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Investigation of the miRNA levels changes to acceptable daily intake dose pesticide mixture exposure on rat mesentery and pancreas

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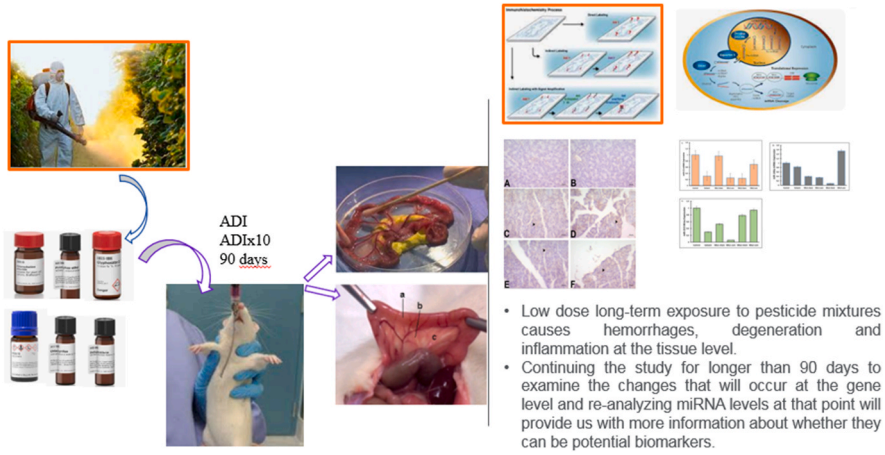
HIGHLIGHTS

- Exposure to a mixture of pesticides at acceptable daily intakes induces degenerative changes in endocrine organs.
- Increased miR-21 in pancreatic and mesentery tissue suggests that it may be linked to pesticide-mediated inflammatory process.
- MiR-223 levels, which suppress proinflammatory cytokines, were significantly reduced in mesenteric tissue.
- In pancreatic tissue, miR-146a expression was increased especially in the Mix-2 commercial group.

GRAPHICAL ABSTRACT



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ABSTRACT

Consumers are constantly exposed to a variety of chemical mixtures as part of their everyday activities and lifestyle. Food, water and commercial products are only some examples of the possible ways people get exposed to these mixtures. However, following federal and local guidelines for risk assessment related to chemical exposure, risk analysis focuses on a single substance exposure scenario and not on a mixture, as in real life.

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Pirimiphos-methyl
Glyphosate
Tebuconazole
Chlorpyrifos-methyl
Deltamethrin

Realizing the pronounced gap of this methodology, the real-life risk simulation scenario approach tries to address this problem by investigating the possible effect of long-term exposure to chemical mixtures closely resembling the actual circumstances of modern life. As part of this effort, this study aimed to identify the cumulative effects of pesticides belonging to different classes and commonly used commercial products on long-term exposure with realistic doses. Sprague Dawley rats were given a pesticide mix of active ingredients and formulation chemicals in a daily acceptable dose (ADI) and 10xADI for 90 days. Following thorough everyday documentation of possible side-effects, after 90 days all animals were sacrificed and their organs were examined. Exposure to pesticides particularly affects the miRNA levels at that point will provide us with more information about whether they can be potential biomarkers.

1. Introduction

A pesticide is a chemical substance or mixture deliberately applied to the environment to control and/or kill populations of insects, weeds, rodents, fungi or other harmful pests. Cereal crops are often sprayed with insecticides (such as pirimiphos-methyl, chlorpyrifos-methyl, deltamethrin, pirimicarb and, dichlorvos) (Bar-L'Helgouac'h, 2004). To reduce the harmful effect of fungi, both strobilurin (azoxystrobin, trifloxystrobin, kresoxym-methyl) and azole fungicides (such as propiconazole, epoxiconazole, tebuconazole) are used. Regarding the application of herbicides/dryers, glyphosate is by far the most commonly used, although others belonging to the phenylurea family have been applied as well. In grain production, plant growth regulators (such as chlormequat, mepiquat, imazaquin) are used to shorten plant stems, that is, to reduce their sensitivity to wind and rain flattening (Tadeo et al., 1996). Thus, it is aimed to increase the productivity and product quality of established crops.

European Union (EU) has a comprehensive monitoring program in place to assess and regulate pesticide residues in food products. This program aims to ensure that food placed on the market complies with established maximum residue levels (MRLs) and does not pose a threat to consumer health. With the analyzes performed on the collected food samples, it is monitored whether the samples are within legally permitted limits. European Multiannual Control Programmes for Pesticide Residues (MACP), EFSA, and national food safety authorities within the EU member states provide detailed information regarding pesticide residues.

The pesticide residues most commonly found in food (such as wheat, rice and rye/oats) between 1999 and 2008 were mostly insecticides, with the percentage of positive grain samples containing multiple residues increasing over time, from 15% in 1999 to 26% in 2007 (Commission, 2006; Authority, 2010). It has been detected that two or more pesticide residues were found in these analyzed samples with MRLs (González-Curbelo et al., 2012). According to current annual reports our six choosen pesticides still detected on foods and livestock animals even if levels exert difference year by year (Government, 2021; Authority et al., 2023; Nations et al.). The ingredients of our mixture (glyphosate, chlormequat chloride, pirimiphos-methyl, tebuconazole, chlorpyrifos-methyl and deltamethrin) were chosen according to highly detected pesticide residues in monitoring programs since 1999. The risk of exposure to these selected pesticides has been clearly identified in various monitoring programs around the World (Government, 2021; Authority et al., 2023; Nations et al.).

Overall, it is clear that since different pesticides are used for different products, consumers may be exposed to multiple chemicals at once rather than a single chemical. Moreover, this exposure usually occurs at doses around or far below the maximum residue limit. However, it is important to state that when two or more chemicals are combined, they can exhibit altered effects than those exhibited by each separate chemical. This altered behavior may be either synergism, potentiation or inhibition. Therefore, when testing a single chemical, it should not be overlooked that unidentified risks and hazards may occur when it is a part of a chemical mixture. Realizing the pronounced gap of this methodology, the real-life risk simulation scenario approach tries to address this problem by investigating the possible effect of long-term exposure to chemical mixtures closely resembling the actual circumstances of modern life (Tsatsakis et al., 2016; Tsatsakis et al., 2017b). Epidemiological and occupational studies have shown that exposure to xenobiotics, even at low doses, can also have effects on a variety of organs resulting in numerous pathologies such as neurotoxicity (Dardiotis et al., 2013; Zaganas et al., 2013; Baltazar et al., 2014), reproductive toxicity (Frazier, 2007), cardiotoxicity (Posnack, 2014, Zafiropoulos et al., 2014), nephrotoxicity (Vardavas et al., 2016), hepatotoxicity (Hernández et al., 2013; Tsitsimpikou et al., 2013), genotoxicity (Hernández et al., 2013; Stivaktakis et al., 2016) and endocrine disruptions (Mrema et al., 2013; Hernández and Tsatsakis, 2017, Tsatsakis et al., 2019a). In detail, as shown by Tsatsakis et al. (Tsatsakis et al., 2019b), Dinca et al. (Tsatsakis et al., 2017b) and Docea et al. (Tsatsakis et al., 2019c) chronic exposure to chemical mixtures, even at very low doses, can have detrimental effects on an organism affecting almost all organs (the liver, the heart, the kidneys, the brain and genital organs) (Docea et al., 2018; Docea et al., 2019; Dinca et al., 2023).

The mesentary contains blood vessels, lymp nodes and nerve cells that could be placed as a target of toxic substances due to its important role in both homeostasis and all body inflammation processes and its relationship with inflammation-based diseases. In recent years, the mesentery has been treated as an organ by the authorities, making this tissue a target organ from a toxicological perspective and taking an important place in the evaluation of target organ toxicity for several toxicants such as pesticides (Hattori et al., 2000; Argikar and Argikar, 2018, Rivera et al., 2019; Spasov et al., 2019; Coffey et al., 2020). There are very limited studies reporting detection of pesticide residues and their toxic effects in mesentery (Madsen et al., 1996; Ye et al., 2015).

The pancreas is the organ responsible for the production of insulin, glucagon and digestive enzymes and is the homeostasis organ that has the most important role in the metabolic axis. Several studies are showing the toxic effects of pesticides on the pancreas and that these effects might be associated with many diseases including diabetes and pancreatic cancer. The functioning of the pancreas is affected by both genetic and environmental factors. Organophosphate pesticides have been reported to be associated with an increased risk of Type 2 Diabetes in both occupational and population exposures (Fryzek et al., 1997; Kamath and Rajini, 2007, Andreotti et al., 2009; Karami-Mohajeri and Abdollahi, 2011, Sevim et al., 2019; He et al., 2020; Leonel Javeres et al., 2020; Brugel et al., 2022; Hoyeck et al., 2022). It has been reported that mesentery and pancreas have metabolic connection in terms of inflammation (Kieffer and Habener, 2000, Coffey et al., 2020).

Exposure to toxic substances can affect the immune system by modulating cellular functionality, and this modulation results in the activation of proinflammatory processes responsible for the accumulation of genetic damage that DNA repair systems cannot adequately eliminate (Fenga et al., 2017). Numerous data suggest that epigenetic modifications may be one of the mechanisms by which pesticides may cause deleterious effects on human health. Even though, the exact role of miRNA in the context of chemical-associated carcinogenesis has not been fully clarified, it is advocated that miRNA may be an important biomarker for carcinogenesis (Costa et al., 2020).

Genetic regulation molecules may alter the mechanisms of pesticide-

related harmful effects on human health. miRNAs are recognized to play pivotal role in patophysological processes of several diseases caused by environmental factors. miRNAs might be accepted as candidate biomarkers for pesticide related inflammatory effects (Sonkoly and Pivarcsi, 2011).

microRNAs are small, evolutionarily conserved 19–24 nucleotide non-coding RNA molecules that regulate gene expression to control a wide variety of biological processes by binding to target mRNAs and inducing their degradation or inhibiting their translation. (Ma et al., 2018). Many microRNAs have been found in a variety of organisms, including humans and mammals, raising curiosity as to whether they can be used as potential diagnostic indicators. Research shows that miRNAs play crucial roles in responses to environmental chemicals and cause a variety of toxicant-related conditions and diseases. In addition, miRNA analyses in pharmacological and toxicological studies may offer potentially useful perspectives in clinical management (Yu and Cho, 2015).

It has been demonstrated that pesticide exposure deregulates the miRNA levels in several tissues. They can also play a role as specific, stable, and sensitive markers of chemical exposures. miR21 is one of the most deregulated miRNA in pesticide exposure scenarios and it is linked to various cellular pathways and diseases (Ruan et al., 2014; Wu et al., 2017; Madhyastha et al., 2021; Valencia-Quintana et al., 2022). It has also been reported that miR21, miR146 a/b and miR223 deregulated with several pesticide exposures (Valencia-Quintana et al., 2022). These three miRNAs also have important regulative role in the inflammation process. miR223 exerts its regulative role on more than 200 target genes in immune response cells and the most known ones are NF-κB , FOXO and STATs. miR146 related with Toll Like receptors (TLRs) and IL1B are important immun response elements. Also, it affects NF-κB and regulates inflammatory cytokines and chemokines expressions (Sonkoly and Pivarcsi, 2011, Jeffries et al., 2019). In this study, we investigated the changes in inflammation parameters as interleukins (IL1β and IL10), biomarkers of inflammation as miRNAs (21, 146a and 223) and histopathological changes upon exposure to pesticide mixtures in pancreas and mesentery.

2. Material and method

2.1. Animals

In this research, a total of 72 male Sprague Dawley rats, aged 8–10 weeks and weighing between 200 and 235 g, were utilized. The rats used in this experimental study were sourced from the Animal Shelter at Ataturk University's Medicinal and Experimental Application and Research Center. All actions and procedures adhered to the national ethical guidelines as approved by the ethical committee. The local ethics committee at Ataturk University approved all procedures (approval number: 36643897-000-E.2000236269). These rats were comfortably accommodated in standard polypropylene cages within an environment that was strictly controlled for temperature (22 ± 1 °C) and humidity (50–60%), maintaining a 12-h light/dark photoperiod. Adequate provisions of standard food and tap water were made available to the animals ad libitum. The standard diet was provided in this study according to the Animal Shelter at Ataturk University's Medicinal and Experimental Application and Research Center's procedures. Animal feed is commercially provided by the research center.

2.2. Reagents

GLY (Glyphosate, CAS number: 1071-83-6), CCC (Chlormequat chloride, CAS number: 999-81-5), PM (Pirimiphos-Methyl, CAS Number: 29232-93-7), TBZ (Tebuconazole, CAS Number: 107534-96-3), CPSM (Chlorpyrifos-methyl, CAS Number: 5598-13-0), and DLM (Deltamethrin, CAS Number: 52918-63-5) were procured through Sigma-Aldrich based in St. Louis, MO, USA. For Roundup Star, containing 441 g/L of water-soluble concentrate of glyphosate potassium salt (comprising 35.5% of glyphosate potassium salt), the source was Monsanto Europe SA/Belgium. Durduran (460 g/L CCC), Activizim 50 EC (500 g/l PM), Reldan 2E (CPSM 22% w/v), and K-Othrine SC 50 (DLM) were purchased from respective suppliers in Turkey: Doğal AS., Safa Tarım, Dow Agroscience Agrochem, and Agrevo Corporation. Additionally, Folicur (TBZ wettable powder, with 0.25 g active ingredient (a.i.) per L^{-1}) was acquired from Bayer CropScience, Portugal.

The acceptable daily intake (ADI) for various chemicals is established by the European Food Safety Authority (EFSA). According to EFSA standards, the ADI is set at 0.04 mg/kg of bw/day for CCC (EFSA, 2023), 0.004 mg/kg of bw/day for PM (Authority, 2011a,b), 0.03 mg/kg of bw/day for TBZ (Authority, 2011a,b), 0.01 mg/kg of bw/day for DLM (McGregor, 2000), and 0.01 mg/kg of bw/day for CPSM (Authority, 2021). As for GLY, the ADI extends up to 0.5 mg/kg of bw/day (EFSA, 2013; Glyphosate,).

2.3. Study design

The rats were distributed into six primary groups, each comprising 12 animals, utilizing a random allocation method. The specifics of these groups, along with their respective dosage regimens, are comprehensively outlined in Table 1. Over 13 weeks, the substances were orally administered to the rats on a daily basis, without exception, spanning all seven days of the week. Solutions of these substances, dissolved in corn oil, were diligently prepared and administered via gavage. The control group did not receive any treatment and solvent group rats were given only corn oil via gavage. The dosages were meticulously calculated based on the established acceptable daily intakes (ADIs) of the substances, as stipulated by the relevant authorities. Throughout the study, all groups were provided with a standard diet comprising water and pellets. After 13 weeks, animals were sacrificed and mesenteric and pancreatic tissues were collected for molecular and pathology analysis.

Individual food and water consumption was meticulously recorded for all the rats in the study, food and water intake in all animal groups were not changed in 90 days in all groups. Animal weights were monitored weekly. There was no significant change was observed in all animal groups in the 90-day period. The liver and kidney are not primary organs for the parameters considered in this study. However, since they are target organs for the toxic effects of many chemicals, the liver and kidney weights were assessed and compared at the end of the study. No statistically significant difference was observed between the groups. These data were included in the supplementary data.

2.4. Gene expression analysis

Total RNA isolation from paraffin-embedded mesenter and pancreatic tissues was performed by using High Pure FFPET RNA isolation kit (Invitrogen- ThermoFisher, USA) according to the manufacturer's

Table 1
In vivo experimental groups and doses.

Groups	Dose	Substance
Control	–	
Solvent	–	Corn oil
Mix-1 chem (Low-dose mix)	1 x ADI	CCC, PM, GLY, TBZ, CPSM, DLM
Mix-2 chem (High-dose mix)	10 x ADI	CCC, PM, GLY, TBZ, CPSM, DLM
Mix-1 com (Low-dose commercial mix)	1 x ADI	CCC, PM, GLY, TBZ, CPSM, DLM
Mix-2 com (High-dose commercial mix)	10 x ADI	CCC, PM, GLY, TBZ, CPSM, DLM

ADI is 0.04 mg/kg bw/day for CCC; 0.004 mg/kg bw/day for PM; 0.5 mg/kg bw/day for GLY; 0.03 mg/kg bw/day for TBZ; 0.01 mg/kg bw/day for CPSM; 0.01 mg/kg bw/day for DLM.(Chem; chemical, Com; commercial).

product manual. cDNA synthesis was performed with cDNA synthesis kit with a fixed amount of RNA from each sample (Invitrogen- Thermo-Fisher, USA). β-actin and miR21, miR146 and miR223 gene expression levels were analyzed with Real-Time PCR, Light Cycler 480 II using LightCycler® 480 Probes Master. Tissue from each animal was studied in 3 replicates. The annealing temperature of all gene regions was 60 °C. The gene expression levels were estimated as ($2^{-\Delta\Delta Ct}$).

2.5. Pathological analyzes

2.5.1. Histopathological method

Samples of rat mesentery and pancreatic tissue were taken and fixed in 10% neutral formalin solution. Tissues were taken into paraffin blocks after routine alcohol-xylol follow-up procedures. 5 μm sections taken on slides were stained with hematoxylin-eosin. In random 6 different fields, mesenterium and pancreatic samples were examined. Vascular injury criteria in the mesentery were assessed according to the modification of the study by Zhang et al. (Table 2) (Zhang et al., 2008)

Degenerative changes in the exocrine pancreas were assessed according to the modification of the study by Wang et al. (Table 3) (Wang et al., 2017).

2.5.2. Immunohistochemical method

5 μm sections taken on polylysine slides were passed through xylol and alcohol series, after washing with PBS, in 3% H2O2 for 10 min endogenous peroxidase inactivation was achieved. To reveal the antigen in the tissues, they were treated with antigen retrieval solution for 2 × 5 minutes at 500 W. Tissues washed with PBS after protein blocking were incubated with IL-1β (Santa Cruz, Cat no. sc-52012) and IL-10 (Santa Cruz, Cat no. sc-8438) primary antibodies at 1/200 dilution at room temperature for 45 min left for incubation. Secondarily; Large Volume Detection System: anti-Polyvalent, HRP (Thermofischer, Catalog no: TP-125-HL) was used as recommended by the manufacturer. DAB (3,3′-Diaminobenzidine) was used as chromogen. After counterstaining with Mayer's Hematoxylin, it was covered with entellan and examined under a light microscope. The percentages of immunopositivity detected in random 6 distinct microscope fields were analyzed using the Fiji Image J program.

2.6. Statistical analyzes

To compare the molecular data across different groups, we utilized one-way analysis of variance (ANOVA) through the statistical software IBM SPSS 20.0. To assess the equality of variances within groups, Levene's test was applied, and the normal distribution within each group was evaluated using the Shapiro–Wilk test. Group distinctions were established through the application of One-way ANOVA, followed by a Duncan multiple range test (DMRT) ($p < 0.05$). The results for each group are presented as the mean ± SD. The intergroup differences in the statistical analysis of histopathologically determined semiquantitative data were assessed using the Kruskal-Wallis test, and the group responsible for the difference was determined using the Mann-Whitney *U* test ($p < 0.05$). The percentages of positive area obtained in immunohistochemical staining were evaluated using One-Way ANOVA and post hoc Tukey test ($p < 0.05$).

Table 2
Severity of mesenteric vascular lesions.

Grade	Vascular and inflammatory changes
1	**Minimal acute microvascular injury (inflammatory cell infiltration in the perivascular spaces of the vessels**
2	**Mild acute microvascular injury (fibrin exudation, and inflammatory cell infiltration in the perivascular spaces of the vessels)**
3	**Predominant vascular hemorrhage in < 30% of mesenteric vessels, in association with minimal inflammation**
4	**Predominant vascular hemorrhage in 31%-60% of mesenteric vessels, in association with moderate inflammation**
5	**Predominant vascular hemorrhage in > 60% of mesenteric vessels, accompanied with very severe inflammation**

Table 3
Degenerations in the acinar cells of the exocrine pancreas.

Grade	Degenerative changes resulting from the light color of the cells
0	No changes
1	Minimal changes, <10% of the total cells in the microscopic field
2	Mild changes, 10%–30% of the total cells in the microscopic field
3	Moderate changes, 30%–50% of the total cells in the microscopic field
4	Severe changes >50% of the total cells in the microscopic field

3. Results

3.1. Histopathological findings

Regarding the pathology findings, statistically significant differences were found in the mesenteric samples between the groups (Fig. 1, $p < 0.05$).

Control and Solvent groups had normal histological appearance. Mild histopathological lesions were observed in the application groups Mix-1 chem, Mix-1 com, and Mix-2 com. In Mix-1 chem and Mix-2 com groups, histopathological findings associated with mild acute microvascular injury were present, characterized by inflammatory cell infiltrations and fibrin exudations in perivascular areas. In the Mix-1 com group, only inflammatory cell infiltrations were observed in perivascular areas. The most severe histopathological findings were observed in the Mix-2 chem group, where widespread hemorrhages and inflammatory cell infiltrations were encountered in approximately half of all mesenteric vessels (Fig. 2).

In the histopathological examination of pancreatic samples, statistically significant differences were found between the groups in terms of degenerative changes (Fig. 3, $p < 0.05$).

. Control group exert normal histological appearance findings. Mild degenerative changes were observed in the acinar cells of the exocrine pancreas in the Solvent group and Mix-1 chem group exerted slightly increased degeneration appearance. In the other treatment groups, moderate degenerative changes were observed in the Mix-1 com group, severe changes in the Mix-2 com group, and very severe changes in the Mix-2 chem group. It was determined that the degenerated acinar cells lost their normal appearance with pale areas (Fig. 4).

3.2. Immunohistochemical findings

Statistically significant differences were found between the groups in the mesentery samples in immunohistochemical staining for IL-1β and IL10 (Fig. 5, $p < 0.05$).

IL-1β and IL-10 immunopositivity could not be observed in the control and solvent groups in the mesenterium samples. In the application groups, IL-1β and IL-10 immunopositivity were mild in the Mix1-chem group and Mix-1 com group, moderate in the Mix-2 chem group, and severe in the Mix-2 com group. Immunopositivity detected were in cells forming inflammatory infiltration (Figs. 3 and 4).

In immunohistochemical staining with IL1β, significant immunopositivity was not detected in the control and solvent groups. However, in the other treatment groups, mild immunopositivity was observed in the Mix-1 chem and Mix-1 com groups, moderate immunopositivity in the Mix-2 chem group. The most pronounced immunopositivity was observed in the Mix-2 com group. The identified immunopositivity was located on the walls of small vessels and in fibrocytes/fibroblasts (Fig. 6).

Similar findings were also observed in the staining with IL10. However, unlike IL1β, positivity was observed in the walls of small vessels in the control and solvent groups as well. In the other treatment

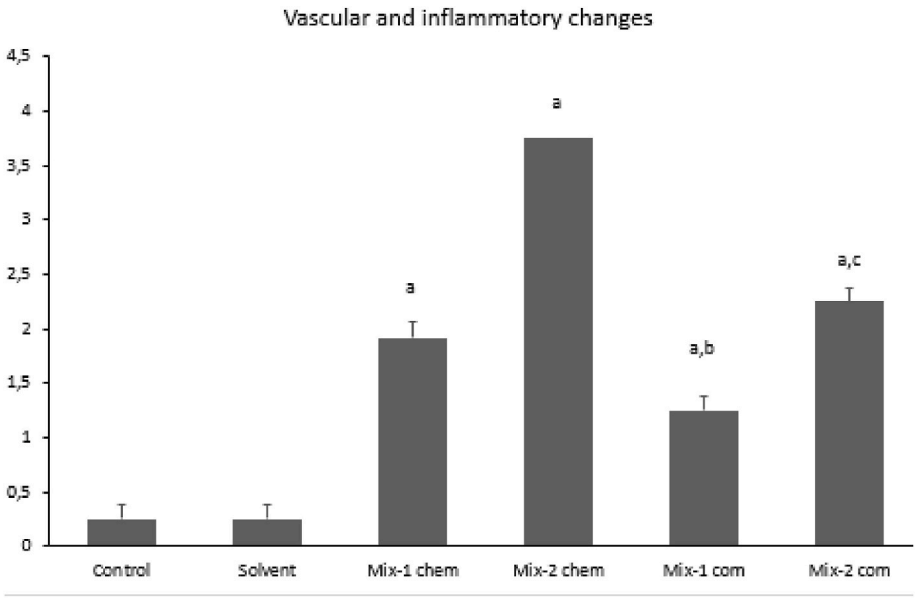

Fig. 1. Vascular inflammatory change levels in mesentery. a; $p < 0.05$ significantly increased compared to control and solvent groups, b; $p < 0.05$ significantly decreased compared to Mix-1 group, c $p < 0.05$ significantly decreased compared to Mix-2 group. (n = 12 animals per group).

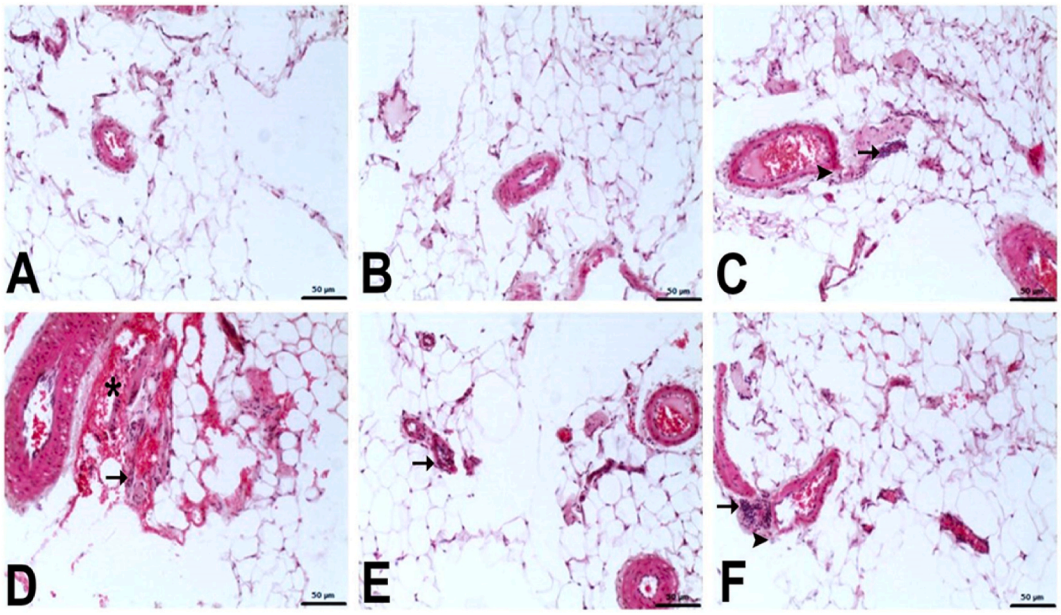

Fig. 2. Histological H&E staining of Mesentery tissue per group (n = 12). A) Control group and B) Solvent group: Normal histological appearance, C) Mix-1 chem group: Grade 2, fibrin exudation (arrowhead), and inflammatory cell infiltration (arrow) in perivascular area, D) Mix-2 chem group: Grade 4, hemorrhage (*), and inflammatory cell infiltration (arrow) in perivascular area, E) Mix-1 com group: Grade 1, inflammatory cell infiltration (arrow) in perivascular area, F) Mix-2 com group: Grade 2, fibrin exudation (arrowhead), and inflammatory cell infiltration (arrow) in perivascular area, Mesentery, H-E.

groups, the intensity of IL10 immunopositivity, in terms of percentage area, was higher than that of IL1β. The changes in immunopositivity among groups paralleled those observed with IL1β. In other words, similar to IL1β, immunopositivity in IL10 was severe in the Mix-2 com group, moderate in the Mix-2 chem group, and mild in the other groups (Fig. 7).

Statistically significant differences were found in pancreatic samples in terms of IL-1β and IL-10 immunopositivity (Fig. 8, $p < 0.05$).

In immunohistochemical staining with IL1β and IL10, significant immunopositivity was not detected in the control and solvent groups. However, varying levels of immunopositivity were observed in the other treatment groups. In the staining with IL1β, the percentage of area covered by immunopositivity was mild in the Mix-1 com group, moderate in the Mix-1 chem group, and severe in the Mix-2 chem and Mix-2 com groups (Fig. 9).

In the staining with IL10, the percentage of area covered by immunopositivity was moderate in the Mix-1 com group, severe in the Mix-1 chem and Mix-2 chem groups, and very severe in the Mix-2 com group (Fig. 10). Both IL1β and IL10 immunopositivity were detected in the acinar cells of the exocrine pancreas.

miRNA-21 mRNA levels in pancreatic tissue in the mix1-chem group; while miRNA-223 did not change in mix2-chem and mix2-com group, miRNA-146a increased in mix2-com group. All miRNA expressions were decreased in the Mix1-com group (Fig. 11).

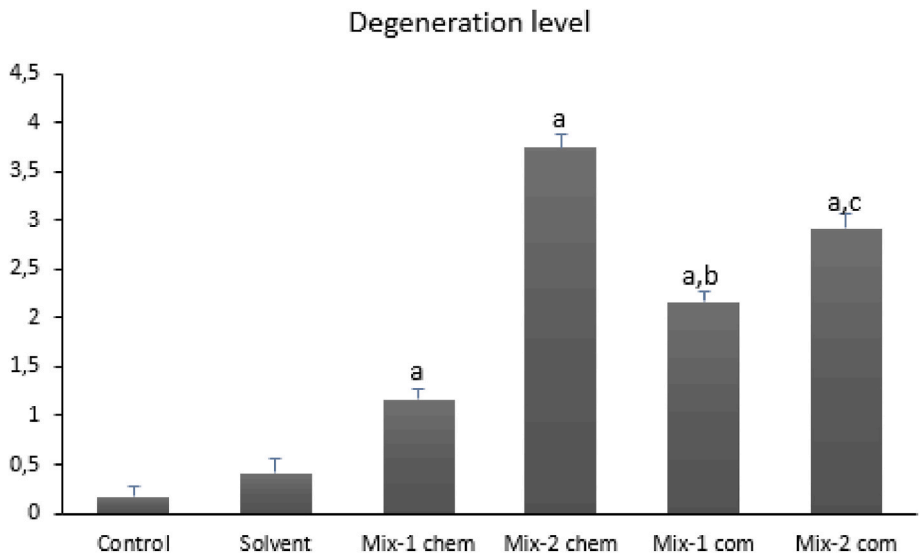

Fig. 3. Degenerative change levels in pancreas tissue (exocrine). a; $p < 0.05$ significantly increased compared to control and solvent groups, b; $p < 0.05$ significantly decreased compared to Mix-1 group, c $p < 0.05$ significantly decreased compared to Mix-2 group. (n = 12 animals per group).

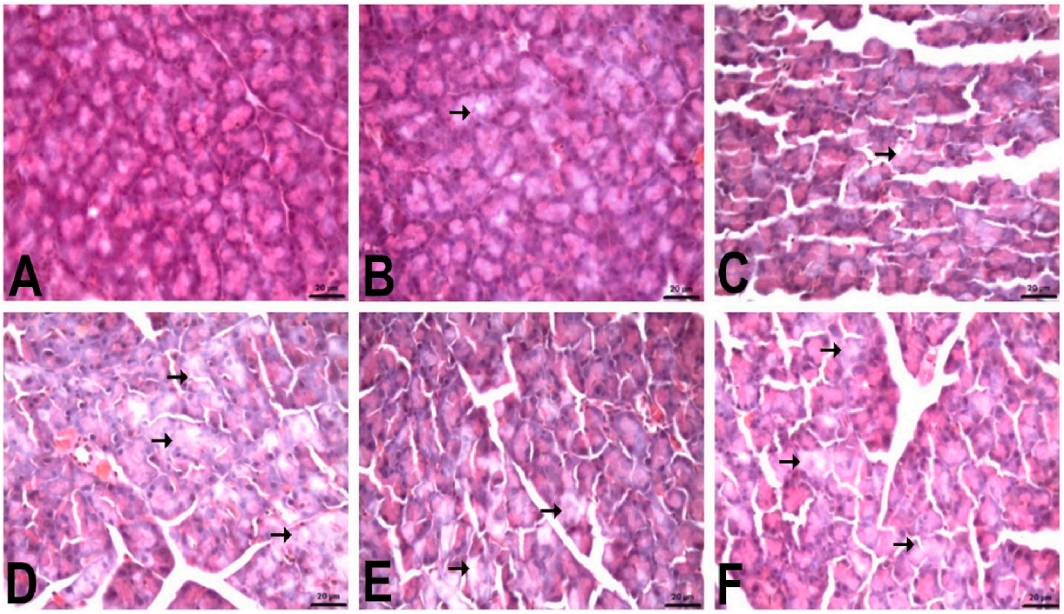

Fig. 4. Histological H&E staining of Exocrine Pancreas tissue (n = 12). A) Control group: Normal histological appearance, B) Solvent group C) Mix-1 chem groups: Mild level of degeneration in the acinar cells (arrows), D) Mix-2 chem group: Very severe level of degeneration in the acinar cells (arrows), E) Mix- 1 com group: Moderate level of degeneration in the acinar cells (arrows), F) Mix-2 com group: Severe level of degeneration in the acinar cells (arrows).

In mesenteric tissue, miRNA-21 levels decreased in Mix-1 chem and Mix-2 com groups, whereas an increase was observed in Mix-1 com group compared to control. miRNA-146a decreased in Mix-1 chem, Mix-1 com and Mix-2 chem groups. miRNA-223 levels decreased in all groups compared to the control (Fig. 12).

4. Discussion

During the last century, an exponential increase in the chemical substances produced by humans has been evidenced. It is well-documented that these substances (or xenobiotics as also described) end up in the human organism either through direct exposure in the setting of domestic, recreational or occupational exposure or through indirect exposure through the food chain. To regulate such an exposure certain legislation has been introduced using data coming from studies where single chemical exposure is used. However, such an approach underestimates the potential synergism, potentiation or inhibition that may arise from the exposure to a mixture of chemicals (as those in real life). For this reason, the Real-Life Risk Simulation Scenario was introduced aiming not only to unveil the potential hazards of chemical mixtures, even at very low doses, but also to aware policy makers and relative agencies in order to change the way they choose to face the topic (Tsatsakis et al., 2016; Tsatsakis et al., 2017a; Tsatsakis et al., 2019a; Tsatsakis et al., 2019b; Dinca et al., 2023). Pesticides have been found to have a disruptive impact on the endocrine system due to their interaction with biochemical processes within the organism. This interaction can lead to alterations in the production of cytokines and chemokines, which are inflammatory mediators. Inflammation, although it can trigger reversible or irreversible events, is typically controlled through self-limiting mechanisms that work in harmony with positive and

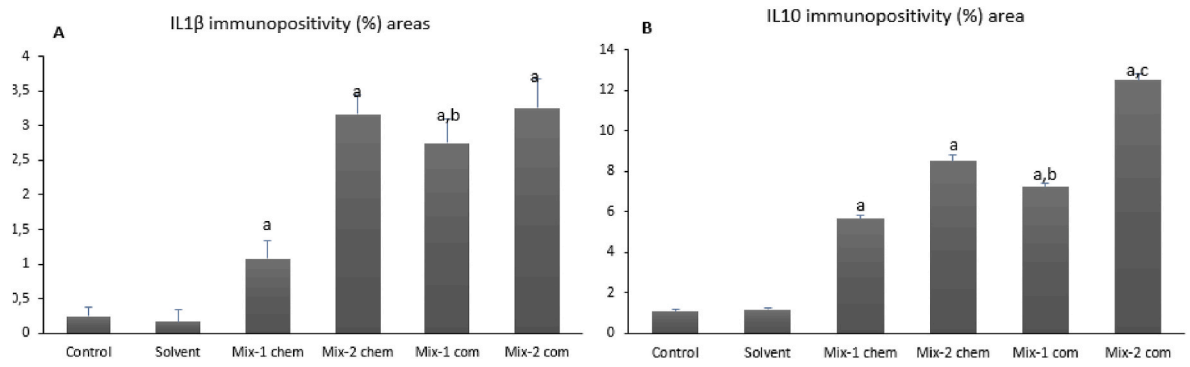

Fig. 5. Immunopositivity (%) areas in mesentery tissues. A) IL1β, B) IL10 (%). a; $p < 0.05$ significantly increased compared to control and solvent groups, b; $p < 0.05$ significantly increased compared to Mix-1 group. (n = 12 animals per group).

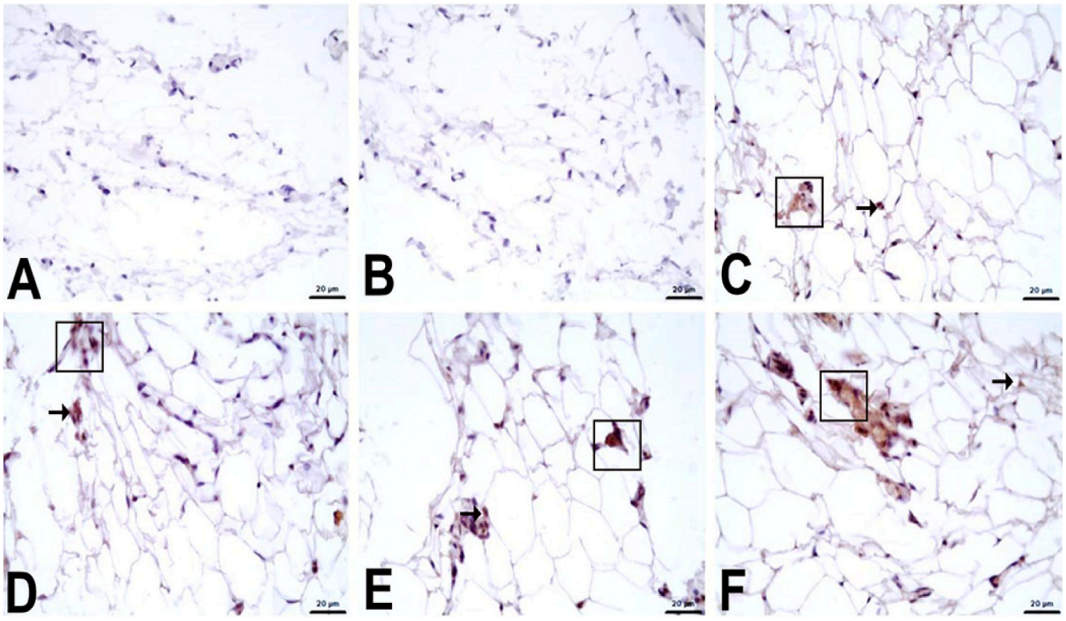

Fig. 6. Immunohistochemical staining of IL1β in mesentery (n = 12). A) Control group and B) Solvent group: IL1β immunonegativity. C) Mix-1 chem group: Mild IL1β immunopositivity, D) Mix-2 chem group: Moderate IL1β immunopositivity, E) Mix-1 com group: Mild IL1β immunopositivity. F) Mix-2 com group: Severe IL1β immunopositivity in walls of small vessels (□), and in fibroblasts (arrows). Mesentery- IHC.

negative regulatory mechanisms (Nathan, 2002, Salimi et al., 2017). When it comes to the regulation of inflammation, miRNAs are believed to play a crucial role. Similarly to cytokines, miRNAs function as key regulators modulating the signaling for the initiation and termination of inflammation. Depending on the specific target, miRNAs can either exacerbate or suppress the inflammatory response. In essence, pesticides have the potential to disrupt the endocrine system, leading to alterations in the production of inflammatory mediators such as cytokines and chemokines (Alam and O'Neill, 2011). miRNAs, on the other hand, serve as important regulators of inflammation, capable of either exacerbating or suppressing its effects depending on the target mRNAs involved. This intricate relationship between pesticides, inflammation, and miRNAs highlights the complex nature of the biological processes affected by these chemicals. (Tahamtan et al., 2018).

Emerging research has shed light on the pivotal role of miR-21 in resolving inflammation through a negative feedback mechanism of inflammatory pathways. Furthermore, miR-21 has been identified as a critical negative regulator of TLR4 signaling, offering potential therapeutic implications (Sheedy et al., 2010; Feng et al., 2014; Lin et al., 2017). Notably, miR-21 has been found to target programmed cell death, enhancing its significance in modulating cellular responses. The overexpression of miR-21 in macrophages has been observed to elicit a reduction in IL-6 secretion, while simultaneously promoting an increase in IL-10 production (Feng et al., 2014). This dual effect highlights the multifaceted nature of miR-21's involvement in immune modulation. Moreover, miR-21 has been shown to exert its anti-inflammatory effects by modulating the TLR4 - NF-κB pathway, thus dampening LPS-induced inflammatory responses in macrophages (Feng et al., 2014). These findings pave the way for further exploration into the therapeutic potential of miR-21 in regulating inflammation. In the results obtained from our comprehensive study, it was observed that there was an increased expression of miR-21 in the mesentery of the Mix1 com group, indicating a potential association between this chemical and the expression levels of this miRNA. Furthermore, the pathology study revealed that the mesentery did not exhibit any signs of hemorrhage, but there was a notable presence of infiltrative cells compared to the control group. This finding suggests a potential link between the Mix-1 com group and the infiltration of cells in the mesentery. Additionally, the analysis also demonstrated the presence of IL1β and IL10 positivity in the mesentery tissues. Moving on to the Mix-1 chem and Mix-2 com groups, our study indicated a decrease in the expression of miR-21 in these groups. Moreover, infiltrative cells were also observed in the

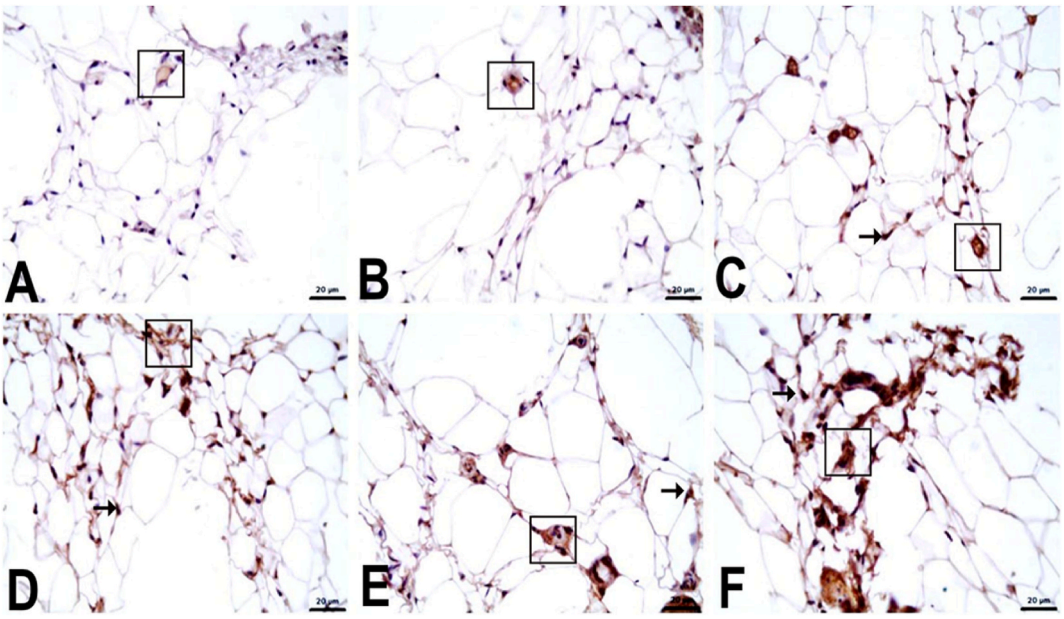

Fig. 7. Immunohistochemical staining of IL10 in mesentery (n = 12). A) Control group and B) Solvent group: Mild IL10 immunopositivity in walls of small vessels (□). C) Mix-1 chem group: Mild IL10 immunopositivity, D) Mix-2 chem group: Moderate IL10 immunopositivity, E) Mix-1 com group: Mild IL10 immunopositivity, F) Mix-2 com group: Severe IL10 immunopositivity in walls of small vessels (□), and in fibroblasts (arrow). Mesentery- IHC.

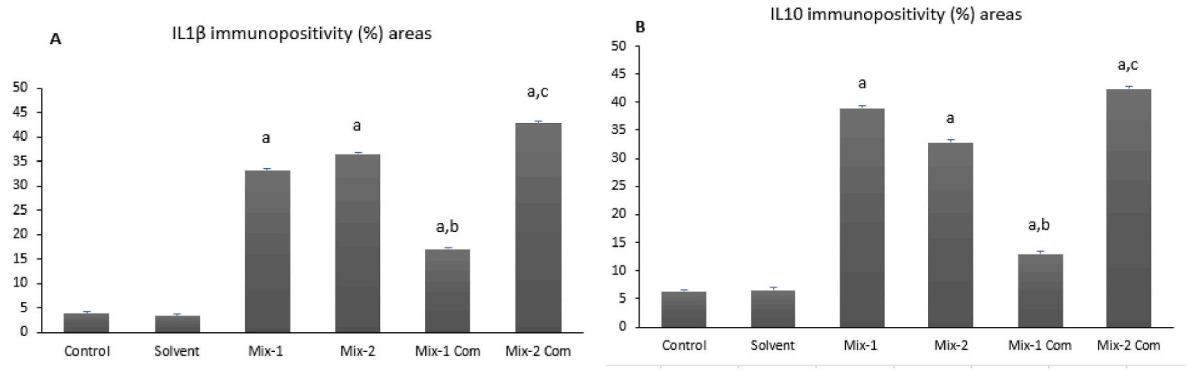

Fig. 8. Immunopositivity (%) areas in pancreas tissues. A) IL1β, B) IL10 (%). a; $p < 0.05$ significantly increased compared to control and solvent groups, b; $p < 0.05$ significantly decreased compared to Mix-1 group, c $p < 0.05$ significantly increased compared to Mix-2 group. (n = 12 animals per group).

mesentery. These findings raise questions about the potential effects of both Mix-1 chem and Mix-2 com groups on the expression of miR-21 and the presence of infiltrative cells in the mesentery. Furthermore, degenerative changes were observed in the pancreatic tissue of these groups, suggesting a potential link between the exposure to Mix-1 chem and Mix-2 com and degenerative changes in the pancreatic tissue.

The miR-146 family includes 2 subgenes: 146-a and 146-b. This family acts as a negative regulator to prevent the spread of inflammation against proinflammatory stimuli. miR-146a and miR-146b expression is also induced in endothelial cells in the activation of the proinflammatory process, including NF-κB, AP-1 and MAPK/EGR pathways, and stops the transcriptional process (Kutty et al., 2013). Studies have shown that it inhibits the biosynthesis of IL-1β, IL-6, IL-8 and TNF-α in lupus nephritis and attenuates chemotactic effects on macrophages through inhibition of TRAF6 activity (Zheng et al., 2017). In a study investigating the protective effects of microRNAs on human chondrocytes in an in vitro osteoarthritis model, it was shown that miR-146a showed protective activity by decreasing IL-6 and IL-8 levels and also downregulated proteins involved in ROS formation such as proapoptotic QORX (Al-Modawi et al., 2019). In another study conducted in patients with paraquat poisoning-induced lung damage, IL-6 and miR-146a levels were investigated in macrophages, peripheral blood mononuclear cells (PBMCs) and serum. IL-6 levels were significantly increased in all samples, while miR-146a levels were significantly decreased. The results suggest that the body's immune system negatively regulates the cleavage of IL-6 by miR-146a and promotes immune responses by increasing the expression of IL-6 (Wu and Li, 2018). In pancreatic tissue, miR-146a was elevated in our mix-commercial group compared to the control (Li et al., 2013). Although degenerative changes were observed in the pancreatic tissue of this group, an increase in IL-10, an anti-inflammatory cytokine, was also observed against the increase in pro-inflammatory cytokines. In mesenteric tissue, despite the absence of hemorrhage, the presence of infiltrating cells and an increase in IL-1 β were found. The studies suggest that it may identify a future therapeutic possibility for the treatment of inflammatory disorders as well as a potential target for the control of viral or bacterial infections through inhibition of immunosuppressive effects.

miR-223 is a key modulator of the differentiation and activation of neutrophils and macrophages and is involved in the activation and function of neutrophils in inflammatory diseases (Zhang et al., 2021). Dysregulation of miR-223 has been associated with many inflammatory diseases, including myocarditis, type II diabetes, atherosclerosis, acute

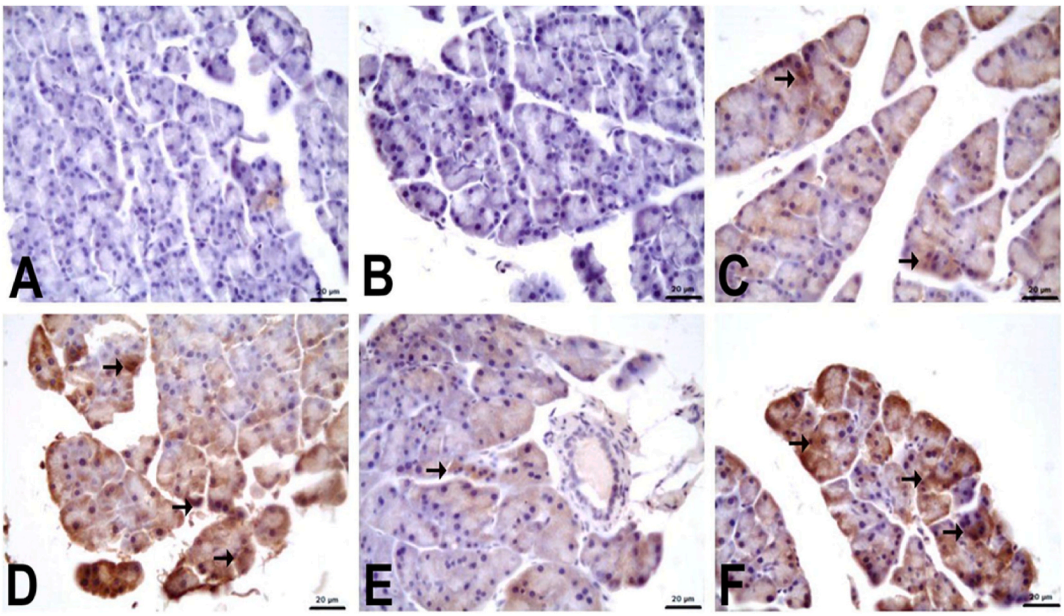

Fig. 9. Immunohistochemical staining of IL1β in pancreas (n = 12). A) Control group and B) Solvent group: IL1β immunonegativity. C) Mix-1 chem group: Moderate IL1β immunopositivity, D) Mix-2 chem group: Severe IL1β immunopositivity, E) Mix-1 com group: Mild IL1β immunopositivity. F) Mix-2 com group: Severe IL1β immunopositivity in acinar cells (arrows). Exocrine pancreas- IHC.

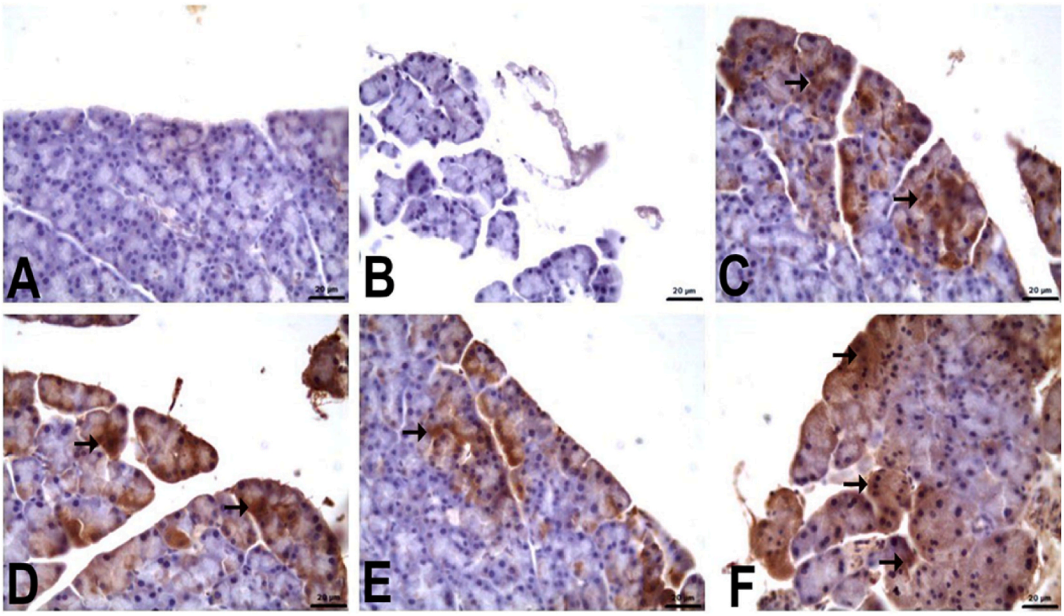

Fig. 10. Immunohistochemical staining of IL10 in pancreas (n = 12). A) Control group and B) Solvent group: IL10 immunonegativity. C) Mix-1 chem group: Severe IL10 immunopositivity, D) Mix-2 chem group: Severe IL10 immunopositivity, E) Mix-1 com group: Moderate IL10 immunopositivity. F) Mix-2 com group: Very severe IL10 immunopositivity in acinar cells (arrows). Exocrine pancreas- IHC.

lung injury (ALI), rheumatoid arthritis, infections and inflammatory bowel disease (IBD). In the inflammatory processes of sepsis, exosomal miR-223 was found to negatively correlate with the production of proinflammatory cytokines such as TNF-a, IL-1b and IL-6 by suppressing Sema3A and STAT3 protein expression in macrophages (Yuan et al., 2021). In a study investigating miRNA profiles associated with occupational exposure to pesticides in urine samples of agricultural workers, miR-223 levels were found to be high, which may be associated with possible pesticide exposure (Valencia-Quintana et al., 2022). In a study investigating the activity of miR-223 in obesity-associated adipose tissue inflammation, it was reported that miR-223 has a critical role in regulating macrophage polarization (Zhuang et al., 2012). In a study investigating whether Cypermethrin promotes lung cancer metastasis through modulation of macrophage polarization, miR-223 may suppress proinflammatory activation of macrophages by suppressing Pknox1 (Huang et al., 2018). In the activation of immune cells, miR-223 downregulation was found to increase IL-1β and IL-6 production at the transcription level (Haneklaus et al., 2013). In our study, a significant decrease in miR-223 levels was observed in mix-1 and mix-2 commercial groups in mesenteric tissue, and pathologically, an increase in IL-1 β and density of infiltrative cells were detected in these groups.

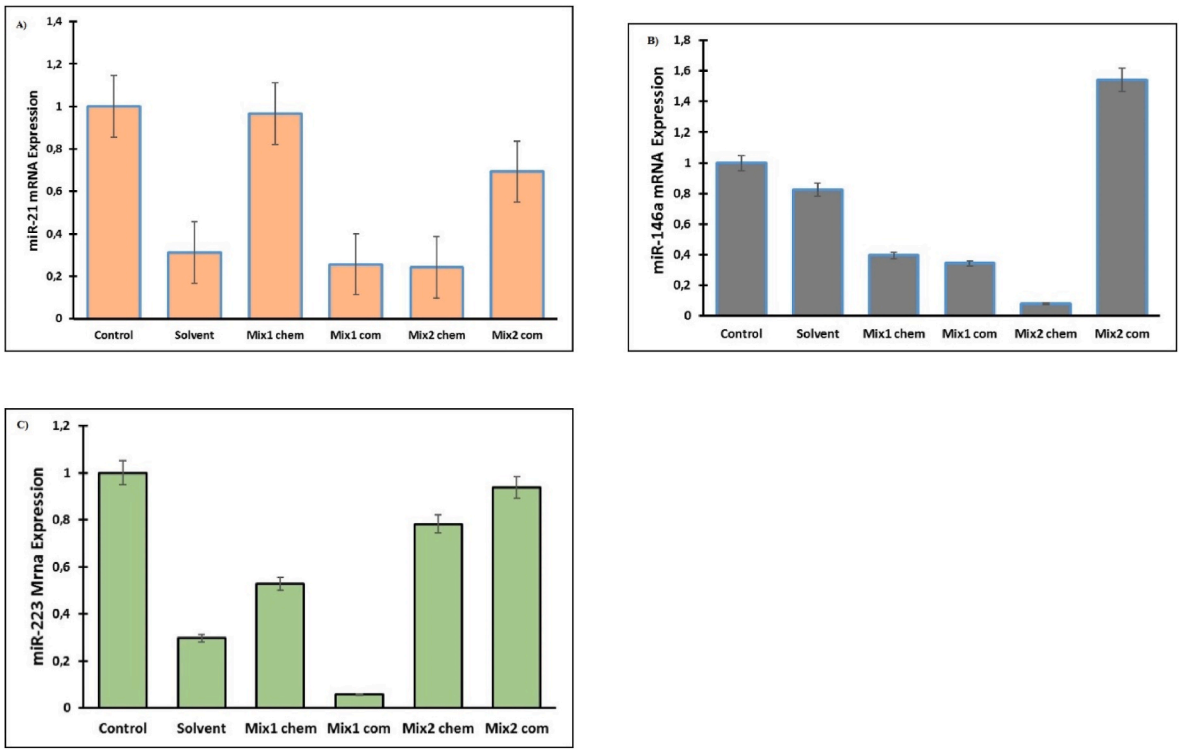

Fig. 11. a) miRNA-21, b) miRNA-146a, c) miRNA-223 mRNA expression analysis results in pancreas tissue. (n = 12 animals per group).

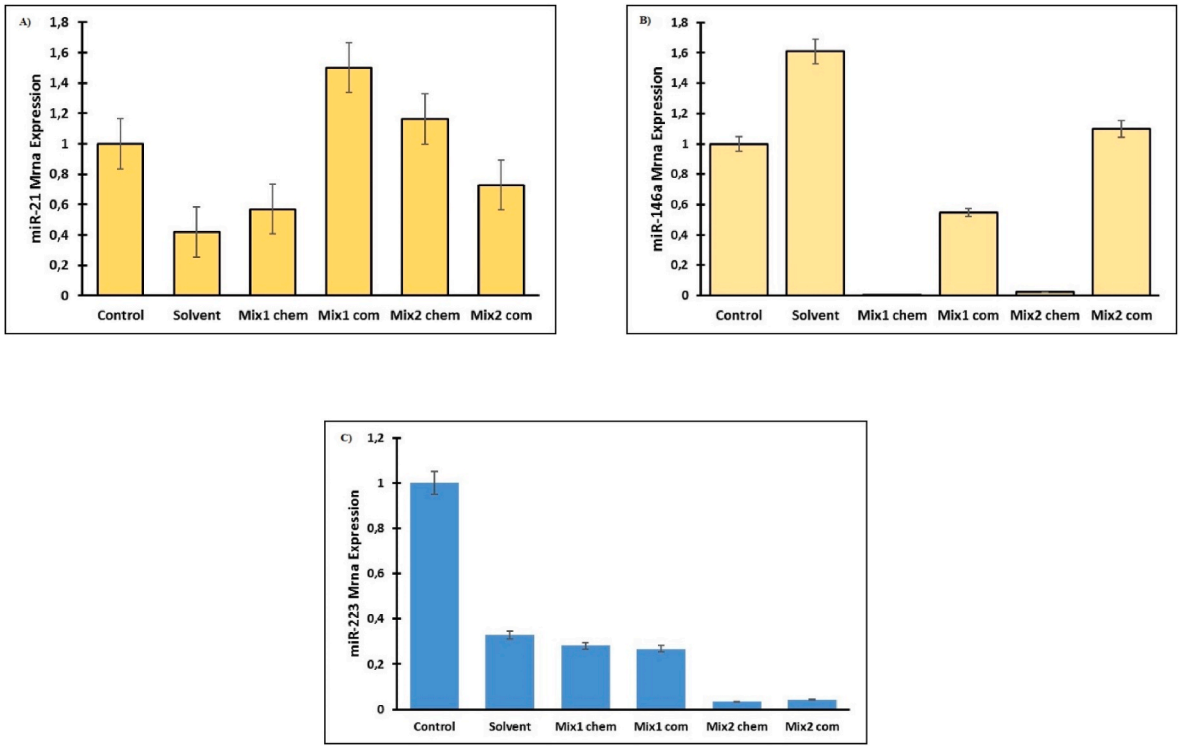

Fig. 12. a) miRNA-21, b) miRNA-146a, c) miRNA-223 mRNA expression analysis results in mesentery tissue. (n = 12 animals per group).

5. Conclusions

Although the level of toxic substances exposed was low, our results exhibited changes both at a structural and at the gene level. Moreover, the necessity of repeated measurements at different time intervals is highlighted to evaluate the potential use of relative biomarkers. In addition to the analyses performed in tissue, analyses performed in serum will be guiding. According to our study, low dose long-term exposure to pesticide mixtures causes hemorrhages, degeneration and inflammation in the pancreas and mesentery tissue. Continuing the

study for longer than 90 days to examine the changes that will occur at the gene level and re-analyzing miRNA levels at that point will provide us with more information about whether they can be potential biomarkers. In toxicology studies, differences in the way males and females react to a particular substance is very important. These differences can be attributed to hormonal variations, metabolic variations, or other physiological factors that distinguish males from females. It's important to note that dimorphic responses can occur in various toxicological contexts, including exposure to environmental pollutants, pharmaceuticals, pesticides and other chemicals. As a limitation of this study, gender-specific differences were not evaluated. Further studies in which dimorphic responses are evaluated at the gene level will enhance the precision and relevance of toxicity assessments. This will provide better protection for diverse populations in assessing the overall toxicity of a substance and for developing appropriate safety guidelines and recommendations.

Credit author statement

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cigdem Sevim reports financial support was provided by Kastamonu University. Cigdem Sevim reports a relationship with Kastamonu University that includes: funding grants.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140712.

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