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Exposure to Toxic Chemicals and Health Outcomes

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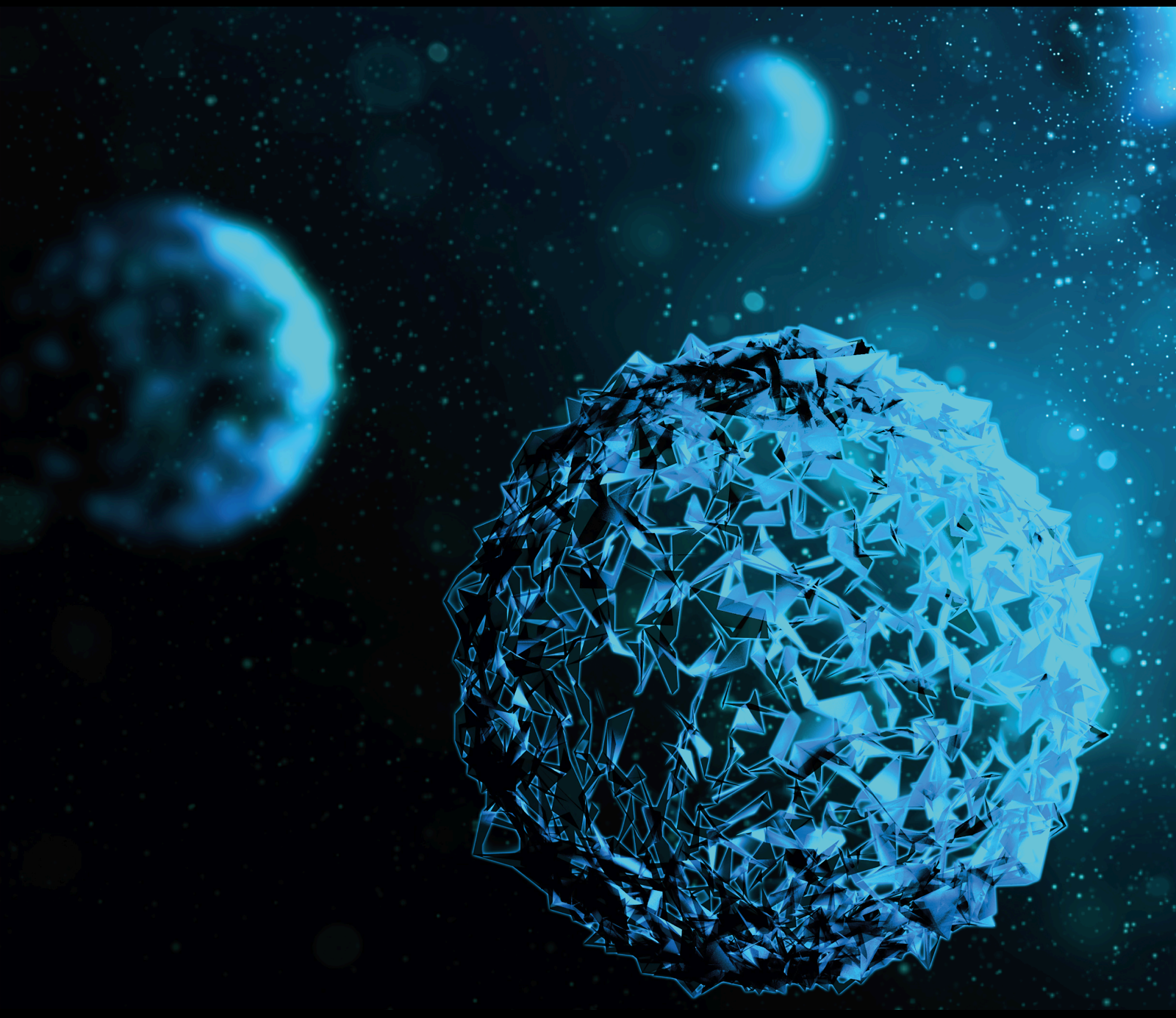

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Research Article

The Prevalence of Asthma and Asthma-Like Symptoms among Seasonal Agricultural Workers

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Guest Editor: Di Jiang

Aim. The aim of the study was to determine the prevalence and risk factors of asthma and asthma-like symptoms in seasonal agricultural workers living in fields with toxic chemical exposure. *Methods*. European Community Respiratory Health Survey (ECRHS) questionnaire was used to assess the prevalence of asthma and asthma-like symptoms in the study. *Results*. Of the study group, 51.1% (267) were male and the age of the study group ranged from 18 to 88 years and the mean (SD) was 45.68 (13.39) years. The prevalence of asthma attacks in seasonal agricultural workers in the last one year (current prevalence) was 11.2%; the prevalence of asthma (cumulative prevalence) was 15.1%. In the study, smoking was found to be an important risk factor for current asthma. The prevalence of cumulative asthma was higher in seasonal agricultural workers with allergic rhinitis ($p < 0.05$ for each). *Conclusions*. Seasonal agricultural workers are exposed to the worst conditions of working groups. These difficult conditions also cause many health problems. Asthma has also been identified as an important health problem among seasonal agricultural workers.

1. Introduction

Seasonal agricultural workers are agricultural workers migrating to places where agricultural demand is high, migrating to their own countries at the end of the season. This group is a vulnerable group because of the inadequacy of living conditions and the inability to reach basic human rights services [1]. The main causes of health problems of agricultural workers can be summarized as education level, low socioeconomic level, bad climatic conditions, safety of working tools, working hours, direct contact with animals and plants, bite, poisoning, parasites, allergies, and chemical products used [2]. Toxic chemicals such as pesticides may contribute to airway reactivity and asthma among agricultural workers. Chemicals may cause exacerbation of asthma. Pesticides may also modulate inflammatory responses to other agricultural exposures, such as endotoxin and allergens [3, 4]. Many temporary or permanent health problems can occur in this group [5].

The main health problems of agricultural workers can be grouped as respiratory diseases, musculoskeletal diseases, infectious diseases, accidents and injuries, dermatological diseases, and psychosocial problems [6]. Seasonal agricultural workers are recognized as a population requiring special attention by the health community. This designation as a "special population" is associated with higher than average occupational risk exposures as well as poorer than average health status [1].

Agricultural workers have been shown to have increased especially rates of respiratory symptoms and diseases due to respiratory irritants, toxic chemicals, and allergens [7]. Respiratory disease is a major health risk for those working in agriculture. The most common cause of asthma among agricultural workers is a result of exposure to agricultural

dust and chemicals. When both are inhaled, they can trigger an allergic reaction in the respiratory system [8]. These problems usually become chronic because of the lack of access to healthcare and repeated exposures [9].

Asthma is a chronic inflammatory disease of the respiratory tract characterized by unstable obstruction of the airflow and severe response of the airways [10]. Asthma is a multifactorial disease in which family, as well as infectious, allergic, socioeconomic, psychological, and environmental factors, play a role [11]. The characteristics of asthma include recurring symptoms, reversible airflow obstruction, and bronchospasm. The symptoms of this disease include wheezing, coughing, chest tightness, and shortness of breath [12].

The aim of the study was to determine the prevalence and risk factors of asthma and asthma-like symptoms in seasonal agricultural workers living in fields with toxic chemical exposure.

2. Material and Method

The study is a cross-sectional study that was conducted on seasonal agricultural workers working in the rural area of Eskişehir (Turkey) in 2017. The questionnaire consists of a sociodemographic questionnaire and European Community Respiratory Health Survey (ECRHS) questionnaire.

ECRHS questionnaire was used to assess the prevalence of asthma and asthma-like symptoms in the study [13, 14]. Based on the ECRHS questionnaire, current asthma is defined as recently taking antiasthma medication or having an asthma attack in the last 12 months, and cumulative asthma is defined as having an attack of asthma at any time in life.

The ECRHS questionnaire includes asthma symptoms: wheezing in the past 12 months, wheezing with shortness of breath in the last 12 months, wheezing in the absence of cold during the past 12 months, feeling tightness in the chest while waking up in the past 12 months, nocturnal cough and dyspnea attacks in the last 12 months, a history of asthma attack in the last 12 months, recent antiasthma medication, and nasal allergies [15].

2.1. Study Sample. In the study, the prevalence of asthma was assumed to be 5% [16–18], the margin of error was taken as 2%, the confidence interval was taken as 95%, and the sample size was calculated at least as 456. A total of 560 seasonal agricultural workers were approached, but only 523 of them agreed to participate. The response rate was 93.3%.

2.2. Ethical Procedure. The approval of Socıal and Human Scıences Ethıcs Committee of Ankara Yıldırım Beyazıt Unıversıty was confirmed.

2.3. Data Analysis Plan. Statistical Package for Social Sciences (SPSS 24.0) was used to evaluate the obtained data on the computer. Chi-squared test was used for univariate analysis, and multivariate logistic regression analysis was used with entering method in multivariate analysis. Results were evaluated at a 95% confidence interval, and $p \leq 0.05$ was accepted as significant for all variables.

3. Results

Of the study group, 51.1% (267) were male and the age of the study group ranged from 18 to 88 years and the mean (SD) was 45.68 (13.39) years. The prevalence of asthma attacks in seasonal agricultural workers in the last one year (current prevalence) was 11.2%; the prevalence of asthma (cumulative prevalence) was 15.1%. In the study, the prevalence of allergic rhinitis was 23.5% and the prevalence of allergic rhinitis was higher in females than in males ($p = 0.034$) in seasonal agricultural workers.

The prevalence of wheezing was higher in males than in females ($p = 0.042$). No correlation was found between gender and wheezing with breathlessness, wheezing without cold, woken up with chest tightness, woken up with shortness of breath, woken up with cough, current asthma, and cumulative asthma among seasonal agricultural workers.

The distribution of asthma and asthma-like symptoms according to gender in univariate models is given in Table 1.

In the study, the presence of family histories of atopy was found to be an important risk factor for wheezing with breathlessness and woken up with chest tightness among seasonal agricultural workers ($p < 0.05$).

In patients with allergic rhinitis, the prevalence of woken up with shortness of breath was 1.527 times higher than those without allergic rhinitis ($p < 0.05$). Smoking was found to be an important risk factor for current asthma ($p < 0.05$). The prevalence of cumulative asthma was higher in seasonal agricultural workers with allergic rhinitis ($p < 0.05$).

Gender, smoking status, pruritus dermatitis and/or eczema, allergic rhinitis diagnosis, and family history of atopy were evaluated as risk factors, and the distribution of asthma and asthma-like symptoms according to risk factors in multivariate models is given in Table 2.

4. Discussion

Allergic diseases, including asthma, rhinitis, and eczema, have emerged as a global public health challenge due to their elevated prevalence [19].

Asthma is a chronic inflammatory disease of the airways that affects approximately 300 million people in the world and 3.5 million people in our country [20]. Agricultural workers are at increased risk for respiratory diseases, including asthma, as a result of exposure to chemicals, grains, animals, and dusts and other agricultural exposures [21], and this population is exposed to bad conditions within the working groups [22, 23]. Pesticides may contribute to asthma among agricultural workers [24].

Differences in living conditions, air and other types of environmental pollution, smoking, and genetic factors may contribute to discrepancy [19]. In a case report made by Çımrın and Karaman, it was found that seasonal agricultural worker developed asthma due to dust exposure [25].

The prevalence of asthma attacks in seasonal agricultural workers in the last one year (current prevalence) was 11.2%; the prevalence of asthma (cumulative prevalence) was 15.1% in our study. The prevalence of asthma varies according to geographical regions.

Table 1: The distribution of asthma and asthma-like symptoms according to gender in univariate models.

Asthma and asthma-like symptoms	Gender		Total	OR (CI 95%)*	p
	Male, n (%)	Female, n (%)			
Wheezing	23 (8.6)	20 (7.8)	43 (8.2)	1.269 (1.090-1.789)	0.042**
Wheezing with breathlessness	5 (21.7)	4 (20.0)	9 (20.9)	1.197 (0.734-1.954)	0.471
Wheezing without cold	8 (34.7)	7 (35.0)	15 (34.8)	0.756 (0.463-1.234)	0.263
Woken up with chest tightness	28 (10.4)	23 (8.9)	51 (9.7)	0.854 (0.606-1.203)	0.366
Woken up with shortness of breath	27 (10.1)	21 (8.2)	48 (9.1)	1.151 (0.816-1.624)	0.422
Woken up with cough	36 (13.4)	32 (12.5)	68 (13.0)	0.800 (0.567-1.128)	0.800
Allergic rhinitis	60 (22.5)	63 (24.6)	123 (23.5)	1.312 (1.083-1.812)	0.034**
Current asthma	33 (12.3)	26 (10.1)	59 (11.2)	1.146 (0.776-1.691)	0.493
Cumulative asthma	42 (15.7)	37 (14.4)	79 (15.1)	1.108 (0.786-1.562)	0.558

*OR: odds ratio; CI: confidence interval. **$p < 0.05$.

Table 2: The distribution of asthma and asthma-like symptoms according to risk factors in multivariate models.

Asthma and asthma-like symptoms	Gender OR (CI 95%)*	Smoking OR (CI 95%)*	Itching dermatitis and/or eczema OR (CI 95%)*	Allergic rhinitis OR (CI 95%)*	Family histories of atopy OR (CI 95%)*
Wheezing	1.238 (0.875-1.753)	0.884 (0.625-1.250)	1.222 (0.865-1.726)	0.853 (0.603-1.205)	0.922 (0.652-1.352)
Wheezing with breathlessness	1.253 (0.758-2.071)	0.995 (0.603-1.641)	1.252 (0.762-2.057)	0.809 (0.492-1.331)	1.861 (1.129-3.069)**
Wheezing without cold	0.785 (0.476-1.293)	0.764 (0.464-1.259)	0.878 (0.535-1.440)	1.517 (0.925-2.489)	0.875 (0.532-1.437)
Woken up with chest tightness	0.819 (0.578-1.162)	0.761 (0.537-1.078)	1.181 (0.835-1.672)	1.115 (0.788-1.578)	1.442 (1.018-2.041)**
Woken up with shortness of breath	1.125 (0.793-1.596)	1.133 (0.800-1.606)	1.067 (0.754-1.511)	1.527 (1.079-2.161)**	0.839 (0.593-1.188)
Woken up with cough	0.791 (0.559-1.120)	1.028 (0.728-1.452)	1.129 (0.799-1.595)	1.056 (0.747-1.491)	1.060 (0.751-1.497)
Current asthma	1.126 (0.758-1.672)	1.446 (1.071-2.154)**	0.921 (0.622-1.365)	0.796 (0.537-1.180)	1.152 (0.777-1.707)
Cumulative asthma	1.143 (0.807-1.620)	1.049 (0.742-1.484)	1.015 (0.718-1.435)	1.495 (1.057-2.114)**	1.030 (0.729-1.457)

*OR: odds ratio; CI: confidence interval. **$p < 0.05$.

In another study conducted in Eskisehir, the prevalence of current asthma, cumulative asthma, and allergic rhinitis was 5.9%, 5.9%, and 37.6%, respectively [26]. In our study in Eskisehir, the prevalence of current asthma, cumulative asthma, and allergic rhinitis was 11.2%, 15.1%, and 23.5%, respectively. We think that the rate of asthma in seasonal agricultural workers is higher than that in the general population, which may be caused by toxic chemicals and environmental exposure.

The prevalence of asthma in different countries has been reported in the range of 2.4-18.4% [27, 28]. In a cross-sectional prevalence study conducted in Iran in 2015-2016 with ECRHS survey, the prevalence of asthma in adults was 8.9% [29]. Similar to our study, in a Swedish study conducted in 2008, the prevalence of asthma was found to be 11.8% [30].

In our study, prevalence of wheezing was higher in males than in females ($p = 0.042$). In a study conducted with the ECRHS survey in Saudi Arabia in 2016, the prevalence of wheezing in the last 12 months was 18.2% and the difference between men and women was not significant ($p = 0.107$) [31].

In the study, the presence of family histories of atopy was found to be an important risk factor for wheezing with breathlessness and woken up with chest tightness among seasonal agricultural workers ($p < 0.05$). In a study by Wang et al., the prevalence of asthma was found to be 3.15 times higher in participants with a history of allergic diseases in the mother than in nonallergic mothers [32]. In another study, if one of the parents has asthma, the risk of asthma in the child increases to 20-30%, and if both parents have asthma, this risk increases to 60-70% [29].

The prevalence of allergic rhinitis was determined as 23.5%, and allergic rhinitis was significantly higher in seasonal agricultural workers in our study ($p < 0.05$). In a study by Yorgancıoğlu et al., the prevalence of allergic rhinitis in our country varies between 8.9% and 27.7% [33]. Increasing prevalence of allergic rhinitis, changing living conditions, environmental and air pollution, childhood infections, longer indoor life, smoking, changes in dietary habits, and some genetic factors are responsible [34]. Allergic rhinitis was found to be 28.3% in a study conducted in Tehran between 2013 and 2016 similarly [35].

In our study, the prevalence of allergic rhinitis was higher in females than in males ($p = 0.034$) in seasonal agricultural workers. Similarly in a study by Nihlén et al., it was reported that allergic rhinitis was more common in women, and the authors stated that the reason for this was that women may be more inclined to write allergy symptoms in the questionnaire [36].

Smoking was found to be an important risk factor for current asthma in our study ($p < 0.05$). In a study by Idani et al., the prevalence of current asthma, asthma symptoms, wheezing, nocturnal cough, and asthma medication was significantly higher in smokers than nonsmokers [37]. In a study consistent with our study, smoking and/or smoke exposure have been shown to lead to exacerbation of lung function in asthmatics, asthma symptoms, and weight gain [38].

Strengths of the study are that the number of cases is higher than the number of samples and the number of women and men is approximate (51.1/48.9).

The limitations of the study are that functional lung monitoring (spirometry, test, PEF monitoring, etc.) procedures that will support the diagnosis of asthma cannot be applied, the study is carried out only in the Eskisehir region, and workers under the age of 18 are not included.

5. Conclusions

Seasonal agricultural workers are one of the occupational groups that are exposed to bad conditions within the working groups. In this group, health problems are frequently encountered throughout the world. Allergic respiratory diseases have also been identified as an important health problem among seasonal agricultural workers. It should not be forgotten that seasonal agricultural workers who apply to the outpatient clinic may have asthma-like symptoms and allergic conditions. Similarly, it should be questioned what the occupation of these patients is in these symptoms, and it should be known that seasonal agricultural workers may have these symptoms. It is estimated that allergic respiratory diseases are highly observed in seasonal agricultural workers due to environmental factors. So respiratory diseases which can be due to toxic chemicals and environmental exposures should be investigated in seasonal agricultural workers.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Adipogenesis Regulation and Endocrine Disruptors: Emerging Insights in Obesity

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Endocrine disruptors (EDs) are defined as environmental pollutants capable of interfering with the functioning of the hormonal system. They are environmentally distributed as synthetic fertilizers, electronic waste, and several food additives that are part of the food chain. They can be considered as obesogenic compounds since they have the capacity to influence cellular events related to adipose tissue, altering lipid metabolism and adipogenesis processes. This review will present the latest scientific evidence of different EDs such as persistent organic pollutants (POPs), heavy metals, "nonpersistent" phenolic compounds, triclosan, polybrominated diphenyl ethers (PBDEs), and smoke-derived compounds (benzo -alpha-pyrene) and their influence on the differentiation processes towards adipocytes in both *in vitro* and *in vivo* models.

1. Introduction

The prevalence and incidence of overweight and obesity worldwide have increased significantly in the last three decades. According to the World Health Organization (WHO), since 1975, obesity has nearly tripled, and every year, at least 2.8 million people die as a result of obesity or overweight. By 2016, 39% of adults were overweight, and 13% were obese; 41 million children under 5 years of age and 340 million children and adolescents between 5 and 19 years of age were overweight or obese [1]. These figures explain the reason why this disease has reached epidemic proportions, and its understanding and intervention are public health priorities.

Diverse studies indicate that the etiology of this chronic disease is multivariate and complex. The predisposing biological factors include genetic characteristics, prenatal determinants, pregnancy, menopause, intestinal microbiota, and viruses. People prone to develop obesity may also be affected by behavioral causes such as excessive energy intake, increased portion sizes, and the practice of a sedentary lifestyle. On the contrary, genetic predisposition to obesity can be influenced by epigenetic triggers such as high availability of food, socioeconomic status, or the presence of chemical contaminants in the environment that could be ingested [2–4].

Due to the deleterious effect of endocrine disruptors on health, it is necessary to characterize the damage by specific dietary exposures of these compounds. This fact implies determining the mechanisms involved in which the food acquires the contaminant. It is also necessary to understand the role as endocrine disruptor and the different physiological and pathological consequences, in particular the relationship with adipogenesis processes.

2. Adipogenesis

In living multicellular organisms, certain cell types have the capacity to originate new and specialized cellular lineages, and this characteristic is called cellular differentiation.

Cellular differentiation is mainly carried out in embryonic development; however, some tissues retain this property of cell differentiation even when the organism is in an adulthood state.

Cell differentiation is activated by a series of signals at the organism level, where these are then translated into specific processes such as differential expression of genes, activation, or inactivation of transcription factors and cellular signaling proteins. The differentiated cell undergoes a series of morphological changes and arrest of cell growth; however, the genetic material of the cell remains unchanged.

Adipogenesis or adipocyte differentiation has been the focus of many studies in recent years. In this process of differentiation, a mesenchymal stem cell (MSC) has the ability to produce mature adipocytes, which are the main constituents of adipose tissue [5, 6].

Adipose tissue was formerly described as a nondynamic tissue whose function was limited to constitute the energy reservoir of the organism, through the accumulation of triglycerides. From the 90s, this tissue begins to attract the attention of scientists, and it is now considered as a highly active and dynamic tissue with a variety of hormonal, immunological, and regulatory functions of energy homeostasis [7, 8]. This is one of reasons why the research of adipocyte differentiation process has acquired great importance, and also due to its relationship with different pathologies such as obesity, diabetes mellitus type II (DM type II) [9], insulin resistance [10], osteoporosis [11, 12], rheumatoid arthritis, and osteoarthritis [13]. On the contrary, MSCs are multipotential cells that can give rise to different cell lineages such as osteoblasts, chondrocytes, myocytes, and adipocytes [14].

The process of adipocyte differentiation occurs throughout the different stages of development of organisms and is controlled by both nutritional factors as well as by genetic and environmental factors. There are different models of cell lines that are widely used by researchers [15, 16] to study the formation of adipocytes and to understand their relationship with obesity. *In vitro* models highlight the use of mouse embryonic cell lines 3T3-L1 and 3T3-F442A that can be induced to differentiate into adipocytes under chemical and hormonal exposure.

In response to an extracellular signal, an MSC undergoes processes of proliferation and clonal expansion that originate preadipocytes and high plasticity cells, which can be ultimately differentiated into a cell with a characteristic-defined phenotype, the mature adipocyte. In the first stage of the process, MSC converges into a preadipocyte that does not differ morphologically or phenotypically from its precursor cell, but in which activation processes that involve transcription factors of the AP1 family occur. Subsequently, the terminal differentiation stage is initiated where the resulting adipocyte acquires the specialized equipment for the secretion and synthesis of proteins and lipids specific to the lineage to which it has differentiated [17].

Different signals that influence adipogenesis have been described, for example, fibroblast growth factor type 1 (FGF1) [18] and insulin-like growth factor type 1 (IGF1) [19] are known for their induction action. On the contrary, there is an inhibitory effect on adipogenesis when the WNT signaling [20] or the hedgehog pathway [21] are activated.

3. Transcription Factors That Regulate Adipogenesis

It is known that the differentiation of adipocytes is a complex process consisting of several stages, and it is widely regulated by both the specific expression of proteins and transcription factors. In the initial phase, adipogenesis is induced by the expression of binding proteins CCAAT/enhancer β (C/EBPβ) and C/EBPδ. The activity of these proteins give rise to a second stage since among their targets are the promoters of the genes that code for peroxisome proliferator-activated receptor gamma (PPARγ) and C/EBPα. PPARγ is considered the master transcription factor of the adipocyte differentiation process since when it is activated by binding to its ligand, morphological changes and the expression of all genes specific for mature adipocytes are induced [22].

PPARγ plays an essential role in the process of adipocyte differentiation of white and brown adipose tissue. Two PPAR isoforms have been described, which are produced by alternative splicing. The isoform 2 of PPAR, expressed mainly in adipose tissue and whose function is to promote the storage of triglycerides [23], has been related to obesity, insulin resistance [24], and dyslipidemia [25]. PPAR isoform 1 is ubiquitous in other cell types besides adipocytes. PPARγ activates the promoter of gene coding for C/EBPα, and reciprocally and inversely, C/EBPα activates the PPARγ promoter, generating a positive feedback loop. Both genes cooperate by binding to sites of promoter regions of various genes that are expressed during the differentiation process, as well as in the mature adipocyte [26]. Some examples of these genes are those that code for proteins involved in insulin sensitivity, lipolysis, and lipogenesis. Krox20 [27] and Kruppel-like factors (KLF) [28] have also been reported in the regulation of differentiation of adipocytes.

4. Endocrine Disruptors

Recently, many researchers have focused their interest on so-called EDs or obesogens, bringing attention to their possible etiological incidence of obesity. Both experimental and epidemiological evidence support the idea that low doses of chemical contaminants have endocrine and metabolic effects. Most of these contaminants are present in the food chain and accumulate in the fat mass after absorption [29, 30]. Secular evidence suggests that some of these EDs may be involved in the global epidemic of obesity, diabetes (diabetogens), as well as in hormone-dependent cancer [3, 4, 31].

The EDs, are a particular group of well-differentiated chemical compounds, defined as "substances exogenous to the organism that alter the function or functions of the endocrine system, being able to cause adverse effects on the health of an organism, its descendants, in the population in general or in a particular subpopulation" [32]. These substances were initially synthesized to fulfill specific functions, such as the control of pests in agriculture, improve the

stability of body lotions, or be part of the structure of certain plastics, but with the time, there have been discovered harmful effects derived from continued exposure to them [33].

Among the characteristics of the obesogens is that they are compounds with very different chemical structures capable of acting at shallow doses, show different mechanisms of action, and be able to alter the hormonal balance [34]. They interfere with the body's ability to regulate growth, its development, metabolism, and other functions. There are hundreds of disruptors in the environment, in food, and everyday products. These can contribute to a variety of diseases and disabilities such as obesity, cancer, diabetes, heart disease, reproductive, or neurodevelopmental problems [34].

The Center for Biomedical Research in Network-Physiopathology of Obesity and Nutrition (CIBERobn), which brings 24 Spanish research groups together, has shown that certain synthetic chemical compounds present in the environment and daily life, associated with pesticides and insecticides, but also perfumes, plastics, or cosmetics, predispose to obesity. These chemical compounds, given their effect on fat gain and obesity [34], are also found in synthetic fertilizers, electronic waste, and food additives that are present in the food chain and products of regular consumption such as food, beverages, personal care products, and household cleaning products [35–37].

The possible causality between this group of compounds and the overweight and obesity etiology has been investigated since the increase in the prevalence of obesity. Other metabolic diseases have been associated with the increase in exposure to EDs, as well.

5. Obesogenic Compounds and Adipogenesis

As previously mentioned, the obesogenic compounds are heterogeneous and come from various sources (Table 1). So far, there is the characterization of some of those and their effects on the processes of differentiation towards adipocyte [42–44] (Figure 1) (Table 2):

5.1. Persistent Organic Pollutants (POPs). POPs are chemical substances soluble in fats and, therefore, in the organism; they bioaccumulate in fat reserve tissues and biomagnify in the food chain [33, 36]. The most important route of human exposure to POPs is the consumption of food, especially those of animal origin. Some studies have identified the presence of POPs in oils and fats, meat, eggs, milk, and fish from freshwater ecosystems [38, 80–82].

This phenomenon is explained by its high resistance to chemical degradation and, therefore, its great persistence in the environment and living beings. There is growing epidemiological evidence that frequent exposure to low doses of sure POPs may be related to obesity and metabolic pathologies in the predisposed genetic population. Exposure during early pregnancy to pesticides can lead to the development of obesity in childhood and have been associated with diseases such as diabetes, hypertension, dyslipidemia, and BMI [33, 83].

POPs include some pesticides such as dichloro diphenyl trichloroethane (DDT) or hexachlorobenzene (HCB) and some industrial chemicals such as polychlorinated biphenols (PCBs). While the use of these chemicals is prohibited in many countries, their presence persists in the environment due to their high stability, and they are still used in some developing countries [37]. DDT was developed in the 1940s as an insecticide and was also used to combat diseases transmitted by insects, such as malaria. Because DDT has been found to be genotoxic and possibly carcinogenic, its use was banned in the USA in 1972 and the Netherlands in 1973. However, in developing countries, DDT is still used for vector control like malaria [84].

DDT may affect the physiology of adipose tissue. The exposure of DDT in cell cultures has a proadipogenic effect, increasing the expression of PPARγ and the binding of the C/EBPδ protein to DNA during adipogenesis [47]. Studies in the NIH3T3-L1 cell line exposed to the pesticide dichlorodiphenyldichloroethylene (DDE) showed no effect on adipogenesis; however, in the presence of DDT, the mature adipocytes expressed more leptin, resistin, and adiponectin. Similarly, Howell and Howell [45] showed that, in human mesenchymal stem cells (hMSC), DDT could significantly increase the process of differentiation into adipocyte and the molecular markers typical of the fat cell, such as PPARγ, leptin, FABP4, and GLUT4 [46]. Additional experimental evidence suggests that the proadipogenic activity of DDT would be through the phosphorylation of the AMPKα protein [48].

5.2. Heavy Metals. In general, many metals have solubility in organic solvents. Human exposure to certain metals such as arsenic, cadmium, and lead has been associated with metabolic alterations such as an increased risk of suffering from DM type II, cardiovascular disease, and obesity. The accumulation of mercury in large fish growing in contaminated water sources, cadmium in cereals and viscera, lead in tubers, or cadmium and arsenic in vegetables have been widely documented [32, 39].

Regarding the influence of heavy metals in the process of adipogenesis, Beier et al. [85] demonstrated through experiments with rats exposed to low concentrations of lead before conception and for 18 months that this heavy metal could stimulate differentiation in mesenchymal cells to mature adipocytes with a concomitant detriment of osteoblastogenesis. This process was further characterized by an inhibition of the cellular signaling pathway of Wnt/β-catenin. More recently, the proadipogenic effect of lead was demonstrated in 3T3-L1 cultures, which involved the activation of the ERK, C/EBPβ, and PPARγ pathways [86].

Studies regarding adipogenesis in zebrafish with exposure to cadmium showed a positive association of this metal with the accumulation of adiposity [87].

Regarding arsenic and its role in adipogenesis, there is scientific evidence of its inhibitory influence on adipocyte differentiation. Hou et al. [49] exposed 3T3 L1 cells to this metal and discovered that it is capable of inhibiting

Table 1: Source and route of exposure in human of endocrine disruptors.

Endocrine disruptor	Source and main route of exposure in human	Reference
Persistent organic pollutants: DDT	Diet (meat, poultry, milk, and fish) and environmental exposition Used as insecticide for vector control like malaria	Srivastava [38], Wong and Durrani [36]
Heavy metals	Work activity and diet with associated industrial activity (water, food, and the environment)	Ferrer [39]
Lead	Large fish growing in contaminated water sources	Arrebola and Gonzales [32]
Cadmium	Cereals and viscera, lead in tubers	Arrebola and Gonzales [32]
Arsenic	Vegetables	Arrebola and Gonzales [32]
"Nonpersistent" phenolic compounds BPA, TBBPA	Linings of canisters, specific plastic containers, thermal printing papers, dental composite fillings, medical devices, polycarbonate, plastic resins and materials used in food containers Inhalation or ingestion of dust; by food intake like fish, milk, eggs, meat, meat products, and breast milk	Fénichel and Chevalier [29]
Phthalates	Used as plasticizers Dispersants, lubricants, emulsifying agents, perfumes and nail polishes Comes from foods that have absorbed the compound from their packaging or the manufacturing process Particulate matter in the air, water, or skin contact with plastics that contain it; plastic food containers may also contain DEHP Water from sources of discharge that have had contact with polymers	Mezcua et al. [33] Azeredo et al. [40]
Triclosan	Used as antibacterial agent Antibacterial soaps, toothpaste, toothbrushes, dental rinses, laundry detergents, kitchen cutting boards and plastics in furniture, toys, and sporting goods	Wong and Durrani [36]
PBDEs	Used as effective flame retardants in plastics, electronics, automobiles, homes, furniture, textiles, and construction materials Butter, fish, and other foods, as well as other foods that contain animal fats	Mezcua et al. [33]
Benzo-alpha-pyrene	Foods cooked on the grill or the barbecue; smoked, roasted or fried at high temperatures foods; oils subjected to repeated heating	Arrebola and Gonzales [32] Franco-Tobón and Ramírez Botero [41]

Table 2: Effect of endocrine disruptor on adipogenesis.

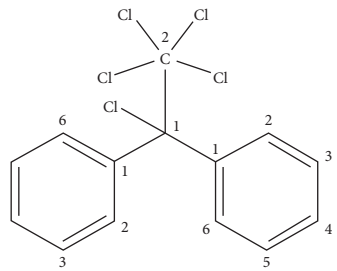
Endocrine disruptor (chemical structure)	Experimental model	Biological effect	Reference
Persistent organic pollutants			
 dichlorodiphenyltrichloroethane	NIH3T3-L1 cells	Proadipogenic effect	Howell and Mangum [45]
	Human mesenchymal cells	Proadipogenic effect, increased expression of PPARγ, leptin, FABP4, and GLUT4	Strong et al. [46]
	3T3 L1 cell culture	Proadipogenic effect, increased expression of PPARγ, and the binding of C/EBPδ protein to DNA, increased phosphorylation of the AMPKα protein	Moreno-Aliaga and Matsumura [47], Kim et al. [48]
Heavy metals			
Lead	Mesenchymal cells from pregnant rat	Proadipogenic effect, decreased osteoblastogenesis Activation of the ERK pathway and expression of C/EBPβ and PPARγ	Hou et al. [49]
Cadmium	Zebrafish	Accumulation of adiposity.	Beezhold et al. [50]

Table 2: Continued.

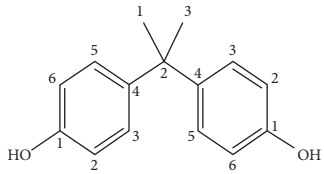
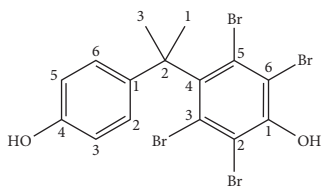
Endocrine disruptor (chemical structure)	Experimental model	Biological effect	Reference
Arsenic	3T3 L1 cell culture	Antiadipogenic effect mediated by activation of CHOP10	Angle et al. [51]
	White adipose tissue, human mesenchymal cells	Inhibition of miR-29b activation-mediated adipogenesis	Miyawaki et al. [52]
"Nonpersistent" phenolic compounds			
 2,2-Bis (4-hydroxyphenyl) propane	3T3 L1 cell culture	Proadipogenic effect Increased expression of PPARγ, leptin, FAS y C/EBPβ To increase size of the mature adipocyte Increase in insulin resistance, increase in proinflammatory interleukins	Masuno et al. [53], Hurst and Waxman [54], Riu et al. [55], Chamorro-García et al. [56], Ariemma et al. [57], Phrakonkham et al. [58]
	Prenatal exposure in a murine model	Increase in food intake, increase in body weight, and adipose tissue Increased expression of PPAR-γ in liver similar to animals with high-fat diets	Angle et al. [51], Miyawaki et al. [52], Wei et al. [59], García-arevalo et al. [60]
	Bone marrow mesenchymal stromal cells.	Decrease of adiponectin Proadipogenic and antiosteogenic effect	Watt and Schlezinger [61]
	Direct exposure of BPA in placenta and milk in rats	Increase in proadipogenic transcripts such as C/EBP-α, PPARγ, and SREBP-1C Increase adipogenesis in a sex-specific way	Somm et al. [62]
	Sheep, gestational exposure to BPA	Increase in adipogenesis in a sex-specific way Proadipogenic effect is independent of PPARγ activation	Pu et al. [63]
	3T3 L1 cell culture	Proadipogenic effect stimulating glucocorticoid receptors (GR) Proadipogenic effect via estrogens receptor (ER) BPA-induced adipogenesis is inhibited by estrogen receptor (ER)	Sargis et al. [64], Boucher et al. [65]
	Human mesenchymal cells	Proadipogenic effect via SREBF1, TR/RXR, and mTOR	Boucher et al. [66]
	Human mesenchymal cells from omental tissue of children exposed to BPA	Increased expression of 11β-hydroxysteroid dehydrogenase type 1, PPARγ, and lipoprotein lipase	Wang et al. [67]
Tetrabromobisphenol A 	NIH3T3-L1	Proadipogenic effect via PPARγ	Riu et al. [55]
	3T3 L1 cell culture	Proadipogenic effect in a dose-dependent manner via PPARγ	Akiyama et al. [68]
	Human mesenchymal cells	Proadipogenic effect mediated by microRNA-103 induction	Woeller et al. [69]

Table 2: Continued.

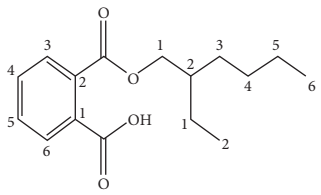
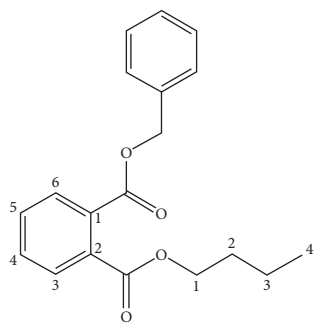
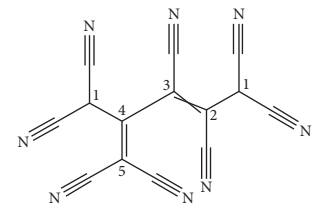
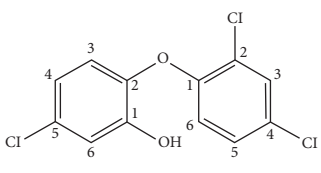
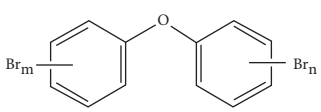
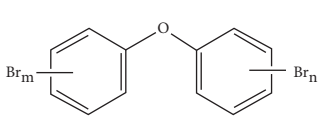
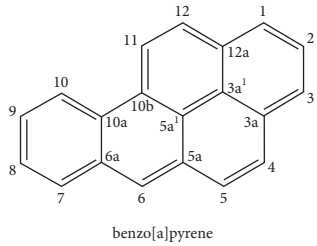
Endocrine disruptor (chemical structure)	Experimental model	Biological effect	Reference
Phthalates			
2-(((2-Ethylhexyl)oxy)carbonyl)benzoic acid 	3T3 L1 cell culture	Proadipogenic effect via PPARγ	Feige et al. [70], Bility et al. [71], Hao et al. [72]
	COS-1 cell culture	Activation of PPARγ, PPARα	Hurst and Waxman [54]
	Fetal exposure in mice	Increase in weight and fat mass in the offspring	Hao et al. [72]
	Human mesenchymal cells	Increase expression of transcripts related to the PPARγ signaling pathway Increase expression of genes involved with lipid metabolism	Ellero-Simatos et al. [73]
Benzyl butyl phthalate 	Bone marrow stromal cells	Decreased differentiation towards osteoblasts, and an increase in adipogenesis vía PPARγ	Chiu et al. [74]
	3T3 L1 cell culture	Proadipogenic effect via PPARγ Proadipogenic effect	Yin et al. [75]
	Mouse mesenchymal cell lines C3H10T1/2.	Epigenetic effect on histones of the PPARγ promoter	Sonkar et al. [76]
2-Dicyanomethylene-1,1,3,4,5,5-hexacyanopentene 	3T3 L1 cell culture	Proadipogenic effect via glucocorticoid receptor (GR)	Sargis et al. [64]
Triclosan  Triclosan	Human mesenchymal cells	Antiadipogenic effect Decrease of adiponectin and lipoprotein lipase	Guo et al. [77]
PBDEs  polybrominated diphenyl ether  PBDE	3T3 L1 cell culture	Proadipogenic effect via PPARγ	Tung et al. [78]

Table 2: Continued.

Endocrine disruptor (chemical structure)	Experimental model	Biological effect	Reference
Benzo-alpha-pyrene  benzo[a]pyrene	Human mesenchymal cells	Antiadipogenic effect via aryl-hydrocarbon receptor	Podechard et al. [79]

adipogenesis through the activation of CHOP10, an inhibitory molecule for the transcriptional activity of C/EBPβ, thus causing the suppression of adipogenesis. CHOP10 is a protein that increases its expression in response to the stress of the endoplasmic reticulum produced by the incorrect folding of proteins. Similarly, Beezhold et al. [50] showed that arsenic can increase the expression of microRNA, miR-29b, involved in the regulation of the cell cycle and in the increase in the expression of cyclin D1, which results in inhibition of the differentiation towards the fat cell.

5.3. Other "Nonpersistent" Phenolic Compounds. Denotes a wide variety of chemical compounds used in industrial applications, with the main characteristic that they suffer a relatively rapid degradation and/or excretion in the body. In spite of this, constant exposure provokes the continual presence of them in biological samples of the general population [32]. Within this group, it is possible to find the bisphenol A (BPA).

BPA is a "nonpersistent" phenolic compound widely used in the manufacture of polycarbonate plastics and epoxy resins. It is present in the linings of canisters, specific plastic containers, thermal printing papers, dental composite fillings, medical devices, polycarbonate, plastic resins, and materials used in food containers among others. It is highly elastic and resistant to heat material [29]. It has been shown that BPA can migrate from food containers and contaminate them, so these can be an important source of exposure to this compound.

The majority of the population is exposed to BPA daily, and there is currently an unprecedented controversy regarding its possible metabolic disruptor effect since experimental studies have shown that exposure to BPA induces an increase in weight in mice, as well as a high risk of DM type II [32]. Through experiments with rats, it has been demonstrated that relatively low doses of BPA—equivalent to daily and frequent exposure levels in large part of the population—act in a similar way to estradiol, the most potent form of estrogen that, among other aspects, influences the distribution of body fat in women. Exposure to these compounds at inadequate levels and certain stages of development—especially in the fetal stage and childhood—exerts a significant influence on both obesity and the development of diabetes [34]. Several epidemiological studies have found that high urinary concentrations of BPA in adults and children were associated with obesity and increased waist circumference [36].

BPA is a compound that has also been widely studied regarding its effect on lipid metabolism and cellular processes of adipocyte differentiation. It has been associated that a high intake of BPA together with a high-fat/high-sucrose diet leads to similar changes in the structure of the intestinal microbial community in mice [35]. Studies in humans have shown that prenatal exposure to BPA is associated with an increase in body fat at 7 years of age or an increase in body mass index at 9 years of age [36]. Regarding the effect of prenatal exposure to BPA, it has been shown that in experimental models with animals, there is an increase in food consumption, body weight, adipose tissue, and decrease in adiponectin concentrations [51, 52, 59]. These results support the hypothesis that exposure to BPA in critical states of adipose tissue development may alter the homeostasis of the adipocyte, thus increasing the risk of developing complications related to obesity [88].

Regarding the influence of BPA on adipogenesis, several studies have shown this correlation. Using cell models of the 3T3-L1 line exposed to BPA, it was observed that the process of adipogenesis is exacerbated [53–55, 89]. In this same line of evidence, Chamorro-García et al. [56] stimulated 3T3 L1 cells with different concentrations of BPA, and at 10 nM, adipogenesis was stimulated. At 100 nM, a significant increase in triglyceride accumulation was observed. Chronic exposure to BPA in cultures of 3T3-L1 preadipocytes for three weeks at concentrations of 1nM increased the proliferation of preadipocytes and produced hypertrophic adipocytes with impaired insulin signal, reducing glucose utilization and increasing the production of proinflammatory interleukins [57].

The target molecule of BPA's action that would play a significant role in this process is not fully elucidated yet;

however, some research groups would bet on the master regulator of adipogenesis: PPARγ. Phrakonkham et al. [58] demonstrated that concentrations of 80 μM BPA are capable of activating PPARγ and adipocyte-specific proteins such as leptin, FAS, and C/EBPβ. Exposure of BPA in prenatal conditions can increase the expression of PPAR-γ in the liver when compared with the control group or with animals fed high-fat diets [60]. Watt and Schlezinger [61] demonstrated a proadipogenic and antiosteogenic effect in mesenchymal stromal cells of bone marrow through the activation of this transcription factor.

Studies aimed to evaluate the adipogenesis *in vivo* demonstrated that direct exposure of BPA in rats through placenta and milk increases adipogenesis in a sex-specific manner since they observed an increase in proadipogenic transcripts such as PPARγ, C/EBP-α, and sterol regulatory element-binding factor 1 (SREBF-1) [62]. In studies with sheep, gestational exposure to BPA overregulates the expression of PPARγ in females; however, in male sheep, it reduced the expression of PPARγ, showing that the effect of BPA can be sex-specific [63].

Additional studies have attempted to show that BPA alters the processes of adipogenic differentiation in a manner independent of the regulation of PPARγ. Experiments by Chamorro-García et al. [56] show that BPA alone is capable of activating adipogenesis in 3T3 L1 cells; however, it fails to stimulate differentiation in stromal cells. When using PPARγ antagonists in the 3T3 L1 cultures, no effect was observed, concluding that the proadipogenic effect of BPA is independent of PPARγ. Sargis et al. [64] showed through luciferase assays in cultures of 3T3 L1 that BPA may possess intrinsic glucocorticoid-like activity, promoting adipogenesis through potentiation of the glucocorticoid receptor (GR) activity.

Studies on the mechanism of BPA in adipogenesis in preadipocytes of donors with healthy BMI showed induction of adipogenesis in the absence of exogenous glucocorticoids. Estradiol had no positive effect on differentiation, but BPA-induced adipogenesis was inhibited by estrogen receptor (ER) antagonists, but not by GR antagonists, suggesting that BPA acts through a nonclassical ER pathway [65].

Microarray assays performed on human subcutaneous preadipocytes cultures exposed to BPA demonstrated that the pathways involved in promoting adipogenesis would be via SREBF1, the TR/RXR receptor, and the mTOR pathway [66].

Wang et al. [67] analyzed the effect of BPA on omental adipose tissues of children during adipogenesis and observed an increase in the expression of 11β-hydroxysteroid dehydrogenase type 1, an enzyme that catalyzes the conversion of cortisone to cortisol, which is a glucocorticoid proadipogenic. It also observed an increase in PPAR-γ and lipoprotein lipase.

Regarding tetrabromobisphenol-A (TBBPA), the brominated analogue of BPA, Riu et al. [55] studied the effect of this compound on cell differentiation towards adipocytes in the NIH3T3-L1 cell line and determined that TBBPA presents specific binding with the protein PPARγ, activating it, which ultimately favored the accumulation of triglycerides in the cell. Similar results were also obtained in the studies of Akiyama et al. [68], who first demonstrated the presence of TBBPA in human milk, but also determined the proadipogenic activity of TBBAP and its brominated derivatives in 3T3-L1 cells.

To understand how TBBPA has a regulatory effect on the process of differentiation towards adipocyte, Woeller et al. [69] propose a mechanism in which TBBPA reduces Thy1 levels, which results in the stimulation of adipogenesis through the induction of microRNA-103.

5.4. Phthalates. With regard to phthalates, these compounds are poorly biodegradable and highly bioaccumulable in the food chain [33]. They are mainly used as plasticizers and, therefore, are present in a large number of everyday objects, such as plastic food containers and medical devices, including tubes for parenteral feeding [32]. They are also found in dispersants, lubricants, emulsifying agents, perfumes, and nail polishes. It has been established that the highest exposure to phthalates comes from foods that have absorbed the compound from their packaging or the manufacturing process [33, 40]. Due to their solubility, they are concentrated in fatty foods such as dairy products, high-fat meat, mayonnaise, fat, fish, and shellfish [30]. Phthalates form noncovalent interactions and can easily leach into the environment. This property, combined with its widespread use in consumer products, allows exposure to these chemicals in the US population [36]. Exposure to high levels of phthalates has been associated with alterations in thyroid hormone levels, insulin resistance, increased risk of obesity or low fertility, and increase in BMI and waist circumference [32, 38]. This phenomenon is explained because ingested phthalates cause dysregulation of glucose metabolism, insulin resistance, and adipogenesis [35]. Phthalates are metabolized by the body and metabolites are usually excreted in the urine [45].

Phthalate metabolites have been shown to activate PPAR receptors and have antiandrogenic effects that may contribute to the development of obesity. Prenatal exposure of mice to phthalate DEHP led to an increase in body weight, as well as an increase in body fat in male offspring. Similar findings were reported in different studies with different animal models [37].

Regarding phthalates and their effect on adipogenesis, it has been shown that (mono-(2-ethylhexyl) phthalate) (MEPH) favors the formation of adipocytes through the activation of PPARγ [70, 71]. Hao et al. [72, 90] demonstrated that this proadipogenic effect was dose-dependent; however, uterus exposure of mice significantly increased weight and fat mass in the offspring, possibly indicating an effect of MEHP on adipogenesis in alive mice.

Ellero-Simatos et al. [73] tested the obesogenic effect of MEHP, by stimulating cultures of human preadipocyte cells during adipogenesis. This research group also carried out metabonomic analyzes with the nuclear magnetic resonance (1H NMR) technique and transcriptome analysis. The results of these experiments showed that MEHP could increase the

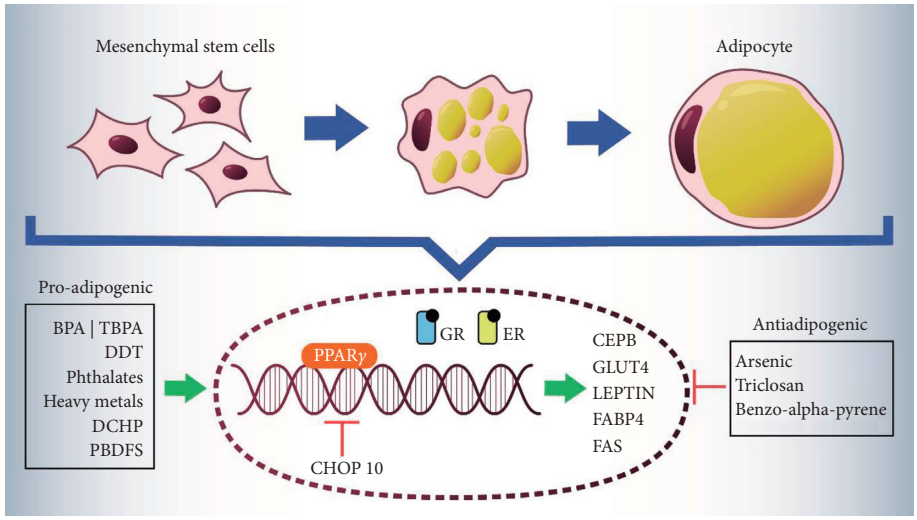


Figure 1: Schematic representation of effects of endocrine disruptors on adipogenic differentiation.

expression of at least 12 transcripts related to the PPARγ signaling pathway, in addition to increasing the expression of genes involved with lipid metabolism: glyceroneogenesis, cytosolic phosphoenolpyruvate carboxykinase, as well as reduction in the release of fatty acids.

Additionally, phthalates have also been related to an alteration in osteoblast homeostasis and adipogenesis in the bone marrow. Studies of Chiu et al. [74], in cell cultures of bone marrow stromal cells exposed to different concentrations of MEHP, showed a decrease in differentiation towards osteoblasts and a concomitant increase in adipogenesis.

Benzyl butyl phthalate (BBP) has also been related to a proadipogenic activity, activating PPARγ, favoring the accumulation of lipids in a dose-dependent manner, and also producing an alteration of lipid metabolism, glyceroneogenesis, and fatty acid synthesis in 3T3-L1 cell cultures [75]. The Sonkar group [76] confirmed the proadipogenic role of BBP, but also suggested a model where BBP produces epigenetic alterations involving the increase of lysine 9 of histone 3 (H3K9), which is typically increased in the promoter of PPARγ in mature adipocyte, which was accompanied by an alteration in histone methylation/acetylation due to the presence of BBP in mouse mesenchymal cell lines C3H10T1/2.

Additionally, Sargis et al. [64] investigated the role of dicyclohexyl phthalate (DCHP) during the process of adipogenesis in 3T3-L1 and discovered that this compound is a glucocorticoid receptor activator, which is a critical regulator in the differentiation towards adipocyte. Based on this result, the group suggests that DCHP could have a proadipogenic effect through a synergistic effect with other adipogenic cell signals.

5.5. Triclosan. It is a widely used antibacterial agent commonly found in antibacterial soaps, toothpaste, toothbrushes, dental rinses, laundry detergents, kitchen cutting boards, and plastics in furniture, toys, and sporting goods. In 2016, the Food and Drug Administration (FDA) issued rules prohibiting the use of triclosan in hand and body antibacterial products, citing the lack of evidence to support its effectiveness as an antiseptic. So far, the disruptive endocrine effects of triclosan are not well understood. Some studies have shown effects on the endocrine system of animals. Studies in animals show that high levels of triclosan interfere with estrogen, androgen, and thyroid hormone. Children who are exposed to triclosan may also be more likely to develop allergic hyperreactivity [36]. Concerning its influence on adipocyte differentiation, it has been shown that triclosan has an inhibitory effect of adipogenesis in a model with hMSCs, and this antiadipogenic effect was concentration-dependent, decreasing the production of typical markers of the cell fat, such as adiponectin and lipoprotein lipase [77].

5.6. Compounds Derived from Smoke (Benzo-Alpha-Pyrene). It is a polycyclic aromatic hydrocarbon compound and a potent carcinogen, which originates in combustion processes and is present in foods cooked on the grill or the barbecue [32, 41]. No obesogenic effect has yet been associated; however, an antiadipogenic effect has been demonstrated in cell cultures of human preadipocytes, and this effect is mediated through a specific receptor, the aryl-hydrocarbon receptor (AHR) [79].

5.7. Polybrominated Diphenyl Ethers (PBDEs). They are a class industrialized chemicals widely used in the manufacturing processes of many materials and currently used as effective flame retardants in plastics, electronics, automobiles, homes, furniture, textiles, and construction materials. Different studies have discovered the connection between PBDE and food, which informs about the presence of PBDEs in butter, fish, and other foods, as well as other foods that contain animal fats [33].

Pentabrominated diphenyl ether is increasing in the tissues and body fluids of individuals in the USA and can be detected in blood, breast milk, and urine samples from

Americans. Diphenyl ether has generally been eliminated in the USA and banned in the European Union. The PBDEs can accumulate in white adipose tissue and are highly lipophilic. These persistent compounds can be released into the blood, especially during weight loss. They have a structure similar to thyroid hormone, and some studies have found that exposure to these can alter the hormonal balance of the thyroid [36]. They have also been implicated in having a proadipogenic effect in 3T3-L1 cell cultures, increasing the accumulation of lipids and the expression of C/EBPα, PPARγ during the differentiation process [78].

6. Conclusion

Adipogenesis is a highly controlled process and is regulated by physiological and environmental conditions. Humans are constantly and chronically exposed to a variety of endocrine disruptors, some with obesogenic activity, which act at shallow doses and showing different mechanisms of action altering the hormonal balance. The exposure to the obesogens can happen continuously during the different stages of development, and in this context, the perinatal exhibition is relevant because the effects can be permanent in the organism.

Endocrine disruptors can alter lipid metabolism, promote fat accumulation, and interfere with processes such as adipogenesis. There is sufficient evidence in models with cell lines, human mesenchymal cells and in rodents that demonstrate that obesogens can have targets of action key molecules of the process of differentiation towards adipocyte, such as molecules that regulate the initial stages of differentiation, C/BPβ and C/EBPδ, or secondary stage proteins such as PPARγ.

There are diverse compounds—originally from food—that are associated with possible obesogenic effects; however, there is no enough scientific evidence to prove such association. However, it is necessary to understand in more detail the complexity of the mechanisms involved in the differentiation of fat cells and the influence of EDs in the adipogenesis and the etiology of obesity.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Gaseous Pollutants and Particulate Matter (PM) in Ambient Air and the Number of New Cases of Type 1 Diabetes in Children and Adolescents in the Pomeranian Voivodeship, Poland

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The increase in type 1 diabetes mellitus (T1DM) incidence in children is worrying and not yet fully explored. It is suggested that probably air pollution exposure could contribute to the development of T1DM. The aim of the study was to investigate the relationship between the concentration of gaseous pollutants including, nitrogen dioxide (NO_2), nitric oxides (NOx), sulphur dioxide (SO_2), carbon monoxide (CO), and particulate matter (PM) in the air, and the number of new cases of T1DM in children. The number of new cases of T1DM was obtained from the Clinic of Paediatrics, Diabetology, and Endocrinology, Medical University of Gdańsk. The number of children of 0–18 years old in Pomeranian Voivodeship was acquired from the Statistical Yearbook. The concentrations of PM_{10} absorbance, NO_2, NOx, SO_2, and CO were measured at 41 measuring posts, between 1 January 2015 and 31 December 2016. It was detected that the average annual concentration of PM_{10} was higher than the value acceptable to the WHO. Furthermore, the average 24-hour concentration of PM_{10} was 92 $\mu g/m^3$ and was higher compared to the acceptable value of 50 $\mu g/m^3$ (acc. to EU and WHO). Moreover, the number of new cases of T1DM showed a correlation with the annual average concentration of PM_{10} ($\beta = 2.396$, $p < 0.001$), SO_2 ($\beta = 2.294$, $p < 0.001$), and CO ($\beta = 2.452$, $p < 0.001$). High exposure to gaseous pollutants and particulate matter in ambient air may be one of the factors contributing to the risk of developing T1DM in children. Therefore, it is important to take action to decrease air pollutant emissions in Poland. It is crucial to gradually but consistently eliminate the use of solid fuels, such as coal and wood in households, in favour of natural gas and electricity. The development of new technologies to improve air quality, such as "best available techniques" (BAT) or renewable energy sources (water, wind, and solar generation) is of critical importance as well.

1. Introduction

According to the World Health Organization (WHO) air pollution contributed to 3.7 million premature deaths in 2012 globally, out of which 280000 were recorded in Europe, which constitutes a significant health problem related to environmental pollution [1, 2]. The concentration of particles with a diameter lower than 10 micrometers (PM_{10}) consisting of various elements of the organic and nonorganic matter is an acknowledged indicator of air pollution. Dust particles: PM_{10}–coarse particles, with a diameter below 10 μm and $PM_{2.5}$–fine particles, with a diameter of 2.5 μm or less, can bond with various chemical compounds, heavy metals, or microorganisms and can be transferred over long

distances, causing negative health effects [2–4]. Air pollution increases the risk of chronic obstructive pulmonary disease (COPD), lung cancer, cardiovascular diseases, strokes, allergic diseases, asthma, diabetes, and autoimmune diseases [5–9]. The source of emissions of air pollutants and suspended dust are primarily fuel combustion processes in the energy sector as well as industrial emissions associated with road transport and heating homes. In Poland, more than 88% of the energy produced comes from coal energy plants, of which nearly 53% use black coal and 35% brown coal. Atmospheric air can also be polluted by the aviation and automotive industry, which involves burning liquid fuel, the use of wearing parts in vehicles, and the rubbing of tires on asphalt surfaces [10–12]. The European Environment Agency (EEA) report on air quality in Europe in 2015 reveals that Greece, Poland, and Bulgaria are the countries where the daily PM_{10} concentration standards are exceeded [13], (Figure 1).

Dobreva et al. emphasize in their work the adverse effects of air pollution on the immune system. The authors demonstrated that air pollution, and $PM_{2.5}$ concentrations in particular can modulate cytokine production and change the balance between Tumour Necrosis Factor α (TNFα) and the anti-inflammatory production of interleukin 10 (IL-10) in teenagers living in cities of the Stara Zagora region in the south-east of Bulgaria [14]. Research conducted in Poland showed that 5.201 asthma symptoms and 234 hospital respiratory admissions were caused annually by air pollution [15]. What's more, epidemiological studies indicate that there is a relationship between T1DM and the concentration of PM_{10} and $PM_{2.5}$ [16–18]. Ciaula analysed data collected from 16 European countries (excluding Poland) emitting pollutants into the air and compared them with the prevalence of T1DM in children. He showed that in the investigated European countries an increase in pollution with PM_{10} corresponds to an increase in the incidence of T1DM [18]. In our preliminary studies, we showed a relationship between PM with the development of T1DM in children in the Lubelskie Voivodeship [19]. It should be added that these are the first such studies in Poland. At present, the relationship between the concentration of PM_{10}, gaseous pollutants in air, and the number of new cases of T1DM in children in the Pomeranian Voivodeship was investigated.

2. Material and Methods

2.1. Geographical Location of the Pomeranian Voivodeship, Poland. The Pomeranian Voivodeship is located in the north of Poland, on the coast of the Baltic Sea. It borders with Russia through the Gulf of Gdańsk (Figure 2). The Pomeranian Voivodeship is one of the most dynamically developing regions in central-eastern Europe. It consists of 16 poviats, including the Tricity, which is an agglomeration of Gdańsk, Gdynia, and Sopot [20, 21]. In the Tricity area, there are industrial plants producing fuels and petroleum-derived products, factories producing chemical fertilizers, feed manufacturing companies, heat and power stations, and thermal power stations and shipyards. There are also two harbours (in Gdańsk and Gdynia) which are the most important transport chain link connecting the Scandinavian countries with the countries of southern Europe [20, 21]. The natural resources in the region include vast green areas, as over 46% of the surface of the Pomeranian Voivodeship is covered by forest (Figure 2).

2.2. Exposure Assessment. Data on the annual average concentrations of nitrogen dioxide (NO_2), nitric oxides (NOx), sulphur dioxide (SO_2), carbon monoxide (CO), particulate matter, particles with a diameter of 10 micrometers or less (PM_{10}), and average 24-hour concentration of PM_{10}, was obtained from the Annual Evaluation of Air Quality 2015-2016, report provided by the Voivodeship Inspectorate of Environmental Protection (WIOŚ) in Gdańsk [22]. Ambient air pollution concentration measurements in Pomeranian Voivodeship were performed with the use of automatic and manual methods. To create the baseline values, the concentrations of PM_{10} absorbance, NO_2, NOx, SO_2, and CO were measured at 41 measuring posts for 2 years, between 1 January 2015 and 31 December 2016. NO_2 and NOx concentrations were measured with the use of chemiluminescence, SO_2 concentrations were measured using UV fluorescence while CO levels were measured with infrared absorption. Using polycarbonate filters, PM_{10} concentrations were determined using gravimetric analysis. The clean filters are conditioned, weighed, and placed in the collector. After 14 days, all filters were removed. In the laboratory, the filters were conditioned and weighed for the second time. The dust concentrations were calculated from the mass difference of the filter, both before and after exposure related to the air flow volume in the collector. Concentrations PM_{10}, NO_2, NOx, SO_2, and CO are given in micrograms per cubic meter ($\mu g/m^3$). The vast majority of data series taken into consideration have completeness in excess of 75%.

In order to assess the quality of air in Poland, the country was divided into zones comprising cities of more than 100000 residents, and other areas located within the borders of the voivodeship.

2.3. The Incidence of Type 1 Diabetes Mellitus in the Years 2015-2016 in the Pomeranian Voivodeship. The number of new cases of T1DM was obtained from the Department of Paediatrics, Diabetology, and Endocrinology, Medical University of Gdańsk. Diabetes was diagnosed according to the Polish Diabetes Association guidelines, which correspond with the guidelines of the WHO [23, 24]. Written informed consent was obtained from all children and adolescents participating in the study, or from their parent or guardian. The study was approved by the Ethics Committee of the Medical University of Gdańsk (No. NKBBN/314/2016) and the investigation was carried out in accordance with the principles of the Declaration of Helsinki as revised in 1996.

The number of children of 0–18 years old in Pomeranian Voivodeship was acquired from the Statistical Yearbook, published by the Polish Central Statistical Office and consent was not required [25].

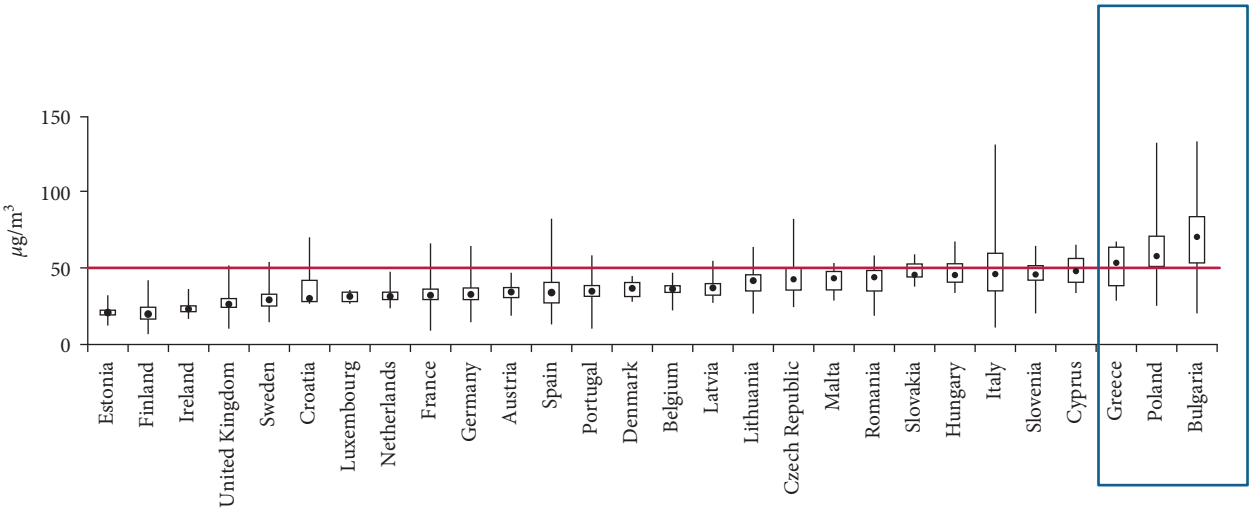

FIGURE 1: PM10 concentrations in relation to the daily limit value in 2015 in the EU. The length of the bars shows the range of reporting air quality data, with the solid black symbol indicating the mean [13].

2.4. Statistical Analysis. The main procedure in the calculation is a generalized linear model–glm. An important feature of this model is the flexibility of glm function. As a consequence, extensions going beyond the simple linear models are feasible. For the data collected in the study, the Poisson error distribution and the natural logarithm as a link function have been determined. The Poisson model is similar to regular linear regression, with two exceptions. Firstly, it assumes that the errors create a Poisson distribution rather than a normal one. Secondly, instead of modelling the dependent variable (y) as a linear function of regression coefficients, it models the natural logarithm of the dependent variable $\ln(y)$.

The equation for Poisson regression is as follows:

$$\ln(y) = \beta 0 + \beta x, \quad (1)$$

where $\beta 0$ and β are regression coefficients and x is an independent variable. In the considered problem, the dependent variable is the number of new cases of T1DM of children and the independent variables are one of the concentrations of gaseous pollutants.

The results have been generated using R statistics language: (A language and environment for statistical computing) version dated 2018 [26]. The assumed significance level is $\alpha = 0.05$.

3. Results

3.1. Concentrations of Gaseous Pollutants and PM_{10} in the Air in Pomeranian Voivodeship in the Years 2015-2016. The annual NO_2 concentrations in the Pomeranian Voivodeship were 15 $\mu g/m^3$, NOx 21 $\mu g/m^3$, SO_2 4 $\mu g/m^3$, and CO 374 $\mu g/m^3$ and were lower than the value acceptable to the EU and WHO [1, 13]. On the other hand, the average annual concentration of PM_{10} was 22 $\mu g/m^3$ and was higher than the value acceptable to WHO at 20 $\mu g/m^3$. Furthermore, the average 24-hour concentration of PM_{10} was 92 $\mu g/m^3$ and was higher compared to the acceptable value of 50 $\mu g/m^3$ (acc. to EU and WHO) as shown in Table 1.

3.2. The Number of Children Aged 0–18 Years Old in the Pomeranian Voivodeship and the Number of New Cases of T1DM. In the years 2015-2016, the number of children of 0–18 years old amounted to 947.362 in the Pomeranian Voivodeship, and the number of new cases of T1DM was 219.

3.3. The Relationship between the Number of New Cases of T1DM, Concentrations of Gaseous Pollutants, and Annual Average Concentration of PM_{10} in the Years 2015-2016. In the Pomeranian Voivodeship, it was found that the number of new cases of T1DM showed a relationship with the annual average concentration of PM_{10} ($\beta = 2.396$, $p < 0.001$) and a relationship was observed between the number of new cases of T1DM and the annual average concentration of SO_2 ($\beta = 2.294$, $p < 0.001$) and CO ($\beta = 2.452$, $p < 0.001$). However, there was no relationship observed between either the number of new cases of T1DM and the mean annual concentration of NO_2 ($\beta = -0.010$, $p < 0.1$) or the number of new cases of T1DM and the annual average concentration of NOx ($\beta = -0.728$, $p < 0.1$) in ambient air in the Pomeranian Voivodeship as shown in Table 2.

4. Discussion

The dynamic increase in the T1DM incidence in children observed in many countries, including Poland, can be associated with the growing pollution of the environment [18, 27–29]. The prevalence of T1DM in children in Poland increased 1.5 times within the 5-year observation period [27]. In order to investigate what may be the cause of the increase in the incidence of T1DM, it was decided to analyse the concentration of PM_{10} and gaseous pollutants (NO_2, NOx, SO_2, CO) in air and the number of new cases of T1DM

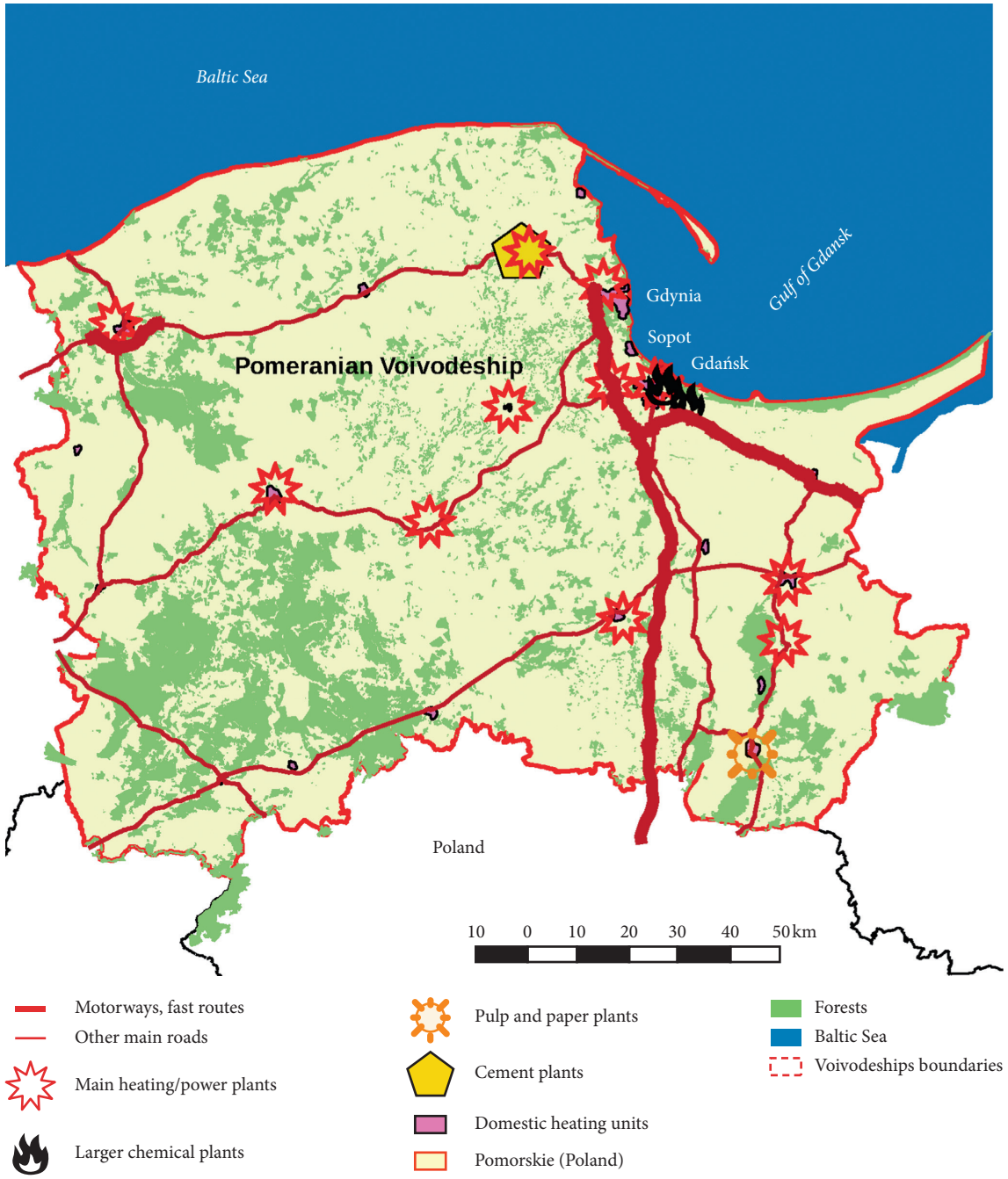

Figure 2: A map showing the potential gas pollution and particulate matter (PM) in the ambient air of the Pomeranian Voivodeship, Poland.

in children in the Pomeranian Voivodeship. Although the annual average concentration of PM_{10} in the Pomeranian Voivodeship does not exceed the acceptable value of 40 $\mu g/m^3$ (acc. to EU), it was higher than the acceptable value recommended by WHO (20 $\mu g/m^3$). Furthermore, the maximum 24-hour concentrations of PM_{10} ($\mu g/m^3$) are higher and exceed the acceptable value of 50 $\mu g/m^3$ (acc. to EU and WHO) at each of the measuring stations located in the Pomeranian Voivodeship.

The chemical factories producing fuels and petroleum-derived products, phosphorus factories, paint factories, heat and power stations or pulp and paper plants located in the Pomeranian Voivodeship can constitute a potential source of PM_{10} and other gaseous products. Furthermore, PM_{10} emissions were mainly coming from roads with the greatest traffic volume. This pertains to the A1 highway running from Gdańsk to the South of Poland, the A7 express road to Warsaw, and the Tricity ring road [22]. The roads listed are shown in Figure 2. In addition to the higher concentration of PM_{10}, the most significant source of SO_2 in the Pomeranian Voivodeship is the emissions from fuel burning processes in the energy industry, chemical industry, and areas where the

Table 1: Comparison of the average concentration of air pollution in the years 2015-2016 in the Pomeranian Voivodeship in Poland with the criteria used by the EU and WHO.

	Mean annual concentration of SO_2 ($\mu g/m^3$)	Mean annual concentration of NO_2 ($\mu g/m^3$)	Mean annual concentration of NOx ($\mu g/m^3$)	Mean 8-h concentration of CO ($\mu g/m^3$)	Mean annual concentration of PM_{10} ($\mu g/m^3$)	Mean 24-hour concentration of PM_{10} ($\mu g/m^3$)
Pomeranian Voivodeship	4	15	21	374	22	92
Acceptable concentration of pollutants ($\mu g/m^3$) acc. to the EU	20	40	30	10.000	40	50*
Acceptable concentration of pollutants ($\mu g/m^3$) acc. to WHO	50	40	—	10.000	20	50**

Abbreviations: EU: European Union; WHO: World Health Organization; SO_2: sulphur dioxide; NO_2: nitrogen dioxide; NOx: nitric oxide; CO: carbon monoxide; PM_{10}: particulate matter 10 micrometers or less in diameter; *not to be exceeded on more than 35 days per year **99th percentile 3 day/year.

Table 2: The relationship between the number of new T1DM cases and the annual average concentration of PM10 and gaseous pollutants in the air in the years 2015-2016.

Parameter	β	Statistical significance
Mean annual PM10 concentration vs. the number of new T1DM cases	2.396	<0.001
Mean annual concentration of sulphur dioxide (SO_2) vs. the number of new T1DM cases	2.294	<0.001
Mean annual concentration of carbon monoxide (CO) vs. the number of new T1DM cases	2.452	<0.001
Mean annual concentration of nitrogen dioxide (NO_2) vs. the number of new T1DM cases	−0.010	0.1
Mean annual concentration of nitrogen oxides (NO) vs. the number of new T1DM cases	−0.728	0.1

PM_{10}: particulate matter 10 micrometers or less in diameter; T1DM: type 1 diabetes mellitus; significance ($p < 0.05$).

majority of houses are heated by low capacity domestic heating units. Carbon monoxide mainly comes from road transport and coal combustion in households, while NOx mainly comes from fuel burning processes in the energy industry and road transport [22].

Finally, when the Poisson regression analysis was applied, it was found that there is a relationship between the number of new cases of T1DM and the annual average concentration of PM_{10}, SO_2, and CO in ambient air in the Pomeranian Voivodeship. The current results are consistent with other authors as well as our previous results [16–19]. In 2017, Michalska et al. conducted studies in the Lubelskie Voivodeship, Poland. Poisson regression analysis showed a correlation between the number of new T1DM cases and the average annual PM10 concentration in the Lubelskie Voivodeship in 2016. However, no correlation was observed between the number of new T1DM cases and the average annual PM_{10} concentration in 2015 [19]. Hathout et al. studied cases of T1DM in children and observed a relationship between T1DM and the concentration of PM_{10} in particular in those below the age of 5 years old [16]. Beyerlein et al. showed that high exposure to the traffic-related air pollutants PM_{10}, NO_2, and possibly $PM_{2.5}$ accelerates the manifestation of T1DM [17].

On the other hand, other findings from studies carried also indicate that particulate matter adversely affects brain structure, decreasing white matter volume or causing neuronal degeneration leading to early Alzheimer or Parkinson's disease [30, 31]. The particulate matter also increases the risk of depressive disorders and suicides [31]. The modelling results indicate that air pollution causes 2800 deaths a year in Warsaw [32]. Moreover, in epidemiological studies conducted in Cracow by Konduracka et al. a correlation was found between air pollution with $PM_{2.5}$ and the incidence of myocardial infarction [33].

The mechanism by which air pollutants contribute to the occurrence of diseases and premature death is not fully known. The in vivo and in vitro studies conducted so far showed that even exposing healthy volunteers to pollutant particles in the air for short periods triggers an inflammatory reaction on several different levels [34–37]. The immune system recognizes the antigens via toll-like receptors that are stimulated directly or indirectly. Signal transduction, which is triggered by the receptors recognizing the antigen, activates transcription factors including Nuclear Factor Kappa B (NFκB), and these in turn trigger the expression of numerous genes responsible for the production of proinflammatory chemokines and cytokines (Tumour Necrosis Factor α, interleukin-6) [36, 37]. Studies indicate that macrophages can capture pollutant particles and trigger an immune response in other lymphatic organs, by means of the presentation to T-lymphocytes by the dendritic cells. The organic chemical compounds contained in the dust can migrate to the systemic circulatory system and cause generalized inflammation of the vessels [4, 34, 36]. In previous studies and the studies by other authors, it was observed that patients with T1DM present with low-level inflammation, which aggravates with time and consequently contributes to the development of chronic vascular complications [36–41].

5. Conclusions

Considering the high level of air pollution in Poland, which exceeds the acceptable limits, and the increase in the incidence of T1DM, efforts should be made to improve the quality of air. It is believed that this can be achieved by limiting road transport in favour of rail and sea transport. In the cities, on the other hand, by replacing conventional combustion engine vehicles with electric or gas-fuelled cars and public transport. It is crucial to gradually but consistently eliminate the use of solid fuels, such as coal and wood in households, in favour of natural gas and electricity. The development of new technologies to improve air quality, such as "best available techniques" (BAT) or renewable energy sources (water, wind, and solar generation) is of critical importance as well.

Abbreviations

GUS: Polish Central Statistical Office
WHO: World Health Organization
EEA: European Environment Agency
EU: European Union
T1DM: Type 1 diabetes mellitus
COPD: Chronic obstructive pulmonary disease
WIOŚ: Voivodeship inspectorate of environmental protection
PM10: Particulate matter 10 micrometers or less in diameter
PM2.5: Particulate matter 2.5 micrometers or less in diameter
PM1: Particulate matter 1 micrometers or less in diameter
SO_2: Sulphur dioxide
NOx: Nitric oxide
NO_2: Nitrogen dioxide
CO: Carbon monoxide
NFκB: Nuclear factor kappa B.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The study was approved by the Ethics Committee of the Medical University of Gdańsk (no. NKBBN/314/2016), and the investigation was carried out in accordance with the principles of the Declaration of Helsinki as revised in 1996.

Consent

No informed consent from the participants was required for the present analysis.

Disclosure

Figure 1 is an exact reproduction of the EEA Air Quality in Europe Report, 2015. The authors were given permission to use the EEA Air Quality in Europe Report, 2015.

Conflicts of Interest

The authors declare that they have no competing interests.

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Research Article

Glycyrrhizae Radix et Rhizoma Processed by Sulfur Fumigation Damaged the Chemical Profile Accompanied by Immunosuppression and Liver Injury

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Glycyrrhizae Radix et Rhizoma (GRER) has been used as a medicinal plant and dietary supplements for its beneficial effect in immunomodulatory effects. Sulfur fumigation (SF) processing was widely used in the storage and maintenance of Chinese medicine because of its convenience and cheapness. However, the disadvantage of SF has been reported, but the systematic study of SF on GRER was deficient. In this paper, the active ingredients, sulfur-fumigated products, immunomodulatory effect, and liver injury of SF-GRER were studied. After SF, the liquiritin decreased from 4.49 ± 0.03 mg/g to 3.94 ± 0.08 mg/g ($P < 0.01$). Compared with the NSF-GRER group, the SF-GRER group showed a decreased immunoregulation in the thymus index, spleen index, and serum IL-6 and SOD levels ($P < 0.05$). After 2 weeks of continuous intragastric administration of SF-GRER in healthy mice, the level of serum aspartate aminotransferase (AST) significantly increased ($P < 0.05$) and the area of liver lesion significantly increased compared with the NSF-GRER ($P < 0.05$) group. The sulfonated products (m/z, 631.13) corresponding to liquiritin apioside (m/z, 551.17) and isoliquiritin apioside (m/z, 551.17) were screened out in SF-GRER by using UPLC-Orbitrap-MS. The sulfonated products provided in this paper were discovered for the first time and could be powerfully applied for the identification of SF-GRER. SF destroyed the chemical composition of GRER, inhibited immunoregulation, and induced liver injury. The feasibility of this processing method needs to be reconsidered.

1. Introduction

Glycyrrhizae Radix et Rhizoma (GRER) has been used as a medicinal plant and dietary supplements since ancient times in China, Japan, Italy, United States, and other different countries and regions [1–4]. Modern research studies showed that GRER had extensive pharmacological effects in antispasmodic, antidiabetic, antiosteoporosis, antidepressive, antitussive and expectorant, hepatoprotective, and memory-enhancing [5–11]. The major bioactive components of GRER were saponins, flavonoid glycosides, and free phenolic compounds [12]. Approximately 250 compounds have been reported from GRER, and more than 151 compounds in GRER have been determined up to present [13]. In the clinical practice of traditional Chinese medicine, there was a saying of "Ten prescriptions and Nine GRER." Therefore, the clinical demand of GRER is very large.

In recent years, sulfur fumigation (SF) has been used to replace traditional sun-drying for various Chinese herbal medicines to prevent pest infestation, mold, browning, and bacterial contamination in a cheap and convenient manner. The raw materials were usually stacked together, and a pot of burning sulfur was placed at the bottom and fumigated in a closed space for 12–24 hours. There have been many reports of sulfur-fumigated traditional Chinese medicine, including ginseng [14], Angelicae sinensis Radix [15], Chrysanthemum morifolium flowers [16], Ophiopogonis Radix [17], Atractylodes macrocephala Koidz. [18], Fritillaria thunbergii Miq. [19], Radix Paeoniae Alba [20], Smilacis Glabrae Rhizoma [21], Gastrodia Rhizoma [22], and lots of others. Numerous

studies have shown that SF-induced dramatic changes in chemical profiles which makes the safety and effectiveness of sulfur-fumigated traditional Chinese medicine have hidden dangers [23]. Therefore, the investigations of SF on chemical profiles, bioactivities, and toxicity of Chinese medicine are urgently needed for reevaluating this potentially harmful processing method. However, the effects of SF on active components, immunomodulation, and liver function of GRER and how to identify sulfur-fumigated GRER are still deficient.

In the present study, firstly, the content changes of main components in GRER before and after SF were detected by HPLC. Secondly, the immunosuppressive mice model and healthy mice model were, respectively, applied to investigate the effects of SF on immunoregulation and liver injury of GRER. Finally, liquid chromatography coupled with linear ion trap hybrid orbitrap mass spectrometry (UPLC-Orbitrap-MS) method was applied to screen the characteristic products in sulfur-fumigated GRER.

2. Materials and Methods

2.1. Ethics Statement. All animal experiments strictly comply with the Guidelines for Animal Experimentation of Jiangsu University (Zhenjiang, China), and the protocol was approved by the Animal Ethics Committee of this institution.

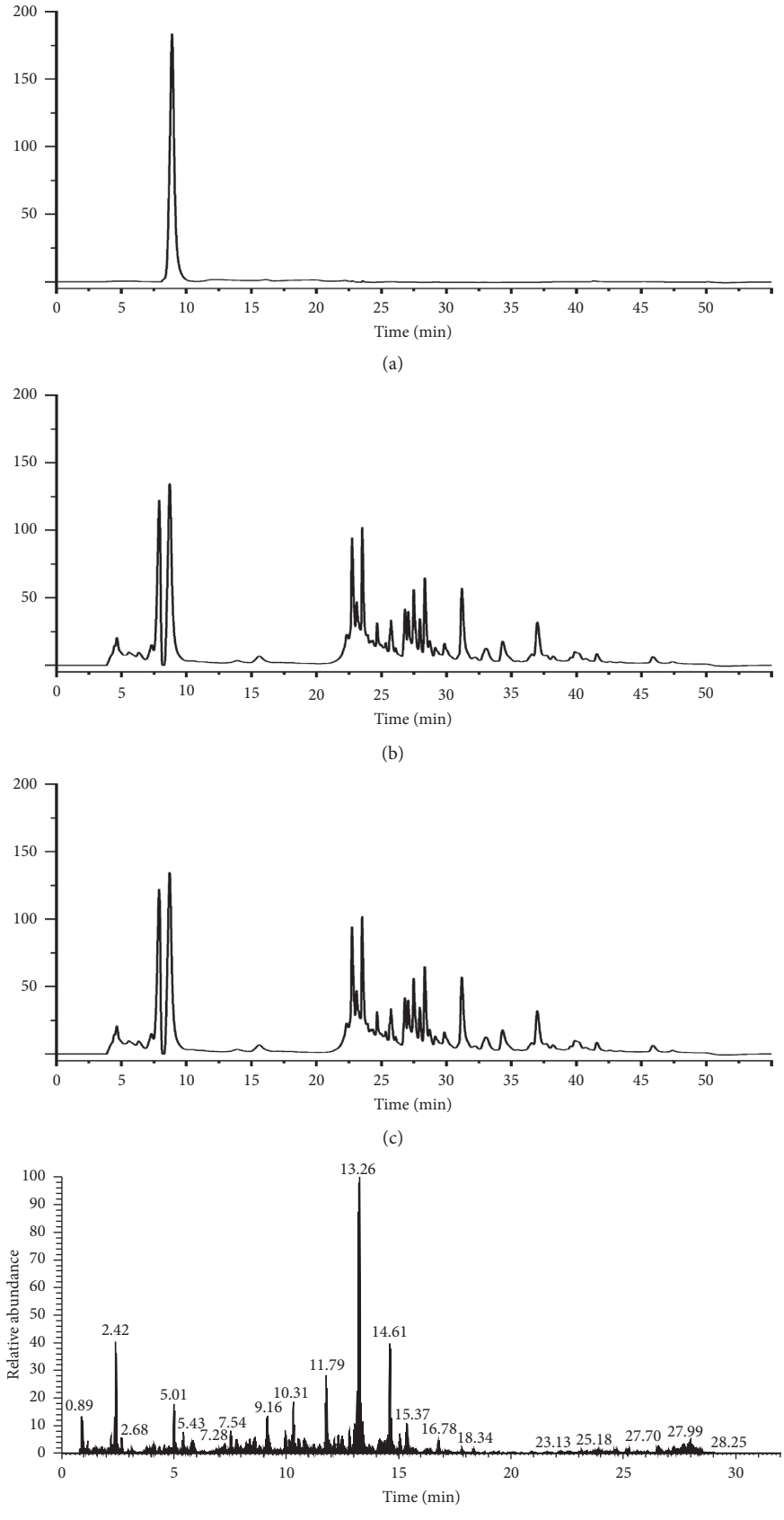
2.2. Materials and Chemicals. GRER was purchased from Gansu Province (Lanzhou Minxian Special Products Co., Ltd.) and identified as the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch. by Professor Wu Chengying in Jiangsu Academy of Traditional Chinese Medicine. Liquiritin (no. K186728, purity > 99.0%) was purchased from Xi'an Kaili Bioengineering Co., Ltd (Xi'an, China). Sublimated sulfur was purchased from Chemical Reagents Co., Ltd. of China Pharmaceutical Group (Shanghai, China). The chromatographic distilled water was made in our laboratory. Chromatographic pure acetonitrile and methanol were purchased from Shanghai Xingke High Purity Solvent Co., Ltd (Shanghai, China). Analytical pure sodium chloride, cyclophosphamide, formaldehyde, and sodium heparin were purchased from Titan Technology Co., Ltd. (Shanghai, China).

2.3. Preparation of Sulfur-Fumigated and Nonsulfur-Fumigated Samples. Sulfur-fumigated GRER (SF-GRER) was prepared according to the method used in literature [24]. The appropriate amount of nonsulfur-fumigated GRER (NSF-GRER) and SF-GRER was weighed and extracted twice with 10 times of water. Combined with the two extracts and concentrating to 100 mL under vacuum, the freeze-dried powder was obtained. For the accuracy of the results, 5 batches of SF-GRER and NSF-GRER were prepared in parallel. 0.2 g of freeze-dried powder of NSF-GRER and SF-GRER were separately weighed in a conical bottle, and 50 mL methanol was added immediately. After 30 min of ultrasound-assisted extraction, the membrane-crossed (0.22 μm) extracting solution was used for HPLC-DAD and Orbitrap-MS analysis.

2.4. Animals and Administration. Male KM mice (20 ± 2 g) were supplied by Laboratory Animal Center of Jiangsu University. All animals were housed under 20–25°C with a relative humidity of 40–70%. All the experimental protocols were approved by the Animal Ethics Committee of Jiangsu University. Animals were divided into two parts. Part I includes mice divided into 6 groups ($n = 10$), and they were normal control group (CON, normal saline, i.g), model group MOD, after continuous intraperitoneal injection of cyclophosphamide (150 mg/kg) for 3 days, SF-GRER low-dose group (SF-L, 2 g/kg/day, i.g), SF-GRER high-dose group (SF-H, 4 g/kg/day, i.g), NSF-GRER low-dose group (NSF-L, 2 g/kg/day, i.g), and NSF-GRER high-dose group (NSF-H, 4 g/kg/day, i.g). Part II includes healthy mice separately gavaged in NSF-GRER or SF-GRER at high or low doses (the same dose as part I, $n = 10$) for 14 consecutive days to investigate the effects of SF-GRER on liver function.

2.5. Collection and Treatment of Mice Samples. The body weight of mice in each group of part I was recorded daily. After 10 days of continuous administration, the whole blood of mice in each group was obtained by eyeball extraction and the serum was separated by centrifugation. Serum SOD and IL-6, thymus index, and spleen index of every group in part I were detected, respectively. For part II, after 14 days of continuous administration, the serum AST was detected and their liver were obtained and fixed by 10% formalin solution. The liver injury was assessed by HE staining and percentage of lesions.

2.6. Sample Analysis. The HPLC coupled with DAD and LCsolution (LC-AT$_{SR,}$ Shimadzu, Japan) was used for the content determination of liquiritin. The chromatographic separation was operated with an Agilent Zorbax SB-C_{18} column (4.6 mm × 250 mm, 5 μm) under column temperature 40°C, detection wavelength 275 nm, flow rate 1.0 mL/min, and injection volume 20 μL. The mobile phase was acetonitrile (A) and water (B) under a gradient elution (0–15 min, 18% A; 15–20 min, 18–60% A; 20–30 min, 60% A; 30–40 min, 60–18% A; and 40–42 min, 18% A; Figures 1(a) and 1(b)). UPLC-Orbitrap-MS was equipped with an electrospray ionization source under positive mode. Chromatographic separation was performed using a Dionex U3000 UPLC system with a Phenomenex Kinetex C_{18} column (2.1 mm × 100 mm, 2.6 μm). The column temperature was set at 30°C. The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% formic acid in water) with gradient program: 0 to 5 min, 5% A; 5 to 15 min, 5–35% A; 15 to 20 min, 35–85% A; 20 to 28 min, 85–5% A; and 28 to 30 min, 5% A (Figures 1(c) and 1(d)). The flow rate was 0.25 mL/min, and the sampling volume was 5 μL. For the MS conditions, the spray voltage was 3.5 kV, the heated capillary temperature was 300°C, the ESI probe temperature was 350°C, the flows of sheath gas and auxiliary gas were 40 units and 15 units, respectively, the scan range was 500–1800 m/z with the Orbitrap analyzer, and target ions selected for fragmentation were obtained by dynamic exclusion for 20 s. All the data analysis was performed using Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, Inc., Bremen, Germany).



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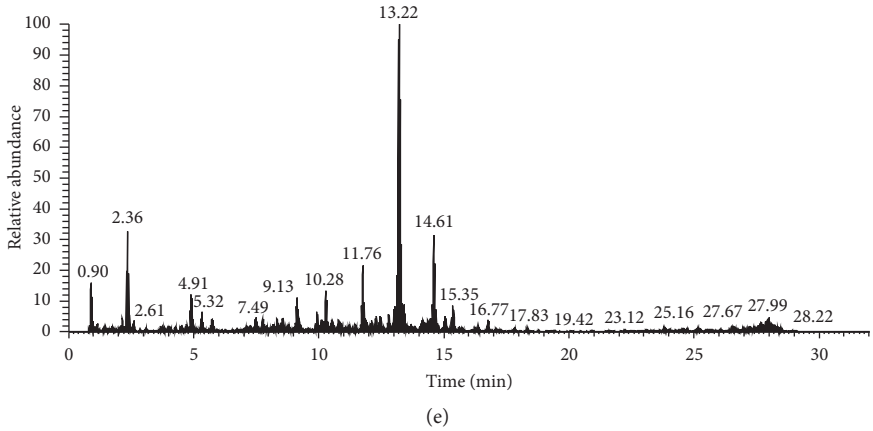

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Figure 1: HPLC-DAD and UPLC-Orbitrap-MS chromatogram: (a) chromatography of liquiritin standard solution; (b) chromatography of freeze-dried powder of NSF-GRER extract; (c) chromatography of freeze-dried powder of SF-GRER extract; (d) total ion chromatography of NSF-GRER; (e) total ion chromatography of SF-GRER.

Table 1: Recovery of liquiritin in NSF-GRER and SF-GRER by HPLC.

Samples	Original quantity (mg)	Added	Determined	Recovery	Average (%)	RSD
	0.449	0.400	0.853	101.10	100.27	1.08
	0.459	0.400	0.857	99.55		
NSF	0.447	0.400	0.846	99.83		
	0.455	0.400	0.862	101.70		
	0.453	0.400	0.849	99.15		
	0.400	0.400	0.795	98.70	100.37	1.47
	0.402	0.400	0.80465	100.65		
SF	0.391	0.400	0.801	102.55		
	0.401	0.400	0.798	99.35		
	0.397	0.400	0.799	100.60		

2.7. Biochemical Indices and Pathological Evaluation. The thymus and spleen index were calculated as follows: spleen (thymus) index = the spleen (thymus) mass (mg)/the body weight (g). Blood samples were collected from eyeballs at intervals of one hour after the last administration. The blood samples were placed in a 2 mL centrifugal tube containing 20 μL of 5% heparin sodium. The supernatant was centrifuged at 3700 rpm for 10 min and frozen (−80°C) in the centrifugal tube. Serum SOD, IL-6, and AST were determined by ELISA [25]. HE staining sectioning (2 μm) was read under optical microscope (OLYMPUS BX41, Japan) [26]. The main examination of the liver was (1) whether there is hepatocyte degeneration (steatosis and edema), (2) whether there is hepatocyte degeneration and necrosis, (3) whether there is inflammatory cell infiltration in the liver lobule, (4) whether there is inflammation or fibrous tissue proliferation in the portal area, and (5) whether there is atrophy of hepatocyte and expansion of hepatic sinuses. Five visual fields were selected for each slice, and then the average percentage of lesion in different groups was calculated.

2.8. Statistical Analysis of Data. Statistical analyses were conducted using one-way ANOVA (GraphPad Prism 5.0) followed by the Tukey test for comparing all pairs of columns. Data were presented as mean value ± SD. The P value <0.05 was considered as statistically significant.

Table 2: Liquiritin in GRER before and after sulfur fumigation ($n = 3$).

No.	NSF (mg/g)		SF (mg/g)	
	Content	Average content	Content	Average content
1	4.524		3.864	
2	4.472		3.934	
3	4.446	4.49 ± 0.03	3.884	3.94 ± 0.08##
4	4.485		3.947	
5	4.506		4.064	

##Compared with NSF, the content decreased significantly ($P < 0.01$).

3. Results

3.1. Methodological Validation. The standard solution was determined according to the chromatographic conditions under item "*HPLC-DAD analysis*." The peak area of liquiritin (Y) was linearly regressed by sample concentration (X). The results showed that the linear relationship of liquiritin was fine in the range of 20–200 μg/mL. The regression equation was $Y = 42438X - 232006$, $r^2 = 0.9998$. The RSD of intra- and interday precision was 0.32% and 0.51%, indicative of a high precision. The RSD of repeatability in SF-GRER and NSF-GRER was 0.67% and 1.98%, respectively. The detection limit (LOD) and quantitative limit (LOQ) of liquiritin was 0.06 μg/mL and 0.21 μg/mL, respectively. The

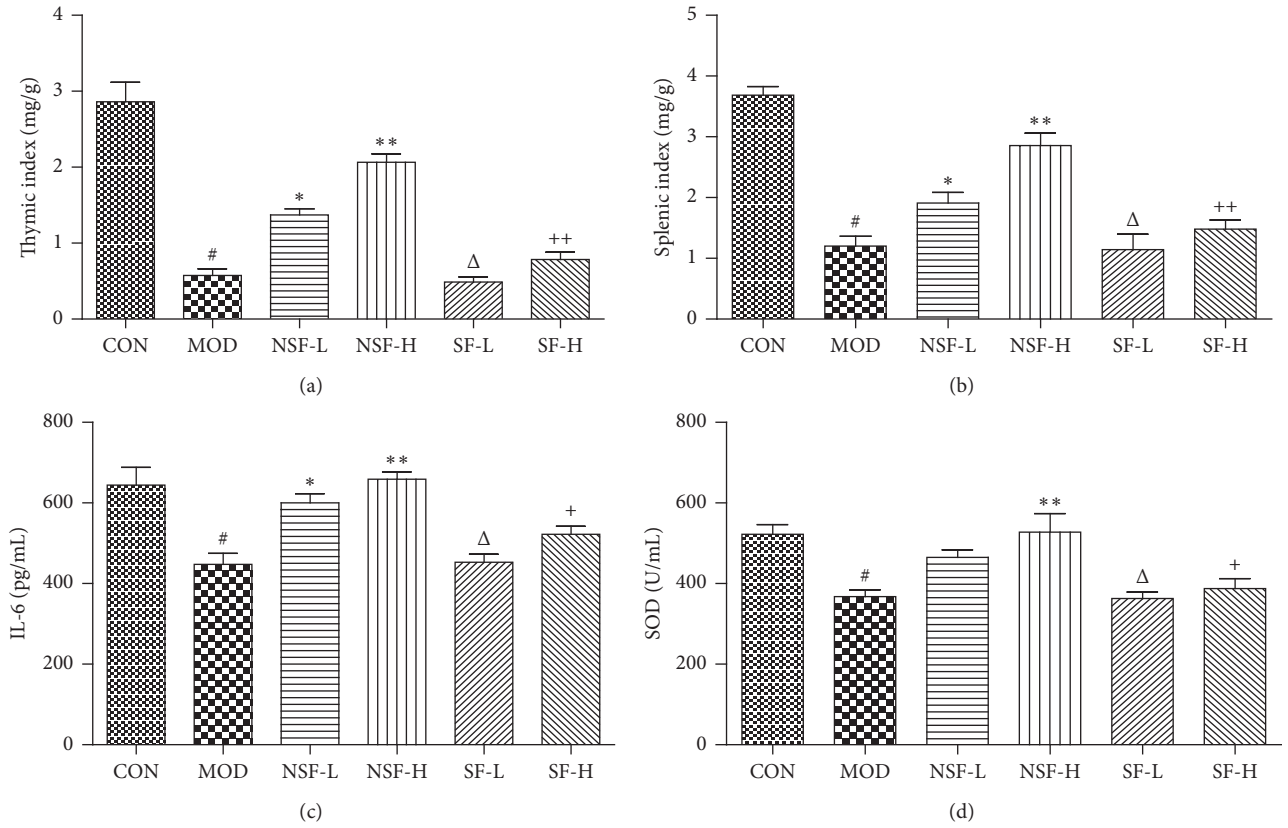


Figure 2: Immunoregulation of GRER before and after sulfur fumigation ($\bar{x} \pm SD$, $n = 10$). $^{\#}$Compared with CON, $P < 0.01$; *compared with MOD, $P < 0.05$; **compared with MOD, $P < 0.01$; $^{\Delta}$compared with NSF-L, $P < 0.05$; $^{+}$compared with NSF-H, $P < 0.05$; $^{++}$compared with NSF-H, $P < 0.01$.

recovery rates of liquiritin in SF-GRER and NSF-GRER were 100.37% and 100.27%, respectively (Table 1). The method had superior stability, reproducibility, and recovery which ensured the accuracy of the quantitative results.

3.2. Liquiritin before and after Sulfur Fumigation. The liquiritin in NSF-GRER was 4.49 ± 0.03 mg/g (0.449%). Compared with NSF-GRER, the liquiritin in SF-GRER decreased significantly to 3.94 ± 0.08 mg/g (0.394%, $P < 0.01$). The liquiritin in GRER before and after sulfur fumigation was shown in Table 2. Our results showed that liquiritin was destroyed or transformed into other structures during sulfur fumigation.

3.3. Immunomodulatory Effects before and after Sulfur Fumigation. Compared with the healthy mice, the thymus index (0.58 ± 0.15 mg/g), spleen index (1.20 ± 0.36 mg/g), serum IL-6 (448.00 ± 54.40 pg/mL), and SOD (367.50 ± 33.67 U/mL) levels of the model group were significantly decreased by cyclophosphamide ($P < 0.01$). Under the intervention of NSF-GRER (low and high dose), the immunosuppression induced by cyclophosphamide could be reversed and the thymus index (1.37 ± 0.16 and 2.06 ± 0.19 mg/g), spleen index (1.91 ± 0.39 and 2.86 ± 0.35 mg/g), serum IL-6 (600.11 ± 46.44 and 658.77 ± 35.99 pg/mL), and SOD levels (465.00 ± 37.97 and 527.50 ± 92.11 U/mL) were significantly increased ($P < 0.05$). However, with the intervention of SF-GRER, the indicators showed an upward trend but no significant difference. It is worth noting that, compared with the NSF-GRER group, the SF-GRER group showed a significant decrease in immunoregulation ($P < 0.05$). Detailed results are shown in Figure 2.

3.4. Effects on Liver Function. After 2 weeks of continuous intragastric administration of SF-GRER and NSF-GRER in healthy mice, the level of AST increased significantly in SF-GRER mice ($P < 0.05$, Figure 3(a)). Therefore, we suspected that SF-GRER may induce liver damage in healthy mice [27, 28]. Subsequent pathological studies showed that SF-GRER significantly increased the area of liver lesion compared with the same dose of NSF-GRER ($P < 0.05$, Figure 3(b)).

3.5. Screening of Sulfur-Fumigated Characteristic Products. UPLC-Orbitrap-MS has been widely used in the identification of characteristic components of traditional Chinese medicine [29, 30]. According to related literature [2, 4, 13, 31–34] and the transformation rule before and after SF, the molecular weight ranging from 500 to 1800 (m/z) was screened one by one under the UPLC-Orbitrap-MS system. Fortunately, liquiritin apioside (LA, m/z, 551.17) and isoliquiritin apioside (ILA, m/z, 551.17) not only had been found in NSF-GRER, their corresponding sulfonated

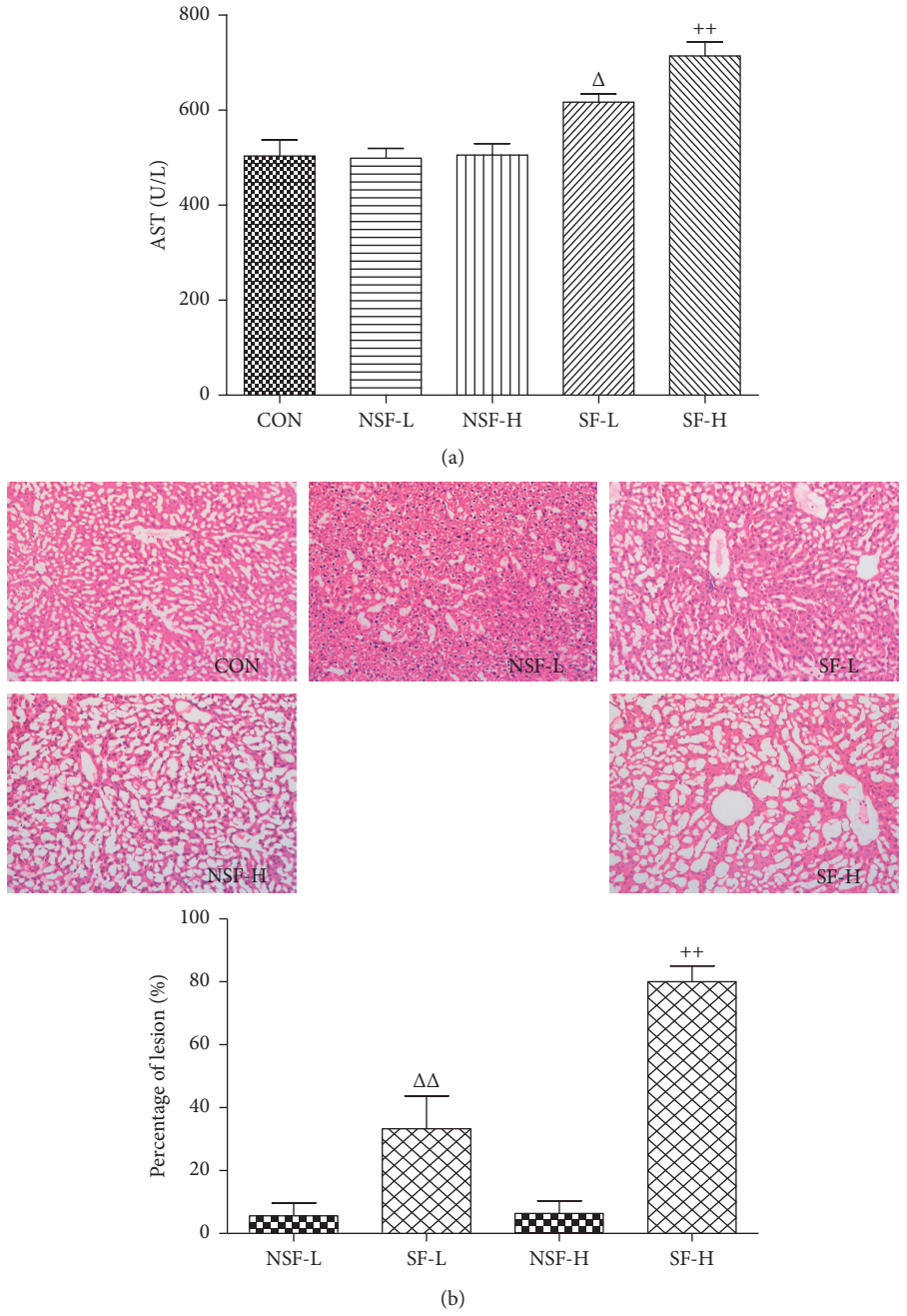


Figure 3: Effects of SF-GRER on liver function in healthy mice ($\bar{x} \pm$ SD, $n = 10$): (a) effect of sulfur-fumigated GRER on the serum AST level in healthy mice; (b) representative photomicrographs (×100) and lesion assessment of livers after prevention of NSF-GRER and SF-GRER. $^{\Delta}$compared with NSF-L, $P < 0.05$; $^{\Delta\Delta}$compared with NSF-L, $P < 0.01$; $^{++}$compared with NSF-H, $P < 0.01$.

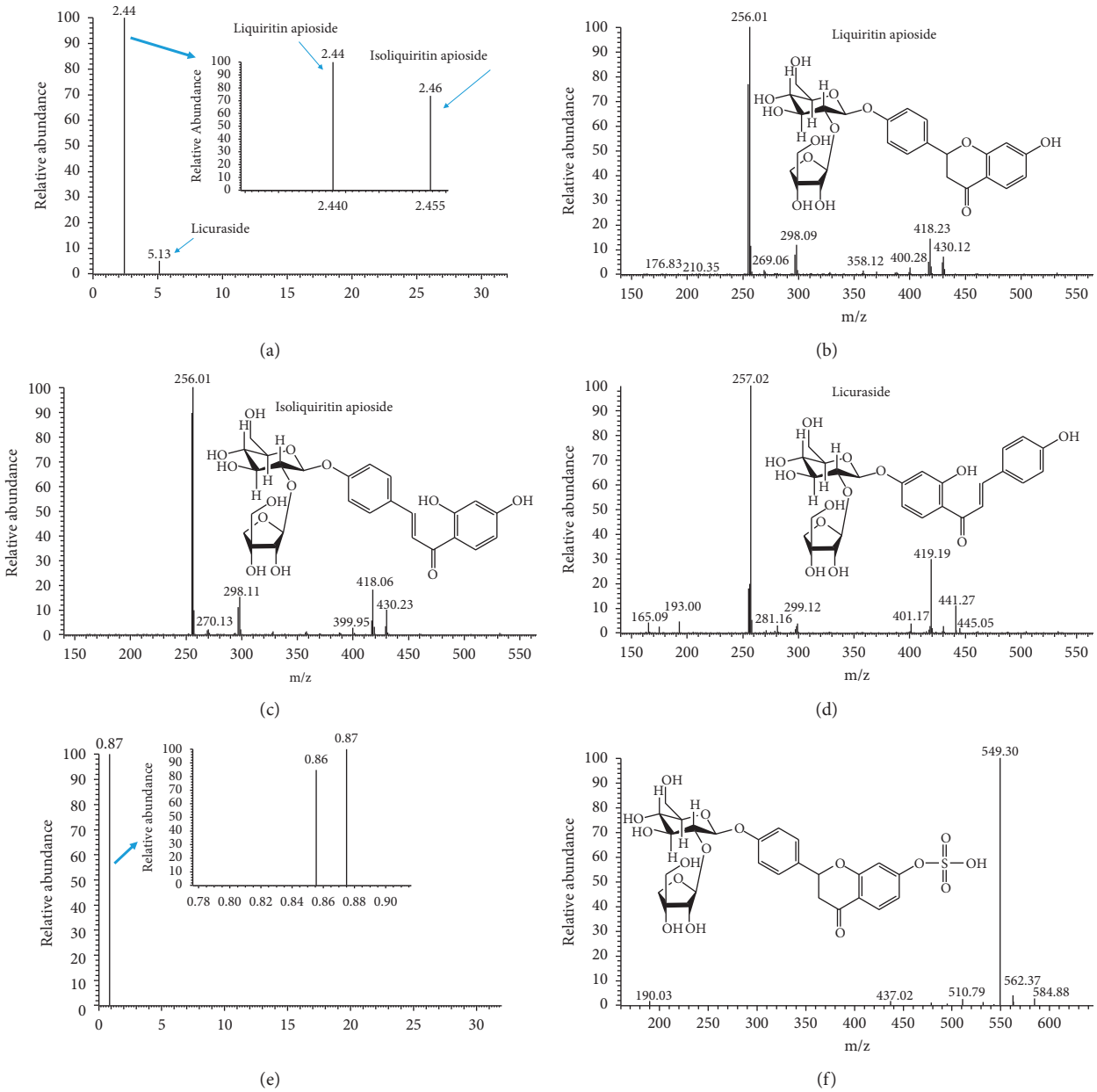
products (m/z, 631.13) also had been screened out in SF-GRER (Table 3 and Figure 4). It is interesting that LA and ILA had same fragmentation ions (m/z, 256.01 and 418.23) and extremely close retention time (2.44 and 2.46 min) because they were isomers. Sulfonation increased the polarity of LA and ILA, so retention time of their sulfonates was advanced to 0.86 and 0.87 min, respectively. The pyrolysis process of LA and ILA in mass spectrometry was first to remove β-apiose (m/z, 418.13 or 418.39) and then β-D-glucose (m/z, 256.07 or 256.25, Figure 5(a)). The pyrolysis process of LA-SO_3 and ILA-SO_3 in mass spectrometry was to remove sulfonic acid groups (m/z, 549.30 or 549.29, Figure 5(b)). There were no other sulfur-fumigated characteristic products found in such a large amount of screening work.

4. Discussion

The advantages to Q-TOF tandem analyzers are their higher mass accuracy and faster scan speeds and duty cycles. However, Orbitrap analyzers do not use magnetic fields to operate, and therefore, cryogenic refrigerants such as liquid

Table 3: UPLC-Orbitrap-MS parameters of characteristic products in SF-GRER.

Name	Retention time (min)	Formula	Precursor ion (m/z)	Mass fragments (m/z)
Liquiritin apioside	2.44	$C_{26}H_{30}O_{13}$	551.17	256.01 254.94 418.23
Isoliquiritin apioside	2.46	$C_{26}H_{30}O_{13}$	551.17	256.01 418.23
Licuraside	5.13	$C_{26}H_{30}O_{13}$	551.17	257.02 419.19 441.27
Liquiritin apioside sulfonate	0.86	$C_{26}H_{30}O_{16}S$	631.13	549.30
Isoliquiritin apioside sulfonate	0.87	$C_{26}H_{30}O_{16}S$	631.13	549.29



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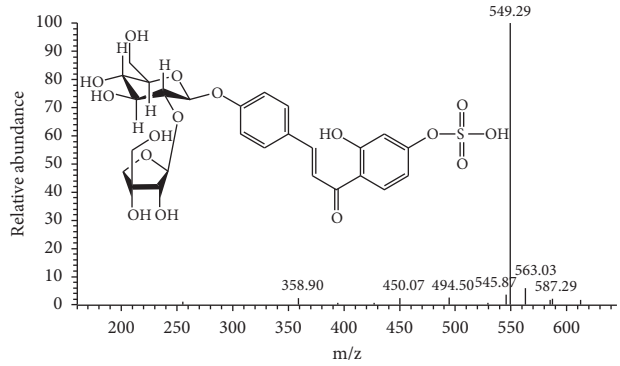
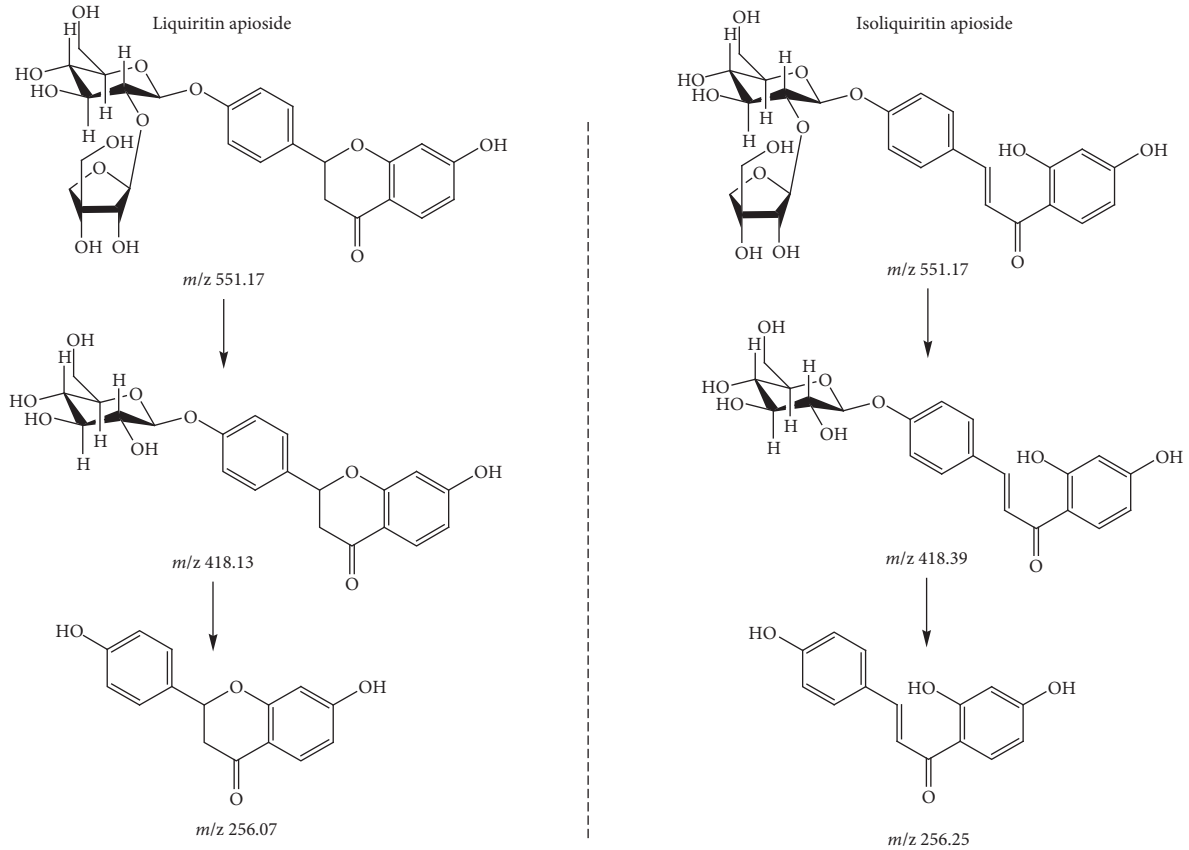


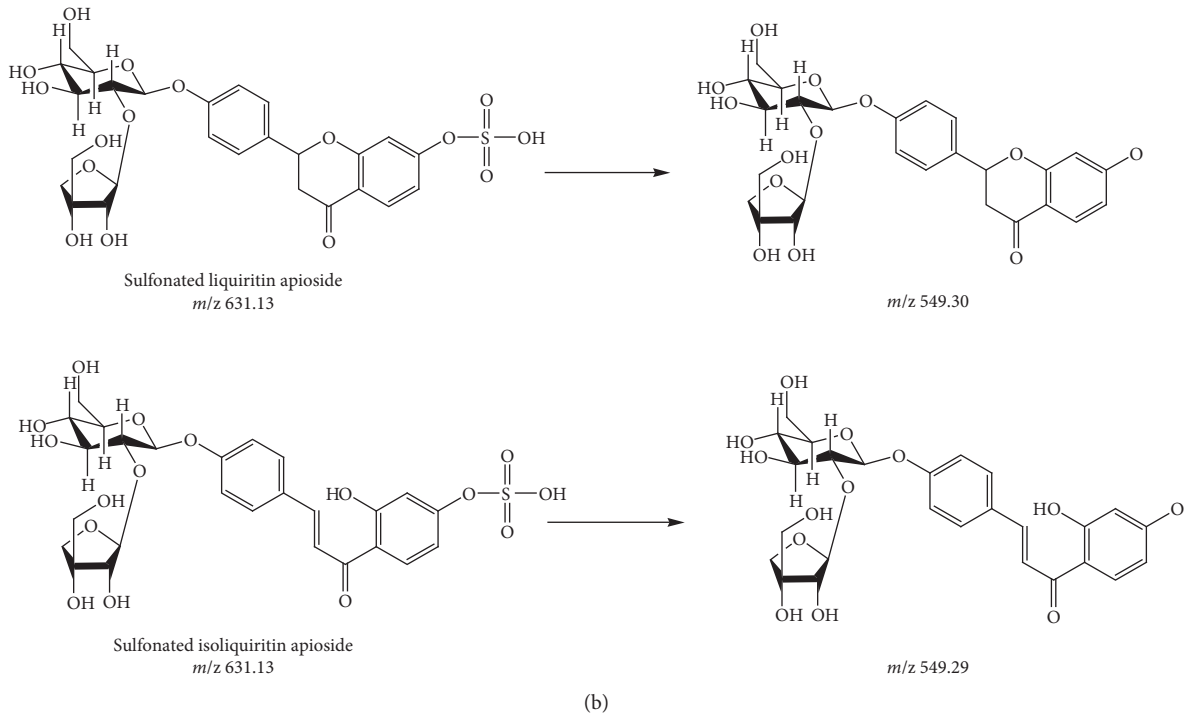
(g)

Figure 4: UPLC-Orbitrap-MS spectrum of characteristic products in SF-GRER. (a–d) MS spectrum of liquiritin apioside, isoliquiritin apioside, and licuraside in NSF-GRER. (e–g) MS spectrum of liquiritin apioside sulfonate and isoliquiritin apioside sulfonate in SF-GRER.



(a)

Figure 5: Continued.



(b)

Figure 5: Pyrolysis process of characteristic products in SF-GRER by using UPLC-Orbitrap-MS: (a) pyrolysis of liquiritin apioside and isoliquiritin apioside; (b) pyrolysis of liquiritin apioside sulfonate and isoliquiritin apioside sulfonate.

helium are not necessary and operating costs are kept low. Furthermore, one of the major advantages of the orbitrap analyzer is its high resolving power [35]. Considering these factors, Orbitrap was applied in this paper.

Liquiritin is one of the main active ingredients in GRER which is also an important index for judging the quality of GRER [36]. Pharmacological studies have shown that liquiritin can enhance immune regulation [37]. Therefore, the liquiritin in GRER decreased significantly after sulfur fumigation, which inevitably leads to the decrease of the immune regulation of GRER. Our results also demonstrated that the decrease of liquiritin in GRER after sulfur fumigation was accompanied by a significant decrease in its reversal of immunosuppressive effect induced by cyclophosphamide in mice. Our results confirm that sulfur fumigation damaged the active ingredients in GRER and reduced its pharmacological activity.

More seriously, the level of serum AST and the area of liver lesion increased significantly after continuous administration of sulfur-fumigated GRER in mice. These results suggested that sulfur-fumigated GRER had potential safety hazards, especially increasing the risk of liver injury. Therefore, whether the sulfur fumigation method can be used in GRER is a question worthy of serious consideration. How to effectively identify sulfur-fumigated GRER which has entered the market is also an important technical problem faced by scientific researchers.

In Chinese Pharmacopoeia, the quality control of sulfur-fumigated Chinese medicines is only to require the control of SO_2 residue less than 400 mg/kg [38], which is an important indicator to test whether the medicine has been fumigated by sulfur or not (NSF-GRER: 43 mg/kg and SF-GRER, 910 mg/kg). However, the residual amount of SO_2 is extremely unstable, which will be significantly reduced with the extension of storage time, even lost more than 50% in 4 months [39]. In order to identify sulfur-fumigated medicinal materials effectively, we suggested that the detection of characteristic products for sulfur-fumigated medicinal materials should be added to the pharmacopoeia. Excitedly, in this study, we successfully used UPLC-Orbitrap-MS technology to screen the characteristic products (m/z, 631.13) of sulfur-fumigated GRER, which can be applied for the identification of sulfur-fumigated GRER.

5. Conclusions

The processing of sulfur fumigation destroyed the active ingredients and attenuated the immunomodulatory effect of GRER. More importantly, the sulfur-fumigated-GRER induced liver damage. In this study, the characteristic products of SF-GRER were first discovered, which provided powerful technical support for its effective identification.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

Authors' Contributions

Jun Jiang and Shichang Xiao equally contributed to this work.

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Research Article

Validity and Reliability of an Assessment Tool for the Screening of Neurotoxic Effects in Agricultural Workers in Chile

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There is a substantial use of pesticides within the agricultural industry of Chile, with neurotoxic effects through mechanisms of acetylcholinesterase inhibition. These pesticides result in deterioration in health, increasing the risk of diseases such as Parkinson's and Alzheimer's in highly exposed occupational population. To date, there are no brief assessment tools to monitor cognitive impairment in agricultural workers chronically exposed to these pesticides. *Method*. 234 agricultural workers and 305 non-agricultural workers were assessed two times (test-retest) through a brief tool which comprised three tests (clock-drawing test (CDT); frontal assessment battery (FAB); trail making tests (TMT) A and B). The full scale of WAIS-IV was administered as a gold standard to 18% of the sample of agricultural workers. Factor analysis was used to evaluate the factor structure, and validity and test-retest reliability were assessed concurrently. *Results*. Cronbach's alpha values were satisfactory or above (>0.60). Test-retest correlations were all significantly correlated ($p < 0.001$). All the tests had a significant correlation with the full scale IQ score of WAIS-IV ($p < 0.05$). The Kaiser–Meyer–Olkin (KMO) measure was 0.74, and the Bartell sphericity test = $p < 0.001$. Three factors explaining 61.62% of the variance were extracted. Two items of the FAB test were dropped of the final factor solution. Normative data transformed into percentile scores and stratified by age and educational level were obtained for Chilean agricultural workers. *Conclusion*. The brief assessment tool has adequate metric properties as a screening instrument. This allows for a simple administration test (10 to 15 minutes) that can potentially be used for the rapid monitoring of cognitive deterioration in the face of occupational exposure to pesticides in agricultural workers.

1. Introduction

Adverse health outcomes from pesticide-related exposure are a global issue impacting both industrialized and developing countries [1, 2]. There is evidence that those most critically impacted by the adverse health implications from pesticide exposure are those in the occupational agricultural setting [3–8].

In Chile, the most frequently sold group of pesticides is the organophosphates (OP), specifically diazinon and chlorpyrifos [9]. The primary mode of action of OP pesticides is via the enzyme acetylcholinesterase [10]. The inhibition of acetylcholinesterase generates an excessive excitation of the muscarinic and nicotinic receptors of the nervous system, causing the acetylcholine neurotransmitter to overaccumulate in the cholinergic synapses. This eventually results in acute or chronic intoxication. Studies found that the neurotoxic effect of pesticides can provoke decreased cognitive performance in developing children and can be considered a precursor to an increased risk of degenerative diseases in chronically exposed workers in occupational settings [3, 11, 12]. In the last decades, increased attention has been paid internationally to the development and administration of tools which can evaluate the

neurobehavioral and cognitive effects of the exposure to neurotoxic substances such as pesticides [5–7]. The administration of these kinds of tools to populations of agricultural workers exposed to chemicals has been developed for decades using methods derived from neuropsychology and experimental psychology. Usually, batteries of a series of behavioral tests have been used to evaluate the effects of neurotoxic pesticides, and the number of tests to be used in these batteries has increased over time [7, 10, 13]. One of the most important tools is the Neurobehavioral Core Test Battery (NCTB) [14]. It is composed of seven behavioral tools: Digit Symbol test, Digit Span test, Simple Reaction Time, Benton Visual Retention test, Santa Ana Dexterity test, Pursuit Aiming II, and the Mood Profile [15, 16]. Noteworthy, the selection of the tests composing the NCTB battery has been performed on the basic rule that these must be proven to be tools sensitive to the changes associated with exposure to chemicals. It means that the tests have shown to be effective in differentiating between groups of workers who had been exposed or not to chemicals, allowing the identification of neurotoxin effects. This implies that batteries also had to comply with the two basic psychometric properties of validity and reliability. In this regard, in 1999 [17], one of the main recommendations for NCTB was the need to establish construct validity in a wide range of different countries. In fact, it had been previously concluded that this battery could effectively assess only the adult population with twelve or more years of education and belonging to North America, Western Europe, and some parts of Asia, while it did not show the same reliability of use on people with less than nine years of education, being additionally discarded its effective use in populations different from the North American and European. In 2013, different aspects of the use of the battery and the selection criteria of the tests for the measurement of neurotoxic effects were discussed again [18]. For example, it was recommended that the elaboration of such type of battery should not include copyrighted tests. This was a relevant point, since most of the tests included in the NCTB were copyrighted; in fact, they should be acquired individually in order to be used legitimately. Instead of this, experts recommend including tests that are of public domain, such as the trail making test (TMT) [19]. A final conclusion regarding the tests is that they should be free of cultural influence. Experts furthermore agreed that it is always necessary to have a control group for comparison in epidemiological studies of neurotoxic exposures. Particularly in the case of the workers, it was pointed out that it was necessary to apply tests in a test and retest context, in order to establish an early monitoring of the occupationally exposed populations with respect to the neurotoxic effect on a follow-up logic. Finally, they concluded that it is necessary to develop a dedicated "screening" assessment tool made up of sensitive tests to be used in the field of human neurotoxicology [18]. Other authors pointed out that the neuropsychological tools that result in a greater effect size with respect to OP pesticides are those that measure working memory/attention, visual memory, psychomotor speed, executive function, and visuospatial ability [5]. Therefore, it can be concluded that the cognitive functions affected to a greater extent by the exposure to OP pesticides are nonverbal abilities, being found in cross-sectional studies a slowdown of reaction time and deficits in the performance in short-term memory and executive function in the most severely affected individuals [5]. Moreover, a constant monitoring of the agricultural workers' neurocognitive functioning is necessary, due to the evidence that pesticides increase the risk for neurodegenerative diseases, such as Parkinson's [20] or dementia and Alzheimer's disease (AD), as it has been observed in longitudinal cohort studies [21].

Different types of neurotoxic disorders have been associated with chronic OP exposure: (1) cholinergic syndrome; (2) intermediate syndrome; (3) OP-induced polyneuropathy; (4) chronical exposure-related neuropsychiatric disorders. The first two disorders are generally detected in case of acute intoxication in the emergency care hospitals and are immediately treated and registered. However, the third and fourth kind of diseases need a detailed monitoring, for example, through a screening assessment tool, and their symptoms may appear later in the individual's life, with a progressive deterioration of the neurobehavioral performance, associated to permanent damages in the central nervous system (CNS), and cognitive deficits, including memory, concentration and learning, attention, information processing, and reaction times.

In Chile, there are few studies in the area collecting evidence of neurocognitive risk in the agricultural workers, the most complete and relevant being the one carried out in the Maule region [22]. In this study, full intelligence scales (Wechsler Adult Intelligence Scale, WAIS-IV) [23] were used to measure neurocognitive effects. The use of this kind of tools, although is advisable in terms of a more specific notion of the performance and the cognitive profile of test takers, implies a longer time of both test administration and further interpretation of the results. These "practical" features make it difficult to adopt them as screening and monitoring tests considering the amount of human and financial resources needed to perform the study, both from an organizational and individual point of view. Therefore, in the Chilean context, nationally validated tools (such as batteries of tests) able to show neurological problems in agricultural occupational populations which are at the same time sensitive and viable for a fast and easy administration are still missing. For this reason, it appears clear that it is necessary to establish an economic test, easy to apply and validated at a national level with agricultural occupational population for the early monitoring of the cognitive effects of exposure to acetylcholinesterase inhibitor pesticides. This test should be directly and specifically focused on the evaluation of these cognitive areas that have been recognized as more sensitive in the adverse effects on workers' health (reaction time, short-term memory, and executive functions), in order to perform an effective surveillance in the context of occupational exposure. Accordingly, the aim of this study is to validate a brief assessment tool for the monitoring of neurotoxic effects in agricultural workers exposed to pesticides, which can be used as a screening

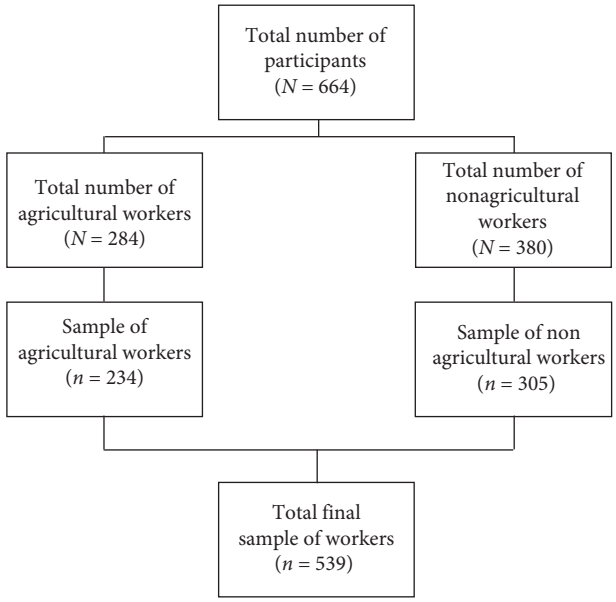

Figure 1: Study participants and sample of agricultural and nonagricultural workers.

measure for the early detection of cognitive impairment in the occupational context.

2. Materials and Methods

2.1. Participants. A sample of 664 workers participated in this study between 2017 and 2018. Of these, 284 were agricultural workers from the Maule region, and 380 were nonagricultural workers from the Metropolitan and Valparaiso regions (see Figure 1). For the reliability analysis, a subsample of 193 agricultural workers and 193 nonagricultural workers (total $N = 386$) had been administered both the test and retest (72% of the total sample). Each participant has signed an informed consent document prior to the beginning of the study. The research protocol and the informed consent documents have been revised and approved by the certified Ethics Committee of the Safety Mutual CChC.

Workers of the exposed group ($n = 284$) were recruited from agricultural companies of the Maule region that accepted to participate in the study, and they were randomly chosen from those who met the inclusion criteria. Inclusion criteria were that participants must be permanent agricultural workers, older than 18 years old, with at least 2 years of occupational exposure to pesticides, and should have normal healthy condition at the moment of the study. Exclusion criteria, on the other hand, were that workers were temporary, younger than 18 years old, and had any health condition (metabolic diseases, or any which could alter the interpretation of results). All this information has been collected through an initial "Pesticides Exposure Questionnaire" already validated in Chile from a previous study [22].

Workers of the control group ($n = 380$) were randomly selected from building companies of other regions (Santiago and Viña del Mar) far from agricultural fields and crops. Inclusion criteria were that they should not be agricultural workers, older than 18, that had not worked (neither temporally) in agriculture for the last 5 years, and that have not been occupationally exposed to any neurotoxic. This information has also been collected by a previous questionnaire.

After a first cleaning process, filtering all the missing data and eliminating subjects that did not meet the inclusion criteria, the sample reached a number of $N = 539$ for the analysis (see Figure 1).

2.2. Materials

2.2.1. Tests Selection. With the aim to select the most appropriate tests for the brief assessment tool, the content validity has been assessed through an accurate literature review and construct identification. We found different tests of processing speed, attention, and visual memory. In a second phase, an evaluation of the tools by three expert referees' with an average work experience in the field of neuropsychological evaluation of 14 years has been carried out. Based on the results of these two phases, the clock-drawing test (CDT), the frontal assessment battery (FAB), and the trail making tests (TMT) A and B have been chosen to be included in the proposed brief assessment tool. Each of these tests is described in the following sections, and a summary of the functions evaluated by them is presented in Table 1.

2.2.2. Clock-Drawing Test (CDT). The clock-drawing test is a simple paper-and-pencil task which takes a short time to be administered, corrected, and scored. Basic instruction given to the participant is to draw an analog clock displaying the 11 : 10. For the scoring, 20 items are considered, giving 0 or 1 points depending on the accomplishment of what is indicated for each item. The maximum total score is 20. Based on the study from Mendez and colleagues [24], the binary items could be grouped in aggregated scores. The authors established three groups of items by correlating the scores with other neuropsychological tests. The first group has been strongly associated with visuoperceptual measures. The second group has been associated with attention measures. The third group has not been associated with any correlated test in the aforementioned study and has been considered with items measuring numerical sequencing. In our study, we attained to this grouping, producing three subscores: visuospatial skills (CDT-VS), attention (CDT-ATT), and numerical sequencing (CDT-NS), plus an aggregated total score based on the 20 total score. Three or more errors have been considered as cognitive impairment, and normal subjects show 2 errors or below.

2.2.3. Frontal Assessment Battery (FAB). The frontal assessment battery is a brief test taking no more than 10 minutes to be administered. It is composed of 6 items exploring different abilities (see Table 1) associated to the

Table 1: Neuropsychological tests and their evaluated functions.

Test	Measured functions
Clock-drawing test (CDT; Mendez et al. [24])	Visuoperceptual skills Self-monitoring Numeric sequencing Motor execution Selective attention
Frontal assessment battery (FAB; Dubois et al.[25])	Abstract reasoning Lexical fluidity and mental flexibility Motor action executive control Self-regulation and interference resistance Inhibitory control Environmental autonomy
Trail making test (TMT-A and TMT-B; Partington and Leiter [19])	Visuospatial skills Processing speed Attention and executive functions (cognitive flexibility)

frontal lobe functions, allowing the identification of executive dysfunctions, and the early detection of diseases such as Alzheimer's, Parkinson's and progressive supranuclear paralysis (PSP). Each item can receive a score from 0 to 3 points. Authors only give a general interpretation of the tool, which is relative to the global score of FAB test, allowing evaluating the severity of the executive dysfunction and obtaining a descriptive pattern of the worse area according to the specific items showing a worse performance.

2.2.4. Trail Making Test (TMT). The trail making test is composed of parts A and B. TMT-A requires the subjects to draw continuous lines connecting 25 scrambled numbered circles following the numeric sequential order (from 1 to 25). Similarly, TMT-B requires the participant to draw a continuous line connecting alternatively the circles with numbers and those with letters following each its own sequence (i.e., 1, A, 2, B, 3, C, etc.). In both versions, the examiner instructs the subjects to never lift the pen from the paper, and if he/she makes an error, he/she has to return to the circle where the error originated and continue. The final score is represented by the total time (seconds) needed to complete the task. Accordingly, a lower score in TMT expresses a better performance, while longer times mean that the subject had problems completing the task. As a cutoff for abnormal performance, commonly 78 or more seconds for TMT-A and 273 or more seconds for TMT-B for completion time are considered.

Besides the tools described above, the Chilean-standardized Spanish version of the Wechsler Adults Intelligence Scale (WAIS-IV [23]) has been administered to the 18% of the agricultural workers sample ($n = 41$) as a gold standard to compare performances obtained with the brief assessment tool. The administration of this test took between 1 hour and half and 2 hours for each worker.

2.3. Procedures. Assistants received an intensive training in the Laboratory of the Neuropsychology and Cognitive Neurosciences Research Center (CINPSI Neurocog) in the Catholic University of Maule (UCM, Talca, Chile) previously to the beginning of all the test administrations.

Before starting the administration of the brief assessment tool, a preliminary pilot assessment including the whole set of tests has been given to 6 agricultural workers in order to fix any problems with the tool's performance. From the analysis of the performances of this preliminary sample, it was verified that the tests worked fine and did not need any change or modification to the compiled administration protocol.

The final assessment protocol has been administered to test takers in two times, with an intermediate lapse of 2–4 weeks. All tests have been given in their workplaces, and each company facilitated an appropriate space (without too much noise or interferences) for test administration. Each assessment was performed by an assistant to a worker at a time. The test taker had to read the informed consent and eventually sign a participation agreement. Then, the assistant explained briefly the sequence of actions he/she was going to perform and answered his/her eventual questions.

2.4. Analyses. Statistical analyses have been performed with R-Studio [26] and SPSS 24.0 [27]. Before starting the analyses, a coresearcher and a research assistant checked 100% of the protocols for recording integrity and correctness of scoring. Firstly, an extensive exploratory analysis has been performed to individuate all the missing data in the examined variables. After that, the variables have been checked for normal distribution with the Shapiro–Wilk test. On the basis of the results of this analysis (all the tests had a p value less than 0.001), nonparametric statistical tests were performed for the following analysis of data. Nonparametric correlations (Spearman's rho) have been performed on the test-retest evaluation, while Cronbach's alpha coefficient has been calculated to measure internal consistency of the brief assessment tool. For the construct validity test, a factor analysis has been performed, by means of which the eigenvalues >1 have been extracted with a maximum of 25 iterations to obtain convergence. Also, a direct oblimin rotation method has been applied to generate the rotated factor solution, considering the same 25 iterations to converge. The Mann–Whitney U test has been performed for the means comparisons between the two groups. An analysis of the convergent validity was made with a subsample of 41 workers to whom the WAIS-IV test was administered as a Gold Standard measure. This test is an intelligence scale composed of 10 subscales, which yields a total score that after being transformed to the standard score represents the intellectual quotient (IQ) of the test takers. In addition to the above, the estimation of the normative values of the brief scale by age group and educational level was made from the percentiles represented by each stratified score.

3. Results and Discussion

3.1. Descriptive Analysis. For this analysis, 539 workers have been considered (agricultural, $n = 234$; nonagricultural, $n = 305$). From the agricultural workers sample, 28% of them

Table 2: Demographic characteristics by group.

	Group	
	Agricultural ($n = 234$)	Nonagricultural ($n = 305$)
Mean age (DS)	46.26 (12.04)	41.13 (13.40)
Female percentage	20.5%	6.2%
Mean monthly income	623 USD	1044 USD
Educational level		
Illiterate	0.9%	1.0%
Primary (incomplete)	25.2%	10.8%
Primary (complete)	34.6%	16.4%
Secondary (incomplete)	12.4%	21.3%
Secondary (complete)	26.9%	44.3%
Technical/professional	0.0%	6.2%

Table 3: Tests scores by group, including mean, standard deviation, median, minimum, and maximum.

	Group											
	Agricultural ($n = 234$)						Nonagricultural ($n = 305$)					
	Obs.	M	DS	Med	Min	Max	Obs.	M	DS	Med	Min	Max
CDT-VS	234	9.49	1.724	10	0	11	305	9.95	1.494	10	1	11
CDT-ATT	234	3.53	0.759	4	0	4	305	3.77	0.584	4	0	4
CDT-NS	234	4.47	1.187	5	0	5	305	4.73	0.823	5	0	5
CDT total	234	17.49	3.175	19	1	20	305	18.45	2.536	19	1	20
FAB1	234	1.93	0.893	2	0	3	304	2.15	0.831	2	0	3
FAB2	234	1.96	0.978	2	0	3	304	2.19	0.853	2	0	3
FAB3	234	2.48	0.819	3	0	3	304	2.67	0.652	3	0	3
FAB4	234	2.56	0.780	3	0	3	304	2.70	0.648	3	0	3
FAB5	234	2.26	0.999	3	0	3	304	2.47	0.915	3	0	3
FAB6	234	2.95	0.282	3	0	3	304	2.97	0.256	3	0	3
FAB total	234	14.13	2.894	15	5	18	304	15.17	2.462	16	5	18
TMT-A	234	63.38	34.412	56	11	285	304	54.13	29.864	48	16	301
TMT-B	193	141.1	63.634	128	31	401	277	122.63	67.953	103	32	301

currently apply pesticides and 37.2% made the last application during 2 years or less. A 28% of this sample applies pesticides only as a temporary job, while 13.7% does it permanently. A 27% applied pesticides since less than 10 years, while 21.4% applied since 10 years or above. A 33.3% declares to have a certification of pesticide applicator. A 57.7% declare to be aware of the health hazards of the application of pesticides, and 50.4% stated that they have received training on these hazards. A 42.3% declare wearing personal protective equipment (PPE) during their work when they mixed the pesticides, and a 44% said that they changed their clothes after apply chemicals at work. Only 7.7% referred that they have been intoxicated and 3.4% that they were hospitalized after pesticides poisoning. With regard to symptoms, a 15% have experienced dizziness, nausea, fatigue, vomiting, and/or salivation while applying pesticides. A 38.5% had a cholinesterase test, and only 3.8% had an exam that indicated severe poisoning. Demographic characteristics of each group composition are shown in Table 2.

Also, descriptive statistics about the scores obtained for each group in each administered test have been calculated (see Table 3).

3.2. Content Validity. As described above, construct domains have been qualitatively identified during the tool development phase through an extensive literature review that allowed to find tests that were brief, were of public domain, and addressed the assessment of the cognitive functions of importance related to the neurotoxic effects of the most used pesticides in Chile. Then, they were revised by a pool of experts on the field of neuropsychology to evaluate their content validity. The expert judges totally agreed that CDT measured visuospatial skills (100% agreement), while partially agreed that this test measures focused attention, planning and organizational abilities, and inhibitory control. With respect to the FAB, they completely agreed (100%) that it measures planning and inhibitory control and partially agreed (75%) that it measures working memory, sustained attention, and organization ability. Regarding TMT-A, there was total agreement (100%) that it measures processing speed and partial agreement (75%) about focused and selective attention, planning, inhibitory control, and visuospatial skills. Finally, in the case of TMT-B, judges agreed (100%) that it consists in a working memory and sustained attention measurement and partially agreed (75%) that it is a self-monitoring measurement. From these interjudge evaluations, the test of the brief assessment tool with strongest relative weight was the FAB, and progressively lower weights resulted for TMT-A, CDT, and TMT-B. The cognitive function of simultaneous processing did not reach agreement for any evaluated test and therefore is not considered

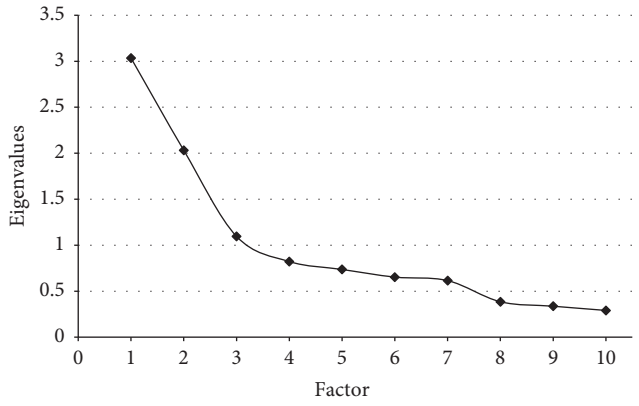

Figure 2: Screen plot with the eigenvalues of each factor yielded from the factor analysis.

as a construct included in the brief assessment tool, while focused attention and self-monitoring reached the lowest interjudge agreement level. On the other hand, the highest agreement values have been reached by visuospatial skills, selective attention, planning, processing speed, and inhibitory control.

3.3. Internal Consistency. Internal consistency analyses have been performed calculating Cronbach's alpha for each subtest, except TMT-A and TMT-B, since their scores represented times of completion and were not comparable. CDT has an alpha = 0.838, indicating a high internal consistency for its 20 items. FAB's initial analysis on all the 6 items had an alpha = 0.606; eliminating the item 6, internal consistency reached alpha = 0.633, considered as satisfactory value. For this reason, in the following analyses, this item has been not considered.

3.4. Construct Validity. Construct validity has been calculated with factor analyses on scores of the entire sample ($N = 539$). A number of 10 variables have been considered as input variables, including the 3 CDT aggregated items (visuospatial, attention, and numerical sequencing), 5 FAB items, and TMT-A and TMT-B scores. The sample obtained a KMO = 0.742 at the Kaiser–Meyer–Olkin value (recommended value is 0.6). The Bartlett's sphericity test was applied and resulted in a value of $\chi^2\ (45) = 1291$, $p < 0.001$, which indicates that the correlation structure is adequate to perform factor analyzes. The principal axis factoring (PAF) was used as the extraction method, since the data of the tools were not normally distributed [28]. Then, the instrument was subjected to a direct oblimin rotation method with a cutoff of 0.30 and using the Kaiser's criterion of eigenvalues greater than 1, which yielded a three-factor solution as the best fit for the data, accounting for 61.62% of the variance. The factors four to ten resulted with eigenvalues below 1 (Figure 2), only explaining between 8% and 2% of variance. The results of this factor analysis are summarized in Table 4. The item 2 of the FAB was eliminated, since it was not integrated into any of the factor solutions tested, with factor weight less than 0.30.

The representation of the resulting three-factor solution was made using the labels that emerged from the evaluation of expert judges with respect to the skills measured by the tests included in the brief assessment tool. After the interpretation of the components as described, the following descriptors were obtained for each one: (a) visuospatial skills and processing speed: this factor had an eigenvalue of 3.03 and accounted for 30.34% of the variance; (b) planning: the eigenvalue of this factor was 2.03 and accounted for 20.31% of the variance; (c) selective attention and inhibitory control: this factor had an eigenvalue of 1.09 and accounted for 10.9% of the variance.

We compared means of each test between groups with the nonparametric Mann–Whitney U test, observing that each scale has significant different scores in both groups ($p < 0.001$). Results and values are shown in detail in Table 5.

3.5. Convergent Validity. To examine convergent validity of the factor solution, the WAIS-IV test was used as a gold standard, and it was administered to 18% of the sample of agricultural workers ($N = 41$). From this sample, it is verified that the instruments have a significant association with the performance assessed by the gold standard test. First off, all the tests of the brief assessment tool showed a significant correlation with the full-scale IQ score. The tools that correlate with all the four factor indexes of the WAIS-IV are the adjusted FAB (FABadj) and the TMT-A. The CDT correlates only with the perceptual reasoning index, and the TMT-B correlates with the perceptual reasoning index as well, but also with the working memory index. The results of the convergent validity analysis are detailed in Table 6.

3.6. Test-Retest Reliability. For the estimation of the brief assessment tool's reliability in terms of its stability in time, a subsample of participants from both groups has been given the brief protocol twice, with an interval period after the first administration of about 2–4 weeks. The correlation between both periods was estimated with Spearman's rho test for each assessment tool, resulting in all the values to be significantly correlated and, hence, stable in time. Detailed values for each test and group are shown in Table 7.

3.7. Normative Data of the Brief Assessment Tool Stratified for Age and Educational Level. Correlations among the demographical variables (age, educational level, and household monthly income) and scores on each of the tests show that only age and educational level were correlated with the scores of all the instruments of the brief assessment tool (see Table 8). Therefore, stratification of the normative data according to these 2 variables has been performed.

In the case of the variable age, it was divided into four groups based on the age distribution of the sample, taking into account the quartiles observed. On the other hand, for the educational level, it was stratified into two groups, the first from 0 to 8 years of education, composed by workers with an educational attainment of elementary educational level, complete or incomplete. The second group is

Table 4: Exploratory factor analysis results for the tests of the brief scale.

Items	Factor*			Dimension
	1	2	3	
TMT-A	−0.855			Visuospatial skills and processing speed
TMT-B	−0.803			
CDT-VS		0.761		Planning
CDT-ATT		0.814		
CDT-NS		0.791		
FAB1			0.433	Selective attention and inhibitory control
FAB2	—	—	—	
FAB3			0.718	
FAB4			0.505	
FAB5			0.494	

FAB items description: FAB1: similarities; FAB2: lexical fluidity and flexibility; FAB3: sequences (programming); FAB4: conflicting instructions; FAB5: go/no go. *Only values of factor weight higher than 0.30 are shown.

Table 5: Group means comparison of performance in each test of the brief assessment tool included in the factor solution.

Test	Group		U	z	p
	Agricultural (exposed)	Nonagricultural (not exposed)			
CDT-VS	239.26	293.58	28492.5	−4.21	0.000
CDT-ATT	242.57	290.23	29266	−4.70	0.000
CDT-NS	256.49	279.51	32523.5	−2.60	0.000
FABadj*	240.8	291.59	28851.5	−3.82	0.000
TMT-A	302.04	244.45	27953	−4.26	0.000
TMT-B	267.27	213.36	20598.5	−4.23	0.000

FABadj includes only items 1, 3, 4, and 5 from the original FAB.

Table 6: Correlations coefficients (Spearman's rho) of each test included in the brief assessment tool and WAIS' FSIQ and indexes.

	FSIQ	VCI	PRI	WMI	PSI	CDT	FABadj	TMT-A
FSIQ	1							
ICV	0.72**	1						
IRP	0.76**	0.28	1					
IMT	0.78**	0.61**	0.55*	1				
IVP	0.63**	0.34*	0.36*	0.29	1			
CDT	0.3*	0.42	0.39*	0.20	0.31	1		
FAB Adj	0.47**	0.32*	0.31*	0.49*	0.33*	0.21*	1	
TMT-A	−0.57**	−0.4*	−0.51	−0.32*	−0.49*	−0.29**	−0.45**	1
TMT-B	−0.41*	−0.3	−0.36*	−0.39*	−0.2	−0.25**	−0.49**	−0.7**

WAIS-IV indexes: FSIQ total = full-scale intelligent quotient; VCI = verbal comprehension index; PRI = perceptual reasoning index; WMI = working memory index; PSI = processing speed index. * = $p < 0.05$; ** = $p < 0.005$.

composed of participants who had between 8 and 12 years of studies, having an incomplete or complete high school educational attainment.

Figures 3 and 4 show the mean scores of the tests included in the brief assessment tool for each age group and according to the educational level, based on the stratification previously described. It is evident that in all the tests there is a detriment in the performance at older age, being more pronounced in the case of the group with lower educational level.

Table 9 shows the normative data of Chilean agricultural workers for the brief validated assessment tool, transformed into percentiles for each age group and educational level. It should be noted that for the older age group, there were no subjects with more than eight years of education. Therefore, for that group are only assumed for people with eight or less years of education.

4. Discussion

We have presented the process and the main results of the validation and reliability study of a brief screening scale to measure cognitive deterioration in occupational populations that are exposed to neurotoxins through the widespread use of pesticides. To date, there have been no similar studies with tools administered in Chile with these characteristics and that allow knowing in a specific way how they work with agricultural workers to monitor health and any signs of cognitive deterioration.

The main purpose has been achieved in terms of developing a tool composed of public domain tests, with no cost, simple to apply, and that could be administered by health and safety technicians due to its ease of administration and punctuation. The abridged tool complies with all

Table 7: Reliability (stability) of test-retest scores in both groups.

Test and groups	Obs.	Rho	p
Agricultural			
CDT-total	196	0.477	0.000
CDT-VS	196	0.475	0.000
CDT-ATT	196	0.408	0.000
CDT-NS	196	0.420	0.000
FABadj	196	0.421	0.000
TMT-A	194	0.659	0.000
TMT-B	194	0.733	0.000
Nonagricultural			
CDT-total	193	0.553	0.000
CDT-VS	193	0.549	0.000
CDT-ATT	193	0.445	0.000
CDT-NS	193	0.440	0.000
FABadj	193	0.384	0.000
TMT-A	193	0.726	0.000
TMT-B	177	0.660	0.000

Table 8: Correlations among age, education, and household income with the tests.

	Age	Education	Family income	CDT	FABadj	TMT-A
Age	1					
Education	−0.60**	1				
Household income	−0.06	0.06	1			
CDT	−0.16*	0.22**	0.04	1		
FABadj	−0.35**	0.37**	0.06	0.12	1	
TMT-A	0.51**	−0.40**	−0.08	−0.24**	−0.39**	1
TMT-B	0.45**	−0.39**	−0.08	−0.21**	0.46**	−0.65**

* $p < 0.05$; ** $p < 0.005$.

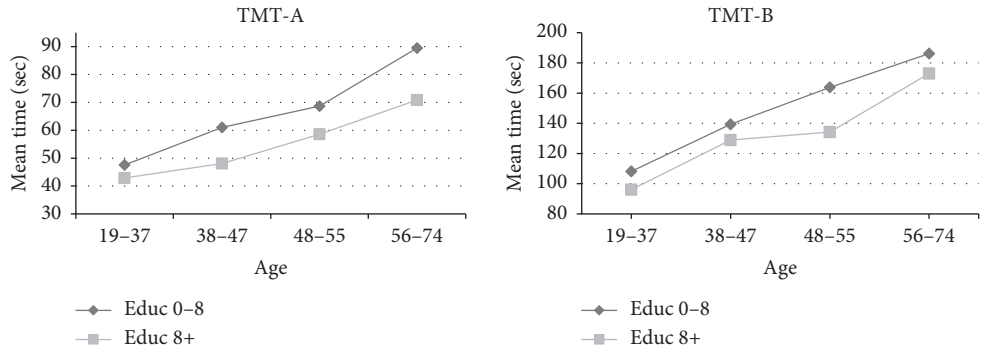

Figure 3: Performance in TMT-A and TMT-B, according to the four age groups and the two educational levels.

the above characteristics and has also been proven to have content and construct validity with respect to the measurement of skills that are associated with those that according to the literature are among the most deteriorated in the face of chronic exposure to acetylcholinesterase inhibitory pesticides, which are the most commonly sold and used pesticides in the Chilean agricultural setting. Previous studies have shown that OP exposure impacts neurological wellbeing such as the executive functions, such as attention (sustained and focused), planning, inhibitory control, and also the visuospatial ability and processing speed [3–7]. Based on the criterion of judges, the content validity was positively verified, since the brief tests included addressed the measurement of these skills and then their pertinence was verified from the underlying factor structure with the three factors that group the different parts of the scale: (1) visuospatial skill and processing speed; (2) planning capacity; (3) selective attention and inhibitory control.

For the study, we administered the gold standard measure (WAIS-IV) to compare the scores obtained from the scale tests and evaluate their convergent validity. Our results showed that in the agricultural occupational population, the TMT-A and the FABadj are the measures most correlated with the level of global intellectual functioning (FSIQ). It is worth mentioning that all the tests included in the brief assessment tool correlate with the FSIQ or with

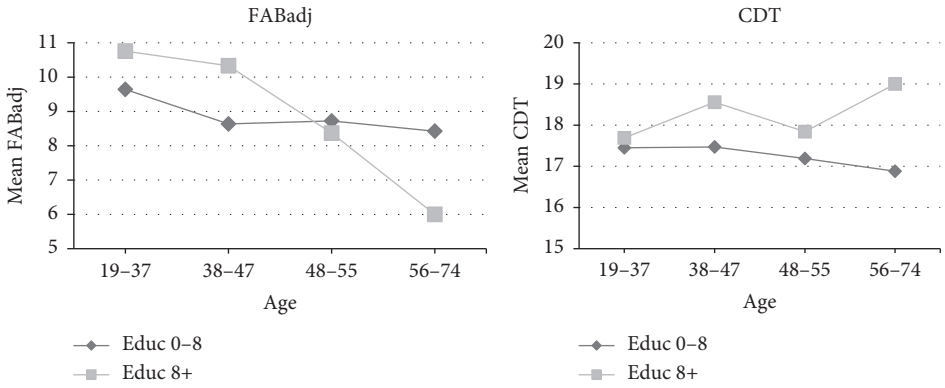

Figure 4: Performance in FABadj and CDT, for the four age groups and the 2 educational levels.

Table 9: Percentiles for the brief assessment tool for each normative group.

Percentile	Education 0–8 years				Education 8 + years			
	CDT	FABadj	TMT-A	TMT-B	CDT	FABadj	TMT-A	TMT-B
19–37 years old ($n = 56$)								
95	20	12	33	72	20	12	22	53
90	20	12	33	72	20	12	26	62
75	19	12	34	75	20	12	32	73
50	19	10	39	94	19	11	50	102
25	17	8	52	124	16	10	58	128
10	10	6	67	143	12	9	63	177
5	9	6	67	143	9	8	69	195
38–47 years old ($n = 63$)								
95	20	12	30	81	20	12	27	54
90	20	12	33	90	20	12	30	59
75	19	11	43	109	20	12	37	81
50	18	9	55	135	19	11	45	121
25	18	7	69	183	18	9	57	146
10	12	3	87	248	14	7	80	216
5	6	2	107	294	13	5	87	318
48–55 years old ($n = 62$)								
95	20	11	39	65	20	11	33	73
90	20	11	42	93	20	11	37	79
75	19	11	49	114	20	11	48	107
50	18	9	62	149	19	8	58	129
25	16	8	79	199	17	6	64	168
10	13	5	93	251	14	5	85	194
5	10	2	109	274	12	4	85	194
56–74 years old ($n = 53$)								
95	20	12	36	95	–	–	–	–
90	20	11	45	104	–	–	–	–
75	19	10	58	116	–	–	–	–
50	18	9	79	168	–	–	–	–
25	16	7	104	220	–	–	–	–
10	12	3	143	311	–	–	–	–
5	7	3	197	370	–	–	–	–

some index of cognitive functioning measured by the Gold Standard test.

The reliability was verified both in terms of internal consistency of the tests, with values from satisfactory to high consistency, and in terms of stability over time of the measurement through test and retest. Even though all the tests of the scale showed a high and significant stability in its administration over time, the Trail Making test stands out especially in this sense, being the test with greater stability as a measure in the agricultural population.

It is expected that in the case of the CDT, the scores obtained will be from 15 points up, with a low difference expected between those cases that show deterioration compared to those who do not. In the case of the FAB, the psychometric analysis itself has led to a decrease in the number of items, because two of its original items (2 and 6)

had little association with the factor solution. To administer this brief assessment tool, the items are maintained in its original composition for the CDT test and for the TMT-A and TMT-B tests. To administer the FAB, a most brief version named "FABadj" here should be used, which considers only 4 of the 6 original items (1, 3, 4, and 5). For the interpretation of the test from the norms reported here, it is advisable to follow the sequence given below:

(1) Set the age group and education level of the evaluated person

(2) Transform the raw score to percentile according to the normative group of the test taker

(3) Report the percentiles obtained for each of the 3 factors that would be measured by each test of the scale: Visuoperceptual ability and processing speed (TMT-A, TMT-B), planning capacity (CDT), and selective attention and inhibitory control (FABadj)

A percentile of 25 or below indicates a cognitive deterioration in the specific factor measured. It should be considered that in the last normative group, the oldest, there were no subjects in the sample with more than eight years of study, so when the scale is administered to people over 56 years old, the data of the only age group available should be used to convert to percentiles. It should also be mentioned that although the TMT-B test is part of the validated scale, in our case, we observed that there were around 15% of cases where it was not possible to complete the administration because it requires knowledge of the sequence of the alphabet. It is expected that when the TMT-B is administered in an agricultural population, this type of situation could be found in workers with a very low educational level. In those cases, it would be convenient to suspend the administration of this part of the brief assessment tool in particular. Also, in cases of the TMT-B, when the test taker has a completion time of more than 300 seconds, it is advisable to suspend their administration because they have exceeded what is conventionally stated as a time limit. This would also be advisable considering the normative data obtained in the present study for agricultural occupational population since there were a minimum percentage of cases (around 1%) that had longer times of completion at that level. Therefore, it is advisable to suspend the administration of TMT-B, because in any case, its performance would be located within the 5% of worst performance according to the norm.

5. Conclusion

It can be concluded that the aim of this work has been met satisfactorily, establishing the validity and reliability of a brief assessment tool for the monitoring of health effects due to permanent occupational exposure to neurotoxic pesticides. Also, for the first time in Chile, there are specific norms for agricultural workers population, with instructions for administration and interpretation with ease for its potential extended use in the prevention of cognitive deterioration in the health of agricultural workers.

Data Availability

The datasets are available from the first corresponding author on reasonable request. The ethical approval granted to the authors does not allow the publication of the dataset online. If readers would like to reanalyse the dataset, additional ethical approvals (on an individual user and purpose basis) will be required.

Disclosure

This work was selected in the Call for Projects of Research and Innovation in Prevention of Accidents and Professional Illnesses 2016 of the Superintendence of Social Security (Superintendencia de Seguridad Social del Gobierno de Chile, SUSESO).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

The Importance of Land Use Definition in Human Health Risk Assessment Related to Lead in Soils

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In many countries, soil contamination and lead exposure is a persistent human and environmental health issue, while in others, it is an emerging concern. Defining the extent of lead contamination and assessing human health risk allow for efficient prevention agendas. The different types of land uses delimit the exposure frequency and hence can influence the evaluation of possible threats. In this study, human health risk assessment is performed under different land use scenarios, after determining the concentration of lead in topsoil of a rehabilitated space. An analytical hybrid method was used to determine the concentrations of the heavy metal. Human health risk indicators, hazard quotient and cancer risk, were subsequently calculated and compared under such scenarios of varying population exposure by land use. Results indicate that an increasing exposure can set health risk indicators above the tolerable levels. Correctly defining the exposure frequency by land use is very important to determine the actual risk levels of a site. Local regulators should take this information into account before designing prevention plans, especially in localities where migration and urbanization are major development factors and since the land use of a public place could change over time and alter the exposure frequency to soil.

1. Introduction

In a never-ending urbanizing and industrializing world, exposure to Pb in soils is an overlooked global concern. In developed countries, there still are several sites where Pb contamination has not been remediated, while in developing countries, Pb contamination is only starting to be reckoned. Migration phenomena between the two types of countries can also contribute to increasing exposure to contaminants since exposure often coincides with development conditions such as access to redeveloped spaces turned into urbanized areas. Since Pb not only causes noncarcinogenic effects on the population but is also considered a class B2 carcinogen by WHO and USEPA [1, 2], its monitoring and evaluation should be continuous in order to avoid repercussions for future generations.

Soils can have high Pb content due to anthropogenic or natural activities. Soils contaminated with Pb can pose risks, since population exposed to the metal by ingestion or inhalation may show health complications such as neurological, renal, and cardiovascular [3]. In order to avoid such exposure, the first step is to carry out an analytical characterization of soils with the goal of detecting high Pb concentrations. USEPA sampling and analytical methods [4–6] are used for most soil characterizations, where the principal techniques recommended for the determination of inorganic analytes include atomic absorption (AA), inductively coupled plasma mass spectrometry (ICP-MS), and X-ray fluorescence (XRF)

[7, 8]. Nonetheless, methods that involve combined analytical techniques have also been successfully used lately in environmental analysis [9, 10].

The second step to minimize the exposure related to Pb pollution is to perform risk assessment. Human health risk assessment seeks to determine carcinogenic risks and noncarcinogenic hazards caused when the population is exposed to toxic chemicals present in soil or other media. One of the parameters calculated in the assessment is the chronic daily intake value (CDI). CDI values represent the amount of chemical substance that a person receives over a period of time. CDI values, and hence risk indicators, depend on a factor called exposure frequency (EF), which is the number of days per year that a person is assumed to be in contact with a toxic chemical substance. EF values vary according to the land use of the study area. For instance, in a scenario of residential land use, residents are in repeated contact with the contaminated soil for ~350 days per year; in a scenario of industrial land use, workers are exposed for ~250 days based on potential equipment use; and in a scenario of recreational land use, adults and children spend only ~40 days in contact with the media since such activities are occasional. This way, different types of land use determine different exposure frequencies and influence the evaluation of risks. Finally, the third step to minimize metal exposure is to socialize results with local regulators to raise awareness and propose possible guidelines.

In this study, human health and ecological risk assessment is performed under different land use scenarios after characterizing Pb across a public area. The main goal is to determine the influence of exposure to Pb in the evaluation of risks since land use scenarios are a determining factor in the calculation of chronic daily intake values. Local and global regulators could benefit from these results since the correct determination of land use and exposure frequency values can improve any future monitoring and prevention plans.

2. Materials and Methods

2.1. Sampling and Analytical Procedures. A public place in Athens, Greece, was referenced by using a GPS and long measuring tape according to Figure 1. A total of 91 superficial soil samples were collected by removing 400 g of topsoil (0–20 cm) with a spatula. Any vegetation residues or gravel were removed from the samples before storing them in commercial polypropylene bags. After transportation to the laboratory, samples were dried for 24 h at 60° and disaggregated in a mortar. Before analytical procedures, samples were quartered and sieved to <250 μm particle size in order to improve homogeneity of the subsamples. Loss of sample soil due to this treatment was insignificant [4, 11, 12]. Samples were collected during the first semester of 2016. The study area is a redeveloped park where people gather for leisure since the 2000s and is surrounded by urbanized streets where commercial and residential infrastructure can be found. The area was a recreational shooting range in the past. It has an area of ~70 ha and is covered by simple vegetation and coniferous trees.

Figure 1: Sampling locations for Pb exposure analysis. Gray squares represent samples analyzed by using XRF only. Crossed grayed squares represent samples analyzed by XRF and ICP-MS.

Analytical techniques used to obtain Pb concentration in soil samples were ICP-MS and XRF. The samples were analyzed both by XRF directly (91 samples) and ICP-MS after total acid digestion (13 samples), in order to obtain 91 corrected XRF measurements that match ICP-MS accuracy following a hybrid methodology [9, 10]. Details on the hybrid methodology can be found in the previous publications [10, 13]. Briefly, to obtain XRF measurements, samples were kept inside their plastic bags and homogenized. The nose of the device was placed on top of the sample keeping a layer of soil of at least 3 cm below. The measurements were taken for 90 s. As part of the quality control process, the following certified reference materials were used: NIST 2710a, NIST 2711a, and CRM 023 [7]. On the other hand, to obtain ICP-MS measurements, samples were sieved to <250 μm particle size, and the resulting fraction was dissolved with nitric acid and subsequently diluted into a 100 mL volumetric flask with deionized water. The quality control process included certified reference materials, blanks, and duplicates [7, 14, 15]. Finally, the data obtained by using XRF were fitted to match the corresponding measurements obtained by using ICP-MS. The linear regression (trend equation) calculated between these data points was applied to the rest of the XRF data points to match accuracies with constant correlation ($R^2 > .090$) [9, 10].

Table 1: Parameters and values used in human health risk assessment.

Parameter	Name	Units	Value
C_{exp}	Concentration of the trace element	ppm	Element dependent
R_{ing}	Ingestion rate	mg/day	200, children 100, adults
EF	*Exposure frequency*	*days/year*	*40, recreational*
ED	Exposure duration	years	6, children 24, adults
BW	Body weight	kg	15, children 70, adults
AT	Averaging time	days	ED × 365
R_{inh}	Inhalation rate	m^3/day	7.5, children 20, adults
PEF	Particle emission factor	m^3/kg	1.36×10^9
SA	Exposed skin area	cm^2/day	2800, children 5700, adults
SAF	Skin adherence factor	mg/cm^2	0.2, children 0.07, adults
ABS	Dermal absorption factor	—	0.001, noncarcinogenic 0.01, carcinogenic

2.2. Reagents and Instrumentation. Reagent-grade chemicals and deionized water were used in the experimentation. Specific acids and stock solutions were obtained from Sigma-Aldrich (St. Louis, MO, USA). Certified reference materials were obtained from NIST (Gaithersburg, MD, USA). Instrumental analysis was carried out by XRF using an Olympus (Newton, MA, USA) Delta Premium 6000 device and ICP-MS using a Thermo Fisher (Waltham, MA, USA) X Series 2 instrument.

2.3. Human Health and Ecological Risk Assessment. Health risk indicators, hazard quotient (HQ) and cancer risk (CR), were calculated for Pb [3, 16–21]. Acceptable levels for total HQ values are smaller than one while tolerable levels for total CR values are in the range of 10^{-6}–10^{-4} [16–18, 20, 21]. Calculations were based on the following parameters:

(1) Chronic daily intake (CDI) value depends on the concentration of the metal across the site. CDI values represent in this case the amount of Pb absorbed by a person over the studied period. Each route of exposure defines its respective CDI value (CDI_{ing}, CDI_{inh}, and CDI_{dermal}) and was calculated according to equations (1)–(3). Specific additional parameters can be seen in Table 1.

(2) Reference dose (RfD) values are 3.5×10^{-3} mg/kg/day for ingestion/inhalation and 5.25×10^{-4} mg/kg/day for dermal contact. RfD represents the maximum daily Pb exposure that would not yield effects on the population. HQ values were calculated according to equation (4). The total HQ value is obtained by adding the correspondent singular values regarding ingestion, inhalation, and dermal contact since effects can be considered accumulative [16].

(3) A slope factor (SF) value of 8.5×10^{-3} mg/kg/day allows the calculation of CR according to equation (5). SF represents the incremental chance of a person to develop Pb-related cancer over a lifetime under the mentioned exposure.

The ecological risk indicator, Potential Ecological Risk Index (RI), was calculated for the study area in the total absence or presence of Pb following equation (6). RI allows an evaluation of potential adverse ecological effect of metals in soil based on toxic response factors (T_r^i) of 10, 2, 30, 5, 1, 5, and 5 for As, Cr, Cd, Cu, Zn, Pb, and Ni [22, 23]. Such metals were analyzed for every sampling point according to the same procedure followed for Pb. B_n values were obtained from Rudnick and Gao [24]. Calculated RI values greater than 150 define a moderate risk, greater than 300 a considerable risk, and greater than 600 a high ecological risk.

$$\mathrm{CDI}_{\mathrm{ing}} = C_{\mathrm{exp}} \times \frac{R_{\mathrm{ing}} \times \mathrm{EF} \times \mathrm{ED}}{\mathrm{BW} \times \mathrm{AT}} \times 10^{-6}, \quad (1)$$

$$\mathrm{CDI}_{\mathrm{inh}} = C_{\mathrm{exp}} \times \frac{R_{\mathrm{inh}} \times \mathrm{EF} \times \mathrm{ED}}{\mathrm{PEF} \times \mathrm{BW} \times \mathrm{AT}}, \quad (2)$$

$$\mathrm{CDI}_{\mathrm{dermal}} = C_{\mathrm{exp}} \times \frac{\mathrm{SA} \times \mathrm{SAF} \times \mathrm{ABS} \times \mathrm{EF} \times \mathrm{ED}}{\mathrm{BW} \times \mathrm{AT}} \times 10^{-6}, \quad (3)$$

$$\mathrm{HQ} = \frac{\mathrm{CDI}}{\mathrm{RfD}}, \quad (4)$$

$$\mathrm{CR} = \mathrm{CDI} \times \mathrm{SF}, \quad (5)$$

$$\mathrm{RI} = \sum_{i=1}^{7} T_r^i \left(\frac{C_n}{B_n} \right). \quad (6)$$

2.4. Lead Exposure Influence. The exposure to Pb in soil from public places occurs when soil particles come in contact with the human body (mouth, nose, or skin). Additionally, the abovementioned indicators of human health risk assessment depend on the EF (exposure frequency) parameter defined in Table 1. However, the definition of exposure to Pb in the study area can be complex since the park's focus is recreational but commercial and residential buildings also surround the whole area. Initially, EF parameter was set to 40 for recreational land use, but people can be exposed to such soils for more than 40 days every year. This way, additional HQ and CR calculations were performed with different EF values (20, 40, 100, 150, 180, 250, and 350) in order to analyze the influence that varying exposure frequencies to Pb would cause. Plots were prepared to show the variation of both indicators, and maps were devised to show the variation in spatial risk for adults and children due to Pb contamination with increasing exposure.

2.5. Statistical Analysis. Descriptive statistics and regression analysis calculated for Pb included median, mean, and standard deviation. Statistical analysis was performed by using software packages XLSTAT® (Addinsoft, Paris, France 2017) and Minitab® (State College, PA, USA). Spatial representation of human health risk assessment was performed by using the SADA software of the University of Tennessee Knoxville (Knoxville, TN, USA).

3. Results and Discussion

Accuracy and precision analyses showed a range of RSD (relative standard deviation) values from 1 to 2%, and mean recoveries calculated were 108%, for XRF. Calculated RPD (relative percentage difference) and mean recoveries for ICP-MS were 8 and 103%, respectively. Pb concentrations in topsoil show a range from 43 to 86,000 mg/kg. Such a wide range of values can be attributed to an extremely heterogeneous metal distribution, which in turn coincides with the abovementioned method of Pb deposition (shooting range). The mean concentration is 7,160 mg/kg, and the standard deviation is 12,689 mg/kg, which confirms a high heterogeneity in the study area [25, 26].

3.1. Human Health and Ecological Risk Assessment. Descriptive calculated parameters resulted as follows.

The total CDI value for adults was 3.94×10^{-3} based on a Pb concentration of 25,067 mg/kg (95% UCL) and as a result of adding partial CDI values 3.92×10^{-3}, 1.15×10^{-6}, and 1.57×10^{-5} for ingestion, inhalation and dermal contact, respectively. Likewise, total CDI was 3.67×10^{-2} for children. It can be noted that the main exposure contributions for Pb follow the order ingestion > dermal contact > inhalation. It can be suggested that at least some policies should be put in place to protect the people who gather around the study area. Children, specially, are prone to play in the dirt and hence would have a greater chance of Pb intake due to high concentrations of the heavy metal. The CDI value for children is almost ten times bigger than the one for adults and hence the calculated risk will be similarly greater.

The total HQ value for adults was 1.15×10^{0}, while the total HQ value for children was $1.07 \times 10^{+1}$. HQ values for ingestion, inhalation, and dermal contact in adults were 1.12×10^{0}, 3.30×10^{-4}, and 2.98×10^{-2}, respectively. The tendency ingestion > dermal contact > inhalation remains. Noncarcinogenic hazard related to Pb contamination in the study area is ten times bigger for children than for adults and is greater than one for both adults and children, meaning that there is a real threat for the residents of the area. Such HQ values are much greater than others reported in parks and playgrounds from Spain, Turkey, Portugal, Montenegro, and China [17, 20, 27–29]. However, these additional reports use different exposure frequency values and hence comparisons are referential only. There appears to be some influence of the exposure frequency in the resulting noncarcinogenic hazard evaluated.

The CR value for adults was 1.14×10^{-5}, while the CR value for children was 2.67×10^{-5}. Carcinogenic risk related to Pb contamination in the study area is two times bigger for children than for adults. In both cases, CR values fall inside the tolerable range of 10^{-6}–10^{-4} but are greater than others reported in parks and playgrounds from China [20] with different exposure frequencies. Once again, the EF value seems to influence the results for carcinogenic risk.

On the other hand, the mean RI value for the study area depended on the mean concentration of As (6.2 mg/kg), Cr (177.9 mg/kg), Cd (not detected), Cu (32.6 mg/kg), Zn (75.0 mg/kg), Pb (7,160 mg/kg), and Ni (92.0 mg/kg) detected in the soil samples. The total RI value calculated was 2,022, which is far above 600, the limit for high ecological risk.

3.2. Lead Exposure Analysis. Figure 2 shows how the calculated hazard quotient (HQ) values increase according to different exposure frequency (EF) values. Reference lines for HQ = 1 and HQ = 10 can be seen for comparison purposes since HQ values greater than one yield risk scenarios. It can be seen that Pb in the study area does not pose a health risk for adults only if exposure frequencies smaller than 40 days/year are considered. This can be the case for scenarios of excavation land use (EF = 20). However, increasing EF values for different land uses yield HQ values for adults of 1.15 (recreational, EF = 40), 7.19 (industrial, EF = 250), and 10.01 (residential or agricultural, EF = 350), which pose a significant health risk. In the case of children, it can be noted that even the smallest EF value, for excavation land use, yields HQ values greater than one and increases linearly to very noteworthy risk values. Properly defining EF values according to land use scenarios is very important in order to accurately perform risk assessment by local authorities for monitoring or possible posterior treatment.

In the same way, Figure 3 shows the calculated cancer risk (CR) values. When exposure frequency (EF) values

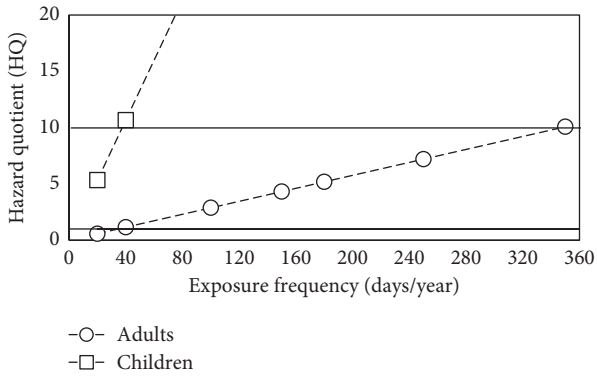


Figure 2: Noncarcinogenic Pb-related hazard values for adults and children based on different exposure frequencies.

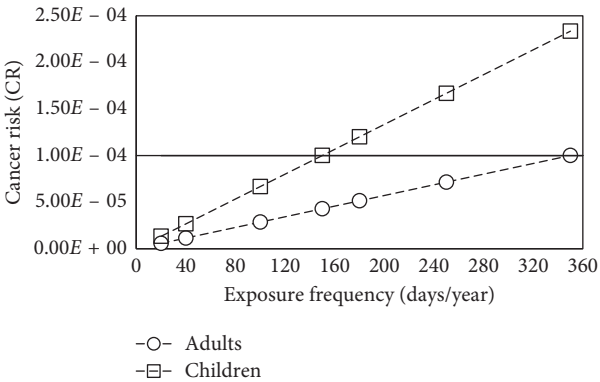


Figure 3: Carcinogenic Pb-related risk values for adults and children based on different exposure frequencies.

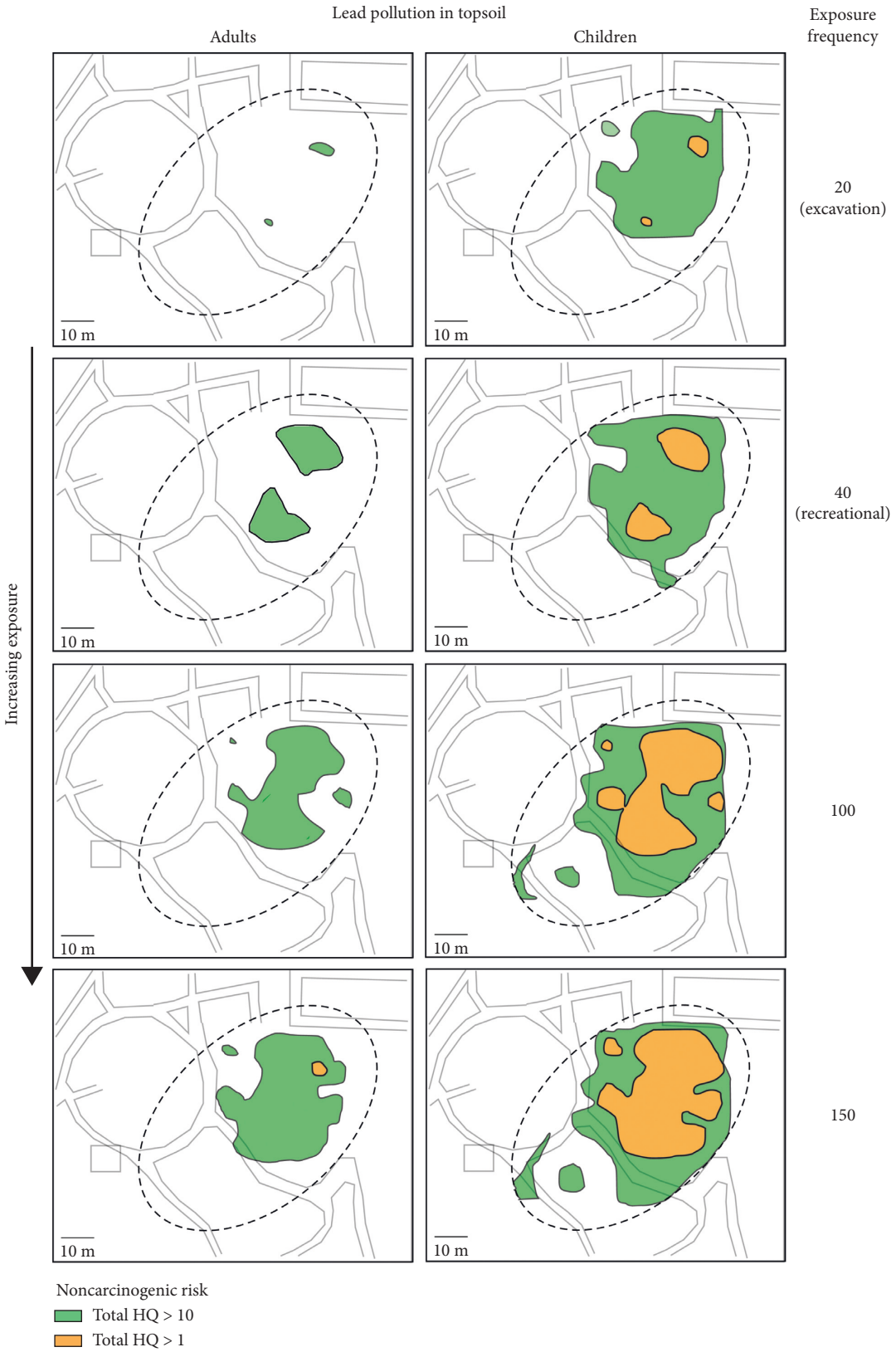
increase, the indicator also increases. A reference line of 1.00×10^{-4} can be seen since the tolerable zone is in the range of 10^{-6}-10^{-4}. As can be noted, the study area sits in the tolerable cancer risk zone for adults. However, an exposure frequency greater than 350, for residential or agricultural land use, would be in the limit between the tolerable area and the cancer risk area. In the case of children, Pb-related cancer risk sits in the tolerable zone for exposure frequencies smaller than 160, which includes excavation and recreational land use scenarios. On the other hand, the CR value becomes present and a real threat for exposure frequencies greater than 160, which includes industrial, residential, and agricultural land use scenarios. It would be important for local authorities, to define the precise exposure frequency that adults and children of the area have with respect to Pb, in order to determine whether a possible cancer risk is feasible.

Once it has been established that human health and ecological risk assessment depend on the exposure frequency chosen for a given study area, it is relevant to analyze such variations on a spatial scale in addition to a general scale. In the previous paragraphs, HQ, CR, and RI values were calculated using a concentration value for Pb in soil that represents the 95% upper confidence limit for measured values. This Pb concentration value was used to generate one indicator that covers the whole site. However, it is understood that every location on the area has a different Pb concentrations and hence should yield different human health and ecological indicators. After calculating HQ, CR, and RI values for every sampling point in the study area, spatial plots were prepared by applying the natural neighbour interpolation method.

Figure 4 shows a spatial representation of Pb-related noncarcinogenic hazard for adults and children, with different exposure frequency values. It can be seen that the risk area increases with similarly increasing exposure. On an excavation land use scenario, the risk for adults is minimal and the risk for children is mostly present in the top-right corner of the area. On a recreational land use scenario, the risk for adults starts to appear and the risk for children covers more than half of the study area with patches of >10 risk. On an industrial land use scenario, the risk for adults is very noticeable and the risk for children has spread out as well as the >10 risk area. Finally, on a residential land use scenario, the risk for adults covers more than half of the study area with patches of >10 risk, and the risk for children almost covers the whole study area with a big proportion being >10 risk area. It can be noted from the figure that the area in green (HQ > 1) increases in size with increasing exposure, as well as spots of higher risk (area in orange, HQ > 10) appear similarly.

There are very noticeable differences between the plots for Pb-related noncarcinogenic hazard in the study area, and there lies the importance of defining the exposure frequency correctly. Situations where heavy metal concentrations yield risk indicators close to the tolerable limits should include a finer analysis of population exposure to determine the most precise risk value. Similarly, study areas where occupational changes will be made in the future due to varying land use or urbanization evolution should take this information into account to prevent threats to the population and the environment.

Regarding ecological risk, Figure 5 shows a comparison of the hypothetical RI spatial distribution excluding and including the presence of Pb in the study area. For this particular site, RI values range from 10 to 84 with a mean of 34 when not considering Pb, which does not result in any risk level. However, when factoring Pb as part of the study area, RI values range from 40 to >10,000 with a mean of 2,022, which results in very high ecological risk. In fact, taking Pb into account, it can be seen that there are only small portions of the area where the ecological risk is <150 suggesting that the majority of the study site suffers from some degree of ecological risk. This public place is an example of the importance of analyzing Pb in topsoil from former shooting ranges since it could comprise risks for both humans and the environment.



(a)

Figure 4: Continued.

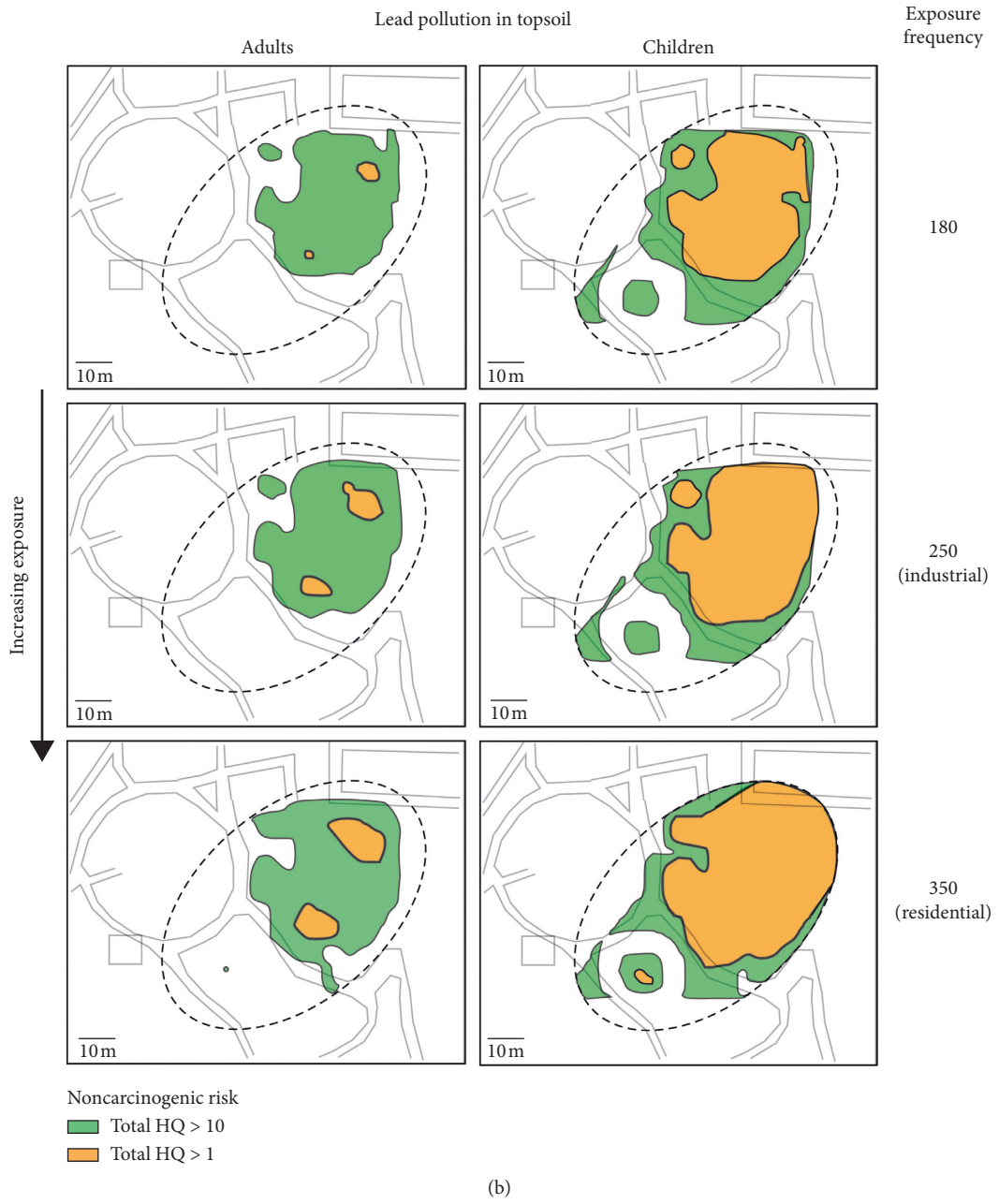


Figure 4: Spatial representation of noncarcinogenic Pb-related risk for adults and children based on different exposure frequencies.

4. Conclusions

This study has analyzed the influence of Pb exposure in the assessment of human health and ecological risk. High concentrations of Pb were found across a study area. For adults and children, human health risk indicators HQ and CR show the presence of Pb-related noncarcinogenic hazard and a tolerable Pb-related carcinogenic risk, respectively. High ecological risk is also found by using the indicator RI. An analysis of the variations in human health risk indicators due to Pb exposure has led to the conclusion that land use scenarios play an important role in the determination of risks both generally and spatially. Situations where metal concentrations yield human health risk indicators close to tolerable zones should define exposure frequencies precisely. This analysis, in turn, should be granular in scenarios of possible land use changes or areas with future urbanization. Appropriately defining exposure values according to land use will allow local authorities and policy makers to perform and design monitoring and treatment procedures suitably.

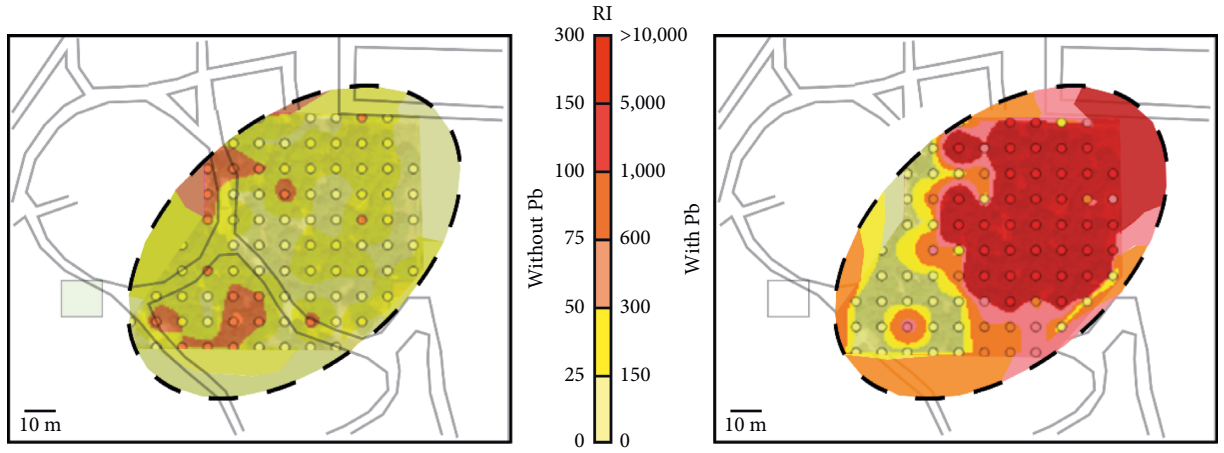


Figure 5: Spatial representation of ecological risk assessment in absence and presence of Pb in the study area.

Data Availability

The metal concentrations and other data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Effects of Pyrene on Human Liver HepG2 Cells: Cytotoxicity, Oxidative Stress, and Transcriptomic Changes in Xenobiotic Metabolizing Enzymes and Inflammatory Markers with Protection Trial Using Lycopene

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Pyrene is one of the major polycyclic aromatic hydrocarbons formed during heat treatment of meat and in car exhausts; however, few studies have investigated pyrene-induced adverse effects on human cell lines. This study aimed at the investigation of pyrene-induced cytotoxicity and oxidative damage in human liver HepG2 cells at environmentally relevant concentrations. Pyrene-induced changes in mRNA expression of xenobiotic metabolizing enzymes (XMEs), xenobiotic transporters, antioxidant enzymes, and inflammatory markers were investigated using real-time PCR. As a protection trial, the ameliorative effects of lycopene, a carotenoid abundantly found in tomato, were investigated. The possible mechanisms behind such effects were examined via studying the co exposure effects of pyrene and lycopene on regulatory elements including the aryl hydrocarbon receptor (Air) and elytroid 2-related factor 2 (RF). The achieved results indicated that pyrene caused significant cytotoxicity at 50 n, with a clear production of reactive oxygen species (ROS) in a dose-dependent manner. Pyrene upregulated mRNA expression of phase I enzymes including CYP1A1, 1A2, and CYP1B1 and inflammatory markers including TNFα and Cox2. However, pyrene significantly downregulated phase II enzymes, xenobiotic transporters, and antioxidant enzymes. Interestingly, lycopene significantly reduced pyrene-induced cytotoxicity and ROS production. Moreover, lycopene upregulated detoxification and antioxidant enzymes, probably via its regulatory effects on Air- and RF-dependent pathways.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of fused-ring aromatic compounds that are released into the environment due to incomplete combustion of organic matter and are found in car exhausts, tobacco smoke, and heated meat [1]. PAHs differ in their physicochemical properties and largely vary in their toxicity, mode of action, and interactions with biological systems [2]. Pyrene is a PAH that consists of 4 fused benzene rings [3]. It is often detected in environmental and food samples at high concentrations and is regarded as one of the best candidates to study the toxicity of PAHs [4]. Pyrene is one of the major 16 PAHs according to the European Food Safety Association [5].

Once humans are exposed to such toxicants, they undergo several metabolic interactions via the xenobiotic metabolizing enzyme (XME) system leading to disruption of xenobiotic metabolic pathways [6]. The major metabolic pathway for PAHs is mainly via an aryl hydrocarbon receptor- (Air) regulated gene battery. Air regulates phase I enzymes such as cytochrome P450 (CYP) 1A1 and 1A2, phase II enzymes such as uridine diphosphate glucuronosyltransferase (UGT) 1A6, and NAD(P) quinone oxidoreductase 1 (NQO1) [7]. Several reports investigated the toxic and mutagenic effects of benzo[a]pyrene as a major promutagenic and procarcinogenic PAH in the human liver and colon cell lines [1, 8]. However, pyrene-induced adverse effects including cytotoxicity and oxidative stress and the mechanisms behind such effects are scarcely investigated.

Lycopene is one of the hydrocarbon carotenoids which is found abundantly in tomatoes, watermelon, and red carrots [9]. It is also the most abundant carotenoid that can be detected in human plasma comprising about 50% of the total carotenoid content in the human body [10]. Lycopene has potential higher antioxidant effects compared with other carotenoids such as β-carotene [11]. Dietary supplementation of lycopene is linked to the reduction of mutagenesis and cancer risk in several reports [12, 13]. However, the mechanisms behind the beneficial effects of lycopene against the mutagenesis and onset of cancer are still unclear. Furthermore, the protective roles of lycopene against pyrene-induced cytotoxicity and oxidative stress and the mechanisms behind such effects have received little attention.

In sight of the previous facts, pyrene was used as a model for PAH exposure and its induced adverse effects including cytotoxicity and oxidative damage were investigated using HepG2 cells as a model. Furthermore, the changes in mRNA expression of XMEs including phase I and II enzymes and xenobiotic transporters were investigated. In addition, pyrene-induced changes in antioxidant enzymes and inflammatory markers were studied. As a protection trial, the ameliorative effects of lycopene against pyrene-induced adverse effects were examined. The mechanisms behind such effects were investigated via examination of coexposure of pyrene and lycopene on regulatory elements including Air and elytroid 2-related factor 2 (RF).

2. Materials and Methods

2.1. Chemicals. Chloroform and isopropanol were of LC/MS grade and were purchased from Wako Pure Chemical (Osaka, Japan). TRI reagent, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), pyrene, lycopene, and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents of analytical grade were purchased from Kanto Chemical Industry (Tokyo, Japan) unless specified.

2.2. Cell Culture Conditions and Treatment. Human liver hepatoma (HepG2) cell lines from Cell Biolabs, Inc. (distributed by Funakoshi Co. Ltd., Tokyo, Japan) were cultured on DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin mixture, in a humidified incubator with 5% CO_2 at 37°C. When confluency was reached, the cells were exposed to pyrene at environmentally relevant concentrations (1, 5, and 50) n for 24 hr. In protection experiments, the cells were coexposed to pyrene at 50 nM and lycopene at 3 concentrations (0.1, 1, and 10 μM) for 24 h. It is worth mentioning that in the preliminary experiments, the cells were exposed to pyrene for 0–48 h. However, there was no significant difference in the pyrene-induced cytotoxicity at both 24 and 48 h; therefore, the protection trials were conducted for 24 h exposure, and results of pyrene exposure for 24 h were shown in the present study. The used incubation time and concentrations for treatments were in accordance with previous reports [1, 14]. Both pyrene and lycopene were dissolved in DMSO, and the final concentration of DMSO in the medium was 0.01%, which did not show any cytotoxicity to HepG2 cells.

2.3. Cell Viability Assay. A CCK-8 assay kit (Dojindo Molecular Technologies, Rockville, USA) was used to determine HepG2 cell viability according to the manufacturer's instructions ($n = 6$ per treatment).

2.4. Reactive Oxygen Species (ROS) Measurement. HepG2 cells treated with pyrene and lycopene were stained with the fluorogenic probe DCF-DA for measurement of ROS production. The fluorogenic probe DCF-DA is used to measure the generalized oxidative stress in the cell produced by many types of reactive oxygen and nitrogen species such as H_2O_2, hydroxyl radicals, and peroxynitrite anions. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a Wallac 1420 ARVO Mx plate reader, PerkinElmer, Tokyo, Japan ($n = 6$ per treatment).

2.5. RNA Isolation and cDNA Synthesis. Total RNA was isolated from HepG2 cells according to a method established before [15]. In brief, cells were lysed using the TRI reagent followed by separation of the RNA upper layer using chloroform combined with centrifugation (15000 g for 20 min at 4°C). Isopropanol was added to the clear RNA layer followed by centrifugation for precipitation of RNA pellets. The pellets were then washed with 70% ethanol and dissolved using RNase-free H_2O. RNA concentrations and qualities were determined using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, Conn., USA). For cDNA synthesis, a ReverTraAce® qPCR RT Master Mix with gDNA remover (Toyobo Co. Ltd., Osaka, Japan) was used as described in the manufacturer's instructions. cDNA samples were stored at −20°C for further analysis.

2.6. Quantitative RT-PCR (qPCR). Gene expression of phase I enzymes including CYP1A1, 1A2, and 1B1; phase II enzymes including UGT1A6, NQO1, and glutathione-S-transferase (GST) A1; xenobiotic transporters including multidrug resistance protein 1 (MDR1) and multidrug

resistance-associated protein 2 (MRP2); antioxidant enzymes including heme oxygenase (HO) 1, superoxide dismutase (SOD) 1, GSTO1, and catalase (CAT); inflammatory markers including cyclooxygenase-2 (COX2) and tumor necrosis factor-α (TNFα); and regulatory elements including Air and elytroid 2-related factor 2 (RF) were determined using real-time reverse transcriptase-PCR (qPCR). The reactions were conducted in a Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA). The PCR mixture contained 2 μL of cDNA (600 ng), 5 μL of Fast SYBR® Master Mix, and 5 μM of each primer, with RNase-free water added to a final volume of 10 μL. The reaction cycle comprised a holding stage for 20 s at 95°C, followed by 40 denaturation cycles for 3 s at 95°C and 30 s at 60°C, and 15 s extension at 95°C. Single amplicon amplification was confirmed using melting curve analysis. The absence of primer dimers and genomic DNA amplification were confirmed by agarose gel electrophoresis. GAPDH was used for normalization by the comparative $^{\Delta\Delta}$Ct method. Each experiment was represented by 6 plates/treatments. Primer sets for the selected targets were designed based on previous work [8] and are presented in Table 1.

2.7. Air Luciferase Assay. A luciferase assay was performed using H4IIE-XRE cells according to the method described previously [16]. In short, cells were seeded in 96-well plates in DMEM supplemented with 10% FBS. In the next day, the cells were exposed to treatments (either pyrene alone or combined with lycopene) or Sudan III (10 μM) (a positive control for the activation of Air [7, 16] for 12 h). Then, the medium was aspirated off and luciferase assay was performed using a Dual Glo luciferase assay system (Promega, Madison, U.S.A.) according to the manufacturer's protocol. The activity was measured using a Wallac 1420 ARVO Mx plate reader.

2.8. RF Reporter Gene Assay. An RF reporter gene assay was conducted based on the protocol described before [17]. In brief, HepG2 cells were seeded into 96-well plates for 24 h with DMEM supplemented with 10% FBS. We transfected pGL4.37 [luc2p/ARE/hygro] and pGL 4.75 [hRluc/CMV] (an internal control for transfection efficiency) vectors at a 20 : 1 mass ratio using lipofectamine 3000 (Life Technologies, Tokyo) according to the manufacturer's protocol. After transfection, the transfection reagent/DNA mixture was removed and the samples solubilized in DMEM without FBS were separately applied to the transfected cells at various concentrations. Luciferase activity was assayed using a dual luciferase system (Promega, Madison, USA) according to the manufacturer's protocol. The activity was measured using a Wallac 1420 ARVO Mx plate reader.

2.9. Statistical Analysis. Statistical significance was evaluated using one-way analysis of variance (ANOVA) with a Tukey–Kramer honest HSD post hoc test (JMP program, SAS Institute, Cary, NC, USA) with $P < 0.05$ considered significant.

Table 1: Primer sequences of the target genes used in this study.

Target	Primer sequence
CYP1A1	F-5′-CTATCTGGGCTGTGGGCAA-3′ R-5′-CTGGCTCAAGCACAACTTGG-3′
CYP1A2	F-5′-CATCCC CCACAGCACAACAA-3′ R-5′-TCCCACTTGGCCAGGACTTC-3′
CYP1B1	F-5′-CTTTCGGCCACTACTCGGAG-3′ R-5′-CTCGAGGACTTGGCGGCT-3′
UGT1A6	F-5′-CATGATTGTTATTGGCCTGTAC-3′ R-5′-TCTGTGAAAAGAGCATCAAACT-3′
GSTA1	F-5′-CAGCAAGTGCCAATGGTTGA-3′ R-5′-TATTTGCTGGCAATGTAGTTGAGAA-3′
NQO1	F-5′-GGATTGGACCGAGCTGGAA-3′ R-5′-AATTGCAGTGAAGATGAAGGCAAC-3′
MDR1	F-5′-GGGAAGAGCACAACAGTCCA-3′ R-5′-ATGTGACTGCTGATCACCGC-3′
MRP2	F-5′-AGAGAGCTGCAGAAAGCCAG-3′ R-5′-CATCTTCCAGGACAAGGGCA-3′
HO1	F-5′-ATGGCCTCCCTGTACCACATC-3′ R-5′-TGTTGCGCTCAATCTCCTCCT-3′
GSTO1	F-5′-AGGACGCGTCTAGTCCTGAA-3′ R-5′-TTCCCTGGGTATGCTTCATC-3′
SOD1	F-5′-GCAGGTCCTCACTTTAATCCTCT-3′ R-5′-ATCGGCCACACCATCTTTGT-3′
CAT	F-5′-TGAAGATGCGGCGAGACTTT-3′ R-5′-TGGATGTAAAAAGTCCAGGAGGG-3′
TNFα	F-5′-GAAGAGTTCCCCAGGGACCT-3′ R-5′-GGGTTTGCTACAACATGGGC-3′
COX2	F-5′-GAGGGCCAGCTTTCACCAA-3′ R-5′-TGTGGGAGGATACATCTCTCCA-3′
AhR	F-5′-ATCACCTACGCCAGTCGCAAG-3′ R-5′-AGGCTAGCCAAACGGTCCAAC-3′
Nrf2	F-5′-CTTGGCCTCAGTGATTCTGAAGTG-3′ R-5′-CCTGAGATGGTGACAAGGGTTCTA-3′
GAPDH	F-5′-TCCAAAATCAAGTGGGGCGA-3′ R-5′-TGATGACCCTTTTGGCTCCC-3′

3. Results and Discussion

3.1. Biological Responses of HepG2 Cells to Pyrene Exposure. The achieved results indicated that pyrene had significant cytotoxic effects on HepG2 cells at 50 n causing 28% reduction in cell viability. Unlikely, benzo[a]pyrene, a promutagenic PAH, did not alter HepG2 cell viability [8, 15]. Pyrene caused a clear induction of oxidative damage, in terms of the production of ROS, in a concentration-dependent manner (Figure 1). A similar observation was recorded after exposure of HepG2 cells to B[a]P [8]. Furthermore, Grauzdytė et al. [18] reported a significant reduction in cell proliferation accompanied by production of oxidative stress in the human bronchial epithelial cells BEAS-2B exposed to PAH extracts. During the metabolism of PAHs, ROS such as superoxide anions, H_2O_2, and hydroxyl radicals could be generated [19].

The liver is considered as the major organ for the metabolism and detoxification of xenobiotics including PAHs [20]. Nevertheless, few reports have investigated the

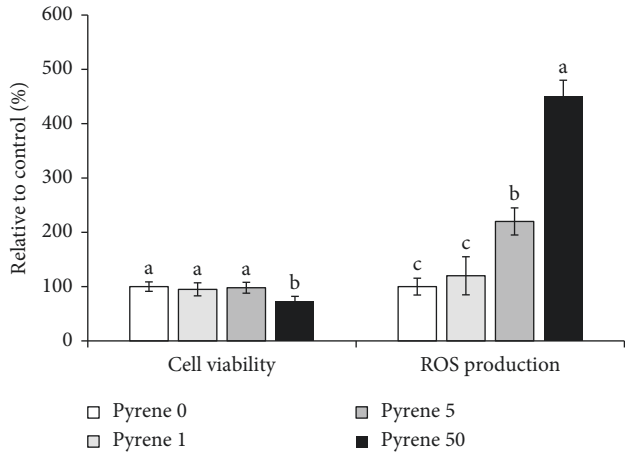

Figure 1: Pyrene-induced cytotoxicity and oxidative stress in human HepG2 cells. Data represent mean ± SD of pyrene-induced cytotoxicity (%) relative to the control using a CCK8 assay and pyrene-produced ROS (%) relative to the control using DCFDI as a substrate, $n = 6$. Columns with different superscript letters are significantly different at $P < 0.05$.

effects of pyrene on xenobiotic-metabolizing enzyme systems, and the obtained results indicated a clear induction of phase I enzymes including CYP1A1, 1A2, and 1B1 in a dose-dependent fashion (Figures 2(a)–2(c)). Consistent with this finding, treatment of the human Caco-2 cell line with PAHs, such as B[a]P, chrysene, phenanthrene, benzo[a]fluoranthene, dibenzo[a,b]pyrene, and pyrene, induced mRNA expression of various XMEs, including CYP1A1 and CYP1B1 [21]. Phase II enzymes are mainly involved in the detoxification of the formed metabolites via conjugation reactions [3]. In the present investigation, pyrene significantly downregulated phase II enzymes including UGT1A6, GSTA1, and NQO1 in a dose-dependent manner (Figures 2(d)–2(f)). In agreement with this finding, pyrene modulated the gene expression of UGT1A7 in human Caco-2 cell lines [21]. MDR1 and MRP2 are among the ATP-binding cassette (ABC) transporters that play important roles in the biodetoxification and active efflux of phase II metabolites of drugs and xenobiotics in the body. In the current study, pyrene reduced mRNA expressions of both MDR1 and MRP2 in a dose-dependent manner (Figures 2(g) and 2(h)). In a similar way, either PAH mixture or B[a]P reduced the gene expression of ABC transporters in human colon and liver cells [8, 21].

In order to investigate the possible reasons behind pyrene-induced oxidative damage, the modulatory effects of pyrene on antioxidant enzymes including HO-1, GSTO1, SOD1, and CAT were investigated. Pyrene had clear inhibitory effects on the tested antioxidant enzymes in a dose-dependent manner (Figures 3(a)–3(d)). Similarly, Ajayi et al. [22] reported that B[a]P induced colonic injury via suppression of antioxidant responses in BALB/c mice with clear inhibitory effects on GST- and CAT-dependent enzyme activities. We further investigated the effects of pyrene on the inflammatory response in HepG2 cells; interestingly, inflammatory biomarkers including Cox2 and TNFα were significantly induced reaching 10.28 ± 1.21- and 9.92 ± 0.89-fold concentration relative to the control (Figures 3(e) and 3(f)). In agreement with this result, Ferguson et al. [23] reported positive associations between PAHs and plasma inflammation marker C-reactive protein and urinary oxidative stress markers 8-hydroxydeoxyguanosine and 8-isoprostane in pregnant women. In addition, Ajayi et al. [22] confirmed the B[a]P-induced inflammatory response in BALB/c mice. From the overall achieved results in the present study, it is clear that upregulation of phase I enzymes and induction of inflammation together with the downregulation of phase II enzymes and xenobiotic transporters might explain the pyrene-induced cytotoxicity and oxidative stress.

3.2. Protective Effects of Lycopene against Pyrene-Induced Adverse Effects in HepG2 Cells. Phytochemicals such as curcumin, resveratrol, quercetin, β-carotene, and retinol had been tested for their protective effects against B[a]P-induced genotoxicity and carcinogenicity in lung and liver cells [8, 24]. Lycopene was used as well in combination with either tocopherol or genistein for protection against 7,12-dimethyl[a]benzanthracene-induced oxidative damage and mammary tumorigenesis in female rats [25, 26]. However, the protective effects of lycopene against the adverse effects of pyrene are less informed. In the current investigation, co exposure of HepG2 cells to pyrene and lycopene at three different concentrations showed clear protective effects against pyrene-induced cytotoxicity and oxidative damage (Figure 4(a)). Interestingly, lycopene had clear inhibitory effects against phase I enzymes, including CYP1A1, 1A2, and 1B1 (Figure 4(b)). Coexposure of lycopene with pyrene led to significant induction of phase II enzymes (Figure 4(c)), xenobiotic transporters (Figure 4(d)), and antioxidant enzymes (Figure 4(e)) and subsequently, reduction in inflammatory biomarkers (Figure 4(f)). In this context, carotenoids such as astaxanthin could alter CYP1A dependent activities via induction of protein expression and inhibition of NADPH P450 reductase-dependent electron transfer in male Wistar rats [27]. Furthermore, carotenoids such as β-carotene protected HepG2 cells against B[a]P-induced mutagenicity and oxidative stress via upregulation of phase II enzymes and ABC transporters [8]. Similar trends occurred in plants, as application of carotenoids alleviated the oxidative stress caused by phenanthrene in wheat [28]. Epidemiological studies showed a clear inverse relationship between tomato intake and a number of chronic diseases and certain types of cancer, and this was attributed to the higher concentrations of lycopene [29]. Furthermore, supplementation with 2 or 4 mg/kg body weight of lycopene can reduce high-fat diet-induced oxidative stress and liver damage in rats [30]. In addition, Wang et al. reported that lycopene concentrations were elevated in the liver upon repeated exposure for 6 weeks at 15 mg/Kg BW/day from 7.0 ± 1.0 to 17.6 ± 1.5 nmol/g tissue [31]. They added that lycopene supplementation significantly decreased cytochrome P450 2E1, inflammatory foci, and mRNA expression of proinflammatory cytokines (TNFα, IL-1, and IL-12), but RF and

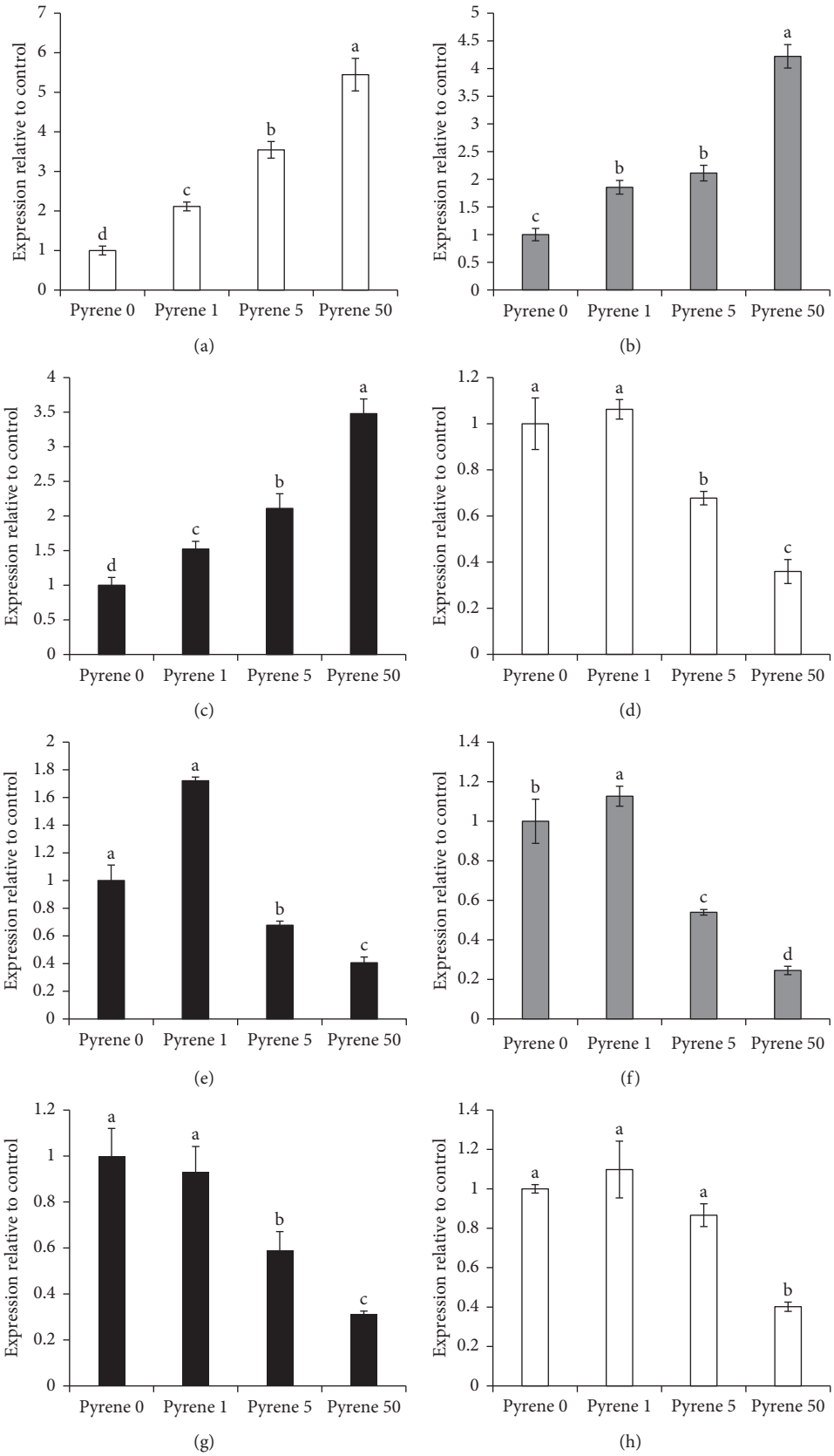

Figure 2: Changes in mRNA expressions of xenobiotic-metabolizing enzymes in HepG2 cells exposed to pyrene. The effects of pyrene (0–50 n) on (a) CYP1A1, (b) CYP1A2, (c) CYP1B1, (d) UGT1A6, (e) GSTA1, (f) NQO1, (g) MDR1, and (h) MRP2 mRNA expression as determined by real-time RT-PCR. Data are presented as mean ± SD ($n = 6$). Columns with different superscript letters are significantly different from each other ($P < 0.05$).

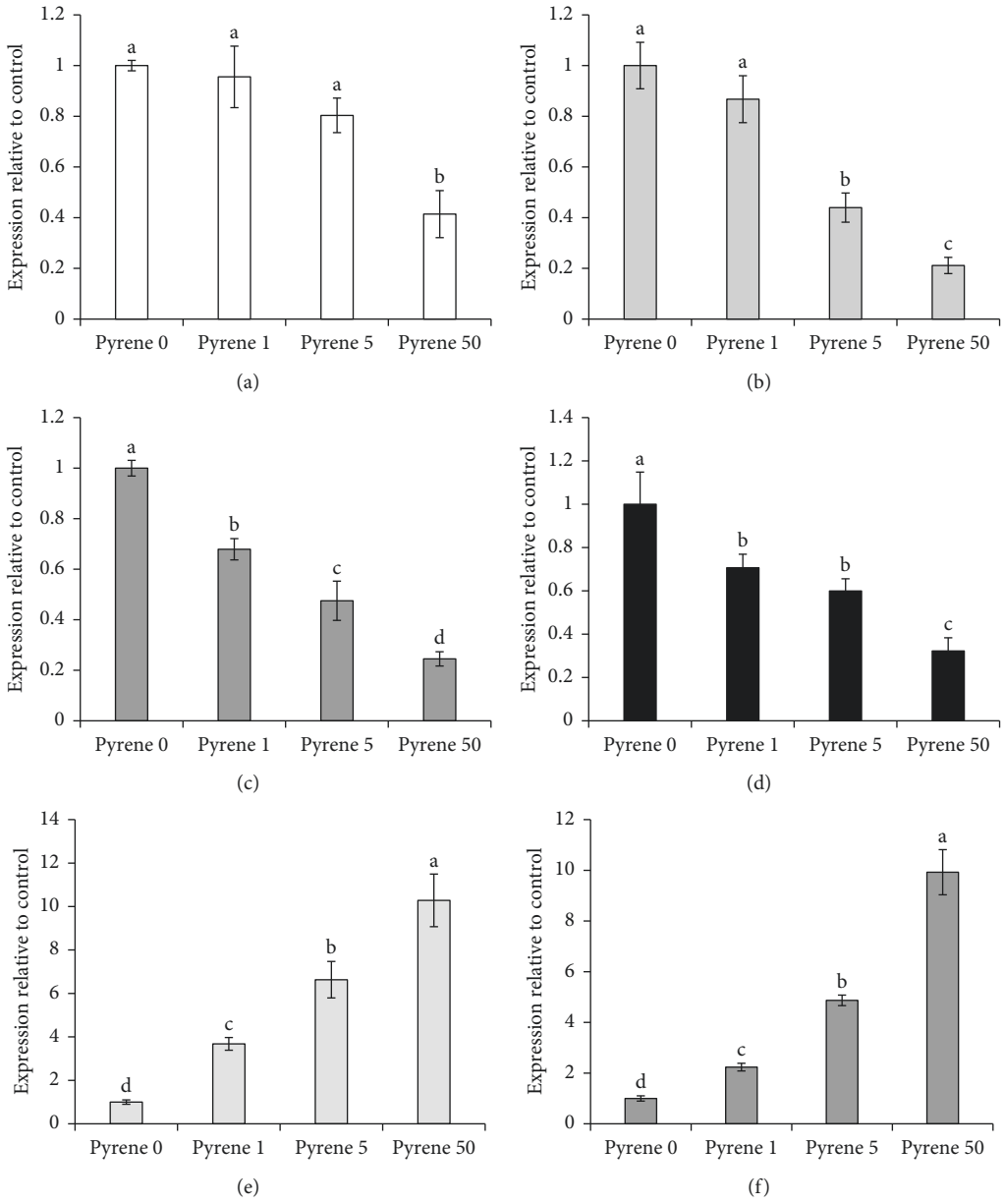

Figure 3: Changes in mRNA expression of antioxidant enzymes and inflammatory markers in HepG2 cells exposed to pyrene. The effects of pyrene (0–50 n) on (a) HO-1, (b) GSTO1, (c) SOD1, (d) CAT, (e) TNF-α, and (f) COX2 mRNA expression as determined by real-time RT-PCR. Data are presented as mean ± SD ($n = 6$). Columns with different superscript letters are significantly different from each other ($P < 0.05$).

HO-1 proteins. Therefore, it could be concluded that lycopene-induced upregulation of phase II and III and antioxidant enzymes might be considered as a protection mechanism against pyrene-induced adverse effects.

3.3. Effects of Pyrene and Lycopene on Regulatory Elements (Air and RF). PAHs are regulated mainly via Air. This ligand-activated transcription factor regulates the cellular responses to environmental pollutants such as dioxins and PAHs. Air is also involved in the regulation of inflammation as well as a variety of endogenous processes [32]. In order to investigate the possible reasons for upregulation of bioactivating phase I enzymes and inflammatory markers, the effects of pyrene on Ahr mRNA expression and reporter gene activity were studied. Pyrene activated Air at both the transcriptional (~4.54 ± 0.19 fold) and functional levels (~6.33 ± 0.88 fold) in human HepG2 cells (Figure 5). Similarly, Air is activated after exposure to PAHs including ketones and quinones [33]. This might explain the upregulation of Air-regulated phase I enzymes including CYP1A1, 1A2, and 1B1 and the inflammatory cytokines including TNFα and COX2. In agreement with this assumption, Øvrevik et al. [34] concluded that Air regulates NF-κB signaling and chemokine responses in human bronchial epithelial cells.

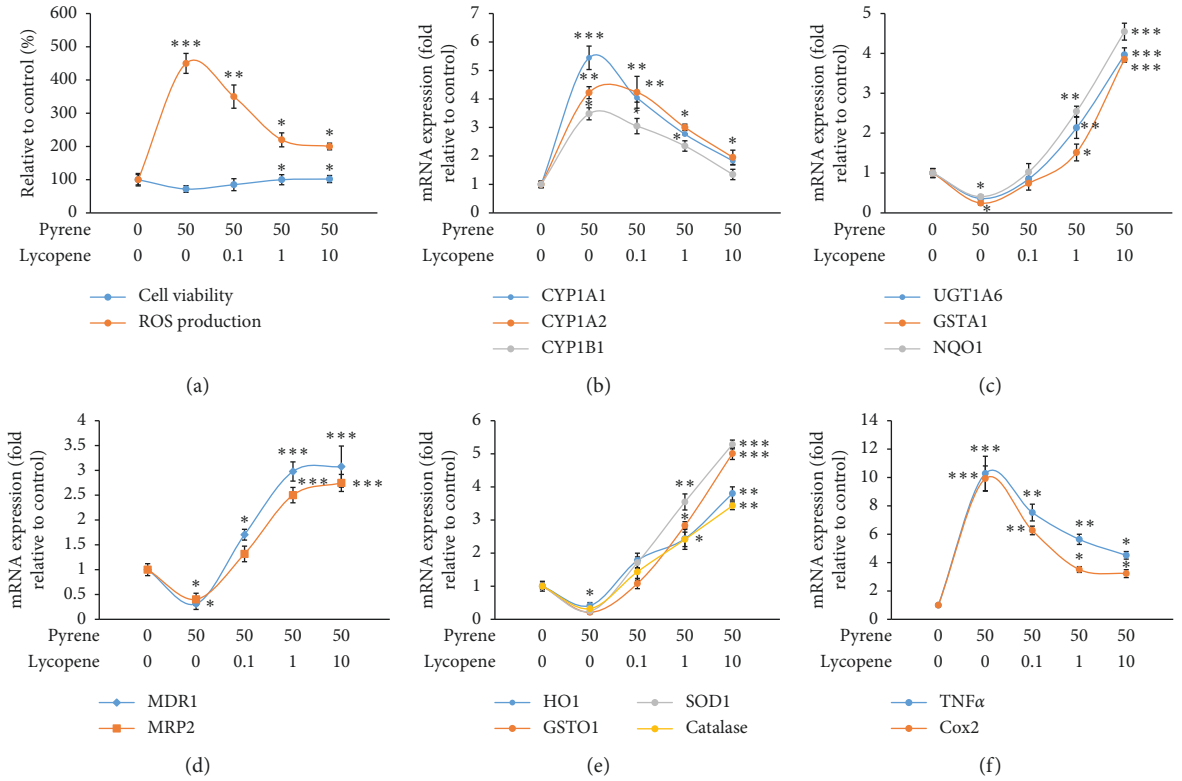

Figure 4: Protective effects of lycopene against pyrene-induced adverse effects in HepG2 cells. Ameliorative effects of lycopene (0–10 μM) on pyrene- (50 n) induced (a) cytotoxicity and oxidative stress, (b) phase I enzymes, (c) phase II enzymes, (d) xenobiotic transporters, (e) antioxidant enzymes, and (f) inflammatory markers. Data are presented as mean ± SD ($n = 6$). Values carrying asterisks (*) are different at $P < 0.05$, (**) are different at $P < 0.01$, and (***) are different at $P < 0.001$, in comparison with the control.

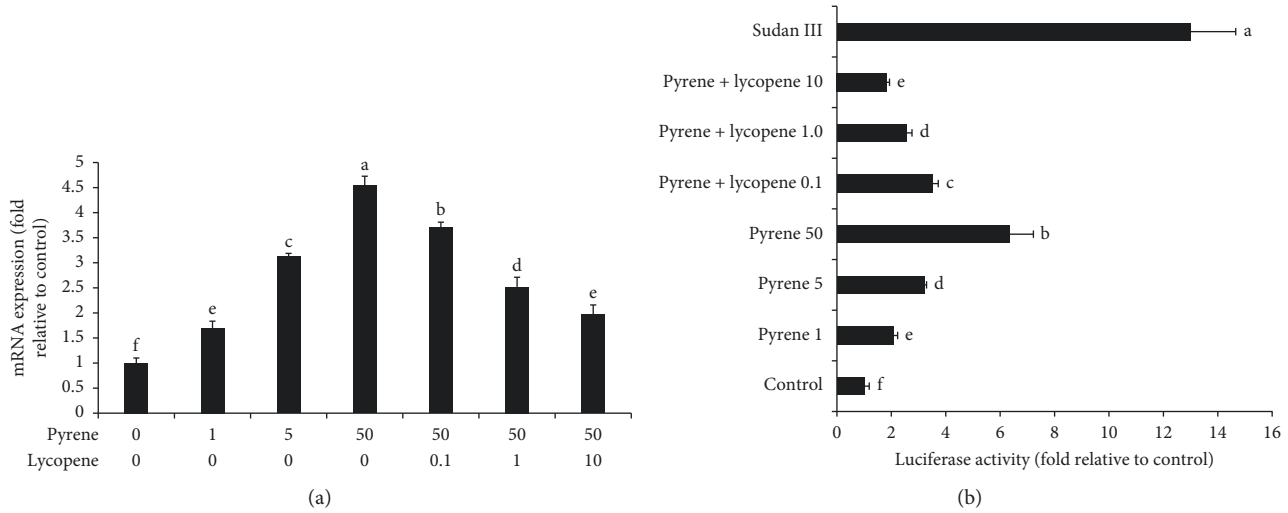

Figure 5: Effects of pyrene and lycopene on Air mRNA expression and luciferase activity. The effects of pyrene and lycopene on (a) Ahr mRNA expression and (b) Ahr luciferase activity. Sudan III was used as a positive control when determining luciferase activity. Data are presented as mean ± SD ($n = 6$). Columns with different superscript letters are significantly different ($P < 0.05$).

RF is a major transcriptional factor that regulates the cellular response to environmental stressors. It plays an important role in the release of antioxidant detoxification enzymes upon exposure to various xenobiotics. In the current study, the effects of coexposure of lycopene and pyrene on the RF mRNA expression levels and its luciferase activity were investigated. Pyrene alone caused significant reduction in both the expression and activity of RF (Figure 6). Similarly, Wang et al. [35] reported that benzo[a] pyrene reduced mRNA expression of the antioxidant

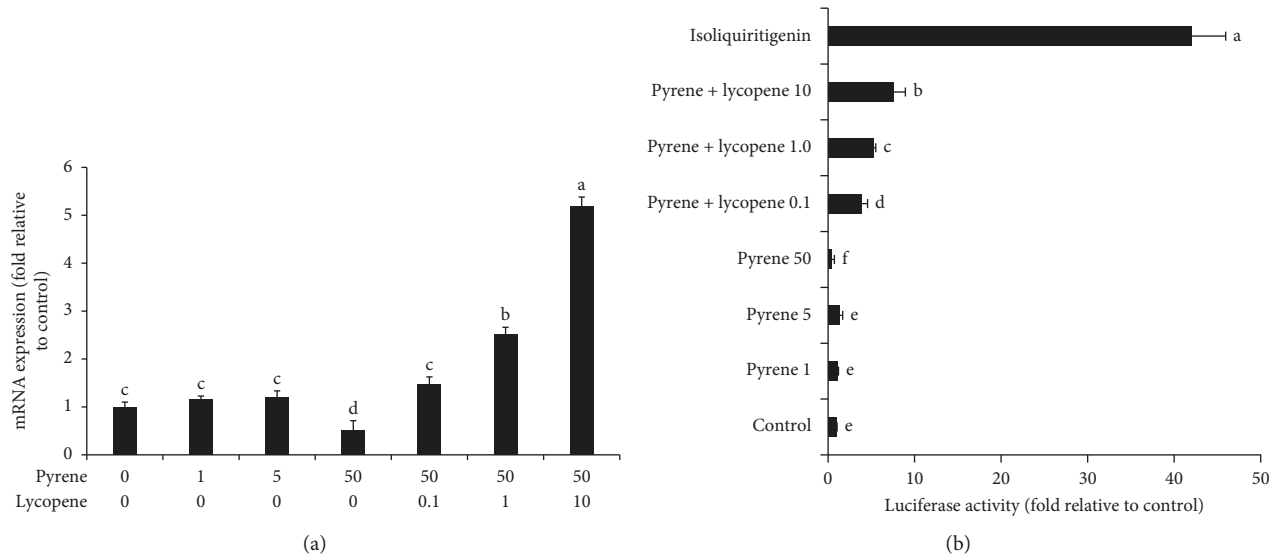

Figure 6: Effects of pyrene and lycopene on RF mRNA expression and luciferase activity. The effects of pyrene and lycopene on (a) RF mRNA expression and (b) RF luciferase activity. Isoliquiritigenin was used as a positive control when determining luciferase activity. Data are presented as mean ± SD ($n = 6$). Columns with different superscript letters are significantly different ($P < 0.05$).

enzymes in the clam *Ruditapes philippinarum* via modulation of the Nrf2-Keap1 signaling pathway. Interestingly, coexposure of pyrene and lycopene could upregulate RF expression and luciferase activity in a dose-dependent manner (Figure 6). These results agree with the results of Abbas et al. [36] who reported that lycopene ameliorates atrazine-induced oxidative damage in the adrenal cortex of male rats by activation of the RF/HO-1 pathway. Furthermore, Yu et al. [37] reported that lycopene attenuates aflatoxin B1-induced renal injury with the activation of the RF antioxidant signaling pathway in mice. Therefore, it is highly suggested that lycopene-induced upregulation of antioxidant enzymes is mechanistically via activation of the RF pathway.

4. Conclusion

Pyrene had cytotoxic effects and oxidative damage on human HepG2 cells. This damage is probably due to downregulation of detoxification and antioxidation enzymes. Lycopene could significantly reduce the adverse effects of pyrene on HepG2 cells. Such protective effects of lycopene are possibly via the activation of the RF pathway and reduction of Air metabolic activation of pyrene. Therefore, dietary supplementation of lycopene is highly recommended for people at a high risk for exposure to PAHs such as pyrene.

Data Availability

All analytical data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

J. M., W. S., and A. E. were responsible for conceptualization. J. M. and W. S. were involved in methodology. W. S., A. E., and M. K. carried out validation. J. M., W. S., and A. E. were responsible for formal analysis. J. M. and W. S. carried out investigation. W. E., J. M., X. L., and X. H. were responsible for resources. W. S., A. E., and M. K. performed data curation. J. M., W. S., A. E., and W. E. wrote the original draft. All authors reviewed and edited the manuscript. X. H. was involved in supervision. X. L. and X. H. carried out project administration. W. E., J. M., X. L., and X. H were responsible for funding acquisition.

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