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ORIGINAL ARTICLE

Pesticide use patterns and their association with cytokine levels in Mexican flower workers

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Abstract

Objective Occupational exposure to pesticides is a known risk for disrupting cellular immune response in flower workers due to their use of multiple chemical products, poor work conditions, and inadequate protection. Recently, the analysis of pesticide use patterns has emerged as an alternative to studying exposure to mixtures of these products. This study aimed to evaluate the association between exposure to different patterns of pesticide use and the cytokine profile of flower workers in the State of Mexico and Morelos, Mexico.

Methods A cross-sectional study was carried out on a population of 108 flower workers. Serum levels of IL-4, IL-5, IL-6, IL-8, IL-10 cytokines were analyzed by means of multiplex analysis, and TNF-α and IFN-γ using an ELISA test. Pesticide use patterns were generated by principal components analysis.

Results The analysis revealed that certain patterns of pesticide use, combining insecticides and fungicides, were associated with higher levels of pro-inflammatory cytokines, particularly IL-6 and IFN-γ.

Conclusion These findings indicate that pesticides may possess immunotoxic properties, contributing to increased inflammatory response. However, further comprehensive epidemiological studies are needed to establish a causal relationship.

Keywords Pesticides · Use patterns · Cytokines · Inflammation · Flower workers · Floriculture

Introduction

Flower production or floriculture is an activity that has become important in different Latin American countries. In Mexico, flowers are grown on a surface of 21,900 hectares, generating 188 thousand permanent jobs, 50,000 temporary jobs, and approximately one million indirect jobs. Most (90%) of flower production in Mexico is carried out in the State of Mexico and this is the only state with exporting capability. The state of Morelos is also one of the main producers of ornamental plants (Andrade-Galindo and Castro-Domingo 2018; Rosales-Salinas et al. 2018; Ramírez Hernández and Avitia Rodríguez 2017; SADER 2019).

The use of pesticides in agriculture, including the production of flowers and ornamental plants has increased considerably since the last century (Damalas and Eleftherohorinos 2011; Schilmann et al. 2010). Most of the pesticides that have been used in Mexico have been proven to be highly hazardous, due to their serious or irreversible adverse effects on human health and on the environment (Bejarano 2017; Ramírez-Hernández and Avitia Rodríguez 2017; Tielemans et al. 2007; Kromhout and Heederik 2005). In the production of flowers and ornamental plants, a great number of pesticides are used, and occupational exposure is considerably greater than that of the general population. Pesticides may be absorbed by several pathways: orally, by inhalation or dermally, during different work activities, such as mixing, carrying, and application, which involve greater contact with those products (Aguilar-Garduño et al. 2017; Bejarano

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2017; Gangemi et al. 2016; García-García et al. 2016; Khan et al. 2013). Also, the multiple chemical products that are used, the high concentrations at which they are applied and the work conditions involving high humidity and temperature, bad ventilation and scarce use of personal protection equipment (PPE) increase the exposure of flower growers to pesticides (Aguilar-Garduño et al. 2017; Mostafalou and Abdollahi 2017; Quevedo-Aguado and Bernaola-Alonso 2014; Ribeiro et al. 2012; Oliveira-Pasiani et al. 2012; Tielemans et al. 2007; del Prado-Lu 2007; Palis et al. 2006).

Exposure to pesticides has been identified as a risk factor for alterations of the immune system, through mechanisms including direct immuno-toxicity, changes in signalling of the cellular immune system generating oxidative stress (carbamates, pyrethroids), mitochondrial dysfunction (neonicotinoid), endoplasmic reticulum stress (pyrethroids, dithiocarbamates), inhibition of esterases and autophagy (organophosphates, organochlorines). Indirect immunotoxicity takes place through endocrine disruption by altering hypophysial (organophosphates, organochlorines), adrenal, thyroid (pyrethroids, dithiocarbamates) and some sex hormone signalling (carbamates, benzimidazoles, triazole), as well as causing antigenicity by generating alergens through the pesticide-protein link (Curl et al. 2020; Gangemi et al. 2016; García-García et al. 2016; Costa et al. 2015; Rajesh et al. 2013). According to the "epithelial barrier hypothesis", these actions could be related to the disruption of the integrity of the epithelial barriers as reviewed by Lima et al (2022). The authors found that chronic exposure to environmental contaminants such as pesticides can affect crucial barrier tissues such as the epithelium of the skin, airways, and intestine. This action of pesticides disrupting the epithelial barrier induces increased permeability and leakage, dysbiosis, and inflammation, with serious implications for metabolism and homeostasis.

There is a delicate control in the balance of the immune system. Particularly, cytokines are a group of proteins that are produced by diverse cell types mainly acting as regulators of the immune response (Zucchini-Pascal et al. 2009). These may be classified as pro-inflammatory or anti-inflammatory. The measurement of cytokine levels offers relevant information on the alteration of the cell immune response and can be used as biomarkers for the evaluation of immunotoxicity (Elsasser-Beile and von Kleist 1993; Costa et al. 2013; Corsini and House 2018; Jacobsen-Pereira et al. 2020).

Prior in vivo and in vitro toxicological studies have looked into the possible association between exposure to specific pesticides and the cellular immune response (Gargouri et al. 2020; Kumar et al. 2014; Massawe et al. 2017; Téllez-Bañuelos et al. 2016). Epidemiological studies on this topic (Costa et al. 2013; Fenga et al. 2014; Jacobsen-Pereira et al. 2020) are scarce and most of them do not take into account workers' exposure to mixtures of pesticides or include limited information about the inclusion of potential confounders in their analyses, such as age, body mass index (BMI), alcohol and tobacco consumption, and/or factors which determine occupational exposure (environmental conditions in greenhouses and the use of personal protection equipment).

Occupational exposure to pesticides does not occur in isolation, instead, flower workers are exposed to a complex combination of multiple chemicals. The analysis of mixtures is useful to summarize the set of pesticides applied in flower growing, identifying those that can interact to lead to certain health effects. The use of different mixtures may be addressed using the analysis of pesticide use patterns (Schilman et al. 2010).

The objective of this study was to evaluate the association between exposure to different pesticides, use patterns, and the cytokine profile, in flower workers in the State of Mexico and Morelos, controlling for potential confounders.

Materials and methods

Study design and population

The information used in this study comes from a cross-sectional study including men that worked in flower farming businesses in the state of Morelos and the State of Mexico. The details of this study may be found elsewhere (Aguilar-Garduño et al. 2017).

The study population was formed by workers who were selected from the records of 103 flower and/or ornamental plant cultivation businesses in the states of Morelos and the State of Mexico. The study was carried out during the rainy season in central Mexico (July–October) in the year 2004. Except for four medium-sized greenhouses, most of them were small businesses with fewer than 10 workers. Male workers between ages 15 and 55 were included, who had at least 6 months of work in this trade. Workers with chronic diseases were excluded from the study (diabetes type 2, renal insufficiency, hepatic alterations, cancer, endocrine diseases, autoimmune diseases or allergies) or with a diagnosis of infertility based on the data collected through a questionnaire (this last one because one of the objectives of the original project was to evaluate reproductive effects). The 136 eligible flower workers received explanations on the study's objectives and those who agreed to participate signed an informed consent form. The project was approved by the Commission for Ethics in Research and was recorded in the Research Commission (RC) with the number "322" of the National Institute of Public Health of Mexico (INSP, Spanish acronym).

In the present analysis, 136 workers were included with sufficient biological sample for cytokine profile/level analysis along with other variables of interest.

Data collection instruments

The information was collected through two types of structured questionnaires:

(1) *General questionnaire*: this questionnaire was applied to each one of the workers and provided information on socio-demographic characteristics (age, marital status, schooling level, and family income), alcohol and tobacco consumption habits, medical history, exposures at home (specific pesticides and other chemical products), and work characteristics (time working in flower production –flowers or ornamental plants, type of work performed in the business, place where activities are carried out, use of personal protection equipment).

Alcohol consumption was estimated in grams consumed per day (g/day) and was categorized as: No alcohol consumption, consumption < 30 g/day, and consumption ≥30 g/day. Tobacco consumption was categorized as never has smoked, ex-smoker, and current smoker.

(2) *Questionnaire on pesticide use at the business*: this questionnaire was applied to those who were responsible for agrochemical purchases at each one of the participating greenhouses (flower-growing businesses) and information was collected on the specific pesticides that were used (commercial names) during the rainy season of 2004.

Also, anthropometric measurements were taken (weight and height) by means of standardized procedures. The BMI was calculated by dividing the weight in kg of the flower workers by the square of their height in meters (kg/m^2) and categorized as normal (17.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2) and obese (≥ 30 kg/m^2), according to WHO criteria (WHO 2000).

The application of questionnaires, as well as anthropometric measurements and the collection of biological samples were done the day after pesticide application at the floriculture business and were carried out by trained nurses who did these procedures in a standardized manner.

Collection of biological samples and laboratory procedures

The workers provided a blood sample in basal conditions, to determine serum cytokine levels. These samples were taken in vacutainer tubes with a red cap, without anti-coagulant. Once collected, the blood samples were allowed to sit for half an hour at room temperature so that a clot would form and then immediately transferred in refrigerated conditions to the Center for Research in Infectious Diseases (CISEI, Spanish acronym) of the INSP in Mexico, where they were centrifuged at 2500 rpm to separate the serum; this was then divided into aliquots that were stored at –70 °C until they were analyzed.

Serum concentrations of IL-4, IL-5, IL-6, IL-8, and IL-10 were determined using the Human cytokine/chemokine Magnetic Bead panel kit, (HCYTMAG-60 K-PX29) (Millipore), according to the provider's recommendations. Fluorescence was quantified and cytokine concentration was calculated using the xPONENT software in the Luminex 200 TM equipment. Serum concentrations of IFN-γ and TNF-α were analyzed by the ELISA method, using the Quantikine Human IFN- Immunoassay and Quantikine Human TNF-Immunoassay (R&D Systems, Inc) kits, respectively. In both cases, cytokine concentrations were reported in pg/mL. Cytokines were categorized according to their median.

Evaluation of pesticide exposure

We used a principal component analysis (PCA) to obtain the pesticide use patterns from the list of pesticides reported by each of the included flower-growing farms. PCA is a multivariate statistical method used to reduce the dimensionality of a data set in which there are many interrelated variables while retaining as much as possible of the variation present in the original data set. The dimensionality reduction is achieved by transforming to a new set of principal component variables which are uncorrelated, and which are ordered so that the first few retain most of the variation present in the original variables.

The criterion for selecting the number of retained principal components is that these have an eigenvalue greater than 1. We observed how much variability is explained by each one of the components individually and jointly. Based on this criterion and looking for an interpretation in the context of the study, we identified six principal components explaining more than 70% of the total variability of the observed data. After the initial extraction, the principal components were rotated to facilitate the interpretation (orthogonal rotation). The determination of the variables that are grouped within the principal component is based on the eigenvectors. To perform comparisons among eigenvectors, these are normalized, creating new component loading scores. A cut-off point was established at 0.30.

The variables that tend to have strong relations do so because they have elements in the eigenvector which tend to be greater in absolute value than the others. To use the results of the principal component analysis in subsequent statistical analyses, we calculated the principal component scores for each one of flower growing farms. These scores allow us to place the observations in the data set with respect to the axes of the principal components. The principal component scores as a continuous variable are difficult to interpret, so we divided the obtained scores into tertiles. In this way, for each one of the six components selected, a categorical variable with three levels was obtained. The low level indicates that the farm had not used the mixture, the medium level shows that some of the pesticides in the use pattern were applied, and the high level represents the use of the mixture during the specific season.

Information on occupational exposure determinants (work activity, use of personal protection equipment, and workplace) was obtained from the questionnaire applied to each flower worker. This questionnaire has proven to be useful for other agricultural workers in Mexico. (Blanco-Muñoz and Lacasaña 2011).

The "work activity" was classified according to the probability of contact with the applied pesticides. The low-contact category included those workers with occasional or no contact with pesticides (administrative work) and workers who used organic production methods. Medium contact included those workers who used pesticides during the work process but who did not carry out any of the tasks included in the following high contact category, which are mixing, application, and filling of the application equipment.

The "correct use of personal protection equipment" (PPE) was defined according to the possibility of reducing pesticide exposure to an acceptable level (use of a face mask with filter plus at least one of the following waterproof garments: overalls, pants, jacket or gloves), medium level of acceptability (use of a face mask with filter or at least one of the following waterproof garments: overall, pants, jacket or gloves) and non-acceptable use which included those workers who did not use a face mask with a filter, nor waterproof overalls, pants, jacket nor gloves, independently of whether they used or not another type of protective garment.

The "workplace" (WP), was classified as being outdoors, a greenhouse or both, supposing that the highest exposure is found in the greenhouse and the lowest exposure is outdoors.

Work activity, use of PPE and workplace are variables that are related to each other, so to summarize the information provided by them an exposure index was created, assigning 1, 2 or 3 points to each category, with 1 corresponding to the lowest exposure, 2 being for medium exposure and 3 for the highest exposure. With respect to workplace, workers who worked outdoors were assigned a value of 1, outdoors and in the greenhouse (both) had a value of 2, and those who worked only in the greenhouse were assigned a value of 3. For the type of activity at work (TAW) a point value of 1 was for the category with lowest contact, 2 for the medium contact category, and 3 for the highest contact category. Finally, for the use of PPE, a value of 1 was considered to be acceptable, 2 was medium acceptability and 3 was for the unacceptable category. Later, points assigned to each worker were added up and punctuations were obtained within a range of 4–9, for which this index was classified as moderate exposure to pesticides (4–6 points) or high exposure to pesticides (≥ 7 points). We omitted the low category since all workers had more than 4 points.

$$\text{Pesticide Exposure Index (PEI)} = \text{TAW} + \text{PPE} + \text{WP}$$

Statistical analysis

The distribution of quantitative variables was tested and according to normality or non-normality, they were described through averages and standard deviations or through medians and ranges, respectively. In the case of cytokines, these were described through percentages, medians, and ranges. Qualitative variables were described through percentages.

To evaluate the association between each one of the pesticide use patterns, categorized in tertiles, and the concentration of each cytokine, categorized as $<$ or $\geq$ median, multiple logistic regression models were constructed, based on the bivariate models and considering the lowest level of each use pattern as the reference category (first tertile).

In the final models, confounders were considered such as the age in years, federal state, BMI (normal, overweight or obese), alcohol consumption (not consuming alcohol, consumption < 30 g/day and consumption ≥ 30 g/day) and tobacco consumption (has never smoked, ex-smoker, current smoker) and, occupational exposure being moderate or high. These variables were selected according to a directed acyclical graph, DAG) and based on prior studies on this topic (Quandt et al. 2006; Mokarizadeh et al. 2015).

As a measure of association, in all models, the odds ratios were estimated and their confidence intervals at 95% (OR CI 95%). The association was considered to be significant if p value ≤ 0.05. All statistical analyses were carried out with R Studio 1.4.1103 and Stata version 15.

Results

General and occupational characteristics of flower workers

The flower workers included in this study were mostly from the State of Morelos (75%), the median age was 33.5 years old, and the median of education was 9 years; 51% of them were overweight or obese, 78% consumed alcohol in different amounts and 80% smoked or had smoked. 11% informed that they worked outdoors, 67% in the greenhouse and the rest at both places. According to the activity they performed, 64.8% were included in the high contact category; only 8% used acceptable personal protection equipment. So 91% were highly exposed to pesticides according to our exposure index (Table 1). The average time spent on the job (seniority) was 12.6 years in flower production work, with average work days of 6.2 h/day and working 6 days per week (data not shown in tables).

Table 1 Characteristics of flower workers in Morelos and State of Mexico

Characteristics	Percentage or median and range[a] ($n = 108$)
State	
Morelos	75
State of Mexico	25
Age (years)	33.5 (15–55)
Education (years)	9 (1–19)
BMI[b] (kg/m^2)	25.1 (15–45)
Normal	49.1
Overweight	37.0
Obesity	13.9
Cigars/day	0.9 (0–20)
Have never smoked	20.3
Ex-smoker	34.3
Currently smoker	45.4
Alcohol (gr/day)	7 (0–144)
Does not consume	21.3
< 30 g/day	63.9
≥30 g/day	14.8
Workplace	
Open air	11.1
Greenhouse	66.7
Both	22.2
Type of activity	
Low contact	3.7
Medium contact	31.5
High contact	64.8
PPE[c]	
Unacceptable use	66.7
Moderately acceptable	25
Acceptable use	8.3
IEP[d]	7.6 1
Moderate pesticide exposure	9.3
High pesticide exposure	90.7

[a] Values represent median and range for continuous variables or percentages for categorical variables

[b] BMI: body mass index

[c] PPE: personal protective equipment

[d] IEP: pesticide exposure index

Pesticide use patterns and cytokine concentrations

Table 2 shows the result of the principal components analysis, the pesticides that were grouped into each use pattern, as well as the chemical family to which they belong.

We selected six principal components within which the following pesticide use patterns (UP) were included: UP1 (Carbendazim, Biphentrine, Imidacloprid, Diazinon, Iprodione, Methyl thiophanate, Permethrine, Triadimefon and Oxamyl); UP2 (Methyl-Parathion, Methamidophos, and Paraquat); UP3 (Propargite, Flufenoxuron, Zineb and Metalaxyl); UP4 (Abamectin, Mancozeb; Triforine and Glyphosate); UP5 (Terbufos, Benomyl, Methomyl, Omethoate, and Carbofuran); and UP6 (Endosulfan, Chlorothalonil, and Captan) (Table 2).

Table 3 shows the cytokine distribution according to median and range. In general, the highest concentrations were observed for proinflammatory cytokines (IFN-γ and TNF-α), whereas IL-6 was the cytokine that showed the lowest concentrations.

Associations between pesticide use patterns and cytokines

The results of the simple logistic regression analysis (Supplementary Table 1) showed significant reductions as well as increases in the possibilities of having cytokine levels that are above the median, according to four (patterns 3, 4, 5, and 6) of the six pesticide use patterns. Like this, compared to low exposure, high exposure according to use pattern 4, reduced the possibilities of having IL-10 (OR 0.63, $p < 0.05$) and TNF-α (OR 0.33, $p < 0.05$) levels above the median. At the same time, high exposure to use pattern 5, significantly reduced the possibility of having IL-4 levels above the median (OR 0.27, $p < 0.05$). Also, for this pattern, negative associations were found with TNF-α levels, for medium exposures (OR 0.43, $p < 0.05$) and for high exposures (OR 0.26, $p < 0.05$). Whereas, high exposure to use pattern 3 and high exposure to use pattern 6, respectively, were associated with IL-6 levels (OR 3.15, $p < 0.05$) and IFN-γ (OR 3.16, $p < 0.05$) that were above the median.

Once adjusted for confounding variables, the exposure index and age, we saw that the possibilities of having IL-6 concentrations that were above the median, in those individuals who had high exposure to use pattern 3, was 414% (OR 5.14, CI 95 1.44–20.85, $p < 0.05$) greater than in those exposed to low levels of this pattern (Fig. 1a; Supplementary Table 2). With respect to pesticide use pattern 1, which was not associated with any of the studied cytokines in the previous analysis, once the adjustment was made the possibilities of having IFN-γ in concentrations above the median increased for those with medium exposure (OR 8.53, CI 95 1.52–66.78, $p < 0.05$) as well as for those who were

Table 2 Main components of pesticides[a] and their conformation

Family	Pesticide use pattern 1	Pesticide use pattern 2	Pesticide use pattern 3	Pesticide use pattern 4	Pesticide use pattern 5	Pesticide use pattern 6
Insecticides						
Carbamates					Carbofuran	
	Oxamyl				Methomyl	
Macrocyclic lactone				Abamectin		
Neonicotinoid	Imidacloprid					
Organochlorine						Endosulfan
Organophosphates	Diazinon	Parathion			Omethoate	
		Methamidophos			Terbufos	
Pyrethroids	Biphentrine		Flufenoxuron			
	Permethrine					
Sulphites			Propargite			
Fungicides						
Aromatic						Chlorothalonil
Amides				Triforin		
Benzimidazoles	Carbendazim				Benomyl	
Carbamate	Thiophanate					
Dicarboxyimides	Iprodione					
Dithiocarbamates			Zineb	Mancozeb		
Phenylamides			Metalaxyl			
Phthalimide						Captan
Thiazoles	Triadimefon					
Herbicides						
Bipyridyls		Paraquat				
Organophosphates				Glyphosate		

[a] The six main components selected accounted for 72% of the data variability

Table 3 Cytokines distribution in flower workers ($n = 108$)

Cytokines (pg/mL)	Median	Range
Anti-inflammatory		
IL-4	2.5	0.1–12.4
IL-10	2.3	0.1–65.3
Pro-inflammatory		
IL-5	1.5	1.2–55.8
IL-6	0.9	0.05–47.6
IL-8	7.6	1.6–44.6
TNF-α	26	0.1–675
IFN-γ	63.2	0.6–250.9

highly exposed to that pattern (OR 6.96, CI 95 1.38–48.99, $p < 0.05$) (Fig. 1b, Supplementary Table 2). Also, the possibilities of having IFN-γ in concentrations above the median, in those who were highly exposed to use pattern 6 was 203% (OR 3.03, CI 95 1.15–8.38, $p < 0.05$) greater than in those exposed to low levels (Fig. 1b, Supplementary Table 2). No associations were found between pesticide use patterns and the other cytokines evaluated (Figs. 1c–g; Supplementary Table 2).

Discussion

Some pesticide use patterns that group different insecticides and fungicides were associated with an increase in pro-inflammatory cytokines, specifically IL-6 and IFN-γ. This is relevant because inflammation is involved in the development of chronic diseases, such as cancer, asthma, type 2 diabetes, cardiovascular disease, immune suppression, neurological disorders, and respiratory diseases (Khan et al. 2013; Mostafalou and Abdollahi 2013).

As far as we know, this is the first epidemiological study carried out in Mexico and one of the few at an international level, to explore the association between exposure to pesticide mixtures and the immune response, in a population that was occupationally exposed to these chemical products. Although several studies have approached the effects of pesticide exposure on cytokine levels, most of

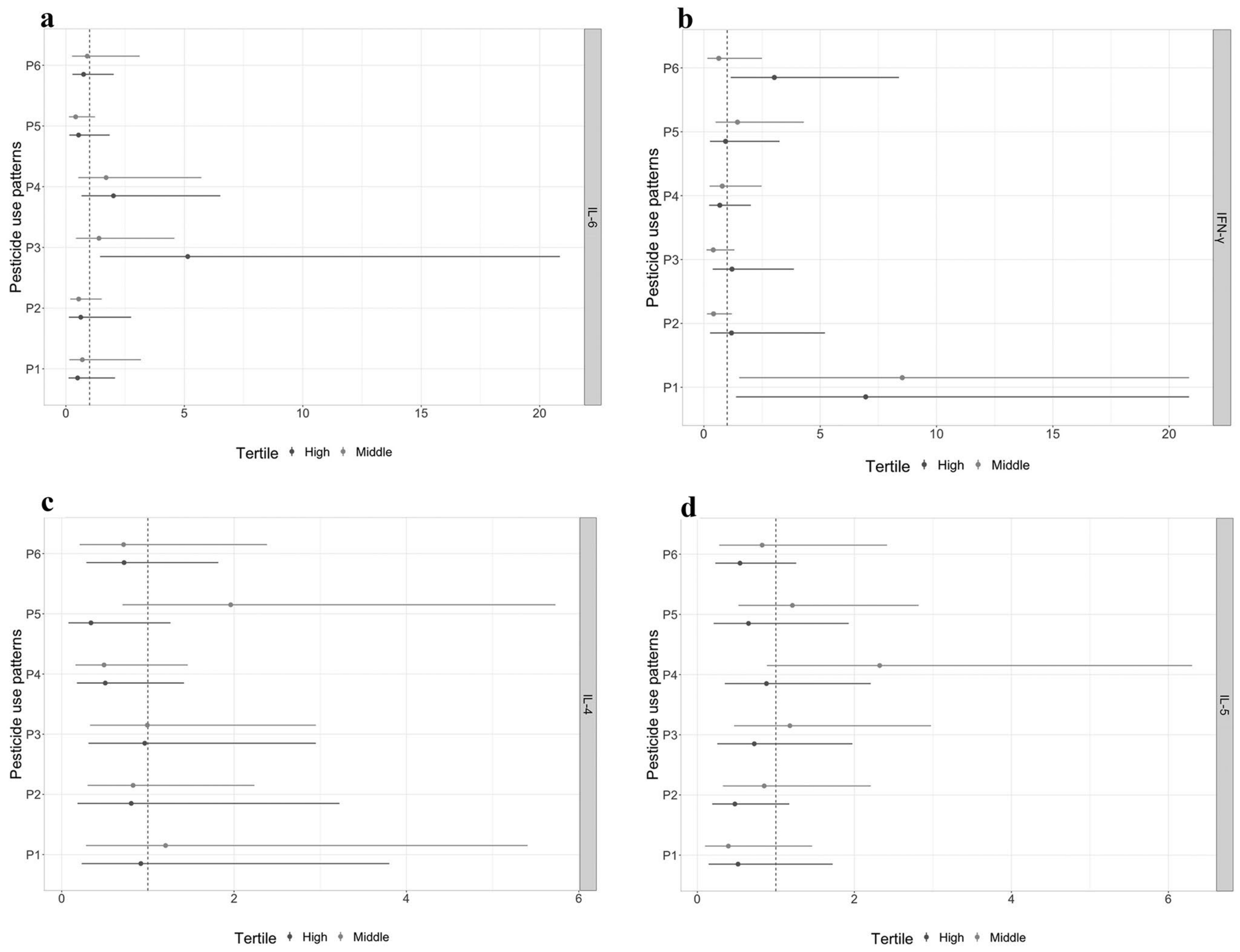

Fig. 1 Association between exposure to pesticide patterns and cytokines in flower workers from the State of Morelos and State of Mexico. Vertical dotted line represent the null value (OR = 1). **a** (OR and CI for the association with IL-6); **b** (OR and CI for the association with IFN-γ); **c** (OR and CI for the association with IL-4); **d** (OR and CI for the association with IL-5); **e** (OR and CI for the association with IL-8); **f** (OR and CI for the association with IL-10); **g** (OR and CI for the association with TNF-α). All models were adjusted for age, status, BMI, smoking, alcohol consumption and exposure index. The reference was always the low exposure category (first tertile)

them are in vitro or in vivo studies that have used cell cultures or animal models, while we have found few epidemiological studies carried out in human populations (Jacobsen-Pereira et al. 2020; Mwanga et al. 2016; Fenga et al. 2014; Costa et al. 2013).

According to our study's findings, flower workers exposed to use pattern 3, which groups propargite (sulphite, insecticide), flufenoxuron (pyrethroid, insecticide), zineb (dithiocarbamate, fungicide) and metalaxyl (phenylamide, fungicide) showed a greater probability of having the highest concentrations of IL-6. This is consistent with the findings of a cohort study carried out in Brazil, where the groups exposed to pesticide mixtures (insecticides, herbicides, and fungicides) had the greatest concentrations of IL-6, compared to those who were not occupationally exposed to these products (Jacobsen-Pereira et al. 2020).

Also, in our study, flower workers with the highest IFN-γ concentrations were the ones who were most exposed to use pattern 1, grouping biphentrin (insecticide, pyrethroid), carbendazime (fungicide, benzimidazole), imidacloprid (insecticide, neonicotinoid), diazinon (insecticide, organophosphate), iprodione (fungicide, dicarboximide), triadimefon (fungicide, thiazole), oxamyl (insecticide, carbamate), thiophanate (fungicide, carbamate), permethrine (insecticide, pyrethroid), and the workers who were most exposed to pattern 6, which grouped endosulfan (insecticide, organochlorate), chlorothalonil (fungicide, organochloride,) and captan (fungicide, phtalimide).

Our results are consistent with those found by Mwanga et al. (2016) in women residing in a rural area of South Africa. In these women, greater exposure to organophosphate (OP) pesticides and pyrethroids was associated with

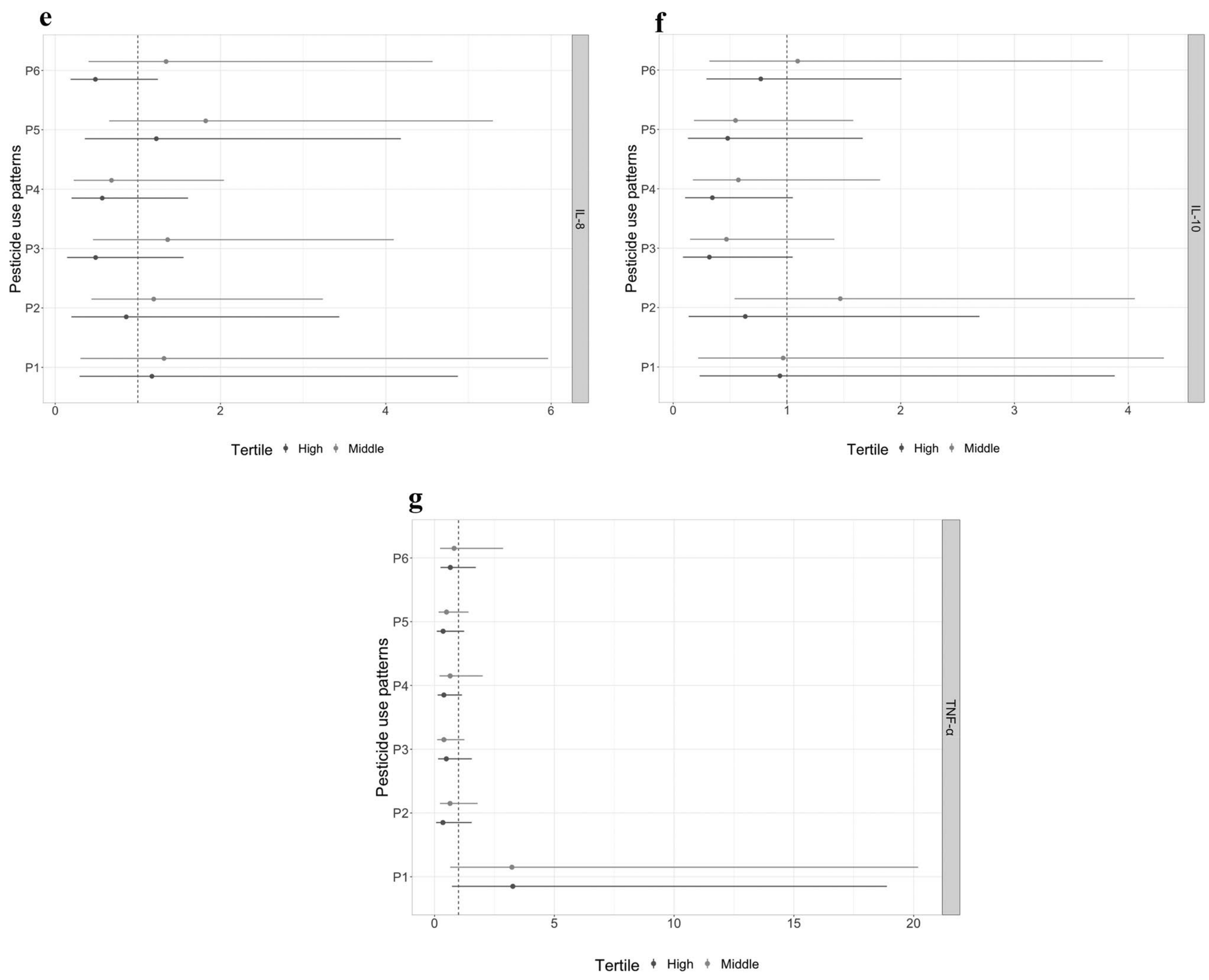

Fig. 1 (continued)

greater possibilities of showing detectable levels of IL-6 and INF-γ. However, our results differ from those of Costa et al. (2013) who found lower concentrations of pro-inflammatory cytokines and, specifically, of INF-γ, in agricultural workers exposed to pyrethroids, when compared to the non-exposed population.

Nevertheless, it is difficult to compare our results with those of other epidemiological studies, due mainly to methodological differences, especially in the way in which the exposure was evaluated, since some only evaluate one or two pesticide families, independently, and/or adjust for potential confounders in the analysis. Like this, in the study by Jacobsen-Pereira et al. (2020), which evaluates the effect of pesticide mixtures on the immune response, the authors did not include potentially confounding factors, such as age, body mass index, alcohol and tobacco consumption, in their analysis. The study by Costa et al. (2013), which evaluated the association with pyrethroids by means of biomarkers, also did not report the inclusion of confounding variables in their analysis, while Mwanga et al. (2016), who did adjust by other variables, evaluated the association with OP and pyrethroids in an independent manner.

With respect to biological mechanisms that could explain the associations found, the OP pesticides and carbamates included in use patterns 1 and 3 inhibit enzymes with esterase activity, altering the lymphocytic cholinergic signalling through the inhibition of acetylcholinesterase. Also, the inhibition of cholinesterase may generate structural and functional changes in the populations of monocytes because these esterases are linked to their cell membrane by monocyte receptors, which may affect IL-6 production (Mokarizadeh et al. 2015). The inhibition of serum esterases by organophosphates in turn inhibits the complement system and that of thrombin, leading to the alteration of the first line of immunological defense, as well as to deregulation of the biosynthesis of the adrenocorticotropic hormone. This inhibits cortisol secretion, the hormone that has an important role in the secretion of diverse cytokines, such as IL-6 and IFN-γ (Corsini et al. 2013; Dhouib et al. 2016; Gangemi et al. 2016; Kang et al. 2019; Hernández-Urzúa and Alvarado-Navarro 2001; Mostafalou and Abdollahi 2017; Pollard et al. 2013; De Camargo et al. 2013; Wang et al. 2018).

On the other hand, the neonicotinoids included in use pattern 1, generate immunotoxicity as they produce oxidative stress, which in turn causes damage to the lymphoid tissue and functional deterioration of the immune system cells; this can be related to the increase in IFN-γ (Pollard et al. 2013; Wang et al. 2018). The Pyrethroids included in use patterns 1 and 3 exert their action through oxidative stress and cytotoxicity (Costa et al. 2013), but also by being endocrine disruptors showing inhibitory effects on androgen receptor transcription (Ding et al. 2020). This could affect some functions of the immune system, altering the liberation of cytokines that are modulated by these hormones (Ben-Batalla et al. 2020). The role of dithiocarbamates has also been described, as immune modulators that influence the maturation and activation of T cells and of natural killer cells (Gangemi et al. 2016).

We found an increase in IL-6 and IFN-γ that was related to exposure to some use patterns. Both are pro-inflammatory cytokines participating in the innate and adaptive response by means of diverse mechanisms (Kang et al. 2019; Pollard et al. 2013). Modulation of IL-6 is done through hepatocytes, monocytes, dendritic cells, and lymphocytes, and that of IFN-γ is mainly through T cells and natural killer cells. In experimental studies, the organic phosphorus pesticide phoxim significantly reduced jejunum villus height and decreased the mRNA expression of junction protein genes, including occludin and claudin-4. In addition, phoxim increased IL-6 and tumor necrosis factor (TNF)-α in jejunum mucosa of Sprague Dawley (SD) rats (Sun et al. 2018). However, the scope of our study is limited and we cannot attribute the differences in cytokine levels to one or some particular pesticides.

One of the main strengths of our study lies in the broad information collected on the types of pesticides that were used, which allowed us to identify use patterns to define the workers' exposure. Other epidemiological studies on this topic have been limited to defining the study population as being exposed or not exposed (Jacobsen-Pereira et al. 2020), or evaluating the effect of a specific chemical family or of some specific pesticides (Mwanga et al. 2016; Costa et al. 2013; Corsini et al. 2005). The adjustment by covariates and by the main variables that could confound the results is another strength of this study.

However, our study has some limitations that we need to mention:

(1) The information about pesticides that were habitually used in the businesses, which was used to find the use patterns, was provided through a questionnaire answered by those who were responsible for pesticide purchases. Although sometimes we managed to have access to the store, to corroborate the information, we cannot dismiss the existence of a possible measurement error. Nevertheless, this would be non-differential because the informants ignored the study hypothesis and the cytokine profile of the workers.

Given the multiple pesticides that are used by flower workers, the measurement of exposure by means of biomarkers is not viable; also, these are not free from measurement error since many pesticide metabolites are not persistent and a series of measurements is required to obtain precise information which is not always possible in epidemiological studies.

(2) Those who carried out the laboratory studies, had no knowledge of the use patterns to which the workers were exposed, so that if measurement errors exist, they are non-differential.

The possible measurement errors that were mentioned above could have led to an underestimation of the associations between exposure to pesticide use patterns and the studied effects.

(3) Although we included important confounders in our analysis, some factors that are related to the immune response as physical and psychological stressors, as well as other environmental contaminants that are frequently found in greenhouses, especially fertilizers and allergens coming from crops, we couldn't control for these for lack of information. Also, some potential confounders measured through the questionnaire that was applied to each flower farmer could have been subject to measurement error; for example tobacco and alcohol consumption, for which we cannot dismiss the possibility of some degree of residual confounding.

(4) Since this is a cross-sectional design, we cannot dismiss the possibility of having a bias of the healthy worker, because it is possible that the workers who were most vulnerable to the immunotoxic effects of pesticides had left the business before the study was carried out. Thus, the association between use patterns and cytokine concentration could have been underestimated. Also, because it is a cross-sectional study, we cannot be sure of the time frame of the observed associations. Nevertheless, we believe there is no "reverse causality" because it would be unlikely that the cytokine profile of the workers would cause changes in the pesticide use patterns used by the businesses.

(5) There is evidence that suggests the existence of gender differences related to the inflammatory response (Klein and Flanagan 2016). Since in this study only men were

included as study population, our results could not be extended to women flower workers.

In spite of the above-mentioned limitations, the study's findings may be a contribution to knowledge on the immunotoxic effects of pesticides in humans and serve as a basis to establish norms for the regulation of pesticide use, particularly in flower growing businesses and the attitudes and practices regarding pesticide use, to reduce occupational hazards for their workers. It is worth mentioning that in Mexico, 183 active ingredients continue to be used, which the FAO/WHO considers to be highly dangerous (Bejarano 2017), 27 of which were considered in this analysis. In addition to, there are still bad practices of using personal protective equipment, flower workers have followed instructions mistakenly on the use of pesticides and overmixing pesticides from the same mechanism of action and family practices that if regularized could help reduce the high exposure that flower growers have to these chemicals (Palis et al. 2006; Olivera-Pasiani et al. 2012).

Conclusion

In conclusion, these results suggest that many pesticide patterns show immunotoxic capacity expressed as an increase in the levels of proinflammatory cytokines IL-6 and IFN-γ. Additional epidemiological studies are required that overcome the limitations of the studies done so far, with a cohort design, and standardized measurements of exposure to pesticide mixtures, which take into account the duration of exposure and the evaluated effects, in larger samples. This would allow for greater validity in determining the association between pesticide exposure and the immune response in humans.

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Author contributions BMG-P: writing—original draft preparation. AS: development or design of methodology; creation of models; reviewing. AL-M: laboratory analysis; reviewing. CE-N: statistical analysis; reviewing. AIB-G: provision of study materials, reagents, and materials. CA-G: reviewing and editing. JBM: conceptualization, Writing-reviewing and editing. ML: funding acquisition; reviewing.

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Data availability The data that support the findings of this study are not openly available due to reasons of sensitivity, to guarantee the confidentiality of the information provided by study participants. Data are available from the corresponding author upon reasonable request. The content is solely the responsibility of the authors and does not necessarily represent the official views of the CONACYT. The funding source had no involvement in study design; in the collection, analysis, and interpretation of data; in the writing of the paper; nor in the decision to submit the article for publication.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human subjects and with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and has been approved by the Commission for Ethics in Research and were recorded in the Research Commission (RC) with the number "322" of the National Institute of Public Health of Mexico (INSP, Spanish acronym).

Consent to participate Written informed consent was obtained from all individual participants included in the study.

Consent to publish Not applicable.

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