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Paper

Synergistic toxicity of 2,4-dichlorophenoxyacetic acid and arsenic alters biomarkers in rats

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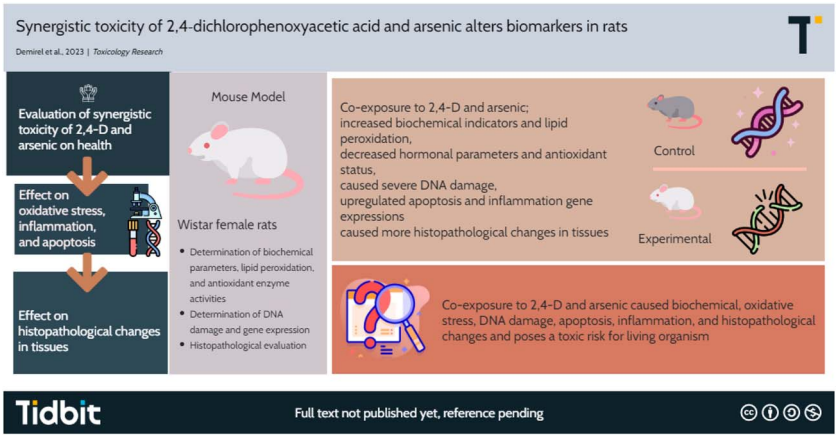
2,4-dichlorophenoxyacetic acid (2,4-D) and arsenic cause severe and extensive biological toxicity in organisms. However, their interactions and toxic mechanisms in co-exposure remain to be fully elucidated. In this study, 28 four-week-old female rats were divided into four groups and exposed to 100 mg/L arsenic or/and 600 mg/L 2,4-D through drinking water for a period of 28 days. As a result, it was revealed that biochemical indicators (ALT, AST, ALP, blood urea nitrogen, and creatinine) were increased and decreased hormonal parameters (FSH, LH, PG, and E2) in arsenic and 2,4-D and arsenic combination-treated groups. Moreover, increased lipid peroxidation (malondialdehyde level) and decreased antioxidant status (superoxide dismutase and catalase activities) were found in the co-exposure groups compared with the individual-exposure groups. Meanwhile, severe DNA damage was observed in co-exposure groups. Additionally, the levels of apoptotic (*Bax, Caspase-3, Caspase-8, Caspase-9, p53,* and *PARP*) and inflammation (*NFκB, Cox-2, TNF-α,* and *TGFβI*) indexes in the co-exposure groups were markedly increased, whereas the levels of anti-apoptosis index (*Bcl-2*) were decreased. It was also observed that co-exposure with 2,4-D and arsenic caused more histopathological changes in tissues. Generally, these results show that co-exposure to 2,4-D and arsenic can seriously cause oxidative stress, DNA damage, apoptosis and inflammation while having toxicological risk for organisms.

Graphical Abstract



Key words: 2,4-dichlorophenoxyacetic; arsenic; DNA damage; inflammation; apoptosis; rat.

1. Introduction

The environmental consequences of polluting soil and water with pesticides raise concerns for human and animal health.[1] 2,4-Dichlorophenoxyacetic acid (2,4-D), one of the pesticides in the herbicide group, is often used instead of resistant herbicides to control broadleaf weeds.[2] 2,4-D causes changes in the actin cytoskeleton in plants and leads to cell death by causing leakage in cell membranes.[3,4] It has been stated that the organism can bioaccumulate 2,4-D quickly during or after the exposure, which can cause stress reactions.[5,6] It has been observed that 2,4-D in many aquatic species, especially fish, can be found in environments that may pose a danger to amphibians, insects, bacteria, rodents, and humans.[7] It has been reported that 2,4-D exposure can induce inflammatory responses in various biological systems,

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lead to nerve damage by causing reactive oxygen species and inhibiting antioxidant defense and cause genotoxicity, autophagy, and carcinogenesis at the cellular level by affecting the normal functioning of the organism.[8] It has also been determined that 2,4-D affects the endocrine system by modulating the production of thyroid hormones and the expression of steroid hormones.[5,7]

Arsenic is a metalloid compound widely found in nature and has a robust carcinogenic effect on the organism.[9] Epidemiological studies have shown that subchronic or chronic arsenic exposure plays a role in developing neurological and cardiovascular diseases and various types of cancer.[10] Toxicity caused by arsenic mainly occurs in cells and tissues by causing oxidative damage to lipid proteins and DNA of cells due to the increase of reactive oxygen species (ROS) through oxidative stress.[11] Arsenic shows its apoptotic effect at the cellular level, especially by triggering the disruption of cell cycle control by cyclin-CDK and the role of *p53* with the inflammatory response formed by *NFκβ* and *TNF-α*.[12] In addition to these effects, long-term arsenic exposure affects the reproductive system in females and causes decreases in hormone levels.[13]

As a result of the studies carried out to date, it has been understood that people or animals are exposed to arsenic and 2,4-D due to consuming contaminated drinking water and foodstuffs or being in areas where these are available.[7,8] Exposure to more than one xenobiotic simultaneously causes the toxicological risk situation to reach dangerous levels. In this respect, it is crucial to reveal the effects of such situations on living things. This study aims to determine biochemical parameters, lipid peroxidation, antioxidant enzyme levels, DNA damage, apoptosis, inflammation, and histopathological changes in tissues in rats exposed to 2,4-D and arsenic and to evaluate the toxicological risk status of this situation.

2. Material and methods

2.1 Chemicals

Ester'H® (CAS Number: 25168-26–71280-20-2, Hektaş, Türkiye) containing isooctyl ester salt equivalent to 480 g/L 2,4-D acid and sodium arsenite (CAS Number: 7784-46-5, Sigma-Aldrich, MO, USA) were used in the study. Other chemicals to be used to determine the parameters to be analyzed were obtained from the relevant commercial companies.

2.2 Experimental design

In the study, 4-week-old 28 Wistar female rats (200 ± 25 g) obtained from Afyon Kocatepe University Experimental Animals Application and Research Center were used. Experimental stages were carried out following universal ethical principles and with the approval of the local ethics committee (49,533,702/155). Rats were kept under standard care conditions (room temperature was 21 ± 2 °C, the humidity was 55–60%, and the light and dark cycle was 12/12 hours) throughout the study and fed with standard rat chow. After a seven-day adaptation period, the rats were randomly divided into four groups of seven. Except for the control group, 600 mg/L 2,4-D[14] and 100 mg/L arsenic[15] were given in drinking water separately or jointly for 28 days. At the end of the experiment, blood and tissue samples were taken from the rats under slight ether anesthesia. Plasma and erythrocyte packages were created from the blood samples.[16] Moreover, some liver, kidney, heart, brain, and ovarian tissues were homogenized in .15 M Tris–HCl buffer (pH 7.4) and used in biochemical analyses. Molecular and histopathological analyzes were performed on other tissue sections.

2.3 Determination of biochemical parameters

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine levels in rat plasma were measured spectrophotometrically (Shimadzu 1601 UV–VIS, Tokyo, Japan) using Biolabo (Medica, Germany) brand commercial kits.

2.4 Determination of lipid peroxidation and antioxidant enzyme activities

Malondialdehyde (MDA) level, which is an essential marker of lipid peroxidation (LPO), were determined according to the methods proposed by Draper and Hardley[17] in whole blood and by Ohkawa et al.[18] in tissues. The methods suggested by Sun et al.[19] and Sinha[20] were used for the determination of superoxide dismutase (SOD) and catalase (CAT) activities in erythrocyte lysate and tissue homogenates, respectively. The amount of hemoglobin (Hb) in lysates was determined using the cyanmethemoglobin method introduced by Drabkin and Austin,[21] while the amount of protein in the tissues was revealed using the colorimetric method proposed by Lowry et al.[22] In these studies, all parameters were measured spectrophotometrically (Shimadzu 1601 UV–VIS, Tokyo, Japan).

2.5 Determination of DNA damage

For Comet analysis conducted to detect DNA damage, slides were coated with 1% agarose (NMA) and frozen. Then, 10 μl of blood sample and 100 μl of .5% LMA were mixed, and the mixture was added to frozen NMA-coated slides and incubated at 4 °C for 5 minutes. Prepared preparations were treated in a lysis solution (100 mM EDTA, 2.5 M NaCl, 10 mM Tris base, 1% Triton X-100 and 10% DMSO) at 4 °C for 1 hour and kept in electrophoresis buffer (10 N NaOH and 200 mM EDTA) at 4 °C for 15 minutes. Afterwards, the preparations were neutralized in .4 M Tris buffer (pH 7.5) for 5 minutes and stained with ethidium bromide (10 μl/ml). The stained preparations were examined by fluorescence microscopy (Zeiss, Germany).[23] Scoring was performed on 100 cells per slide in three repetitions under minimal illumination. The DNA damage score was evaluated according to the size of the comet (0-no damage, 1-slight, 2-moderate, 3-severe, or 4-most damaged) and expressed as arbitrary units (AU).[24]

2.6 Determination of gene expression

Procedure on RNA isolation from liver, kidney, and brain tissues was performed using an RNA purification kit (GeneJet, Thermo Scientific, USA), and the kit's quality was demarcated with a microplate photometer (MultiskanTM FC, Thermo Scientific, USA). Primers specific to *Rattus norvegicus* were designed according to NCBI (Table 1) and named by FastPCR 6.0. *β-actin, p53, Bcl-2, Caspase-3, Caspase-8, Caspase-9, NFκB, TGFβI, TNF-α, Bax, Cox-2,* and *PARP* gene expression levels were determined with PCR analysis (Number of cycle and temperature of each gene is 40) in Real-Time PCR Detection System (CFX Connect™, Bio-Rad Lab, USA) and results were got through CFX Maestro. Analyses were conducted in three repetitions, and mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.[25]

2.7 Histopathological evaluation

Tissue samples of liver, kidney, heart, brain, and ovarium were fixed in 10% buffered formalin solution for histopathological examinations. For this purpose, tissue samples were thinned to 2–3 mm and placed in tissue tracking cassettes. After washing the samples overnight, they were kept in absolute alcohol of

Table 1. Oligonucleotide sequence, product size, and gene bank number of genes.

Gene	Oligonucleotide sequence	Product size (bp)	Gene bank number
β-Actin	F: GAGGGAAATCGTGCGTGACAT	452	NC_005111.4
	R ACATCTGCTGGAAGGTGGACA		
p53	F TGCAGAGTTGTTAGAAGGCCCA	397	NM_030989.3
	R GTCACCATCAGAGCAACGCTC		
Caspase-3	F ACCCTGAAATGGGCTTGTGTA	427	NM_012922.2
	R GCCATATCATCGTCAGTTCCAC		
Bcl-2	F GGGTATGATAACCGGGAGATCG	508	NM_016993.1
	R ACTCAGTCATCCACAGAGCGA		
NFκB	F TCCCCAAGCCAGCACCCCAGC	334	NM_199267.2
	R GGCCCCCAAGTCTTCATCAGC		
Caspase-8	F TTGCTGAACGTCTGGGCAACG	502	NM_022277.1
	R TCGTCGATCCTTCCCAGCAAGC		
Caspase-9	F AGAAACACCCAGGCCGGTGGA	327	NM_031632.1
	R ACCACGAAGCAGTCCAGGGCAC		
TGFβ1	F ATACGTCAGACATTCGGGAA	294	NM_021578.2
	R AGCAAAGATAATGTACTCCAC		
TNF-α	F CGAGTGACAAGCCCGTAGCC	368	NM_012675.3
	R GGATGAACACGCCAGTCGCC		
Bax	F AGGACGCATCCACCAAGAAGC	363	NM_017059.2
	R CAGTGAGGACTCCAGCCACAA		
Cox-2	F GAGTACCGCAAACGCTTCTCC	395	AF233596.1
	R CTTGCGTTGATGGTGGCTGTC		
PARP	F GTATCCCCAAGGACTCGCTCC	537	NM_013063.2
	R CTTCACTCTTGACTGCCGGGA		

50, 70, 80, and 96 with xylol, paraffin with xylol and paraffin melted at 56–58 °C for 2 hours and then blocked in paraffin. Paraffin blocks were cut with a microtome (Leica, RM 2245) at a thickness of 5 μm and transferred to microscope slides. The prepared sections were dried in a drying oven for 10 minutes before being used in histopathological methods. All sections were subjected to absolute, 96, 80, 70 and 50 alcohol series and xylol series before staining with hematoxylin–eosin (H&E). The stained tissue preparations were examined with a binocular headlight microscope (Nikon Eclipse, Tokyo, Japan); the damaged areas were detected, and their photographs were taken (Nikon DS FI3, microscopic digital camera systems, Tokyo, Japan).

2.8 Statistical analyses

The obtained data was statistically analyzed by using the SPSS 22 software package. Before the analysis, the compliance of the data with the typical distribution pattern was tested, and it was determined that the data were within the normal distribution. A one-way ANOVA test was used to evaluate the findings, and Duncan's posthoc test was used to compare statistically different results. Data were expressed as "mean ± standard deviation," and the statistical significance level was considered <.05.

3. Results

3.1 Effects on biochemical parameters

It was determined that the activities of ALT (Fig. 1A), AST (Fig. 1B), and ALP (Fig. 1C), which are liver enzymes, increased in the groups given 2,4-D and arsenic separately, and this increase was higher in the group given the two substances jointly ($P < .05$). In addition, BUN (Fig. 1D) and creatinine (Fig. 1E) levels, which are kidney function parameters, did not change much in the 2,4-D-treated group but increased in the arsenic-treated group. It was determined that the combination of 2,4-D and arsenic increased these parameters more than the amounts given alone ($P < .05$). It was determined that E2 (Fig. 2A), PG (Fig. 2B), LH (Fig. 2C), and FSH (Fig. 2D) levels, which are among the parameters indicating hormonal status, did not change in the 2,4-D-treated group, decreased in the arsenic-treated group, and these values decreased more in the group in which 2,4-D and arsenic were given together ($P < .05$).

3.2 Effects on antioxidant status

MDA levels (Table 2) in whole blood, liver, kidney, heart, brain, and ovarium tissues ($P < .05$) were found to be high levels in the 2,4-D and arsenic groups compared to the control group. Moreover, it was observed that when 2,4-D and arsenic were given jointly, the MDA values were at the highest level compared to those given alone. Additionally, when 2,4-D or arsenic exposure was alone, it decreased the SOD (Table 3) and CAT (Table 4) activities as antioxidant enzymes in all tissues of the rats ($P < .05$). On the other hand, these activities were found to be low levels in the 2,4-D and arsenic combination group compared to those given alone.

3.3 Effects on DNA damage

DNA damage was determined with the Comet assay, and it was found to be higher in the 2,4-D (3.69 ± .53 AU) and arsenic (4.32 ± .62 AU) groups compared to the control group (1.54 ± .26 AU) ($P < .05$). Besides, DNA damage was observed to be higher in the 2,4-D and arsenic combination group (6.64 ± .58 AU) than those which take these substances separately (Fig. 3A).

3.4 Effects on mRNA expression levels

When the results of mRNA expressions of apoptotic genes in the liver (Fig. 3B), kidney (Fig. 3C), and brain (Fig. 3D) tissues were evaluated, it was determined that the expression levels of *Bax*, *Caspase-3*, *Caspase-8*, *Caspase-9*, *p53*, and *PARP* were higher in the 2,4-D and arsenic-treated groups when compared to the control

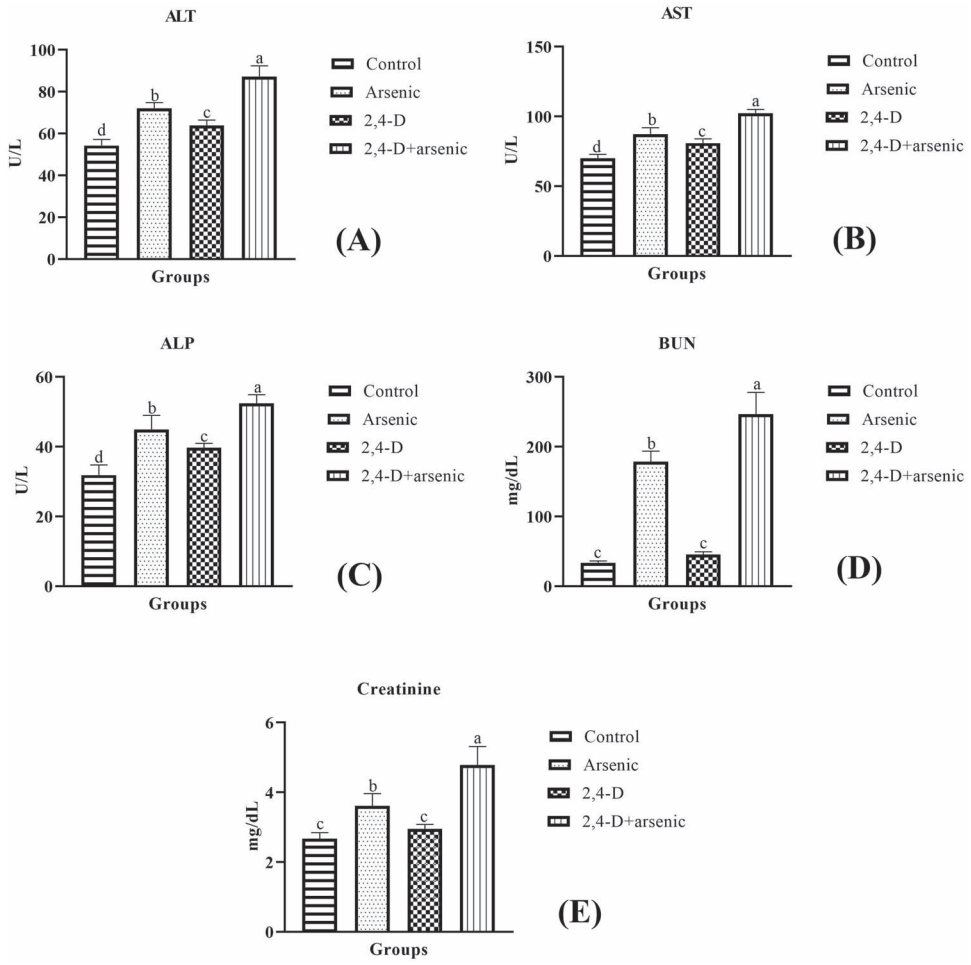

Fig. 1. Alone and co-exposure effects of 2,4-D and arsenic on rat plasma AST (A), ALT (B), ALP (C), BUN (D), and creatinine (E) levels. Different letters [a,b,c] are statistically significant ($P < .05$).

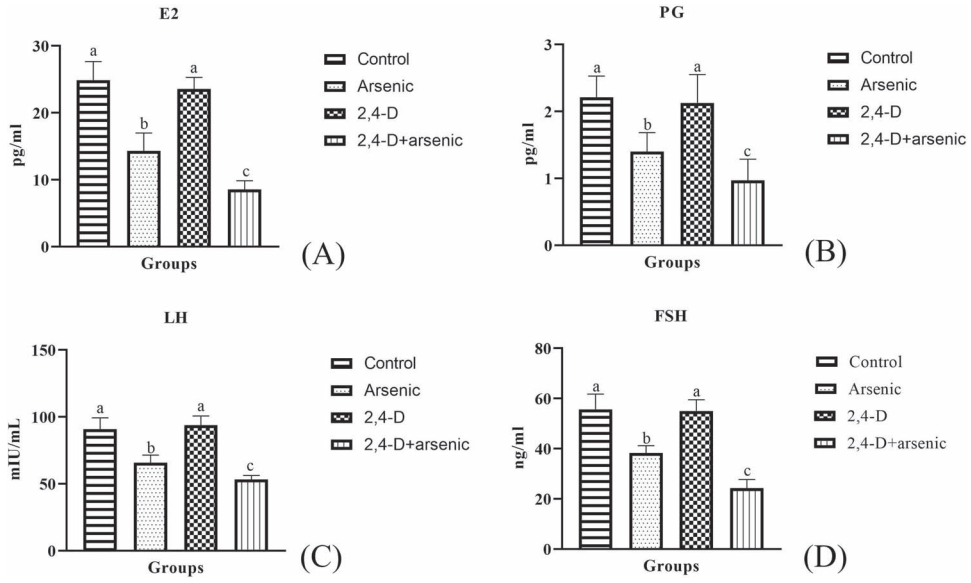

Fig. 2. Alone and co-exposure effects of 2,4-D and arsenic on E2 (A), PG (B), LH (C), and FSH (D) levels. Different letters [a,b,c] are statistically significant ($P < .05$).

group ($P < .001$), and the fold increases were even higher when the exposure of these two substances was jointly. On the other hand, it was determined that the *Bcl-2* mRNA expression levels in these tissues decreased in the 2,4-D and arsenic-treated groups, while the decrease was more in the groups treated by the substances together ($P < .001$). It was observed that the mRNA expressions

Table 2. Alone and co-exposure effects of 2,4-D and arsenic on MDA levels of blood, liver, kidney, heart, brain, and ovarium tissue homogenates.

Groups	Blood (nmol/ml)	Liver (nmol/g tissue)	Kidney (nmol/g tissue)	Heart (nmol/g tissue)	Brain (nmol/g tissue)	Ovarium (nmol/g tissue)
Control	4.01 ± .41^{c}	6.82 ± 1.08^{c}	4.77 ± .66^{c}	4.23 ± .62^{c}	3.49 ± .31^{b}	3.50 ± .27^{c}
Arsenic	4.69 ± .51^{b}	10.57 ± 1.62^{b}	8.99 ± 1.41^{b}	8.30 ± 2.01^{b}	4.54 ± .58^{b}	4.92 ± .51^{b}
2,4-D	4.25 ± .45^{c}	9.54 ± 1.15^{b}	4.90 ± .61^{c}	4.51 ± .36^{c}	4.26 ± .34^{b}	4.41 ± .34^{b}
2,4-D + arsenic	5.47 ± .41^{a}	14.75 ± 2.89^{a}	12.26 ± 2.17^{a}	11.68 ± 3.62^{a}	8.38 ± 2.71^{a}	8.78 ± .85^{a}

Mean ± standard deviations; *n*: 7 ^{a,b,c}values with different letters in the same column are statistically significant ($P < .05$).

Table 3. Alone and co-exposure effects of 2,4-D and arsenic on SOD activity of blood, liver, kidney, heart, brain, and ovarium tissue homogenates.

Groups	Erythrocyte (U/mgHb)	Liver (U/μg protein)	Kidney (U/μg protein)	Heart (U/μg protein)	Brain (U/μg protein)	Ovarium (U/μg protein)
Control	21.22 ± 1.32^{a}	4.30 ± .30^{a}	4.06 ± .57^{a}	2.94 ± .34^{a}	3.58 ± .20^{a}	1.72 ± .34^{a}
Arsenic	18.53 ± 2.27^{b}	2.02 ± .44^{b}	2.22 ± .23^{c}	2.13 ± .24^{b}	2.94 ± .18^{b}	1.19 ± .23^{b}
2,4-D	18.60 ± 1.62^{b}	2.20 ± .31^{b}	3.19 ± .69^{b}	2.17 ± .36^{b}	3.05 ± 1.16^{b}	1.44 ± .32^{ab}
2,4-D + arsenic	1.80 ± 1.45^{c}	1.36 ± .25^{c}	1.30 ± 1.24^{d}	1.13 ± .23^{c}	2.54 ± .25^{c}	0.80 ± .22^{c}

Mean ± standard deviations; *n*: 7 ^{a,b,c,d}values with different letters in the same column are statistically significant ($P < .05$).

Table 4. Alone and co-exposure effects of 2,4-D and arsenic on CAT activity of blood, liver, kidney, heart, brain, and ovarium tissue homogenates.

Groups	Erythrocyte (U/mgHb)	Liver (U/μg protein)	Kidney (U/μg protein)	Heart (U/μg protein)	Brain (U/μg protein)	Ovarium (U/μg protein)
Control	75.49 ± 5.66^{a}	17.60 ± 2.32^{a}	8.96 ± 1.08^{a}	6.44 ± .43^{a}	7.63 ± 1.36^{a}	3.17 ± .71^{a}
Arsenic	52.61 ± 4.52^{b}	10.48 ± 1.27^{b}	6.53 ± .61^{b}	5.37 ± .43^{b}	4.82 ± 1.07^{b}	1.86 ± .41^{b}
2,4-D	54.43 ± 5.59^{b}	12.35 ± 1.67^{b}	6.06 ± .76^{b}	5.41 ± .85^{b}	4.96 ± .72^{b}	1.88 ± .25^{b}
2,4-D + arsenic	40.41 ± 4.65^{c}	7.70 ± 1.45^{c}	4.37 ± .33^{c}	3.50 ± .46^{c}	2.45 ± .82^{c}	1.10 ± .92^{c}

Mean ± standard deviations; *n*: 7 ^{a,b,c}values with different letters in the same column are statistically significant ($P < .05$).

of *NFκB*, *Cox-2*, *TNF-α*, and *TGFβI* genes, which are associated with inflammation, were higher in 2,4-D and arsenic-treated groups compared to the control group ($P < .001$) and that these fold increases were even higher when the two substances were given together ($P < .001$).

3.5 Effects on histopathologic changes

In the determined 2,4-D-treated, arsenic-treated and 2,4-D + arsenic-treated groups, degenerative changes in neurons in brain tissue, areas of focal gliosis, vacuolization formations in neurons, and hyperemia in vessels were observed (Fig. 4B-D). Areas of hyaline degeneration in cardiac muscle cells in heart tissue, focal areas of mononuclear cell infiltration between cardiac muscle cells and focal areas of hemorrhage between cardiac muscle cells were seen (Fig. 5B-D). In the liver tissue of the rats, hyperemia in the vessels, dual-nucleated hepatocytes in the periportal areas, an increase in the number of bile ducts in the periportal area, sinusoidal dilatation and areas of hyperemia were observed (Fig. 6B-D). In the kidneys of the rats, narrowing of the Bowman's space in the glomerulus, hyperemia and vacuolization formations, hemorrhage in tubulus lumens, hyaline cylinder formations in tubulus lumens and degenerative changes in tubular epithelial cells were observed (Fig. 7B-D). Moreover, congestion in the vessels of the ovaries, hemorrhage in the stroma, degenerative changes in the appearance of the corpus luteum, areas of mononuclear cell infiltration in the interstitial region, and an increase in fibrous tissue in the interstitial region was seen (Fig. 8B-D). When this situation was examined, it was determined that 2,4-D caused less damage to tissues than arsenic and that the damage occurred more severely after exposure to substances together than in singular exposures. Besides, histopathological changes in the brain (Fig. 4A), heart (Fig. 5A), liver (Fig. 6A), kidney (Fig. 7A), and ovariun (Fig. 8A) tissue were ordinary in the control group. Additionally, the evaluation of histopathological changes is presented in Table 5.

4. Discussion

Exposure to more than one xenobiotic creates severe toxic effects on the organism and increases possible risks.[26] It has been reported that liver function parameters (AST, ALT, and ALP) increase in rats exposed to 2,4-D and arsenic,[8,27,28] and that BUN and creatinine values increase with arsenic exposure[15] while the 2,4-D exposure does not affect these parameters much.[29] Similarly, this study has revealed that the co-exposure of these two substances induces liver and kidney function parameters more than when treated alone. These results show that the co-exposure of 2,4-D and arsenic synergistically causes liver and kidney damage.

In an in vitro study of the toxicokinetics of 2,4-D (this study was performed according to Tier 1 of the United States Environmental Protection Agency's Endocrine Disruptor Screening Program), it has been reported that 2,4-D treated at a maximum of 10^{-4} M does not have the potential to interact with estrogen, androgen or steroidogenesis pathways in vitro.[30] However, it has been reported that feeding rats 100, 300, 600 (female), or 800 (male) mg/kg of 2,4-D for 28 days do not cause reproductive toxicity, developmental neurotoxicity, and immunotoxicity. In addition, considering that the non-observed adverse effect level (NOAEL) of 2,4-D is 16.6 and 20.6 mg/kg/day for men and women, it has been stated that the

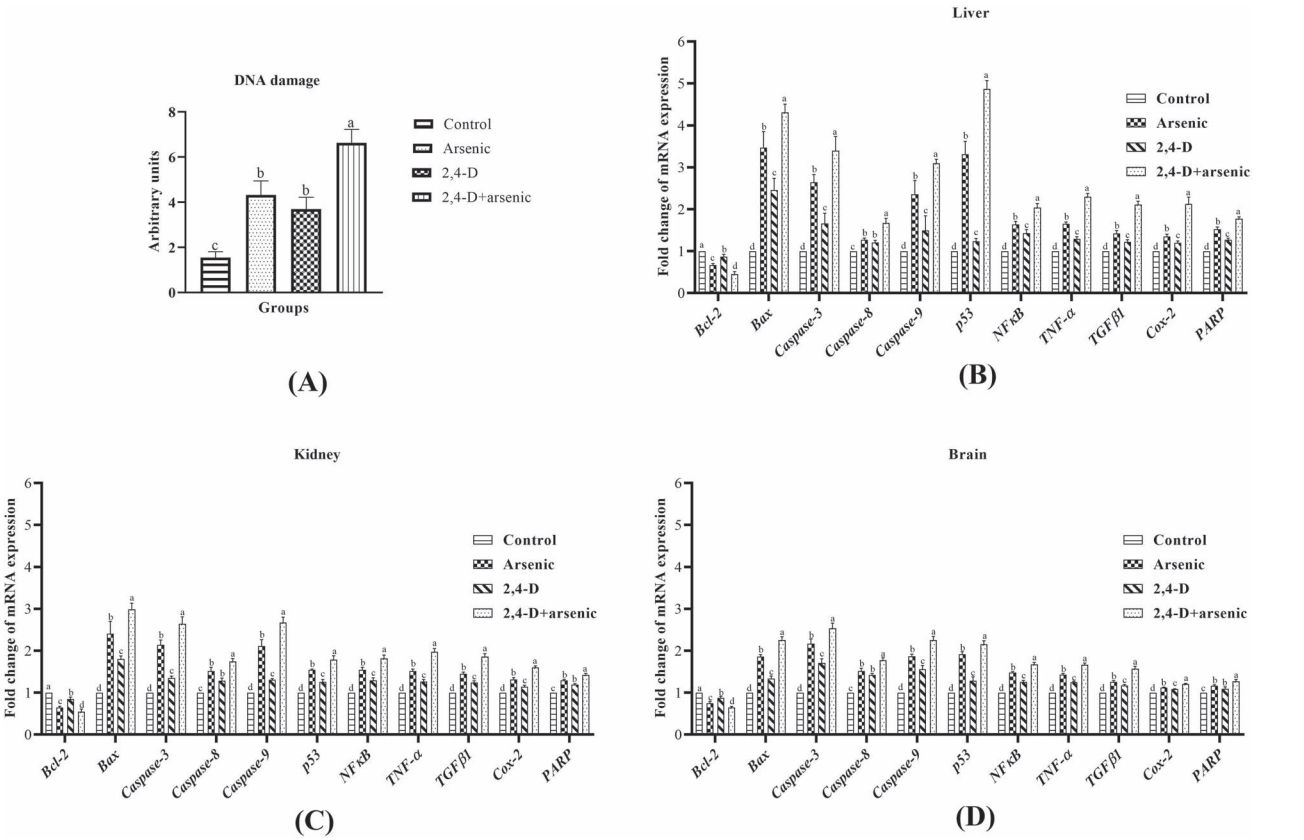

Fig. 3. Alone and co-exposure effects of 2,4-D and arsenic on DNA damage (A), gene expression in liver (B), kidney (C), and brain (D) tissues. Different letters [a,b,c] are statistically significant ($P < .05$).

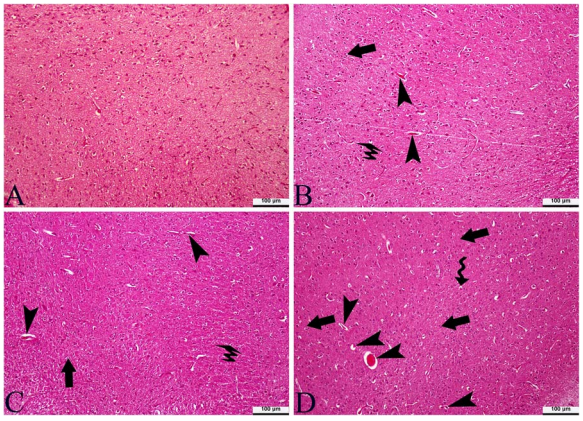

Fig. 4. Histopathological alterations in rat brain tissue created by the exposure of 2,4-D and arsenic, both separately or jointly. Arrow: neuronophagia and focal gliosis, arrowhead: congestion in the veins, and curved arrow: vacuolization in neurons. A: Control; B: 2,4-D; C: arsenic; D: 2,4-D + arsenic. All figures are stained with H&E. 20x and 100 μm were used as the original magnifications.

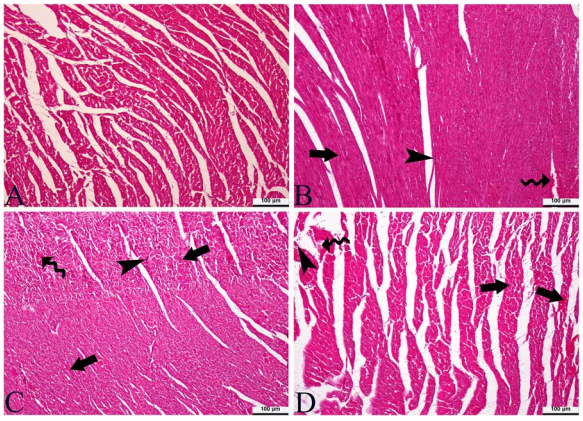

Fig. 5. Histopathological alterations in rat heart tissue created by the exposure of 2,4-D and arsenic, both separately or jointly arrow: hyaline degeneration areas in muscle cells, arrowhead: mononuclear cell infiltration areas in the myocard, and curved arrow: Hemorrhage areas in myocard. A: Control; B: 2,4-D; C: arsenic; D: 2,4-D + arsenic. All figures are stained with H&E. 20x and 100 μm were used as the original magnifications.

doses given to rats are much higher than these.[31] Therefore, the findings obtained from this study and the studies above show that 2,4-D has a minimal (possible) endocrine activity potential. In a study examining the adverse effects of arsenic given to female rats at different doses (50, 100, and 200 ppm) in drinking water for 28 days, it was reported that the uterine structure was adversely affected and dose-related decreases in plasma levels of E2, PG, FSH, and LH existed.[32] In another study in which female rats were exposed to arsenic at a dose of 4 μg/ml for 28 days, it was reported that there was a decrease in both LH and FSH levels and a dose- and time-dependent decrease in circulating E2 levels.[13] This study, which was conducted in parallel with these results, revealed that the exposure of arsenic reduced the circulating levels of steroid hormones, and the effect of arsenic exposure together with 2,4-D was even more substantial, and the disorder possibly caused this situation in the endocrine system due to excessive ROS production.

Table 5. Alone and co-exposure effects of 2,4-D and arsenic on histopathological alterations in brain, heart, liver, kidney, and ovarium tissue of rats (*n*: 7).

Tissue	Histopathological changes	Control	Arsenic	2,4-D	2,4-D + arsenic
Brain	Congestion in the veins	−(7/7)	+(2/7) ++(5/7)	+(5/7) ++(2/7)	++(4/7) +++(3/7)
	Vacuolization in neurons	−(7/7)	+(2/7) ++(1/7) +++(4/7)	+(4/7) ++(3/7)	++(5/7) +++(2/7)
	Neuronophagia and focal gliosis	−(7/7)	+(5/7) ++(2/7)	−(6/7) ++(1/7)	++(1/7) ++(3/7) +++(3/7)
Heart	Hyaline degeneration areas in muscle cells	−(7/7)	−(1/7) + (4/7) ++(2/7)	−(2/7) + (5/7)	+(1) ++(3/7) +++(3/7)
	Mononuclear cell infiltration areas in the myocard	−(7/7)	−(1/7) + (3/7) ++(3/7)	−(3/7) + (3/7) ++(1/7)	++(3/7) +++(4/7)
	Hemorrhage areas in myocard	−(7/7)	+(2/7) ++(5/7)	−(4/7) + (1/7) ++(2/7)	++(3/7) +++(4/7)
Liver	Hyperemia of the vein	−(7/7)	−(1/7) + (4/7) ++(2/7)	−(3/7) + (4/7)	+(2/7) ++(5/7)
	Binaural hepatocyte formations in periportal areas	−(7/7)	−(1/7) + (4/7) ++(2/7)	−(5/7) ++(2/7)	+(1/7) ++(4/7) +++(2/7)
	Increase in the number of bile ducts in the periportal areas	−(7/7)	+(5/7) ++(2/7)	−(6/7) ++(1/7)	+(1/7) ++(3/7) +++(3/7)
	Sinusoidal dilatation and hyperemia	−(7/7)	+(5/7) ++(1/7) +++(1/7)	−(2/7) + (5/7)	+(4/7) ++(2/7) +++(1/7)
Kidney	Congestion and narrowing of Bowman's space	−(7/7)	++(4/7) +++(3/7)	−(4/7) ++(3/7)	++(5/7) +++(2/7)
	Hyperemia and vacuolization formations in the glomerulus capillary ball	−(7/7)	−(2/7) + (4/7) ++(1//7)	−(3/7) + (4/7)	+(2/7) ++(5/7)
	Hyaline cylinder formations in tubular lumens	−(7/7)	+(1/7) ++(1/7) ++(5/7)	−(3/7) + (4/7)	+(2/7) ++(3/7) +++(2/7)
	Degenerative changes and focal necrosis areas in tubular epithelial cells	−(7/7)	+(4/7) ++(3/7)	−(3/7) + (4/7)	+(2/7) ++(4/7) +++(1/7)
	Hemorrhage in tubulus lumen	−(7/7)	−(5/7) + (2/7)	−(7/7)	+(4/7) ++(1/7)
Ovarium	Hyperemia in the veins	−(7/7)	−(2/7) + (3/7) +++(2/7)	−(4/7) + (3/7)	+(1/7) ++(3/7) +++(3/7)
	Areas of mononuclear cell infiltration in the stroma	−(7/7)	+(3/7) ++(4/7)	−(1/7) + (4/7) ++(2/7)	+(3/7) ++(3/7) +++(1/7)
	Areas of fibrosis formation in the stroma	−(7/7)	++(3/7) +++(4/7)	+(5/7) ++(2/7)	++(2/7) +++(5/7)

Symbols in the table; −: None, +: Mild, ++: Moderate, +++: Severe.

In in vivo studies, it has been determined that 2,4-D and arsenic cause an increase in the level of MDA, which is an indicator of lipid peroxidation, and a decrease in the activities of SOD and CAT, which are antioxidant markers.[15,28,33,34] As a result of the study, it was understood that the co-exposure of 2,4-D and arsenic led to a further increase and/or decrease in the values of these parameters due to the synergistic effect and increased oxidative stress. Studies on rodents have reported that acute and/or chronic exposure of 2,4-D at moderate and high doses causes DNA damage and can cause genotoxicity.[8,35] Likewise, it has been

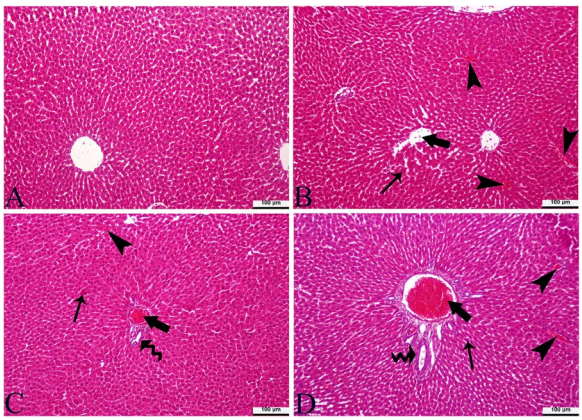

Fig. 6. Histopathological alterations in rat liver tissue created by the exposure of 2,4-D and arsenic, both separately or jointly. Arrow: hyperemia of the vein, arrowhead: Sinusoidal dilatation and hyperemia, curved arrow: Increase in the number of bile ducts in the periportal areas, and thin arrow: Binaural hepatocyte formations in periportal areas. A: Control; B: 2,4-D; C: arsenic; D: 2,4-D + arsenic. All figures are stained with H&E. 20x and 100 μm were used as the original magnifications.

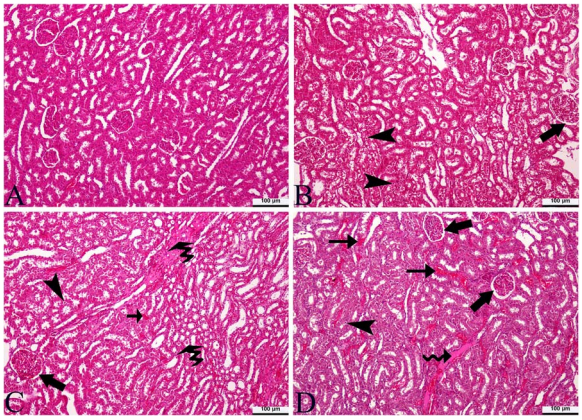

Fig. 7. Histopathological alterations in rat kidney tissue created by the exposure of 2,4-D and arsenic, both separately or jointly. Arrow: congestion and narrowing of Bowman's space, hyperemia and vacuolization formations in the glomerulus capillary ball, arrowhead: degenerative changes and focal necrosis areas in tubular epithelial cells, curved arrow: hyaline cylinder formations in tubular lumens, and thin arrow: hemorrhage in tubulus lumen. A: Control; B: 2,4-D; C: arsenic; D: 2,4-D + arsenic. All figures are stained with H&E. 20x and 100 μm were used as the original magnifications.

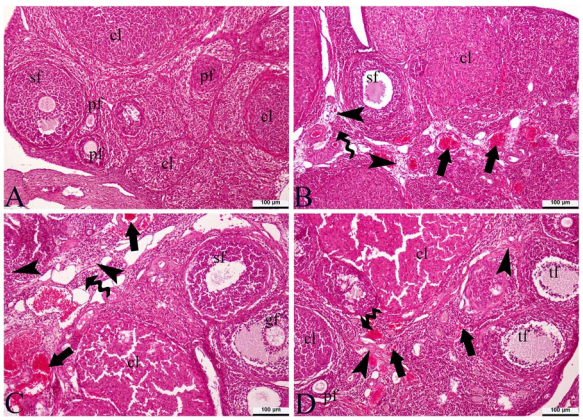

Fig. 8. Histopathological alterations in rat ovarium tissue created by the exposure of 2,4-D and arsenic, both separately or jointly arrow: hyperemia in the veins, arrowhead: areas of fibrosis formation in the stroma, and curved arrow: areas of mononuclear cell infiltration in the stroma. Also, the corpus luteum (cl), the primary follicle (pf), the secondary follicle (sf), the tertiary follicle (tf), and the graff follicle (gf) were not found any alterations. A: Control; B: 2,4-D; C: arsenic; D: 2,4-D + arsenic. All figures are stained with H&E. 20x and 100 μm were used as the original magnifications.

determined that arsenic exposure in rats in drinking water and orally causes DNA damage.[15,36] Similarly, in this study, it has been revealed that both 2,4-D and arsenic cause DNA damage, which is more in co-exposure cases, and it can be stated that this situation is caused by the direct effects of the compounds on DNA, as well as increased lipid peroxidation and decreased antioxidant activity.

In a study investigating the synergistic effects of arsenic and nicotine, rats were co-exposed to lower concentration of arsenic (25 ppm in drinking water) or nicotine (.25 mg/kg, subcutaneously) for five months; afterwards, it was reported that the expression levels of *Bax* and *Caspase-3*, which are apoptotic markers, increased in liver and brain tissues (especially in brain tissue).[37] As a result of the arsenic exposure at a dose of 10 mg/mL to rats for three months, *Caspase 3, Caspase 8, Caspase 9,* and *PARP* expression levels determined from liver tissue and an increase in *Bax/Bcl-2* ratio were observed; and it was reported that arsenic caused DNA fragmentation and thus apoptosis.[38] Similarly, some studies have reported that arsenic causes apoptotic cell death in tissues.[39,40] Moreover, recent in vivo studies have shown that 2,4-D plays an active role in the apoptotic process.[8,41] In parallel with these results, in this study, it was determined that the apoptotic process increased with the synergistic effect through the exposure to arsenic and 2,4-D substances separately, as well as the joint exposure.

As a result of two different studies in which arsenic was exposed to rats through drinking water (100 mg/L) for 30 and 60 days, it was reported that increased expression levels of *IFN-γ, IL-1β, TNF-α,* and *NFκB* mRNA, which are inflammatory cytokine markers, were observed in kidney, liver, and brain tissues.[15,33] Ince et al. have found that *NF-κB, Cox-2, TNF-α,* and *MCP-1* mRNA expressions, which cause inflammation in liver, kidney, and brain tissues, increase when ethanol and 2,4-D are exposed singularly and jointly.[8] As a result of the exposure of 75 mg/kg 2,4-D to neonatal rats for four weeks, it has been understood that the expression levels of TNFα, *IL-6, IL-1β,* and *IL-18* protein and mRNA increase, indicating inflammation in the brain tissues.[41] This study determined that the singular exposure of arsenic and 2,4-D caused an increase in the mRNA expressions of *NFκB, Cox-2, TNF-α,* and *TGFβI* genes and led to inflammation. It was also understood that exposure increased the severity of inflammation with a synergistic effect.

Tayeb et al. have reported that there are degeneration areas in the livers, degeneration of neurons in the brain tissue, glomeruli in the kidneys, damage in the tubules and hyperemia in the blood vessels of rats given 15, 75, or 150 mg/kg 2,4-D daily for four weeks.[42] In parallel, Ince et al. have stated that histopathological changes occur in the liver, kidney, heart, lung, testis, and brain tissues after exposure to 2,4-D at a dose of 5 mg/kg for eight weeks.[8] According to Arslan-Acaroz et al. when rats were exposed to 100 mg/L arsenic for 60 days, neuronal degeneration and focal gliosis in the brains of rats, thickening of the interalveolar septal tissue and mononuclear cell infiltration in the lungs, hyaline degeneration in the heart, degenerations in the liver, enlargement and leukocyte infiltration in the sinusoids, hydropic degenerations in the kidney tubule and shrinkage of the Bowman's capsule

were detected.[33] In addition, Kucukkurt et al. found that when male and female rats were exposed to arsenic through 100 mg/L drinking water for 28 days, both male and female rats in the arsenic group experienced liver, kidney, heart, and brain tissue damage.[11] Similarly, our study observed visible histopathological changes in tissues, especially the liver and kidneys of rats, following exposure to 2,4-D and arsenic; however, after the co-exposure to these substances, it was determined that the damage to the tissues was more severe.

5. Conclusion

This study is the first to suggest that these substances have a possible synergistic effect on rats exposed to arsenic and 2,4-D jointly. This synergistic effect was determined based on 2,4-D and arsenic biochemical, oxidative stress, DNA damage, apoptosis, inflammation, and histopathological changes. Therefore, it has been understood that exposure to both arsenic and 2,4-D, separately or jointly, poses a toxic risk at the current dose and time, and, as a result, it has been understood that there are dangerous consequences for living things.

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Author contributions

Hasan Huseyin DEMIREL: Supervision, Visualization, Writing-original draft, Writing–review & editing, Methodology, Funding acquisition, Project administration, Conceptualization; Fahriye ZEMHERI-NAVRUZ: Investigation, Methodology; Damla ARSLAN-ACAROZ: Data curation, Investigation, Methodology; Ismail KUCUKKURT: Data curation, Investigation, Methodology; Ali TUREYEN: Data curation, Investigation, Methodology; Sinan INCE: Data curation, Investigation, Methodology, Writing-review & editing.

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