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Effects of long-term direct exposure to pesticides towards immune function: a cross-sectional study

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Abstract

Exposure to pesticides is an important risk factor for genotoxicity in farmworkers. Once there is a possibility that genotoxicity is related to the autoimmunity process, the present study aimed to characterize genotoxicity and possible immunological alterations related to chronic and direct exposure to a complex mixture of pesticides. It employed Interleukin-6 (IL-6), Interferon-gamma (IFN- γ), IL-12 and IL-10 as biomarkers of cellular immune function. DNA damage was greater in the exposed group ($p < 0.05$). Both IL-6 and IL-12 were significantly increased in the exposed group ($p < 0.05$ and < 0.01 , respectively); on the other hand, IL-10 levels were decreased ($p < 0.01$). Evaluation of the association between cytokine levels and epidemiological variables showed a correlation between alcohol consumption and higher levels of IL-12 ($p < 0.05$). In addition, negative correlation between % DNA and IFN- γ ($p < 0.05$) as well as % DNA and IL-6 ($p < 0.05$) and positive correlation was found between IL-6 levels and absolute number of T CD8+ lymphocytes ($p < 0.05$) as well as between IL-10 levels and absolute number of T CD4+ lymphocytes ($p < 0.05$). No association between all the evaluation parameters was found in the non-exposed group. The observed alterations related to chronic and direct exposure to a complex mixture of pesticides may allow the monitoring of these workers to minimize the negative impact caused on their health.

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Introduction

Occupational exposure to chemical agents, including pesticides, is a significant public health problem due to its broad exposure, making it one of the conditions potentially associated with the development of diseases such as cancer, Parkinson's, Alzheimer's, autoimmune diseases, hematological and liver disorders, hormonal changes, and diseases linked to reproduction (Abdollahi et al. 2004; Salazar-Arredondo et al. 2008; Singh et al. 2011; Miranda-Contreras et al. 2013; Freire et al. 2015; Zeljezic et al. 2015; Piccoli et al. 2016).

Among the various harms to human health, our research group has shown an association between occupational exposure to pesticides and the occurrence of genotoxicity, as evidenced by the comet assay (Khayat et al. 2013; Franco et al. 2016; Godoy et al. 2019; Ramos et al. 2020). Specifically, in our recently published study (Ramos et al. 2020), exposure to a mixture of pesticides presented higher levels of DNA damage, inflammatory cells and changes in genotype distribution of TNF- α . However, our study group did not find an association between CD4 subsets and DNA damage.

The immune system plays an essential role in the body's homeostasis, being precisely regulated at different levels. The complex network of regulatory mechanisms of the immune system makes it susceptible to different changes associated with environmental exposure, justifying the importance of studies in the immunotoxicology field, more specifically in the context of disease assessment (Rocha et al. 2012; Rocha-Parise et al. 2014; Kreitinger et al. 2016; Andersen et al. 2021). Some studies have shown that exposure to pesticides can lead to immunotoxicity and loss of immune cell function (Sunyer et al. 2010; Corsini et al. 2013; Mokarizadeh et al. 2015). On the other hand, exposure to these chemicals was found to be a risk factor for the activation of the immune system, evidenced by the development of autoimmune conditions such as systemic lupus

erythematosus (Cooper and Parks 2004), rheumatoid arthritis (Parks et al. 2011), autoimmune thyroiditis (Langer 2010) and multiple sclerosis (Parrón et al. 2011). The fact is that the response to exposure to pesticides is heterogeneous and complex, especially considering the time and period of exposure, the association with other risk factors and the type of pesticide involved.

Concerning the lack of association between DNA damage and immune alterations in pesticide-exposed individuals, it cannot be considered integrally without evaluation of other immune alterations such as other T cell subtypes and cytokine production. Furthermore, since was found a positive correlation was found between genomic instability, evidenced by the comet and micronucleus assay, and autoimmune thyroid diseases and type I diabetes mellitus in children (Mihaljevic et al. 2018), this field needs to be better explored.

In this regard, the measurement of cytokine levels is an important goal since they reflect the activity profile of the immune system, thus being used as biomarkers to indicate or monitor disease or its progress (Kany et al. 2019). However, the effects of exposure to pesticides in human cytokine networks remain to be elucidated.

Based on the exposure above, this study proposes to evaluate the effects of occupational exposure to a complex mixture of pesticides on the levels of pro- and anti-inflammatory cytokines as well as in T helper cells to verify if there are changes resulting from such exposure. These results could support the knowledge of the field of immunological diseases and environmental impacts, aiming to minimize the negative impact on health and contribute to the formulation of public health policies.

Material and methods

Study population and data collection

A cross-sectional study was conducted with fourteen pesticide-exposed individuals and twelve

individuals with no direct contact or closer exposure to pesticides (living nearby crops and, therefore, environmentally exposed to pesticides). The exposed and non-exposed individuals were matched by age, gender, and lifestyle.

This study was conducted with a restricted group of pesticide-exposed individuals due to the eligibility criteria that demanded a cohort of individuals that had over ten years of direct exposure to pesticides (occupationally exposed to various pesticides during storage, mixing, loading, and pesticide spraying activities) and that was continuously attended by the local health service, also a service responsible for investigating the conditions of the work environment using epidemiological data in conjunction with the Health Surveillance. These individuals were selected to guarantee the reliability of the data related to exposure to pesticides and the possibility of collection of new data, also more biological samples and following-up the health status over time.

All the studied individuals were fully informed about the procedures and the aims of the study as well as signed informed written consent before participation. The performance of this research was approved by the Research Ethics Committee of the Federal University of Goiás (#2.648.494). The employed procedures were performed according to the principles of the regulatory guidelines and standards described in Resolution No. 466/12 of the National Health Council, which approves the regulatory guidelines and standards for research involving human beings in Brazil.

Sociodemographic data were collected by applying to the studied individuals a questionnaire with open-ended and closed questions. The questionnaire included questions about age, gender, smoking habit, alcohol consumption, overweight, sedentary lifestyle and inflammatory comorbidities, as well as the occupational history of pesticide exposure, use of personal protection equipment (PPE), history of acute intoxication, daily time and years of exposure.

Sample collection

A total volume of 15 mL of peripheral blood was obtained from exposed and non-exposed individuals in vacuum tubes containing EDTA (ethylenediaminetetraacetic acid). Samples were processed, frozen, and stored at -20°C until analysis.

Determination of DNA Damage

DNA damage was evaluated by alkaline single cell gel electrophoresis (comet assay) according to Singh et al. (1988) with slight modification, as described before (Ramos et al. 2020). The slides were examined under a fluorescence microscope (Axio Imager 2®, Carl Zeiss) with the Comet Imager version 2.2 software (MetaSystems GmbH). The percentage of DNA in the tail (% DNA) was measured in one hundred randomly selected nuclei per individual.

Lymphocyte cell count

The absolute number of lymphocytes was determined in the whole blood samples obtained from each pesticide-exposed individual, by an automated hematological counter ABX micros 60 (Horiba ABX Diagnostics, France).

Peripheral blood mononuclear cells obtaining

In order to obtain peripheral blood mononuclear cells (PBMC), density-gradient centrifugation with Ficoll-Paque™ Plus (density 1.077 g/mL, GE Healthcare, Uppsala, Sweden) was performed. For each blood sample in a 3 mL volume previously diluted 1:1 in phosphate-buffered saline (PBS), 3 mL of the Ficoll-Paque PLUS (GE Healthcare) were placed into 15 mL centrifuge tubes and then diluted blood was carefully placed over the Ficoll-Paque gradient. The tubes were then centrifuged at 1500 rpm for 30 min at room temperature to obtain PBMC pellets.

Immunophenotyping

PBMC pellets, obtained as described above, were carefully recovered by Pasteur pipette and placed in 15 mL tubes containing PBS. The tubes were then centrifuged twice for 10 min at 1500 rpm. The supernatant was discarded to be added 1 mL of PBS enriched with 1% of bovine fetal serum (BFS). The viable cells were counted by using Trypan blue coloration 1:9 (10 μ L of the sample in 90 μ L of Trypan blue) in a Neubauer chamber. Aliquots of 100 microliters containing 1×10^6 cells each were prepared to immunophenotype the T CD4 and CD8 lymphocytes, by using BV480-conjugated anti-human CD3, PerCP-conjugated anti-human CD4 and APC-CY7- conjugated anti-human CD8 (BD Biosciences – San Diego, CA, USA). Unstained cells were used as a negative control. The cells were incubated at 4°C for 20 min in the dark. After, the cells were washed twice in PBS with 1% BFS and analyzed using BD FACSDiva™ software (BD Biosciences, USA). At least 10,000 lymphocytes for each sample were acquired and analyzed.

Lymphocyte activation and culture supernatant recovering

The separated PBMCs from each sample at the concentration of 1×10^6 /mL was added to culture bottles in duplicate and then to RPMI culture medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin (Flow lab.-USA), 12.5 mM of HEPES buffer (pH=7.4), 0.2% NaHCO₃ and 10% BFS. Then, 100 μ L of phytohemagglutinin (PHA-Sigma, USA), a mitogen, was added and incubated at 37°C for 72 hours.

Cytokine measurement

The levels of IL-6, IL-10, IL-12 and IFN- γ cytokines were measured in the culture supernatant, obtained as described above, by

sandwich enzyme immunoassay (ELISA) (Uncoated ELISA, Invitrogen TM, Waltham, Massachusetts, USA), according to manufacturer's specifications. Briefly, plates were sensitized overnight (18 to 24 hours) with capture antibody, the background was eliminated, standard curve and samples were incubated overnight and detection antibody, streptavidin-HRP complex and chromogen were added. Optical density was measured in a spectrophotometer at 450 nm (Celer®, Polaris).

Statistical analysis

Continuous and categorical variables were presented as mean and standard deviation and percentages, respectively. Statistical analysis was performed using GraphPad Prism software version 8.0 and SPSS software version 26.0. Shapiro-Wilk and Levene tests were done to verify normality and homoscedasticity, respectively. Chi-square test for categorical variables and Mann-Whitney U test for continuous variables were used. Spearman's correlation test was also used to correlate immunological parameters with continuous variables. Statistical significance was considered when $p < 0.05$.

Results and Discussion

The epidemiological characteristics of both groups are shown in Table 1. No difference was shown regarding age, sex, smoking habits, alcohol consumption, overweight, sedentary lifestyle and inflammatory comorbidities. Concerning exposed individuals, 57.1% did not refer to any use of PPE and 21.4% reported a history of acute pesticide poisoning. The mean time of exposure was 6.0 ($\pm$ 3.8) hours per day for 20.1 ($\pm$ 8.5) years.

It is important to emphasize that the absence of a significant association between the epidemiological parameters in the evaluated groups is essential to minimize confounding factors in the context of immunological studies

since several clinical and epidemiological factors can interfere with the immune function (Dowd and Aiello 2009; Brodin and Davis 2017).

Table 1. Epidemiological variables from the study population

Variable	Groups		p-value
	Exposed (n = 14)	Non- exposed (n=12)	
Age (years)	50.1 ($\pm$ 13.4)	54.7 ($\pm$ 10.9)	0.462 ^a
Sex			
Male	11 (78.6%)	7 (58.3%)	0.265 ^b
Female	3 (21.4%)	5 (41.7%)	
Smoking habits			
Yes	4 (28.6%)	1 (8.3%)	0.192 ^b
No	10 (71.4%)	11 (91.7%)	
Alcohol consumption			
Yes	10 (71.4%)	7 (58.3%)	0.484 ^b
No	4 (28.6%)	5 (41.7%)	
Overweight			
Yes	11 (78.6%)	7 (58.3%)	0.265 ^b
No	3 (21.4%)	5 (41.7%)	
Sedentary lifestyle			
Yes	3 (21.4%)	3 (25.0%)	0.829 ^b
No	11 (78.6%)	9 (75.0%)	
Inflammatory comorbidities			
Yes	1 (7.1%)	1 (8.3%)	0.910 ^b
No	13 (92.9%)	11 (91.7%)	

^ap-value associated to Mann-Whitney test

^bp-value associated to chi-square test

Immunological parameters of exposed and non-exposed individuals are presented in Figure 1. Exposed individuals presented lower levels of IL-10 ($p < 0.01$) and higher levels of IL-6 ($p < 0.05$) and IL-12 ($p < 0.01$). IFN γ also showed a slight increase, but not significant ($p = 0.781$) in the exposed group.

Interleukin (IL)-6 is an inflammatory cytokine that plays an important role in chronic inflammation as well as in cellular differentiation and proliferation (Holdsworth and Can 2015). Higher levels of IL-6 have been associated with autoimmune diseases,

such as rheumatoid arthritis and systemic lupus erythematosus (Tanaka et al. 2014). In this study, IL-6 levels were higher in pesticide-exposed individuals when compared to control subjects. IL-6 release was shown to be increased by different human immune cell lineages after in vitro exposure to two different tested pesticides (Martin et al. 2019). Moreover, increased IL-6 levels were detected in the sera of chronic pesticide-exposed individuals (Jacobsen-Pereira et al. 2020).

Corroborating IL-6 elevation, exposure to pesticides also showed leading to a higher release of IL-12, another inflammatory cytokine with a central role in Th1 responses (Holdsworth and Can 2015). Surprisingly, this cytokine is barely evaluated in the literature regarding pesticide exposure and most of the published studies showed a decrease in IL-12 levels in pesticide exposure individuals as well as a dysfunction of immune cells (Luty et al. 2000; Pruett et al. 2006; Khayat et al. 2013; Ge et al. 2021).

This difference may be because the referred studies employed animal models (Luty et al. 2000; Pruett et al. 2006; Ge et al. 2021), and also because other biological fluids such as sera (Luty et al. 2000; Pruett et al. 2006; Khayat et al. 2013; Ge et al. 2021) and intraperitoneal fluid (Pruett et al. 2006) were analyzed. Furthermore, except for two of the cited studies, it was not evaluated chronic exposure effect and none of the studies evaluated exposure to a complex mixture of pesticides. Still, based on the fact that one of these studies showed an association between immune spleen cell apoptosis with a reduction of immune function (Ge et al. 2021). Considering that, chronic exposure to a mixture of pesticides lead to a different response when compared to acute and subacute exposure to only one pesticide.

Concerning IL-10, an important anti-inflammatory cytokine that contributes to immune regulation (Holdsworth and Can 2015), the effects of pesticide exposure related to this cytokine remain contradictory, with studies suggesting that this

exposure leads to decreased IL-10 levels (Esquivel-Sentíes et al. 2010; Neta et al. 2011) and

others revealing an increase (Jorsaraei et al. 2014; Taghavian et al. 2016).

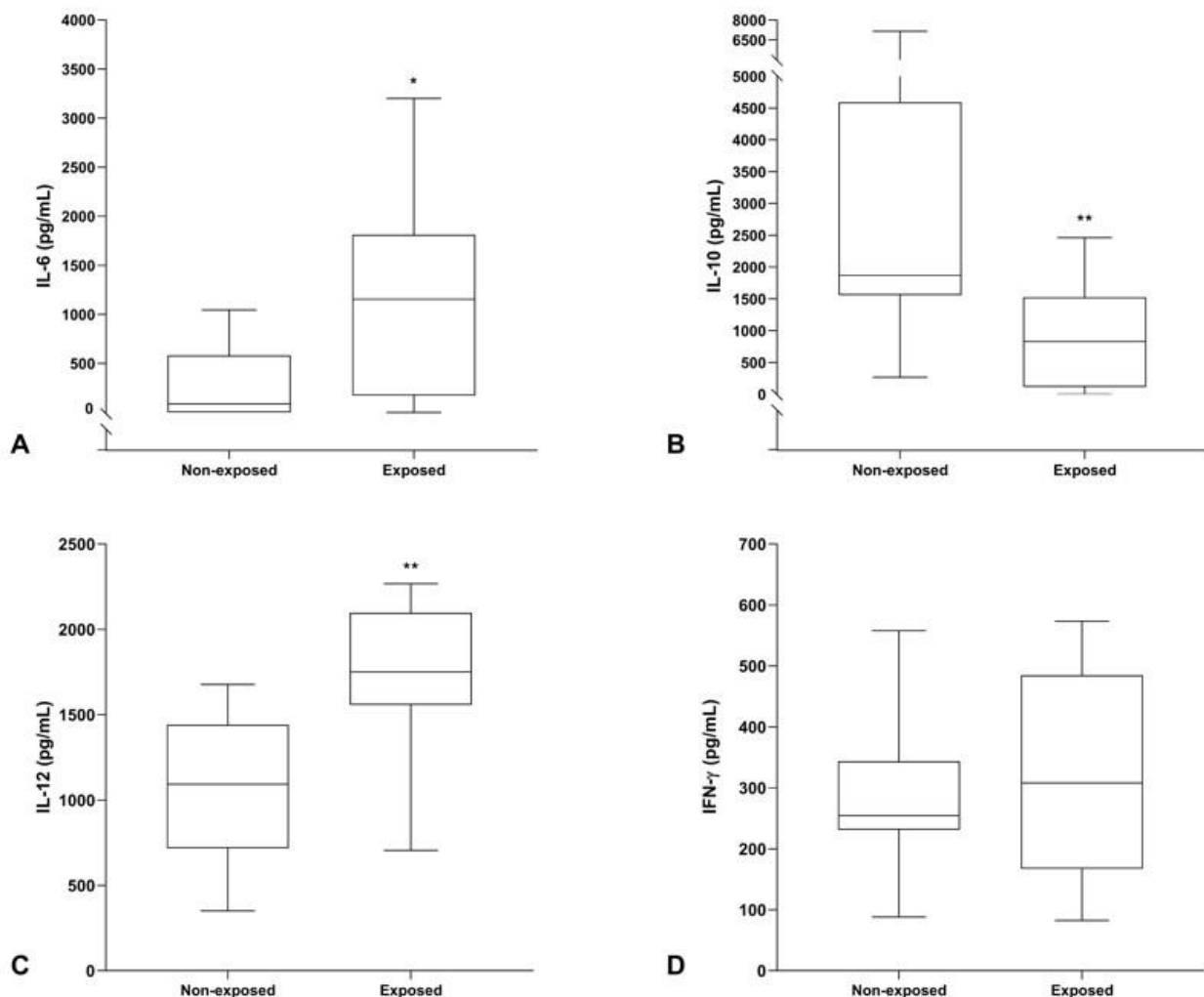


Figure 1. Cytokine levels of experimental groups. * $p < 0.05$; ** $p < 0.01$.

Considering that the exposed individuals of the present study were also evaluated in the study from Ramos and co-workers in 2021, which showed a TCD4 regulatory (TREG) cell decrease in comparison with control subjects, IL-10 lower levels may be related to this, since TREG cells are mainly regulatory due to the ability to secrete this cytokine (Laidlaw et al. 2015; Fujio et al. 2017). Based on the observed cytokine levels, it was performed an evaluation of the association

between cytokine levels and epidemiological variables, described in Table 2.

The only variable that showed a correlation with altered cytokine levels was alcohol consumption. Exposed individuals that reported alcohol consumption presented higher levels of IL-12 ($p < 0.05$). In addition, no association between epidemiological and immunological parameters was found in the non-exposed group (data not shown).

Table 2. Association between epidemiological and cytokine levels of exposed individuals

Variable	Cytokine levels			
	IL-6 (pg/mL)	IL-10 (pg/mL)	IL-12 (pg/mL)	IFN- γ (pg/mL)
Sex				
Male	1148.4 $\pm$ 868.4	1089.9 $\pm$ 766.3	1812.1 $\pm$ 387.9	326.9 $\pm$ 192.9
Female	1235.0 $\pm$ 1702.2	234.6 $\pm$ 328.6	1181.9 $\pm$ 495.9	304.9 $\pm$ 51.7
Smoking habits				
Yes	1663.4 $\pm$ 807.2	835.5 $\pm$ 809.4	1757.7 $\pm$ 22.6	351.2 $\pm$ 198.1
No	968.4 $\pm$ 1055.1	935.1 $\pm$ 804.0	1644.8 $\pm$ 566.5	310.5 $\pm$ 168.7
Alcohol consumption				
Yes	1342.1 $\pm$ 969.6	1033.8 $\pm$ 798.4	1852.6 $\pm$ 323.6*	315.7 $\pm$ 173.7
No	729.1 $\pm$ 1128.2	588.7 $\pm$ 709.2	1238.4 $\pm$ 549.5	338.3 $\pm$ 187.5
Overweight				
Yes	1205.7 $\pm$ 1081.4	674.7 $\pm$ 621.7	1578.4 $\pm$ 478.4	301.2 $\pm$ 143.4
No	1024.9 $\pm$ 889.7	1757.1 $\pm$ 769.7	2038.9 $\pm$ 262.5	398.9 $\pm$ 274.1
Sedentary lifestyle				
Yes	2107.2 $\pm$ 950.5	1453.3 $\pm$ 937.8	1705.2 $\pm$ 561.2	412.7 $\pm$ 156.5
No	910.6 $\pm$ 904.0	757.5 $\pm$ 700.8	1669.4 $\pm$ 478.9	297.5 $\pm$ 172.7
Use of PPE				
Yes	1244.6 $\pm$ 811.3	1202.7 $\pm$ 873.8	1891.4 $\pm$ 233.3	394.1 $\pm$ 182.8
No	1108.7 $\pm$ 1195.2	684.6 $\pm$ 662.7	1516.3 $\pm$ 557.0	268.2 $\pm$ 149.7
History of acute pesticide poisoning				
Yes	1448.9 $\pm$ 837.6	1114.1 $\pm$ 719.2	1760.4 $\pm$ 26.9	410.3 $\pm$ 194.7
No	1090.1 $\pm$ 1079.2	850.0 $\pm$ 813.2	1654.4 $\pm$ 538.4	298.1 $\pm$ 165.2

* p < 0.05 in Mann-Whitney test.

Since chronic alcohol consumption is shown to be associated with increased IL-12 levels (Laso et al. 1998), our studied population may be under a chronic consumption status. This pattern of alcohol intake is often described in epidemiological studies that evaluate rural workers (Girish et al. 2010; Ganesh Kumar et al. 2013). Still, once this relation was also found only in the exposed group, it is possible that both alcohol consumption and pesticide exposure act synergically to increase IL-12 levels.

Exposed individuals included in this study presented higher DNA damage when compared to non-exposed individuals (25.9 $\pm$ 6.0 and 20.0 $\pm$ 7.6, respectively; p < 0.05).

This finding is following studies from our research group that reported an association between DNA

toxicity and pesticide exposure (Khayat et al. 2013; Ramos et al. 2020; Nascimento et al. 2021). As presented in Figure 2, exposed individuals presented a negative correlation between % DNA and IFN- γ (p < 0.05) and % DNA and IL-6 (p < 0.05). Additionally, a positive correlation was found between IL-6 levels and an absolute number of T CD8+ lymphocytes (p < 0.05) and between IL-10 levels and an absolute number of T CD4+ lymphocytes (p < 0.05). Non-exposed individuals did not present any correlation between cytokines, immune cells and DNA damage.

The positive correlation between IL-6 levels and an absolute number of CD8+ lymphocytes in exposed individuals may be related to the findings of previous studies that highlight IL-6 contribution to TCD8+ lymphocytes proliferation (Gagnon et

al. 2008; Lee et al. 2016; Zhou et al. 2016). Taken together, these results suggest that exposure to pesticides stimulates the IL-6/CD8⁺ lymphocytes pathway, which could be a risk factor for

autoimmune diseases associated with autoreactive T CD8⁺ lymphocytes, such as autoimmune thyroiditis, type 1 diabetes and multiple sclerosis (Deng et al. 2019).

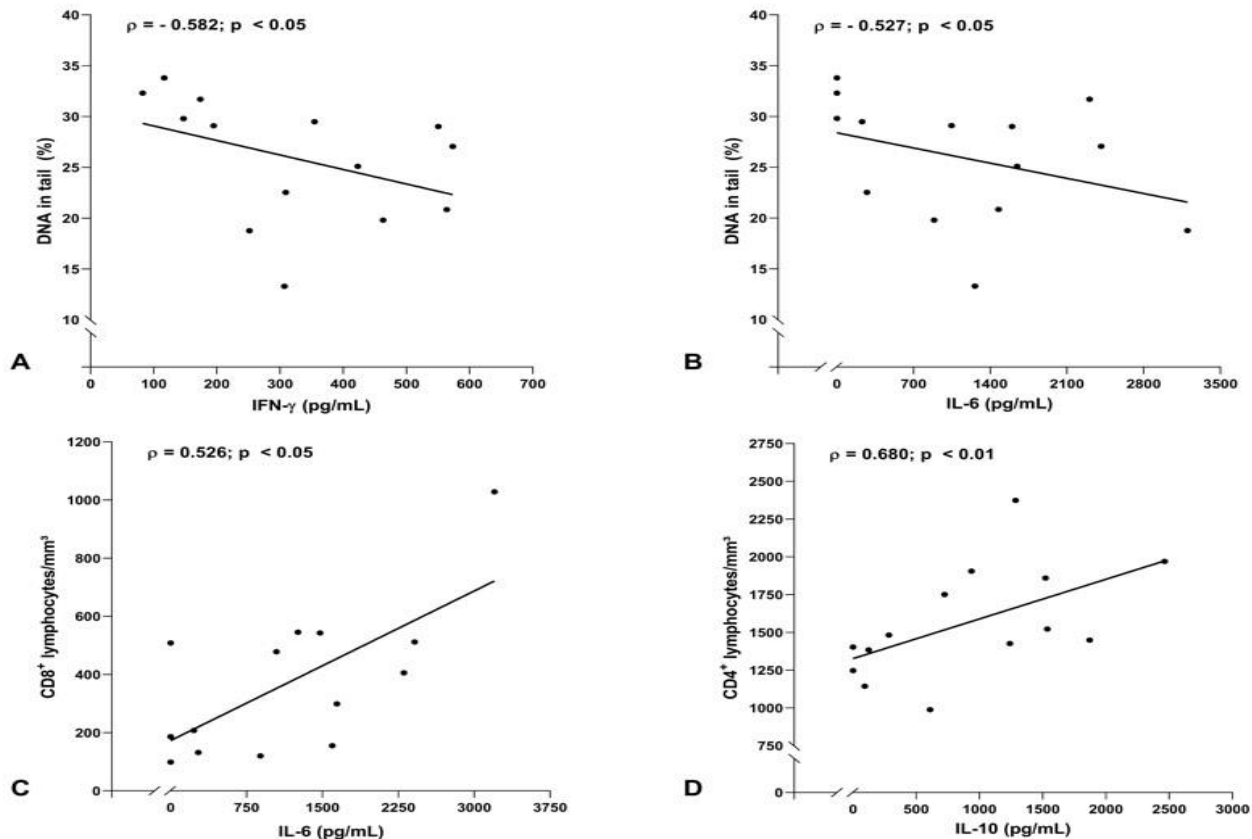


Figure 2. Correlation between cytokine levels, DNA damage and total CD8⁺ and CD4⁺ lymphocytes

Additionally, a positive correlation was observed between the number of CD4⁺ lymphocytes and IL-10 levels. This result probably reflects the duality of immune responses to immunogenic stimuli, considering that inflammatory conditions also elicit regulatory responses to control exacerbated immune responses and to maintain the body's homeostasis (Cronkite and Strutt 2018).

Considering the results presented above, pesticides may present heterogeneous immunogenic properties, being the inflammatory response sustained according to the immunogenicity of the pesticide, which demands a specific ability of

immune cells to recognize as well as eliminate it. Another plausible explanation is that there may be a certain level of DNA damage that exceeds their threshold of tolerability and repair, leading to decreased cellular function and exhaustion. However, to test both presented hypotheses, new studies should be conducted to verify the immunogenicity of different pesticides and to set the threshold of DNA damage tolerated by immune cells.

Conjuring overall data, this study points to an inflammatory profile in individuals exposed to pesticides, corroborating our previous findings

(Ramos et al. 2020). Additionally, it is important to note that this inflammatory profile observed in this study could contribute to the autoimmune process. Antigenic mimicry between pesticides and self-antigens, endocrine disruption and loss of tolerance mechanisms may be a reason for this involvement (Mokarizadeh et al. 2015). However, the mechanisms of increased risk of autoimmunity in pesticide-exposed individuals remain unclear. The effects of pesticide exposure on the immune system are difficult to evaluate considering that several intrinsic and extrinsic factors impair the immune response (Corsini et al. 2013; Gangemi et al. 2016). In addition, it is important to note that pesticides present different mechanisms of action and the consequences of the exposure could differ according to the pattern of pesticide exposure (i.e. the type, amount, variety and mixtures) (Pedlowski et al. 2012; Nascimento et al. 2021).

Conclusion

This study reinforces the association between pesticide-exposure and DNA damage and finds an association with immune alterations, also present in the exposed individuals. So, chronic exposure to a complex mixture of pesticides may comprise the arsenal of different environmental risk factors for immune dysfunction and the development of autoimmune diseases. However, the fact that our study employed a limited number of individuals and the scarce studies in the literature demands further studies employing larger cohorts aiming the evaluation of biomarkers of autoimmunity need to be developed to better understand the direct effects of pesticide exposure in the autoimmune process. Additionally, *in vitro* and *in vivo* studies should be carried out in order to verify the immunogenic properties of pesticides and to set thresholds for DNA damage deleterious rates that could directly and or indirectly impact the function of the immune cells.

Conflict of interest

Authors declare no conflict of interest.

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