Journal of Environmental Health and Sustainable Development

Effect of Polycyclic Aromatic Hydrocarbons Extracted from Indoor Air of Coffee Shops on Oral Squamous Carcinoma Cell (KB/C152): An in Vitro Approach

Shahnaz Sargazi [1, 2], Ali Asghar Ebrahimi [1], Seyed Mehdi Tabatabaei [2*], Mohammad Hassan Ehrampoush [1], Ramin Saravani [3,4], Mohammad Javad Zare Sakhvidi [5,6], Hossein Fallahzadeh [7], Sheida Shahraki [3]

[1] Environmental Sciences and Technology Research Center, Department of Environmental Health Engineering, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[2] Health Promotion Research Center, Zahedan University of Medical Sciences, Zahedan, Iran.
[3] Cellular and Molecular Research Center, Research Institute of Cellular and Molecular Sciences in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran.
[4] Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran.
[5] Occupational Health Research Center, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[6] Department of Occupational Health, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[7] Center for Healthcare Data Modeling, Departments of Biostatistics and Epidemiology, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

ARTICLE INFO

ORIGINAL ARTICLE

Article History:
Received: 12 August 2023
Accepted: 20 October 2023

Corresponding Author:
Seyed Mehdi Tabatabaei

Email:
zu.healthdeputy@gmail.com

Tel:
+98 54 33438801

Keywords:
PAHs,
Air Pollution, Indoor,
DNA Damage,
KB/C152.

ABSTRACT

Introduction: The two main causes of indoor air pollution (IAP) are cooking and smoking. Toxic substances, including polycyclic aromatic hydrocarbons (PAHs), recognized as human carcinogens, are present in cooking and tobacco smoke. This study aims to determine the cytotoxicity and genotoxicity of PAHs collected from the indoor air of coffee shops on human cell line (KB/C152) in vitro approach.

Materials and methods: Sampling pumps at a 2 l/min flow rate for 5 hours were applied to collect indoor air samples. Next, KB cells in cell culture medium were exposed to different concentrations of extracted PAHs using Methyl Thiazolyl Tetrazolium (MTT) test. Finally, terminal dUTP nick-end labeling (TUNEL) test and cell cycle assessments were both examined using flow cytometry.

Results: The MTT test revealed a significant cytotoxic effect on KB cells depending on the PAHs concentrationas compared to the control cell line. The lethal concentration 50 (LC_{50}) value against KB cells was 100 ± 3.09 µg/ml. Accordingly, exposure to extracted PAHs resulted in an arrest in the cell cycle at the sub-G1 checkpoint. The extracted PAHs suppressed the cell cycle in the sub-G1 phase, damaged DNA, and arrested KB cells from proliferating. Additionally, a statistically significant increase in DNA cleavage percentages ($p \leq 0.05$) was seen in the TUNEL test, which also showed a dose-dependent increase in DNA damage.

Conclusion: Extracted PAHs caused DNA damage and arrested in the cell cycle in epidermoid carcinoma of the mouth cells (KB/C152) in vitro mechanisms. This evaluation highlights mechanisms of exposure to extracted PAHs and their detrimental health effects.

Citation: Sargazi Sh, Ebrahimi AA, Tabatabaei SM, et al. ***Effect of Polycyclic Aromatic Hydrocarbons Extracted from Indoor Air of Coffee Shops on Oral Squamous Carcinoma Cell (KB/C152): An in Vitro Approach*****.** J Environ Health Sustain Dev. 2023; 8(4): 2113-24.

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[DOI: 10.18502/jehsd.v8i4.14437]

Introduction

One of the main issues that humans confront is air pollution. The use of fossil fuels and population density in some cities are rising daily due to the uncontrolled expansion of industrial activity. The community health has been negatively impacted by air pollution, which increases the risk of cancer, bronchitis, cardiovascular disease, and other respiratory illnesses. The World Health Organization (WHO) estimates that seven million people worldwide die every year from diseases linked to indoor and outdoor air pollution[1]. Indoor air quality (IAQ) has received increased attention in recent years from governmental institutions, worldwide scientific communities, and environmental authorities due to the fact that it enhances the well-being of residents and employees. Studies have looked at the issue's quantitative (indoor air) and qualitative changes, focusing on the increase in pollutants and their concentrations in certain areas. Ninety percent of people's time is spent indoors, including in their homes, gyms, offices, schools, public transit, recreational facilities, etc. Therefore, it is crucial to consider how IAQ generally affects people's health and quality of life. Minerals and organic biological particles include chemicals like carbon monoxide, ozone, radon, gases, particulate matter (PM) and fibers, volatile organic compounds (VOCs), bacteria, fungus, and pollen. Organic pollutants like polycyclic aromatic hydrocarbons (PAHs) can also have an effect on IAQ [2–7].

The broad category of chemical compounds known as PAHs includes substances with two to seven benzene rings [8,9]. Due to the incomplete combustion of organic materials, such as fossil fuels, many environmental contaminants are produced [10]. Some PAHs are teratogenic, mutagenic, and carcinogenic[11].

PAHs are a severe health threat, causing growing concerns regarding the prevalence of PAHs inside and outside [11]. PAHs substances may cause systemic inflammation, nausea, vomiting, skin, and eye irritation. However, its long-term health impacts include cataracts, DNA, liver, kidney damage, gene mutation, and cardiovascular mortality [12,13]. Long-term health impacts include a range of malignancies (including on skin, lung, bladder, and gastrointestinal), as well as liver, kidney, and skin cancers. PAHs substances affect DNA, RNA, proteins, lipids, and other molecules to cause cancer. PAHs create volatile organic substances, aldehydes, and alkanes by the peroxidation of lipids, which then interact with the protein of the cell membrane when there are too many active aldehydes. PAHs create breaks in DNA and mutations in RNA, which alter how the chromosomes are distributed throughout cell division. Additionally, it stops producing protein, deactivates the enzyme, damages the membrane, and destroys the cells. Cell carcinoma results from these compounds [14]. According to the International Agency for Research on Cancer, formaldehyde, PAHs, 1,3-butadiene, and benzene are carcinogens. These substances are found in indoor air pollution (IAP), and carcinogenic risk assessment should be done on these substances [15].

Smoking and cooking are the two main sources of IAP with PAH compounds. It is generally known that IAP contributes to the development of lung cancer [9]. However, relatively few studies have been conducted on the relationship between IAP and other diseases, including oral cancer. Therefore, molecular studies employing in vitro and in vivo animal models would aid in improving researchers' understanding of the long-term effects of IAP exposure on the oral mucosa. Deciphering the molecular mechanism of IAP-mediated carcinogenesis may help identify early diagnostic indicators and possible therapeutic targets. This study sought to establish the cytotoxicity and genotoxicity of PAHs collected from the indoor air of coffee shops in the city of Zahedan on a human cell line in a dose-dependent manner. The KB line was widely used in studies on viral infection, cancer chemotherapeutic screening, tumorigenicity, and cell nutrition and metabolism. Therefore, this study aimed to identify the in vitro mechanisms, by which PAHs damage DNA, induce cell cycle arrest in KB/C152 cells, and cause toxicity.

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Materials and Methods

Air sampling and chemical analysis

The SKC standard (224-44TX) sampling pumps equipped with dichotomous sampling media, at a 2 l/min flow rate for 5 hours, were applied to collect indoor air samples. For particle and gaseous phases PAHs samples, polytetrafluoroethylene (PTFE) filters, and XAD-2 adsorbent tubes were utilized, respectively [16]. Twenty three air samples were taken from February to March 2021. The filters were placed in the filter holder immediately after sampling, and then the filters and adsorbents were individually wrapped in foil. They were transferred to the laboratory and stored at -2°C for 72 hours before analysis. Samples on filters and absorbents were extracted using dichloromethane solvent, according to the NIOSH method (5515)[16]. The PTFE filters and the contents of the front and back of the absorbent were placed separately in dark colored glass vials. Each jar was filled with five milliliters of dichloromethane and then for 30 minutes, it was affected by the waves using an ultrasonic device to introduce the pollutant into the solvent. In the next step, the extracts were concentrated to less than 1 ml by carefully blowing a medium flow of nitrogen gas on the vials. The extracted items were transferred to special vials for analysis using gas chromatography-mass spectrometry (Agilent 7890)[17]. The resulting solution was freeze-dried for concentration before being dissolved in Dimethyl Sulfoxide (DMSO) at a concentration of 10 mg/ml.

Cell culture

An epidermoid carcinoma in the mouth of an adult Caucasian male was used to create the KB/C152 cell line. This cell line was obtained from Pasteur Company (Tehran, Iran). Cells were cultured in RPMI 1640 media supplemented with 1 mmol/L of L-glutamine, 10% (v/v) fetal bovine serum (FBS), and 1% (w/v) penicillin and streptomycin. After daily examination with an inverted microscope, the cells were placed in an incubator at 37 °C, which was humidified and contained 5 percent carbon dioxide[18].

Cell viability assay

An assay known as the Methyl Thiazolyl Tetrazolium (MTT) assay was utilized to determine whether or not cells are viable. This assay is predicated on the mitochondrial dehydrogenases ability to convert MTT into formazan crystals. Cells from KB/C152 were plated in 96-well plates at a density of 3×10^4 cells per ml for 24 hours before being given the all-clear to adhere. Following the completion of the log phase of growth, the cells were concurrently exposed to various concentrations of the extracted PAHs. Extracted PAHs (3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL) were added to the wells for 24, 48, and 72 hours. MTT (20 µl) was then added to each well. The Supernatant solution was withdrawn after two hours of incubation at 37°C, and the resulting formazan was dissolved in DMSO. An ELISA Microplate Reader (Awareness Technology Stat Fax 2100) was used to measure absorbance at 570 nm. Viability was obtained by dividing the amount of dye light absorption produced by treated cells against untreated cells multiplied by 100. The cytotoxicity of PAHs on KB/C152 was quantified using LC_{50} values. All experiments were replicated three times[18].

Cell cycle distribution

The cells were cultivated on plates containing six wells. After a 24-hour exposure period, cells from 6-well plates were collected (those that were not treated with PAHs and those that were treated with PAHs). Cells were harvested and then fixed for ten minutes on ice in ethanol at a 70% concentration. The cells were then given two rinses in phosphate-buffered saline (PBS). Following fixation, 0.5 mL of PBS containing propidium iodide at a concentration of 50 µg/ml and RNase at 100 µg/ml was added to the cell pellet, which was then suspended for 15 minutes. Using flow cytometry, the proportional amount of cells present in each phase was determined (the cell cycle percentages in the G0/G1, S, and G2/M stages were calculated) [19].

TUNEL assay

With some modifications, the terminal dUTP nick-end labeling (TUNEL) assay for DNA

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damage was carried out in accordance with the instructions previously published by Lisa et al. [20]. Extracted PAHs were added to 1×10^5 cells, which were subsequently fixed in 4% PFA, rinsed in PBS at room temperature, and permeabilized with 0.1 percent Triton X-100. To make TUNEL reagent, 5 µl of the enzyme solution and 45 µl of the label solution were combined. Then, 50 µl of the TUNEL reaction mixture was added. Parafilm was used to cover the mixture, which was then kept at 37 °C for an hour. After that, the TUNEL reagent was eliminated by washing with PBS, and the cells were exposed to Hoechst 33342 at 100 ng/ml concentration in PBS while incubating in the dark. PBS was used to clean the cells first, and water was used to rinse them. TUNEL-stained cells were assayed by flow cytometry (Becton-CellQuest Dickinson's software is being used in conjunction with the FACS Calibar).

Statistical analysis

The experiment was done in triplicate for each group. The Shapiro-Wilk tests and quantile-quantile plots were used to assess the data normality. One-way analysis of variance (ANOVA) test was used to conduct the statistical study. Then, to compare the two groups, either the least significant difference (LSD) test or Dunnett's test was used. The relationship between total PAH concentrations and different levels of genetic damage and other data was investigated using Pearson rank correlation test. Pearson's correlation coefficients of 0.00 to 0.19 were considered very poor, 0.20 to 0.39 poor, 0.40 to 0.59 moderate, 0.60 to 0.79 strong, and 0.80−1.0 extremely strong. For each test, a significant difference was defined with a p-value of less than 0.05. In each phase of the data analysis process, desktop version of the statistical analysis program SPSS 25.0. (SPSS Inc., Chicago, IL, USA) was used.

Results

There was a concentration of 0.34 mg/m^3 extracted from PAHs in indoor air of coffee shops. The sample contained 20.69 $µg/m^3$ benzo[a] pyrene, 9.95$µg/m^3$ benzo[e]pyrene, 40.37$µg/m^3$ naphthalene, 21.28$µg/m^3$ benzo[b]fluoroanthene, 19.56 $µg/m^3$ benzo[k]fluoroanthene, 24.72 $µg/m^3$ acenaphthene, 27.23 $µg/m^3$ acenaphthylene, 23.59 $µg/m^3$ phenanthrene, 23.92 $µg/m^3$ anthracene, 34.10 $µg/m^3$ fluorene, 20.86 $µg/m^3$ fluoranthene, 16.61 $µg/m^3$ benzo[a]anthracene, 27.42 $µg/m^3$ pyrene, 2.07 $µg/m^3$ dibenzo[a,h]anthracene, 0.56 $µg/m^3$ benzo[ghi]perylene, 5.44 $µg/m^3$ Indeno[1,2,3-cd]pyrene, and 18.99 $µg/m^3$ chrysene. After the freeze-drying process, a 10 mg/ml combination of PAHs in DMSO solution was created and employed for cell culture.

The KB and control cells were exposed to extracted PAHs in the concentration range of 3.125-400 µg/ml for 24 and 48 hours to differentiate between their effects. Figure 1 displays the morphology of treated and control cells. The cell population decreased after co-culturing the cells for more than 48 hours with different PAH doses. Additionally, treated KB cells showed morphologic changes in comparison with control cells. The reduction in cell volume, the number of live cells, and the rounding of cells were among the modifications performed, so that the nucleus comprised the majority of the cell's total volume. The cells were treated for no more than 48 hours due to their extreme sensitivity.

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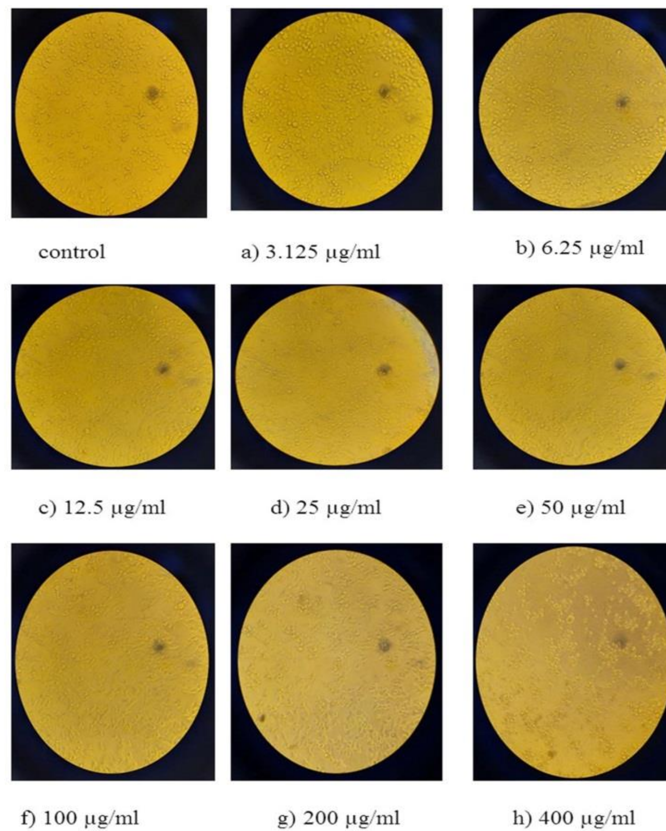

Figure 1: Morphological changes in cells treated with various concentrations of extracted PAHs in the KB cell line

The impact of PAHs on cell viability was assessed after 24 and 48 hours exposure time. At 400 µg/ml of PAHs concentrations, KB cells viability significantly declined after 24 and 48 hours of treatment. At any measured dose, PAHs had no significant impact on the viability of control cells (*P ≤ 0.05) (Figure 2).

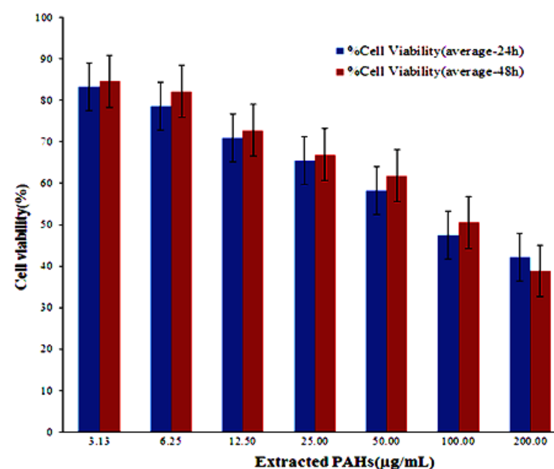

Figure 2: MTT assay of cell viability in KB cells exposed to extracted PAHs. For 24 hours and 48 hours, the cells were treated with increasing concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml). For each data point, the results are expressed as mean ± SD of three separate experiments. Values are significantly different from untreated controls: *$p < 0.05$.

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Dunnett test was run on control samples, 24-hour cell culture data, and 48-hour cell culture data from cell culture. The MTT assay statistical analysis revealed that the mean difference between control and 48-hour treated cells was equivalent to 56.12. Since the P-value was 0.001, there was a significant difference between the groups. Table 1 displays the statistical analysis of this section. The LC_{50} dose for KB cells was established to be 100 ± 3.09 µg/ml at 48 hours.

Table 1: Dunnett test results related to MTT assay data

(I) group	(J) group	Mean difference (I-J)	Std. Error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
Control	48h	56.12*	7.012	0.000	39.30	72.94
24h	48h	-1.61	7.012	0.962	-18.43	15.20

* The mean difference is significant at the 0.05 level.

To determine if the PAH-induced growth inhibition of the cells was connected to cell cycle arrest, flow cytometry was utilized to look at the cell cycle profile. At 200 µg/mL, PAHs increased the population of subG1 (M1 region) cells in the cell cycle test assessment from 5.54% in the control sample to 89.87% in the treatment sample (Figures 3 and 4). The Dunnett test was used to statistically analyze the results of cell cycle experiments using control, 24-hour, and 48-hour cell culture data. In 48-hour cell culture data, the mean difference between control and treated cells was positive at 53.36. There was a significant difference between the groups, as shown by the P-value of 0.003. Table 2 displays the statistical analysis of this section. The proportion of M1 area was significantly linked with the time spent in cell culture. It was discovered that when time increased, the phase percentage also increased, which is compatible with the study findings (Table 3). Meanwhile, the G0/G1 (M2) S and G2/M (M3 and M4) cell populations had a falling tendency when the extracted PAHs increased (Figures 3 and 4).

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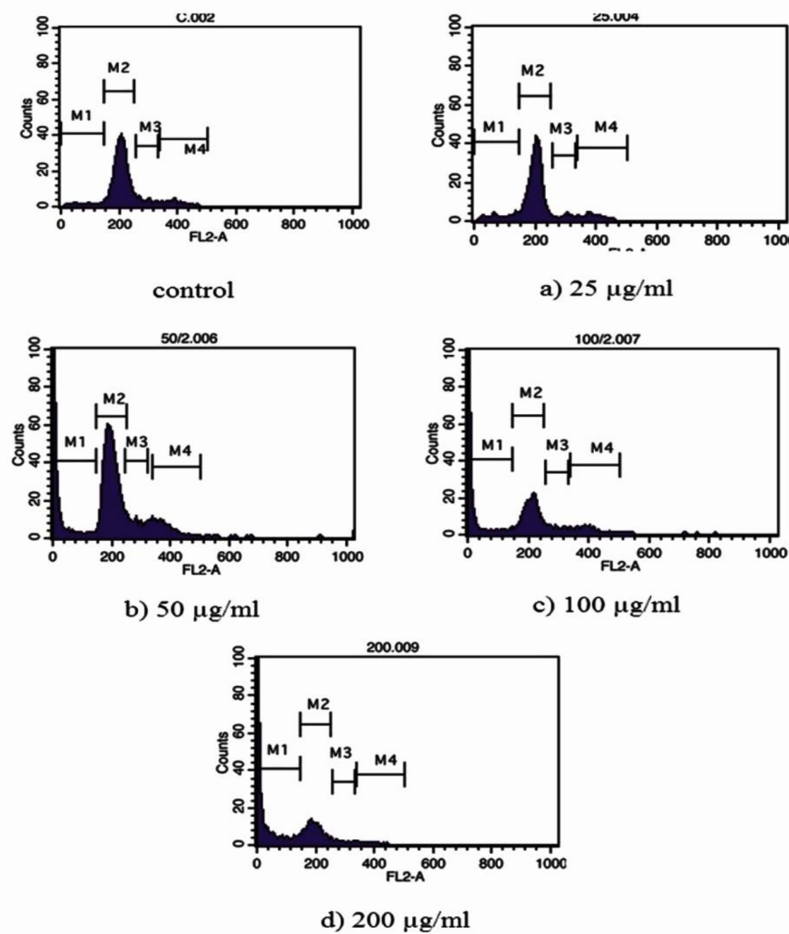

Figure 3: Cell cycle distribution in extracted PAHs-treated KB cells, as shown in representative histograms. Propidium iodide staining was used to determine cell cycle distribution. (a) control, (b) treated KB cells at 25µg/Ml, (c) 50 µg/ml, (d)100 µg/ml, and (e) 200 µg/ml.

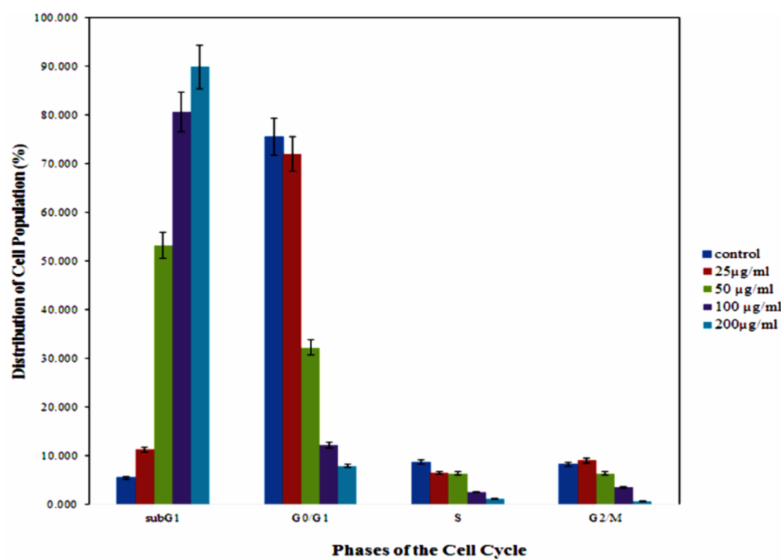

Figure 4: Induction of cell cycle arrest in KB cells by PAHs. Cells were cultured in different concentrations of extracted PAHs for 48 hours.

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Table 2: Dunnett test results related to cell cycle assay data

(I) group	(J) group	Mean difference (I-J)	std. error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
Control	48h	-53.36*	1.61	.016	-95.43	-11.27
24h	48h	-29.33	1.61	.173	-71.41	12.75

* The mean difference is significant at the 0.05 level.

TUNEL test was used to examine DNA damage in KB cells exposed to PAHs. Figure 5 shows representative TUNEL test pictures of treated and untreated cells stained with Hoechst 33342. As indicated by the DNA fracture percentage, significant levels of DNA strand breaks were discovered in KB cells treated with various dosages of PAHs compared to the control group ($p \leq 0.001$). The degree of damage to DNA and chromosomes may be greatly increased by short-term in vitro exposure to different concentrations of extracted PAHs from coffee shop indoor air, which was strongly connected with total PAH concentrations. It is consistent with the findings of the statistics used to create this section, since $p < 0.01$ and $r = 0.986$ (Table 3).

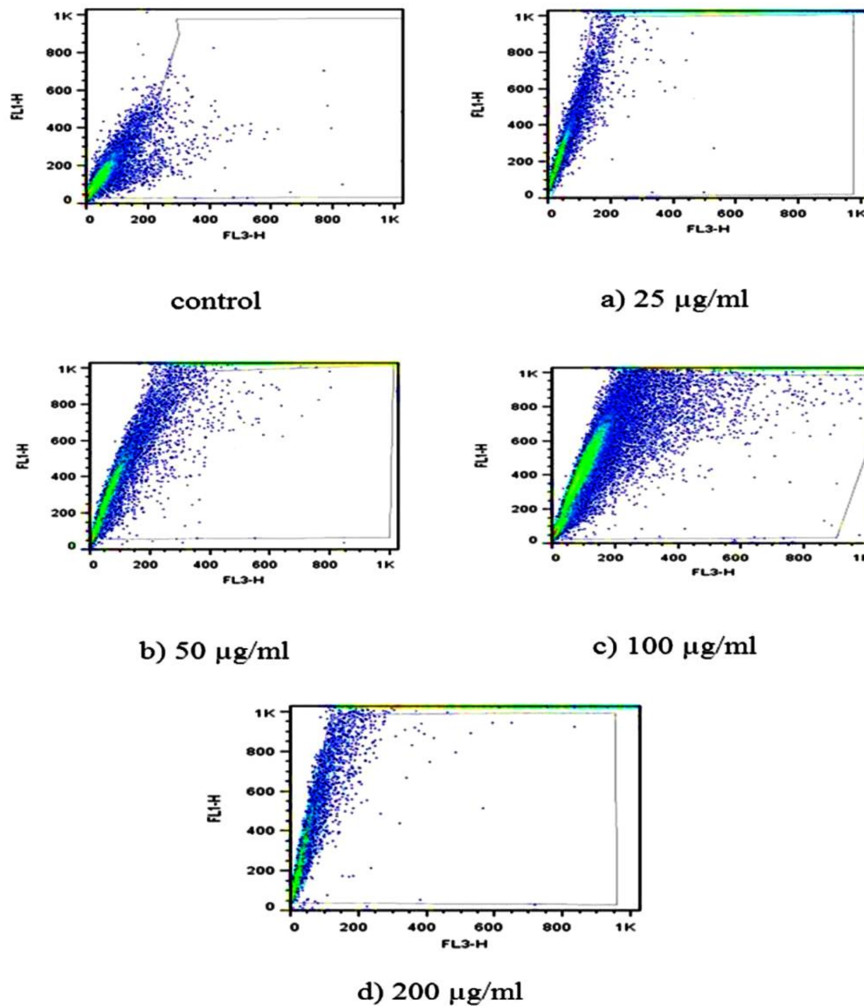

Figure 5: TUNEL assay images of (a) control cells, (b) treated KB cells at 25µg/Ml, (c) 50 µg/Ml, (d)100 µg/Ml, and (e) 200 µg/mL (the percentages of DNA damage at 25, 50, 100, and 200 µg/mL were 8.79%, 11.8%, 13.4%, and 18.7%, respectively).

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Table 3: Pearson's correlation coefficients (r) between parameters

		Viability rate	Concentration of PAHs	m_1 (subG1 checkpoint of cell cycle)	Group (24h and48h)	Percentage of DNA damage
Viability rate	Pearson Correlation	1	-0.167	-0.520	-0.778**	-0.083
Concentration of PAHs	Pearson Correlation	-0.167	1	-0.878	-0.530	0.986**
m_1 (subG1checkpoint of cell cycle)	Pearson Correlation	-0.520	-0.878	1	0.741**	-0.909*
Group(24h and48h)	Pearson Correlation	-0.778**	-0.530	0.741**	1	-0.603
Percentage of DNA damage	Pearson Correlation	-0.083	0.986**	-0.909*	-0.603	1

** Correlation is significant at 0.01 level (2-tailed).

* Correlation is significant at 0.05 level (2-tailed).

Discussion

PAHs, particularly benzo[a]pyrene (B[a]P), have been investigated as potential environmental carcinogens. PAHs have been shown to be genotoxic in both Vivo and in Vitro studies. In chronic exposure to PAHs in vivo, DNA strand breakage, DNA-PAH adduct formation, damage to genetic material, the formation of micronuclei, activation of unscheduled DNA synthesis, and mutation of DNA structures were observed [21]. Although the majority of in vitro studies that are currently available have concentrated on the genotoxic effects of one or more PAHs identified as a result of PM in urban air [21], it is unknown how effective PAHs are in complex combinations. The findings of the present study will help in figuring out how different PAH chemicals interact with one another.

Extracted PAHs significantly slowed KB cell proliferation in a dose and time-dependent manner. Consequently, treated cells had a considerable reduction in number compared to control cells, and cytotoxicity increased by increasing doses. On the other hand, there was not a strong correlation between the viability rate and the length of treatment for cell culture. In fact, when the duration increased, a decline in the viability rate was seen, which is consistent with previous findings [22].

Cell development and differentiation are strongly regulated by the cell cycle distribution process [23]. Changes in the cell cycle distribution may be related to apoptosis and cell differentiation. An increase in the number of cells at the sub G1 checkpoint and a decrease in the number of cells in the S and G2/M phases of the cell cycle are signs that PAHs cause cell cycle arresting at this checkpoint. The stimulation of the cell's signaling route and the presence of DNA damage from oxidative stress may be the factors in the arresting of the cell cycle. DNA damage dose sets off several checkpoints, which eventually stop the progression of cell cycle. In addition, different types of damage may trigger various checkpoints [22]. The results of this study are in line with those of earlier research, showing that cells arrested at the G0 checkpoint are associated with DNA damage and higher levels of the protein p53 in the cells [24]. Furthermore, according to the chemical composition, PM-induced cell cycle arrest may occur in the G1 [25] or G2/M phases [26]. Also, the quantity and kind of PAHs were probably the main contributors to mitotic arrest [27].

A trustworthy technique for examining DNA damage caused by chemicals is the TUNEL test [28]. As it was predicted, the DNA damage worsened as the concentration of PAHs rose. This high induction rate may be attributed to high levels of PAHs, especially carcinogenic PAHs. The Olive tail moment was at its least significant in the group with the lowest Coke Oven Emissions (COE) exposure and at its most prominent in the group

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with the greatest COE exposure, according to previous studies on coke ovens [29]. Similar findings were found with the Olive tail moment with urine 1-hydroxypyrene (1-OHP), representing the internal exposure dosage of PAHs. This was seen in employees using coke ovens [30]. Finally, Table 3 shows that the correlation between the sub-G1 (M1 region) population and DNA damage was 0.909, indicating that both conditions derived from the presence of organic materials in the environment [21].

Conclusion

The effects of PAHs, one of the potentially hazardous chemical compounds, were investigated at the level of cells and molecules. Accordingly, PAHs impeded the spread of KB cell line by generating DNA damage, arresting the cell cycle at sub-G1checkpoint, inducing apoptosis, and fragmenting DNA. Extracted PAHs substantially affected KB cells (apoptotic, genotoxic, and cytotoxic). Accordingly, the present study clarified the processes behind the harmful effects of extracted PAHs and their associated adverse health impacts. The current investigation demonstrated how extracted PAHs are hazardous and detrimental to health.

Acknowledgment

This research was supported by Shahid Sadoughi University of Medical Sciences and Zahedan University of Medical Sciences.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This project is funded by Shahid Sadoughi University of Medical Sciences (ID: 7248).

Ethical Considerations

All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors and are aware that with minor exceptions, no changes can be made to authorship once the paper is submitted.

Code of Ethics

The present study has been approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd (IR.SSU.SPH.REC.1399.101).

Authors' Contributions

All the authors contributed to the study's conception and design. Preparation of material, data collection, and analysis were performed by Shahnaz Sargazi, Ramin Saravani, Hossein Fallahzadeh, and Sheida Shahraki. The first draft of the manuscript was written by Seyed Mehdi Tabatabaei, Ali Asghar Ebrahimi, Mohammad Hassan Ehrampoush, and Mohammad Javad Zare Sakhvidi. All the authors commented on the previous versions of the manuscript. All the authors read and approved the final manuscript.

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