-85 wheat, pre-treated to non-uniform SMF in various times with and without 60 gL^{-1} PEG6000 or 100 mM NaCl.

The Part of Organs	Experimental Groups	GSH nmolmg^{-1}protein	GSSG nmolmg^{-1}protein	GSH/GSSG
Leaves	**Control**	111.78^{a*}	29.57^{a}	3.78^{a}
	2.2 s	167.33^{b}	51.65^{b}	3.24^{a}
	19.8 s	174.31^{b}	54.30^{b}	3.31^{a}
	60 gL^{-1}PEG6000	329.25^{c}	127.86^{c}	2.57^{b}
	2.2 s+60 gL^{-1} PEG6000	269.05^{d}	97.13^{bc}	2.76^{b}
	19.8 s+60 gL^{-1} PEG6000	264.67^{d}	95.66^{bc}	2.77^{b}
	100 mMNaCl	352.75^{c}	142.81^{c}	2.47^{b}
	2.2 s+100 mMNaCl	259.79^{d}	93.78^{bc}	2.77^{b}
	19.8 s+100 mM NaCl	255.88^{d}	93.39^{bc}	2.74^{b}
Roots	**Control**	143.58^{a*}	37.59^{a}	3.82^{a}
	2.2 s	185.21^{b}	57.52^{b}	3.22^{a}
	19.8 s	192.22^{b}	59.14^{b}	3.25^{a}
	60 gL^{-1}PEG6000	374.11^{c}	178.15^{c}	2.10^{b}
	2.2 s+60 gL^{-1} PEG6000	297.43^{d}	127.63^{bc}	2.33^{b}
	19.8 s+60 gL^{-1} PEG6000	301.09^{d}	126.64^{bc}	2.38^{b}
	100 mMNaCl	393.91^{c}	192.15^{c}	2.05^{b}
	2.2 s+100 mMNaCl	306.55^{d}	131.00^{bc}	2.34^{b}
	19.8 s+100 mMNaCl	309.11^{d}	133.26^{bc}	2.32^{b}

* Different letters indicate significant differences ($p<0.05$) among the experimental groups according to one-way ANOVA, post hoc LSD tests.

Table 14. Statistical significance of F value (three-way ANOVA) for individual and combined effect of static magnetic field, abiotic stress and cultivar for antioxidant defence parameters of leaf samples from 5-day-old *in vitro* germinated wheat.

Treatments	df	T-SOD	Fe-SOD	Mn-SOD	Cu/Zn-SOD	POX	CAT	APX	GR	GSH	GSSG	GSH/GSSG
SMFs	2	8.263^{**}	18.372^{***}	4.841^{ns}	6.236^{*}	5.642^{*}	15.159^{***}	8.360^{*}	62.291^{***}	18.742^{***}	60.082^{***}	1.077^{ns}
Abiotic Stressors	2	23.820^{***}	86.621^{***}	0.466^{ns}	13.164^{***}	154.049^{***}	16.426^{***}	35.936^{***}	617.324^{***}	345.666^{***}	367.473^{***}	86.140^{***}
Cultivars	1	0.755^{ns}	0.720^{ns}	0.296^{ns}	0.660^{ns}	2.402^{ns}	0.022^{ns}	1.781^{ns}	0.513^{ns}	0.392^{ns}	0.109^{ns}	0.070^{ns}
SMFs x Abiotic Stresseors	4	66.461^{***}	33.512^{***}	1.574^{ns}	33.568^{***}	62.272^{***}	43.698^{***}	13.834^{***}	50.232^{***}	33.297^{***}	52.872^{***}	16.126^{***}
SMFs x Cultivars	2	0.514^{ns}	0.066^{ns}	0.055^{ns}	0.941^{ns}	0.068^{ns}	0.311^{ns}	0.125^{ns}	0.065^{ns}	0.332^{ns}	0.193^{ns}	0.298^{ns}
Abiotic Stressors x Cultivars	2	0.711^{ns}	0.402^{ns}	0.048^{ns}	0.399^{ns}	0.098^{ns}	0.039^{ns}	0.252^{ns}	0.046^{ns}	0.147^{ns}	0.825^{ns}	1.015^{ns}
SMFs x Abiotic Stressors x Cultivars	4	0.098^{ns}	0.137^{ns}	0.020^{ns}	0.103^{ns}	0.163^{ns}	0.165^{ns}	0.160^{ns}	0.031^{ns}	0.037^{ns}	0.512^{ns}	0.235^{ns}

^{ns}Not Significant;* $p<0.05$; ** $p<0.001$; *** $p<0.0001$.

Table 15. Statistical significance of F value (three-way ANOVA) for individual and combined effect of static magnetic field, abiotic stress and cultivar for antioxidant defence parameters of root samples from 5-day-old *in vitro* germinated wheat.

Treatments	df	T-SOD	Fe-SOD	Mn-SOD	Cu/Zn-SOD	POX	CAT	APX	GR	GSH	GSSG	GSH/GSSG
SMFs	2	60.495^{*}	27.543^{*}	19.948^{*}	10.729^{*}	201.380^{*}	33.912^{*}	39.661^{*}	1.129^{ns}	49.866^{*}	66.830^{*}	1.971^{ns}
Abiotic Stressors	2	614.390^{*}	307.754^{*}	75.676^{*}	235.072^{*}	107.813^{*}	125.888^{*}	148.822^{*}	13.340^{*}	437.658^{*}	988.932^{*}	662.576^{*}
Cultivars	1	0.561^{ns}	1.617^{ns}	1.597^{ns}	0.078^{ns}	1.441^{ns}	0.420^{ns}	13.054^{*}	0.554^{ns}	0.023^{ns}	1.259^{ns}	4.067^{ns}
SMFs x Abiotic Stresseors	4	99.145^{*}	23.786^{*}	37.293^{*}	61.005^{*}	145.196^{*}	18.124^{*}	90.169^{*}	1.632^{ns}	45.340^{*}	50.720^{*}	7.847^{*}
SMFs x Cultivars	2	0.058^{ns}	0.148^{ns}	0.239^{ns}	0.424^{ns}	0.060^{ns}	0.129^{ns}	4.387^{ns}	0.067^{ns}	0.158^{ns}	0.084^{ns}	0.554^{ns}
Abiotic Stressors x Cultivars	2	0.044^{ns}	0.494^{ns}	0.393^{ns}	0.100^{ns}	0.024^{ns}	0.225^{ns}	13.697^{*}	0.009^{ns}	0.058^{ns}	0.115^{ns}	1.468^{ns}
SMFs x Abiotic Stressors x Cultivars	4	0.009^{ns}	0.135^{ns}	0.128^{ns}	0.215^{ns}	0.302^{ns}	0.166^{ns}	5.879^{*}	0.208^{ns}	0.024^{ns}	0.153^{ns}	0.368^{ns}

^{ns}Not Significant; * $p<0.001$

Discussion

In this study, we investigated interactive effect of artificial SMF pre-treatments and abiotic stressors on the germination stages of two different wheat cultivars with assessing growth parameters (germination rate, seedling vigour index, average plant and root fresh weights and average plant and root heights) and some biochemical parameters (chloroplast pigments (Chl*a+b,a, b* and CAR), free radicals ($^{\bullet}OH$ and H_2O_2), FRAP content and antioxidant defence parameters including activities of SOD isozymes, CAT, POX, APX and GR and GSH and GSSG contents).

According to previous reports, SMF pre-treatment increased germination performance or growth in tomatoes (De Souza et al. 2006), soybeans (Shine et al. 2012) and cotton (Bilalis et al. 2013) with different combinations of MF density, frequency and exposure time. For this reason, some researchers suggested that MF pre-treatment under appropriate conditions is a more environmentally-friendly approach than fertilizer to increase yield performance (Bilalis et al. 2013). MF affects radical-pair mechanisms in the cell (Galland and Pazur 2005) and influences cell membrane structures by changing cell membrane permeability, ion transportation and mineral uptake (Shine et al. 2012). Common opinion among the scientist, these effects could be biological responses in cells (Podleśny et al. 2005; De Souza et al. 2006; Wang et al. 2008; Shine et al. 2012; Bilalis et al. 2013), and in another general view harsh environmental conditions (salinity and drought) decrease growth parameters in plants (Wang et al. 2009; Bhardwaj et al. 2012). Our study was consistent with previous ones in terms of seed germination ratios, seedling vigour indexes, average plant and root fresh weights and average plant and root heights. These measures of growth decreased with salinity and drought compared to the control, but increased with individual and combined applications of SMF for both abiotic stressors.

Metabolically active cells produce ROS due to biological functions, such as mitochondrial respiration, chloroplast electron transport chain and peroxisome/glyoxisome activity. Bailly (2004) and El-Maarouf et al. (2008) reported that cell-controlled ROS accumulation could play a beneficial role in germination and growth by changing gene expression during seed development, dormancy and germination, protecting against pathogen-attacks, elongating cell walls, regulating of redox signalling and interacting with abscisic acid and gibberellins transduction pathways. Drought and salinity strongly induce ROS accumulation in germinated seeds. If these accumulations exceed a controlled amount, ROS restrict plant development and reduce crop yields (Wang et al. 2009; Pratap and Sharma 2010). The energy level of MF is not enough to break molecular or chemical bonds for creating ROS in the cell, but it can influence nuclear and electron spins of ROS via Zeeman splitting, hyperfine interaction, electron exchange and dipole-dipole interaction (Galland and Pazur 2005). As a result, the kinetics and yield of chemical reactions are affected due to spin precession rates of unpaired electrons and consequent effects on the lifetime or concentration of free radicals (Timmel et al. 1998). Like us, Podleśny et al. (2005), Shine et al. (2012) and Bhardwaj et al. (2012) measured increasing ROS concentrations in pea, soybean and cucumber seedlings under MF pre-treatment. Additionally, Hajnorouzi et al. (2011) detected an increase in total antioxidant capacity via the DPPH method in maize under MF treatment.

Increase in photosynthetic capacity is another important factor for biomass increase during plant growth (Shine et al. 2012). Thylakoid membranes of chloroplasts are the major source of singlet oxygen ($^1O_2$) as a result of photo-excitation of chlorophyll-to-chlorophyll triplet state, which then reacts with O_2. An increased-chlorophyll-triplet-state in photosynthetic machinery reduces chlorophyll content in plant leaf because of the degradation in chlorophyll molecules. Hakala-Yatkin et al. (2011) reported that external MF application protects plants against strong light by slowing oxygen production. Carotenoid, which are located within the thylakoid membranes of the chloroplast, are vital for detoxifying $^1O_2$ and

triplet chlorophyll, besides absorbing light in appropriate wavelengths and transferring it to the chlorophyll. There was an increase in ratios of Chl*a*+*b*, Chl*a* and *b* content for potato, mung bean and maize leaves under MF conditions (Chen et al. 2011; Rakosy-Tican et al. 2005; Shine and Guruprasad 2012). Additionally, Rakosy-Tican et al. (2005); Shine and Guruprasad (2012), reported that MF treatment increased CAR content in the cell. In the presented study, we have found similar results about Chlorophylls and carotenoids contents.

As we mentioned above, ROS play a dual role during plant development. Lower concentrations of ROS are involved in cell signaling, acclimation and cross-tolerance while their higher concentrations are extremely harmful to cellular components (Reddy and Raghavendra 2006). For example, H_2O_2 is involved in programing cell death, somatic embryogenesis, response to wounding, root gravitropism and ABA-mediated stomata closure, besides damaging of cellular macromolecules. It can be formed from $O_2^{\bullet-}$ by SOD as well as by spontaneous dismutation in the cell.

In our study, total-SOD enzyme activities increased under 2.2 and 19.8 s SMF pre-treatment in both wheat cultivars. The activity of Fe-SOD, Zn/Cu-SOD and Mn-SOD changed, and Fe-SOD was the most affected. POX is one of the key enzymes in plants and has a dual-function depending on the site of production and developmental stage of the plant, such as cytosolic POX (Compound I) detoxified H_2O_2, whereas the Compound III of POX catalyses the generation of $^{\bullet}OH$ from $O_2^{\bullet-}$ derived H_2O_2 via the *hydroxylic cycle* (Liszkay et al. 2003). POX is also involved in cell wall construction, differentiation and plant response to biotic and abiotic stress (Ghamsari et al. 2007). CAT is another important ion contained in the H_2O_2-scavenging enzyme within the cell (Scandalios et al. 1997). The combination of APX and GR can also remove H_2O_2 via the Ascorbate-Glutathione (AsA–GSH) cycle. GSH and GSSG are other important components, which are involved in the AsA–GSH cycle, an H_2O_2-scavenging pathway. GSH can react chemically with $O_2^{\bullet-}$, $^{\bullet}OH$ instead of H_2O_2 and therefore plays an important role in intracellular defence against ROS. GSH–GSSG is one of the crucial redox pairs in the cell, and the balance between them is central to maintaining a state of cellular redox. This ratio is maintained by GR (Foyer and Noctor 2011). Sumugat (2004) reported lower activities of SOD POX, APX and GR decreased seed germinability in the stored seed; the post-harvesting activity of priming increased these enzyme activities. This result showed that seed vigour is highly correlated with these antioxidant enzymes activities.

In our study, the enzyme activities of POX, CAT, APX and GR and the content of GSH and GSSG were enhanced while the ratios of GSH/GSSG decreased under SMF pre-treatment in all groups. MF treatment increased CAT activity by up to 95% in soybean seedlings (Shine et al. 2012) and 69% in *Chlorella vulgaris* (Wang et al. 2008), SOD activity up to 87% in tobacco suspension-culture (Sahebjamei et al. 2007) and 124% in *Chlorella vulgaris* (Wang et al. 2008), cytosolic POX activity less than 50% in *Chlorella vulgaris* (Wang et al. 2008) and up to 27% in soybean (Shine et al. 2012), but decreased CAT and APX activities in tobacco suspension-culture (Sahebjamei et al. 2007) and SOD and APX activities in soybean (Shine et al. 2012).

The general assumption is that tolerance or sensitivity to drought or salinity in plants is well correlated with inherent antioxidant responses. In this study, the activity of antioxidant enzymes (total-SOD, Fe-SOD, Mn-SOD, Cu/Zn-SOD, CAT and POX), which are responsible for ROS detoxification, increased in leaf and root samples of both wheat cultivars under PEG 6000 and NaCl-treatment. Under stress conditions, the activities of APX and GR and the contents of GSH and GSSG increased while GSH/GSSG dramatically decreased. APX, GR, GSH and GSSG are important components of AsA–GSH cycles in the intracellular compartment. Wang et al. (2009) detected that a treatment of 35% PEG or 200 mM NaCl, induced H_2O_2 content and SOD and APX activity in Xinmu No. 1 and Northstar alfalfa varieties. In black gram, the highest rate of PEG 6000 treatment

significantly decreased germination percentage, seedling growth parameters, amylase activity, chlorophyll and carotenoid contents but increased the activities of SOD, CAT and POX (Pratap and Sharma 2010).

In our study, the combined effect of 2.2 and 19.8 s SMF pre-treatment with 60 gL^{-1} PEG6000 or 100 mMNaCl significantly stimulated the activity of total-SOD, Fe-SOD, Cu/Zn-SOD, CAT and POX in all groups. As for AsA–GSH cycles' components (APX, GR, GSH and GSSG), their activities and contents increased remarkably with 2.2 and 19.8 s SMF and 60 gL^{-1} PEG6000 or 100 mM NaCl. The ratio of GSH/GSSG increased slightly in all groups, between 6.37–14.15%, compared to the application of only 60 gL^{-1} PEG6000 or 100 mMNaCl. In *Cucumis sativus* L., Piacentini et al. (2001) detected that EMF treatment enhanced growth parameters and the activities of SOD, CAT and GR and also senescence-delay effects.

Conclusion

This study addressed effects of artificial SMF pre-treatments on abiotic stress in the germination stages of two different wheat cultivars depending on assessing the physiological (germination rate, seedling vigour index, average plant and root fresh weights and average plant and root lengths) and biochemical (chloroplast pigments (Chl*a+b,a, b* and CAR), free radicals ($^{\bullet}OH$ and H_2O_2), FRAP content and antioxidant defence parameters including activities of SOD isozymes, CAT, POX, APX and GR and GSH and GSSG contents). Appropriate SMF pre-treatment times affected changes in the intercellular redox in the cell. Perturbed antioxidant defence systems are important to combat abiotic and biotic stress factors during plant development and improving crops against harsh environmental conditions is a top priority for breeders. But the breeding process takes quite a long time, and sometimes breeders need temporary and quick solutions to combat harsh environmental conditions. These results show that static magnetic field pretreatment compensated for the negative effects of drought and salinity stresses on the growth parameters in both cultivars duo to stimulating the antioxidant defence system, especially in an agriculturally important crop, in order to reduce yield losses and to provide breeders with temporary and quick solutions.

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Research Article

Investigation of cytotoxic effects of some novel synthesized iminothiazolidinone derivatives on HeLa cell line (CCL2)

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Abstract

In medicinal chemistry, the thiazolidinones are a practical framework which can be leaned as a pharmacophore in a large diversity of biologically active compounds. Furthermore, they build up the base of antibacterials, anti-convulsant, anti-tumorals, antivirals, anti-diabetic, anti-inflammatory, anti-HIV compounds in many other therapeutic agents. In this study, the cytotoxic effects of some novel synthesized iminothiazolidinone derivatives (Compound A-E) on HeLa (3) cell line (CCL2) which arising from human cervical carcinoma were studied. Accordingly, kinetics parameters of proliferation rate, mitotic and the labeling index were determined upon the application of the iminothiazolidinone derivatives. 1 x 10^{-6}, 5 x 10^{-6}, 10 x 10^{-6} M concentrations of the derivatives were implemented for 72 hours to find out the optimum concentrations, and the result was explicated by reproduction rate analysis. Parameters of the mitotic and labeling index of the cells which were treated with the optimum concentrations of the uniquely synthesized iminothiazolidinone derivatives for 0-72 hours were calculated. The results indicated that the tested compounds caused a remarkable decrease in the propagation of HeLa cell cultures and the 10 x 10^{-6} M concentration was found to be the most effective concentration of the iminothiazolidinone derivatives regarding reducing the reproduction rate. Drugs can be obtained from these derivatives will offer a promising treatment modality in cervix carcinoma in the future.

Key words: Iminothiazolidinone, HeLa cells, viability, mitotic index, labelling index.
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Bazı yeni sentezlenmiş iminotiazolidinon türevlerinin HeLa hücre hattı üzerindeki sitotoksik etkilerinin araştırılması

Özet

Tıbbi kimyada, tiazolidinonlar, büyük bir çeşitlilik barındıran biyolojik olarak aktif bileşikler içinde farmokofor olarak kullanılabilecek kapasiteye sahiptir. Ayrıca, birçok terapötik ajanlarda antibakteriyel, anti-konvülsan, anti-tümöral, antiviral, anti-diyabetik, anti-inflamatuar ve anti-HIV bileşiklerinin tabanını oluştururlar. Bu çalışmada, bazı yeni sentezlenmiş iminotiazolidinon türevlerinin (A-E Bileşikleri) insan servikal karsinomu kaynaklı HeLa (3) hücre hattı (CCL2) üzerine sitotoksik etkileri araştırılmıştır. Buna göre iminotiazolidinon türevlerinin uygulanması üzerine yayılma hızı, mitotik ve işaretlenme indeksinin kinetik parametreleri belirlenmiştir. Optimum konsantrasyonların bulunması için $1x10^{-6}$, $5x10^{-6}$, $10x10^{-6}$ M konsantrasyonlarında tiazolidinon türevleri 72 saat süreyle uygulanmış ve sonuç, üreme hızı analizi ile açıklanmıştır. 0-72 saat boyunca benzersiz şekilde sentezlenmiş iminotiazolidinon türevlerinin optimum konsantrasyonları ile muamele edilen hücrelerin mitotik ve işaret indeks parametreleri hesaplanmıştır. Sonuç olarak, test edilen bileşiklerin HeLa hücre kültürlerinin çoğalmasında, kayda değer bir düşüşe neden olduğu ve $10x10_{-6}$ M konsantrasyondaki iminotiazolidinon türevlerinin çoğalma hızının düşüşüne ilişkin en etkili konsantrasyon olduğu bulunmuştur. Bu türevlerden elde edilecek ilaçların gelecekte serviks karsinoması tedavisinde umut verici bir model sunması ümit edilmektedir.

Anahtar kelimeler: İminotiazolidinon, HeLa hücreleri, canlılık, mitotik indeks, işaretlenme indeksi

Introduction

Nowadays, 4-thiazolidinones are one of the most praised compounds in medicinal chemistry. Its small heterocyclic molecular structure and medicinal chemists properties, as well as its structural and therapeutic diversity, have encouraged the researches (Hamama et al. 2011). 4-thiazolidinone is a promising scaffold for the search of new potential antibacterial, antiviral, antidiabetic and anticancer agents (Oleh et al. 2012). Thiazolidinone ring system has been participated in a wide range of biologically effective compounds, either as a substituent group or as a revision of other ring, which has excited the researchers to synthesize various compounds including this moiety (Chavan and Pai 2007; Jain et al. 2012). In addition, thiazolidinones and their derivative products have a momentous position in the area of heterocyclic compounds based on their presence in the structures of macrocyclic complex medications. They also have some applications in industry and pharmaceutical area because of their biological particulars (Verma and Saraf 2008; Hamama et al. 2008; Jain et al. 2012). Due to their reaction capabilities both as N-nucleophiles and S-nucleophiles, thioureas are successfully used in the synthesis of biologically active compounds. As a result of comprehensive research made in recent years, new thiourea compounds having antiarthiritic, anti-inflammatory, antiviral, antidiabetic, antithyroidal, fungicidal, bactericidal, insecticidal and pesticidal have been discovered (Rahman et al. 2005; Jain et al. 2012; Liu et al. 2012). Against imino analogues of thiazolidinones are less widespread, even the accurate possibility to infer an additional manage for chemical variety and hereby allow of examination of unconjugated regions of a thiazolidinone-based pharmacophore (Laurent et al. 2004). On the other hand, anticancer action, antiproliferative effect, antimicrobial, antituberculosis and antibacterial activities of iminothiazolidinone compounds have been observed in their biological and pharmacological activity studies (Küçükgüzel et al. 2008; Kumar et al. 2014; Kaminskiyy et al. 2016). In addition, the existence of anti-inflammatory (Ottana et al. 2005) and anti-fungal (Liu et al. 2000) effects of these compounds have also been shown.

In this study, the antiproliferative properties and cytotoxic effects of the newly synthesized iminothiazolidinone derivatives was evaluated on HeLa cell line (CCL2) arising from human cervix carcinoma. In order to achieve these purposes, cell kinetic parameters such as propagation assay, mitotic and labeling index analysis were determined. 1 x 10^{-6}, 5 x 10^{-6}, 10 x 10^{-6} M concentrations of iminothiazolidinone derivatives were adopted to cells for 24, 48 and 72 hours. It was shown that all applications in all experiential series ($p<0.05$) showed a perceptible decrease in mitotic index, labeling index and cell proliferation.

Materials and methods

Chemistry

Iminothiazolidinones were synthesized in two steps in this study. In the first step, substituted thioureas which were planned to use as substrates in the principal reactions, were prepared by the reaction of substituted phenyl isothiocyanate with substituted hetaryl amines. In the second step which is the main part of the study, the cyclocondensation of each of the previously prepared thioureas with chloroacetic acid and substitute thiophene-2-carboxaldehyde were achieved by the technique of one-pot multicomponent reaction; and newly synthesized 5-substitued-2-imino-4-thiazolidinone compounds (A-E) were obtained (Tuğcu 2009). The confirmation of the structures of newly synthesized compounds were carried out by the use of (1) H NMR, (13) C NMR and HR-MS data.

Cell culture

In this study, the employed HeLa cell line (CCL-2) was obtained from American Animal Cell Culture Collection. Cells were grown in Medium-199 (M-199, Sigma, USA) including 0.1 mg/ml streptomycin (Streptomycin sulphate, I. E. Ulugay), 10% foetal bovine serum (FBS, Gibco Lab), 100 IU/ml penicillin (Pronapen, Pfizer), amphotericin B (Sigma, USA) and 0,002 M glutamine, pH 7.2, at 37ºC in 5% CO_2 moisturized air.

Compound concentrations

Compound concentrations that used in the present study were determined according to previous *in vitro* and clinical studies. At first, a 1000 µM stock solution was prepared with M-199 supplemented with 10% FBS. Three different concentrations were prepared by dilution of the stock solution. These compounds were called as concentration 1 (D1= 1 x 10^{-6} M), concentration 2 (D2= 5 x 10^{-6} M), concentration 3 (D3=10 x 10^{-6} M). The experiments were carried out by using these three compound concentrations. HeLa cells were treated with the above compound concentrations for the time periods of 24, 48 and 72 hours.

Preparing of ^{3}H-Thymidine

Nine ml deionized water was added to a vial containing 1 mCi/ml ^{3}H-Thymidine (TRA-120, Amersham, England) and stock solution was prepared. Then 1 mCi/ml solution was diluted to 1 µCi/ml with cell culture medium. The cells will be labeled with this solution.

Cell proliferation assay

The effects of iminothiazolidinone derivates (Compounds A-E) on cell proliferation of HeLa cells were measured by MTT assay. Method is based on the competency of live cells to transform tetrazolium salt into purple formazan. HeLa cells were emplaced into 96-well plates at a density of $2x10^{4}$ cells per well and incubated overnight. Then the cells were cultivated with the concentrations of 1 µM, 5 µM and 10 µM iminothiazolidinone derivates (Compounds A-E). The medium in each well was removed and 40 µl fresh MTT solution (5 mg/ml in PBS) were added into each well, and the content was incubated at 37 ^{o}C for 4 h at the end of the experimental period. Then, 160 µl DMSO (Dimethyl Sulfoxide, Sigma) was added into each well and cells were shaken thoroughly for 1 h on a shaker. Then, the absorbance of the samples was measured against a blank using an Elisa reader (µQuant, Bio-Tek Instruments Inc) at 450-690 nm.

Determination of optimal concentration with cell viability analysis

To determine the most effective concentrations of iminothiazolidinone derivates on HeLa cells, three different concentrations (D1= 1 μM, D2= 5μM, D3= 10μM) were applied to cell culture for 72 h. The cytotoxic effects of the different concentrations were evaluated with MTT assay. At the end of this application, the most effective concentration which was determined according to the absorbance values was used for the following parameters.

Mitotic index analysis

For mitotic index analysis, HeLa cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well and incubated overnight. Then the cells were treated with 1 μM, 5 μM and 10 μM iminothiazolidinone derivates (Compounds A-E) concentrations. At the end of the experimental period cells were fixed with Carnoy's fixative (3:1 methanol-acetic acid). Mitotic index were determined by the methods of Feulgen. The cells were processed with 1 N HCl at room temperature for 1 minute and then hydrolyzed with 1 N HCl for 10.5 minutes at 60°C after treated with Feulgen. After lamellas were treated with Feulgen, they were washed in distilled water and for 3 minutes stained with 10% Giemsa stain solution pH 6.8, and then washed twice in phosphate buffer. After dyeing, the coverslips were rinsed in distilled water. And then they were air dried. Mitotic index was calculated for each drug concentration and control group by counting metaphases, anaphases and telophases. Finally, the mitotic index was calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control. At least 3000 cells were examined from each slide for determination of the mitotic index by the same scorer. At least three thousand cells were viewed from each cover glass for the mitotic index.

^{3}H-thymidine labeling index analysis

For ^{3}H-thymidine labeling index analysis which determine cells in the synthesis phase, HeLa cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well and incubated overnight. Then the cells were treated with 1μM, 5 μM and 10 μM iminothiazolidinone derivates (Compounds A-E) concentrations. At the end of the experimental period, cells were treated with medium containing 1 μCi/mL ^{3}H-thymidine for 20 min to evaluate the labeling index and the labeled cells.

Autoradiography

After labeling, the cells were fixed with Carnoy's fixative (3:1 methanol-acetic acid) and the remaining radioactive material was washed twice with 2% perchloric acid at 4°C for 30 min. The slides were prepared and were coated with K.2 gel emulsion (Ilford) prepared with distilled water at 40°C to determine thymidine labeling index. After 3 days exposure at 4°C, autoradiograms were washed with D-19 b developer (Kodak) and fixed with Fixaj B (Kodak). The slides were evaluated after being stained with Giemsa for 3 min. The same person evaluated all the slides by counting at least 3000 cells from each slide. The labeling index was calculated by examining 100 areas on each slide at a magnification of ·12.5. At least three thousand cells were examined from each coverslip.

Statistics

Values of proliferation rate, MI and AI were evaluated relative to controls and to each other in order to find how significant they are. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by DUNNETT's test, and the significance between experimental groups was found by Student's t-test.

Results

Synthesis of iminothiazolidinone derivatives (Compounds A-E)

A blend of the appropriate amine (1 mmol) and substitute phenylisothiocyanate (1.2 mmol) was ruffled in CH_2Cl_2 at room temperature for 24 h. The crude product was concentrated under vacuum and recrystallized from ethanol. The

appropriate thiourea (1 mmol), chloroacetic acid (1.2 mmol) and substitute thiophene-2-carbaldehyde (1 mmol) were stirred with a magnetic stirrer at 40 ºC for 24 h. The crude product was purified by column chromatography ($CHCl_3$ or CH_2Cl_2) as explained by Kasmi-Mir et al. (2006). At the end of the process, five iminothiazolidinone derivatives were obtained.

Compound A

2-[(5-chloropyridin-2-yl)imino]-3-phenyl-5-(thiophen-2-ylmethylidene)-1,3-thiazolidin-4-one: Yellow solid, m.p. 284-5 ºC;

FTIR (near) $\gamma_{max/cm}^{-1}$**:** 3082, 3017, 2961, 1704, 1585, 1562, 1544, 1458, 1364, 1353, 1157, 862, 832, 794; ^{1}H NMR (500MHz, $CDCl_3$)**:** δ **(**ppm) 7.01 (1H, d, J=8.4 Hz), 7.20 (1H, dd, J=4.8; 4.0 Hz), 7.40 (2H, brd, J=7.3 Hz), 7.44 (1H, brd, J=3.7 Hz), 7.53 (1H, d, J=7.7 Hz), 7.54 (2H, dd, J=7.7; 7.3 Hz), 7.55 (1H, dd, J=8.4; 2.7 Hz), 7.60 (1H, brd, J=5.1 Hz), 8.03 (1H, s), 8.50 (1H, brs); ^{13}C NMR (100MHz, $CDCl_3$)**:** δ **(**ppm) 121.81, 122.71, 125.59, 128.05, 128.50, 128.72, 129.02, 129.37, 131.53, 132.99, 135.55, 137.97, 138.56, 145.75, 154.23, 156.23, 166.24; MS (m/z)**:** 399 M^{+2}, 398 M^{+1}, 397 M^+, 396 M^{-1}, 230, 140, 112, 96, 76.

Compound B

3-(2,4-dimethylphenyl)-5-[(3-methylthiophen-2-yl)methylidene]-2-(phenylimino)-1,3-thiazolidin-4-one **:** Yellow solid, m.p. 155-6 ^{0}C; FTIR (near) $\gamma_{max/cm}^{-1}$**:** 3083, 3068, 3032, 2977, 2952, 2922, 1711, 1633, 1592, 1495, 1356, 1272, 1172, 1155, 870, 820, 770; ^{1}H NMR (500MHz, $CDCl_3$)**:** δ **(**ppm) 2.27 (3H, s), 2.37 (3H, s), 2.42 (3H, s), 6.96 (2H, brd, J=8.6 Hz), 6.97 (1H, d, J=7.8 Hz), 7.18 (1H, d, J=4.7 Hz), 7.19 (1H, m), 7.20 (1H, s), 7.21 (1H, d, J=7.8 Hz), 7.35 (2H, dd, J=8.2; 7.4 Hz), 7.48 (1H, d, J=4.7 Hz), 8.06 (1H, s); ^{13}C NMR (100MHz, $CDCl_3$)**:** δ **(**ppm) 14.70, 17.93, 21.49, 118.79, 121.40, 122.98, 124.99, 128.17, 128.56, 129.40, 129.62, 131.27, 131.76, 132.25, 132.66, 135.98, 139.80, 142.77, 148.67, 150.55, 166.54; MS (m/z)**:** 406 M^{+2}, 405 M^{+1}, 404 M^+, 403 M^{-1}, 389, 371, 312, 222, 202, 154, 121, 118, 91, 77.

Compound C

3-(4-butylphenyl)-5-[(3-methylthiophen-2-yl)methylidene]-2-(phenylimino)-1,3-thiazolidin-4-one**:** Yellow solid, m.p. 284-5 ^{0}C;

FTIR (near) $\gamma_{max/cm}^{-1}$**:** 3061, 3032, 2957, 2922, 2854, 1702, 1632, 1590, 1510, 1359, 1297, 1269, 1167, 1146, 874, 835, 775 ; ^{1}H NMR (500MHz, $CDCl_3$)**:** δ **(**ppm) 0.95 (3H, t, J=7.4 Hz), 1.41 (2H, m), 1.65 (2H, m), 2.42 (3H, s), 2.67 (2H, t, J=7.8 Hz), 6.97 (1H, d, J=5.1 Hz), 6.99 (2H, brd, J=8.6 Hz), 7.17 (2H, brd, J=8.6 Hz), 7.36 (5H, m), 7.47 (1H, d, J=5.1 Hz), 8.06 (1H, s); ^{13}C NMR (100MHz, $CDCl_3$)**:** δ **(**ppm) 14.15, 14.68, 22.68, 33.49, 35.66, 118.78, 121.39,122.92, 124.36, 125.02, 127.91, 129.46, 129.66, 131.26, 132.65, 132.73, 134.23, 142.68, 143.87, 148.59, 151.11, 166.76; MS (m/z)**:** 434 M^{+2}, 433 M^{+1}, 432 M^+, 431 M^{-1}, 389, 250, 207, 154, 121, 77.

Compound D

3-(4-butylphenyl)-5-[(5-methylthiophen-2-yl)methylidene]-2-(phenylimino)-1,3-thiazolidin-4-one **:** Yellow solid, m.p. 160-1 ^{0}C;

FTIR (near) $\gamma_{max/cm}^{-1}$**:** 3066, 3027, 2955, 2926, 2852, 1710, 1638, 1591, 1512, 1359, 1266, 1171, 1146, 830, 805, 773; ^{1}H NMR (500MHz, $CDCl_3$)**:** δ **(**ppm) 0.95 (3H, t, J=7.4; 7.0 Hz), 1.41 (2H, m), 1.65 (2H, m), 2.52 (3H, s), 2.67 (2H, t, J=7.8 Hz), 6.80 (1H, d, J=4.7 Hz), 6.99 (2H, brd, J=7.4 Hz), 7.17 (2H, brd, J=7.4 Hz), 7.35 (5H, m), 7.38 (1H, d, J=5.5 Hz), 7.91 (1H, s); ^{13}C NMR (100MHz, $CDCl_3$)**:** δ **(**ppm) 14.18, 16.10, 22.70, 33.52, 35.67, 118.11, 121.39, 124.74, 124.99, 127.24, 127.91, 129.49, 129.52, 132.58, 133.05, 136.35, 143.91, 147.15, 148.66, 151.20, 166.71; MS (m/z)**:** 434 M^{+2}, 433 M^{+1}, 432 M^+, 431M^{-1}, 389, 250, 207, 154, 121, 77.

Compound E

2-[(4-butylphenyl)imino]-5-[(5-methylthiophen-2-yl)methylidene]-3-phenyl-1,3-thiazolidin-4-one**:** Yellow solid, m.p. 137-8 ^{0}C;

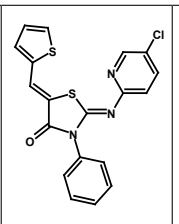
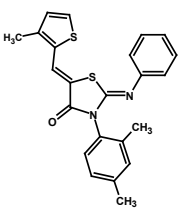
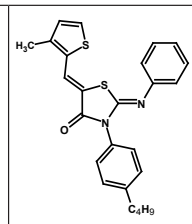
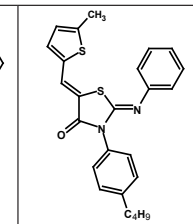
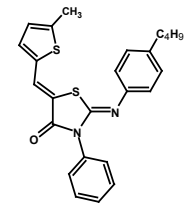
FTIR (near) $\gamma_{max/cm}^{-1}$**:** 3065**,** 3029, 2957, 2886, 2867, 2851, 1712, 1641, 1599, 1496, 1364, 1270, 1183, 1145, 854, 831, 802; ^{1}H NMR (500MHz, $CDCl_3$)**:** δ **(**ppm) 0.95 (3H, t, J=7.4 Hz), 1.37 (2H, m), 1.62 (2H, m), 2.53 (3H, s),

2.62 (2H, t, J=7.8 Hz), 6.81 (1H, d, J=4.7 Hz), 6.90 (2H, brd, J=8.6 Hz), 7.16 (2H, brd, J=8.2 Hz), 7.17 (1H, d, J=4.7 Hz), 7.44 (1H, m), 7.47 (2H, m), 7.52 (2H, brd, J=7.0 Hz), 7.92 (1H, s); ^{13}C NMR (100MHz, $CDCl_3$)**:** δ **(**ppm) 14.18, 16.08, 22.58, 33.80, 35.38, 118.26, 121.13, 124.61, 127.24, 128.30, 128.95, 129.40, 132.96, 135.25, 136.40, 139.65, 145.98, 147.05, 150.34, 166.58; MS (m/z)**:** 434 M^{+2}, 433 M^{+1}, 432 M^{+}, 431M^{-1}, 389, 250, 207, 154, 121, 77.

Determination of optimal concentrations with mitochondrial dehydrogenase enzyme activity analysis

In this study, three different concentrations (D1=1μM, D2=5μM, D3=10μM) of iminothiazolidinone derivatives (Compound A-E) were applied to HeLa cell culture for the period of 24, 48 and 72 h. The absorbance values of each concentration for 72 h are shown in Table 1. The absorbance differences between control and experimental groups were found statistically significant ($p<0.05$). In addition, significant differences among the experimental groups were also noted ($p<0.05$). The results revealed that 10 μM concentration is the optimum concentration for the tested compounds (Table 1). The results from all parameters shown that the tested compounds caused a significant decrease in the proliferation of HeLa cell cultures in a concentration-dependent manner. Viability values of HeLa cells treated with iminothiazolidinone derivatives (Compound A-E) were shown in Figure 1 a-e. The results showed that compound E (2-[(4-butylphenyl) imino]-5-[(5-methylthiophen-2-yl) methylidene]-3-phenyl-1,3-thiazolidin-4 one) is the most effective compound among the tested compounds.

Table 1. Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with different concentrations of iminothiazolidinones for 72 h (±SD, $p<0.05$).

Experimental Groups					
	Compound A	**Compound B**	**Compound C**	**Compound D**	**Compound E**
Control	**457,67×10^{-3*} ±0.09**	**462,12×10^{-3*} ±0.05**	**464,12×10^{-3*} ±0.08**	**447,28×10^{-3*} ±0.06**	**464,58×10^{-3*} ±0.02**
D_1	**445,33×10^{-3}±0.07**	**385,04×10^{-3}±0.02**	**417,29×10^{-3}±0.09**	**395,34×10^{-3}±0.03**	**479,29×10^{-3}±0.05**
D_2	**403,57×10^{-3}±0.09**	**447,29×10^{-3}±0.02**	**453,54×10^{-3}±0.11**	**302,44×10^{-3}±0.07**	**345,32×10^{-3}±0.04**
D_3	**345,00×10^{-3}±0.04**	**326,52×10^{-3}±0.04**	**411,33×10^{-3}±0.08**	**284,22×10^{-3}±0.04**	**285,08×10^{-3}±0.01**

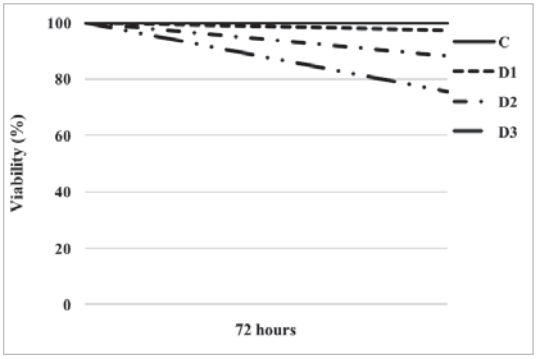


Figure 1a. Viability (%) values of HeLa cells treated with 1 μM, 5 μM and 10 μM concentrations of compound A for 72 h (450- 690 nm) ($p<0.05$).

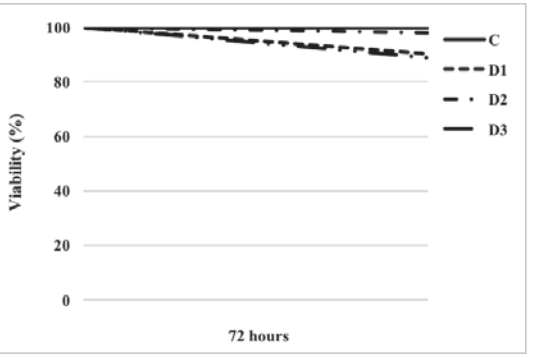


Figure 1b. Viability (%) values of HeLa cells treated with 1 μM, 5 μM and 10 μM concentrations of compound B for 72 h (450- 690 nm) ($p<0.05$).

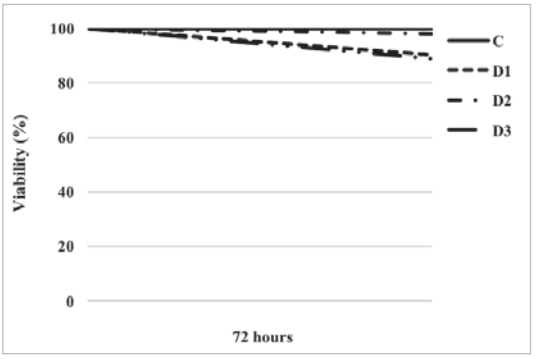


Figure 1c. Viability (%) values of HeLa cells treated with 1 µM, 5 µM and 10 µM concentrations of compound C for 72 h (450- 690 nm) ($p<0.05$).

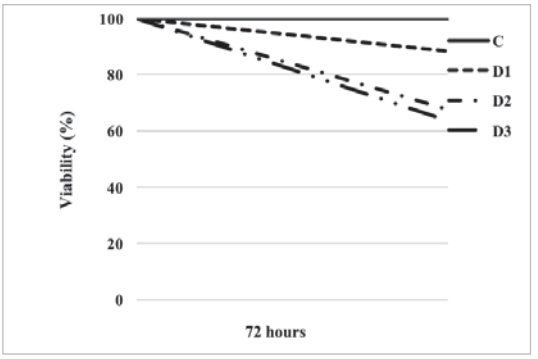


Figure 1d. Viability (%) values of HeLa cells treated with 1 µM, 5 µM and 10 µM concentrations of compound D for 72 h (450- 690 nm) ($p<0.05$).

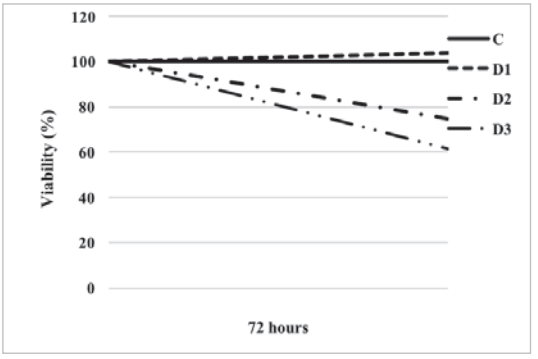
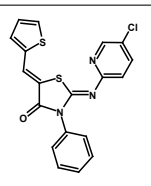
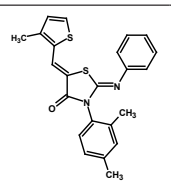
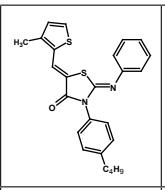
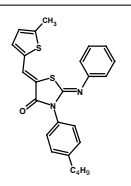
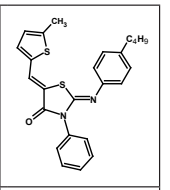


Figure 1e. Viability (%) values of HeLa cells treated with 1 µM, 5 µM and 10 µM concentrations of compound E for 72 h (450- 690 nm) ($p<0.05$).

Mitotic index

After application of five different imino-thiazolidinone derivatives (Compound A-E) to HeLa cell line for 24, 48 and 72 h, antimitotic effects of all treatments were evaluated with mitotic index analysis. As a result of this analysis, cells which were in mitosis or not were counted. Mitotic index (MI) values of the cells in a time-dependent manner were decreased significantly in all experimental groups (Table 2). In addition, statistically significant difference was noted among the all experimental groups ($p<0.05$). In a time-dependent manner, MI (%) values of HeLa cells treated with compound E (2-[(4-butylphenyl)imino]-5-[(5-methylthiophen-2-yl)methylidene]-3-phenyl-1,3-thiazolidin-4-one) at a concentration of 10 µM were shown at Fig. 2. The differences between the control and the each of experimental group were also found significant ($p<0.05$). As seen in Fig. 2, MI of compound E compared with the control group decreased from 7.67% to 3.78% at 24 h; 7.27% to 3.16% at 48 h and 6.98% to 2.27% at 72 h for a concentration of 10µM. It is obvious that the proliferation of HeLa cell lines was effectively inhibited by compound E.

Table 2. Mitotic index (%) values of HeLa cells treated with 10 µM concentrations of the tested compounds for 0-72 h (±SD, $p<0.05$).

Experimental Groups						
		Compound A	**Compound B**	**Compound C**	**Compound D**	**Compound E**
Mitotic Index (%)						
	Control	**D_3**	**D_3**	**D_3**	**D_3**	**D_3**
24 Hour	**7.67±0.18**	**6.73±0.33**	**6.07±0.34**	**7.08±0.43**	**3.89±0.17**	**3.78±0.36**
48 Hour	**7.27±0.28**	**5.98±0.17**	**5.82±0.32**	**6.59±0.21**	**3.08±0.18**	**3.16±0.17**
72 Hour	**6.98±0.37**	**5.06±0.23**	**4.75±0.25**	**5.97±0.13**	**2.89±0.14**	**2.27±0.41**

Labeling index

Labeling index (LI) values of HeLa cell line for 24, 48 and 72 h time period were shown in Table 3. It was observed that the iminothiazolidinone derivatives exhibited toxicity against HeLa cell lines compared to control group and especially compound E (2-[(4-butylphenyl)imino]-5-[(5-methylthiophen-2-yl)methylidene]-3-phenyl-1,3-thiazolidin-4-one) among the all treatments was found to have the most effective IC_{50} at 10 µM. LI values of the cells were increased significantly while decreasing from 24 h to 72 h for the control group. As seen in the Fig. 3, LI decreased from 6.24% to 3.03% at 24 h; 7.68% to 1.54% at 48 h and 8.11% to 0.82% at 72 h for 10µM concentration of compound E. The differences between the control and the experimental groups were found to be significant ($p<0.05$).

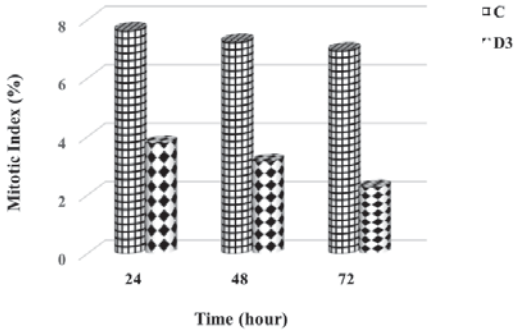


Figure 2. Mitotic index (%) values of HeLa cells treated with 10 µM of the compound E (2-[(4-butylphenyl)imino]-5-[(5-methylthiophen-2-yl)methylidene]-3-phenyl-1,3-thiazolidin-4-one) for 0-72 h ($p<0.05$).

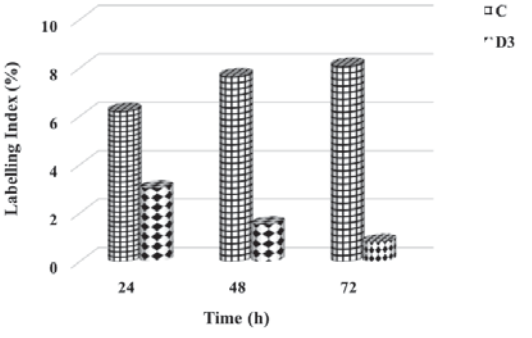
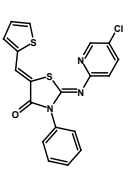
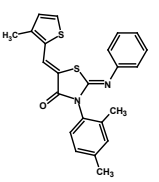
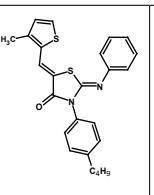
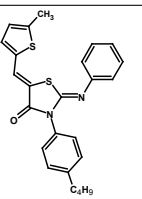
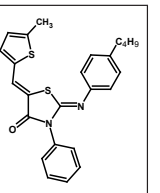


Figure 3. Labelling Index (%) values of HeLa cells treated with 10 µM of compound E (2-[(4-butylphenyl)imino]-5-[(5-methylthiophen-2-yl)methylidene]-3-phenyl-1,3-thiazolidin-4-one) for 0-72 h ($p<0.05$).

Table 3. Labeling index (%) values of HeLa cells treated with 10 µM concentrations of iminothiazolidinones for 0-72 h (±SD, $p<0.05$).

Experimental Groups						
		Compound A	**Compound B**	**Compound C**	**Compound D**	**Compound E**
Labelling Index (%)						
	Control	**D_3**	**D_3**	**D_3**	**D_3**	**D_3**
24 Hour	**6.24±0.52**	**6.02±0.22**	**5.21±0.43**	**6.19±0.34**	**2.97±0.24**	**3.03±0.39**
48 Hour	**7.68±0.32**	**5.24±0.31**	**4.72±0.14**	**6.08±0.32**	**1.66±0.33**	**1.54±0.12**
72 Hour	**8.11±0.41**	**4.86±0.15**	**3.33±0.23**	**5.75±0.53**	**0.94±0.11**	**0.82.±0.30**

Discussion

Thiazolidinones are thiazolidine analogues due to having a sulfur atom at position 1, a nitrogen atom at position 3 and a carbonyl group at position 2, 4, or 5. Its analogues are applicable for the mostly studied moiety and its existence in penicillin was the first diagnosis of its availability in nature. Recently, 4-thiazolidones and the related heterocyclic compounds have been demonstrated to be a perspective source of innovative anticancer agents (Havrylyuk et al. 2009, 2010; Panchuk et al. 2012). These compounds are known for their broad biological activity, including antimicrobial, fungicidal, antiviral and anti-inflammatory activities (Lesyk et al. 2003; Lesyk and Zimenkovsky 2004). Novel pharmacological effects of 4-thiazolidones have been also found, such as antidiabetic and anticancer.

The aim of this study was to evaluate the effects of five newly synthesized 4-thiazolidinone derivatives on HeLa cell line (CCL2) originated from human cervical carcinoma by comparing the parameters of cell kinetic, labeling index and mitotic index. At the first stage of this work, the MTT-1 assay was applied to identify the cytotoxicity of iminothiazolidinones on the growth rates of HeLa cells after 24, 48 and 72 h of exposure. For this, the IC_{50} index was employed, calculated as a lethal concentration of compounds, which kills 50% cells in comparison with a control culture. All drugs, extracts, inhibitor applications carry out based on IC_{50} in experiments. This is optimum dose. It was found that 10 µM concentrations was the optimum concentration and compound E (2-[(4-butylphenyl) imino]-5-[(5-methylthiophen-2-yl) methylidene]-3-phenyl-1,3-thiazolidin-4 one) was the most effective compound among the tested compounds. The most effective compound and effective inhibiting concentration were evaluated. In Fig. 1a-e viability % values of HeLa cells treated with D1, D2 and D3 concentrations of five different compounds were shown. It was found that all compounds under study inhibited proliferation of CCL2 in a dose-dependent mode in 72 h after their addition to the culture medium.

Chandrappa et al. (2009) reported the synthesis of 2-(5-((5-(4-chlorophenyl)furan-2-yl) methylene)-4-oxo-2-thioxothiazolidin-3-yl) acetic acid derivatives and evaluation of their cytotoxic activity. The compounds with electron donating groups at C-terminal of the phenyl ring concluded an increase in activity by urging cell death while compounds with electron with drawing groups (CN, F, CF3) showed decreased activity (Chandrappa et al. 2009).

According to the results of our study, the decrease in mitotic index (MI) and proliferation rate of cells were achieved at the dose level of 10 µM, especially at 72 h. It was observed that percentage of synthesis phase of HeLa cells treated with 10 µM concentration of tested compounds significantly reduced, and almost none of the cells experienced the synthesis phase (Table 2). Also, MI was 2.74% for the

experimental group and 6.98% for the control group after administration of compound E at D3 for 72 h. MI was significantly different between the control and experimental group ($p<0.001$). These data suggested that compound E had an antitumoral effect on HeLa cell line and arrested cell division G2/M phase in vitro.

In the current study, the application of 10μM of compound E for 72 h decreased the LI to 0.82% vs. 8.11% in the control group ($p<0.001$). This decrease was regarded as a significant result for the inhibition of tumor growth. The results revealed that treatments of compound E lower the percentage of the cells at S phase.

In the present study, changes in the cell cycle of HeLa cells caused by five newly synthesized iminothizolidones were investigated and, to our knowledge, this is the first investigation on this topic. The five newly synthesized iminothiazolidinone compounds had cytotoxic effect according to MTT test. The results showed that decrease in viability, mitotic index (MI) and labeling index (LI) of cells and the decline in proliferation rate were achieved at the dose level of 10 μM, especially at 72 h. According to the results, all of the tested compounds showed good antitumoral activity on HeLa cell line comparing to controls. We found that the compound E had the highest activity in reducing the reproduction rate of the cells. As a result, compound E effected cell kinetic parameters on HeLa cell line significantly ($p<0.001$). The possible anticancer effect of the compound E found in the current study may give us inspiration for the further research in the experimental animal models.

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