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Immunomodulatory effect of metribuzin and Tribenuron-methyl in male rabbit ITELV/98

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ABSTRACT

Male rabbits of ITELV/98 strains, about 3 months old and weighing an average of 2200±14.96g, were used in this exposure to pesticides, and experiments reveal symptoms related to the intake of pesticides into the water. Thus, a difficulty breathing and coughing were observed in the groups that consumed Metribuzin and Tribenuron-methyl at the same concentration of 2 µg/l. Metribuzin used at concentrations of 0.1 ; 1 and 2 µg/l had no effect on the weight of the animals tested. Indeed, during the treatment of animals by Metribuzin, the groups receiving 0.1 and 1 µg/l showed a change in weight (2200±14.96g at 5225±29.64g and 2140±108.66g at 5331.5±104,11g) comparable to that of the control (2200±14.96g at 5398±58.62g) (P<0.05). The use of a higher dose of 2µg/l of Metribuzin did not modify the weights of this group compared with the control group and the group receiving 1µg/l of Metribuzin (P <0.05). The use of Tribenuron-methyl at the same concentrations did not alter the weight evolution of the experimental rabbits. Indeed, the groups of rabbits receiving 0.1 and 1 µg/l of this herbicide show a variation in weight (2200±14.96g at 5166±108.14g and 2117±105.18g at 5325±78.79g) respectively Remains comparable to that of the control (P <0.05). The evaluation of anti-ovalbumin rabbit IgG by previously optimized non-competitive ELISA showed a decrease in their rate in all experimental groups. In groups consuming metribuzin or tribenuron-methyl at 1 µg / l, a decrease in the level of IgG is observed, which becomes more pronounced at the concentration of 2 µg / L (P <0.05) and shows the effect Immunorepressor of these two pesticides.

***Key Words:** Rabbits, Metribuzin, Tribenuron-methyl, weight, ELISA, anti-ovalbumin IgG, immunosuppression*

INTRODUCTION

The major concern of the developing countries remains the food self-sufficiency to break with increasingly heavy dependencies. As a result, agriculture needs to intensify production, and this requires the increased use of pesticides and other chemicals. The use of pesticides, however, is accompanied by contamination of terrestrial and aquatic ecosystems [1,2,3]. The toxicity of pesticides requires that they be limited or even avoided in food and water, as well as strict handling rules [4,5]. The health risk associated with occupational exposure to pesticides is best known and most certainly proven [6,7]. Several studies on occupational exposures indicate that some pesticides (dibromochloropropane, chlordecone, carbaryl, dibromoethylene and 2,4-dichlorophenoxyacetic acid) have deleterious effects on male fertility [8,9,10]. The genotoxic potential of pesticide mixtures has also been reported [11]. Carbamate pesticides are generally neurotoxic and inhibit acetylcholinesterase. Some have been associated with negative effects on human development, especially in babies and children [12]. Complex mixtures of organochlorine pesticides are a determining factor in the risk of breast and prostate cancer [13,14]. The neurobehavioral effects of long-term exposure to pesticides also demonstrate their involvement in neurovegetative diseases or cognitive decline [15,16,17]. Algeria is classified as a country that uses large quantities of pesticides with 400 approved crop protection products, of which 40 are widely used by farmers [18]. The current state of affairs reveals the importation of products with summary labeling which does not mention anything during a visual inspection and which are prohibited in the countries of origin. The concentration of certain organochlorine molecules in water in the Algiers region (lindane, 2,4 'and 4,4' DDT, 2,4 'and 4,4' DDE) and organophosphates (diazinon, parathion) Exceeds the guideline values recommended by the WHO in 30% of samples [19]. In Algeria, the phytosanitary practices of greenhouse growers and farmers in general are bad and potentially harmful to the health of applicators, consumers and the environment [20,21]. Many pesticides cause, in humans or in different species, immunosuppression reactions [22,23] hypersensitivity [24,25] autoimmunity [26,27] Biochemical and Hematological Toxicity [28].

The objective of this work was to evaluate the immunotoxic risk of two pesticides, Metribuzin and Tribenuron-methyl used respectively for the cultivation of potatoes and cereals in Algeria. Both pesticides were used in ITELV/98 rabbits, causing acute toxicity. The evolution of the weight of the animals was measured and the immunological state was evaluated by indirectly optimized non-competitive enzyme linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Animals and Food

M Rabbit farming is carried out under conditions that meet the guide to good practice in rabbit production [29]. Male rabbits (n = 30) strain ITELV/98 about 2 months old and weighing 2108.3 ± 48.31g were provided to us by the national institute of breeding of Sidi Bel-Abbes. They are used and reared in a battery, with separators, equipped with feeders with a temperature of 20 ± 2°C and a lighting of 12 hours per day [30]. The animals, arranged in seven groups of five rabbits each, a control group and six experimental groups, perceived ad libitum of water and a standard rabbit diet "El alf" in the form of granules (20% barley, dehydrated alfalfa 35%, Soybean meal 13%, wheat bran 30%, salt 0.5%, mineral complex and vitamin 1%) throughout the experiment.

Consumption of pesticides

Metribuzin marketed in Algeria as Sencor® (70% Metribuzin) is a herbicide of the triazine family, which inhibits photosynthesis by blocking the d1 protein of photosystem II and is used for weed control in cultivation of potatoes [31]. Tribenuron methyl, sold under the name Granstar 75 DF (75% pure Tribenuron methyl), is also an herbicide of the sulfonylurea family, inhibitor of the enzyme acetolactate synthase (ALS), leading to the synthesis of branched amino acids and used as a selective cereal herbicide

[32,33]. These two pesticides were provided to us by the agricultural services department of the town of Sidi Bel-Abbés (Algeria). Animals were watered ad libitum with water supplemented with both pesticides at the calculated concentrations of 0.1; 1 and 2 µg/l which were respectively 1; 10 and 20 times higher than the quality standard for water intended for human consumption. This standard sets the quality limit for each type of pesticide at 0.1 µg/l and the quality limit for the total pesticide concentration at 0.5 µg/l [34].

Immunogens and immunization of animals

The ovalbumin, immunogen (SIGMA, A 5253, lot 60K0844), was dissolved in the amount of 0.6 mg/ml in physiological water. To perform the first injection, a volume of Freund's complete adjuvant (ACF) was mixed with an equal volume of the antigenic ovalbumin solution. For recall immunizations Freund's incomplete adjuvant (AIF) was used under the same conditions. ACF (Sigma F 5881, lot 029K8708) and AIF (Sigma F 5506, lot 098K8724) were supplied to us in oily form in small 10 ml vials by Sigma Aldrich, Germany. The AIF used contains 0.85 ml of paraffin oil and 0.15 ml of mannide monooleate emulsified with 0.85% of sodium chloride. The ACF also contains koch bacilli killed. Immunization of the animals was performed subcutaneously, the most common way of avoiding sterile abscesses following Freund's complete adjuvant injection [35,36]. The thighs and shoulders of the rabbits were shaved in order to target the different injection sites and the syringe was inclined at the time of its introduction so as not to damage other organs. Freund's complete adjuvant was heated to 37 °C for 1 to 2 minutes and then vortexed to suspend mycobacteria [37]. The immunization solution was prepared by mixing 0.5 ml of the ACF with 0.5 ml of the antigenic solution containing approximately 300 µg of ovalbumin with a vortex. A volume of 1 ml was injected deeply into four sites at the thighs and shoulders of each rabbit of the experimental batches. Booster injections were performed at the 15th, 30th and 45th day using the antigen in the presence of Freund's incomplete adjuvant under the same conditions.

Blood sample

Ten days after the third immunization call (55th day), a last boost type immunization was followed on the 60th day by prelevment of a 5 ml blood sample. The animal was heated in the light of a lamp to cause peripheral vasodilatation. The blood was taken by incision from the marginal vein of the rabbit ear. The incision was made perpendicular to the vessel and the vein was compressed after sampling to obtain hemostasis [38,39]. The blood collected in a dry tube was centrifuged at 1500 g for 15 minutes. The serum was then aliquoted and frozen at -20°C until use.

Optimization of ELISA test for the determination of anti-ovalbumin IgG

Chemical products and reagents

Gelatin, Tween 80, ovalbumin, goat anti-rabbit IgG antibody labeled with peroxidase, o-Phenylenediamine were supplied to us by Sigma-Aldrich (Germany). All other chemicals and solvents were of analytical grade.

Principle of the ELISA test

The analysis protocol chosen was that of the ELISA conceptualized and developed by Engvall and Perlmann in 1971 [40]. This was an indirect, non-competitive ELISA technique based on the sandwiching of the anti-ovalbumin antibody between the antigen (ovalbumin) and the goat anti-rabbit IgG antibody labeled with peroxidase. The enzymatic activity was measured by adding o-Phenylenediamine (OPD), a colorless substrate whose oxidation provides an orange product appreciated by colorimetry at 492 nm.

Different steps of the ELISA technique

Coating of the Ovalbumin antigenic solution

Each well of the immunoplate receives 100 µl of an antigenic dilution in 0.15 M phosphate buffered saline (PBS) pH 7.4. After incubation for 2 h at 37 °C, the plate was washed three times with PBS-Tween buffer and reversed drying on Joseph paper in order to remove any traces of residual liquid.

Saturation of adsorbent sites (surcoating)

Distribute in each well 100 µl of a blocking solution, PBS-Tween-Gelatin. The plate was incubated for 1 hour at 37 °C in a humid chamber then washed 3 times with PBS-Tween buffer.

Incubation with rabbit antiserum (AS) anti-ovalbumin

Distribute 100 µl of each rabbit AS dilution in PBS-Tween-Gelatin. After incubation for 2 hours at 37 °C in a humid chamber, during which the antibodies bind to the antigens adsorbed on the plate, this was washed 5 times with PBS-Tween buffer and dried by turning over on joseph paper.

Incubation with Conjugate Solution

Distribute 100 µl of the conjugate, goat antibody, anti-rabbit IgG, labeled with peroxydase in PBS-Tween-Gelatin. Cover the plate and incubate for 1 hour at 37 °C in a damp chamber. Wash 5 times with PBS-Tween buffer to remove any unbound conjugate and reverse-dry on joseph paper.

Addition of revelation buffer and reading

Dissolve extemporaneously 6 mg of orthophenyldiamine (OPD) in 12 ml of sodium citrate / citric acid 0.1 M pH 5.5 and add 100 µl of oxygenated water H_2O_2 3% extemporaneously.

Dispatch quickly 100 µl per well and cover the plate with aluminum foil. Incubate for 30 min at 37 °C in a humid chamber. During incubation, the bound enzymatic conjugate converts the colorless chromogen to yellow. The addition of 50 µl of the stop solution (H_2SO_4 2N) causes the chromogen to turn from yellow to orange. The optical densities of the chromogen were read at 492 nm using an ELISA microplate reader (Tecan Sunrise, Austria GmbH).

Determination of the optimal dilutions for titration of the various reagents by ELISA

The optimal proportions of each of the reagents included in the enzyme immunoassay were determined systematically, namely, Rabbit Anti-Ovalbumin Polyclonal Antibody, ovalbumin antigen, and peroxidase-conjugated goat anti rabbit IgG. The adsorption

capacity of the microplate conditions the fixed quantity, which in turn controls the cascade of subsequent reactions. The experiment was carried out in the form of a series of cross-tabulations.

In order to determine the optimum titration conditions, well series of a microtiter plate (from A to H) were sensitized by a series of ovalbumin concentration of 10; 5; 2.5; 1.25; 0.625; 0.3; 0.1 and 0.07µg/ml. Each series of wells (1 to 11) corresponding to a given dilution of ovalbumin was reacted with a range of dilutions of the immunoserum (Is) of the anti-ovalbumin rabbits (1/75, 1/150 , 1/300, 1/450, 1/600, 1/750, 1/1200, 1/2400, 1/4800, 1/9600 and blank).

The previously sensitized well series were crossed with dilutions of 1/2000 and 1/3000 of the peroxidase-conjugated goat anti rabbit IgG.

Statistical analysis

The results were expressed as a mean followed by the standard deviation. The statistical analysis of the data of the different groups was carried out by the Student "t" test, this parametric statistical test was adapted to a comparative analysis between the means of the experimental groups and that of the control group. The probability P <0.05 was considered significant.

RESULTS AND DISCUSSIONS

Weight evolution of animals treated with Metribuzin

After eight (08) weeks of subacute toxicity, by ingestion of pesticides, weighing showed that the weight change of the rabbits in the control group (2094±14.96g to 5398g±58.62) was comparable to that of experimental animals group receiving 0.1 µg/l of Metribuzin (2140±108.66 g to 5331.5±104.11 g) and of the group receiving 1 µg/l of Metribuzin (2133.33±47.17 to 5225 g±29.64) (P <0.05). For the experimental group receiving 2 µg/l of Metribuzin, the evolution of the weight of the animals was also comparable to that of the control (2109.17±53.52 to 5444 g±54 g) (P <0.05). In a teratology study where pregnant rabbits received large doses of metribuzin, a decrease in body weight was observed for the 135 mg/kg bw dose [41]. The work of Chiali et al., 2013 [42], showed that exposure to Metribuzin induced a significant reduction in weight, food consumption and a disruption of biochemical parameters in the Wistar rat. According to Merhi, 2008 [43], the treatment of male and female mice with the mixture of 11 pesticides does not appear to affect food consumption or the normal evolution of the weight of these animals compared to the controls. In our case the dose of Metribuzin used even 10 times that recommended by Directive 98/83 / EC of 03/11/98 [34] does not seem to affect the weight of the animals. According to McKinley et al, 2008 [44], Metribuzin is a thyroid hormone disrupting herbicide that is subject to a marketing authorization for agricultural use. In our experimental conditions, the probable disturbances of thyroid hormones, strongly implicated in the regulation of metabolism, growth and development, in particular of the central nervous system of mammals [45] have not been demonstrated by simple weight measurements. However, in the group consuming 2µg/l of Metribuzin, respiratory difficulties, cough and aggressiveness were noted. These symptoms were reported in the document by Praznoczy and Pepin, 2008 [46] which reported the acute effects of pesticides on human health. According to Bleeke, 1985 [47] rats given radioactively labeled metribuzin removed about 95% after

the second day. Deaminometribuzin mercapturate was found in urine and toxic metabolites such as deaminated, diketo and diketo deaminated in tissues. According to MSNBS, 1989 [48] consumption of Metribuzin by beagle dogs at a dose of 1500 ppm results in reduced food consumption and weight gain. In our case the dose used of 2 µg/l (20 times the norm) does not have a significant effect on the normal weight development of the animals.

Weight evolution of animals treated with Tribenuron-methyl

This compound is a biocide for domestic use, an estrogen hormone-disrupting herbicide that requires marketing authorization for agricultural purposes [44]. When administered at a dose of 0.1 and 1 µg/l in drinking water for 8 weeks, there was no change in the weight of animals receiving 0.1 µg/l (2117±105.18 g to 5325±78.79 g) and 1 µg/l (2115.5±72.30 to 5166g±108.14) of Tribenuron methyl, compared to the control group (P<0,05). Administration of this herbicide to Crl: CD-1 (ICR) BR mice for 4 and 18 weeks showed no observed effect level (NOEL) of 500 and 20 ppm, respectively, with decreased weight in both [49]. A study conducted by the same agency in the New Zealand white rabbit defines a no-observed-effect level (NOEL) of 20 mg/kg bw/day of tribenuron methyl in the mother and fetus with teratological effects. From 80 mg/kg bw/day, weight and appetite losses as well as deaths in mothers and fetuses were reported. In our experiment in the group receiving 2µg/l of Tribenuron-methyl a loss of a rabbit occurred on the 58th day. This loss seems to be related to the consumption of this pesticide but may be due to a probable physiological maladjustment of this rabbit who succumbed to this high dose despite a normal weight change that goes from 2089.5±38.89 to 5427g±49, 85 and which remains comparable to that of the control.

Evaluation of the IgG immunoglobulin level by indirect non-competitive ELISA

Determination of optimum titration conditions by ELISA

The results of figure 1 show the influence of the rabbit antiserum dilution on the curve of the optical densities as a function of the ovalbumin concentrations.

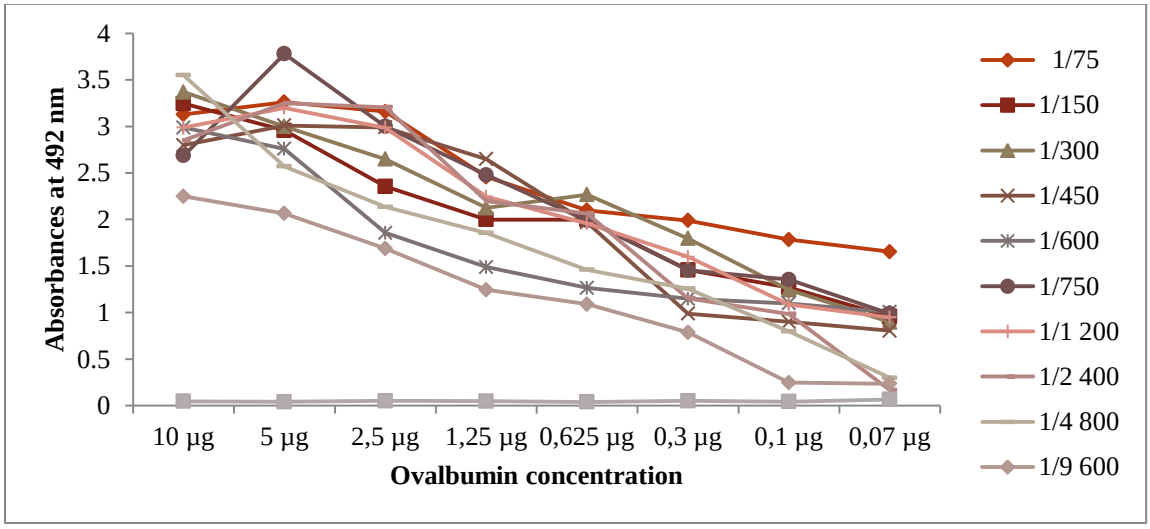

Figure 01: Influence of rabbit antiserum anti ovalbumin dilutions as a function of the variation of antigen concentrations at 1/2000th dilution of the conjugate.

The sensitivity of the test increases at low dilutions of rabbit anti-ovalbumin antiserum and low concentration of ovalbumin. The optical densities range from 1.58 to 0.045. Thus, from these results we opted for dilution 1/750 of antiserum rabbit anti ovalbumin. At this dilution, the absorbance has a maximum value of 1.58 units at the concentration of 5 µg of antigenic solution (ovalbumin). Finally, the 1/750 dilution of rabbit anti-ovalbumin antiserum and the concentration of 5 µg of this antigen were retained for the rest of our work. Therefore, dilution to 1/2000th was used for the determination of immunosera in the range of dilutions adopted compared to that using 1/3000th which gave low optical densities.

IgG determination of the different experimental groups

These same experimental conditions were adopted for the ELISA test allowing the enzyme immunoassay of rabbit anti-ovalbumin IgG. Figure 2 shows that the level of immunoglobulins expressed in units of optical density was decreased in the animals receiving Metribuzin compared to the control. This decrease was dose-dependent.

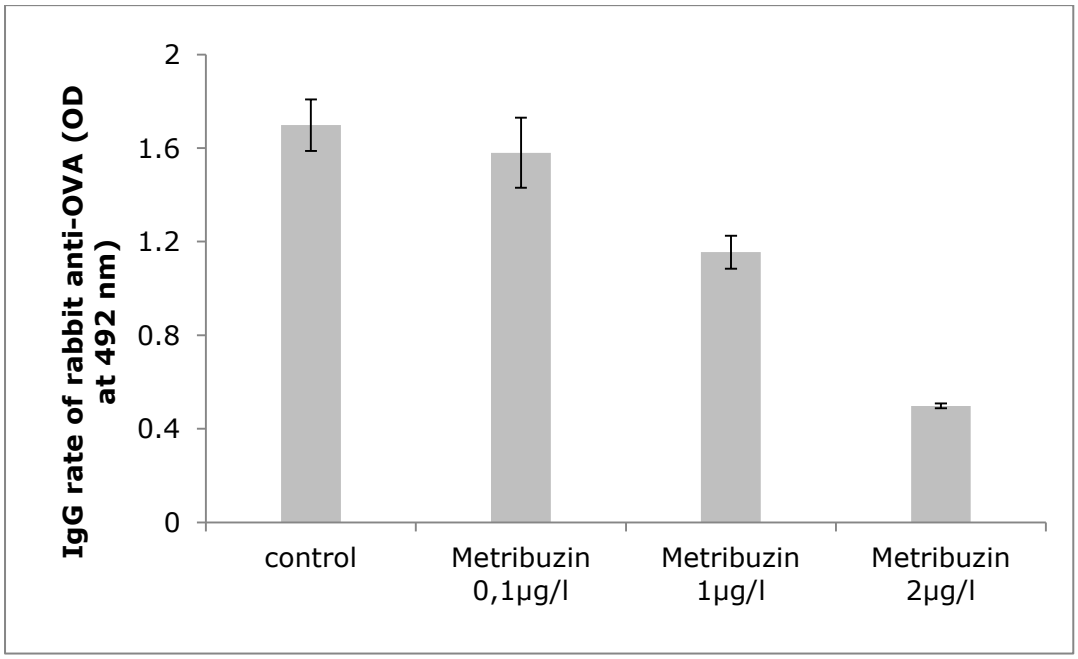

Figure 2: IgG level of rabbit anti-ovalbumin in the control group and those consuming metribuzin at 0.1; 1 and 2 µg/l. (Values are expressed as mean ± standard deviations).

The same applies to animals receiving the same doses of Tribenuron-methyl (Figure 3). Thus the injection of ovalbumin in rabbits caused a reaction of the immune system by the implementation of an innate immunity then specific resulting in the synthesis by the plasmocytes of IgG directed against the ovalbumin. This is argued by many researchers who are discussing the response of the immune system to a compound foreign to the organism [50,51,52]. Immunosuppression may result in blocking the presentation of the antigen which does not allow maturation and/or migration of the dendritic cells. It can also inactivate proliferation, migration and tissue infiltration by lymphocytes. It can also be obtained by lymphocyte depletion [53]. In our case,

there is a pronounced dose-dependent decrease in the synthesis of immunoglobulins in contaminated animals. In vivo studies have shown that some pesticides act essentially in utero by altering macrophage activity [54] and by decreasing the amount of lymphocytes in the spleen and thymus fetuses [55], but also in adult animals, resulting in decreased immunoglobulin production and proliferation of T lymphocytes [56].

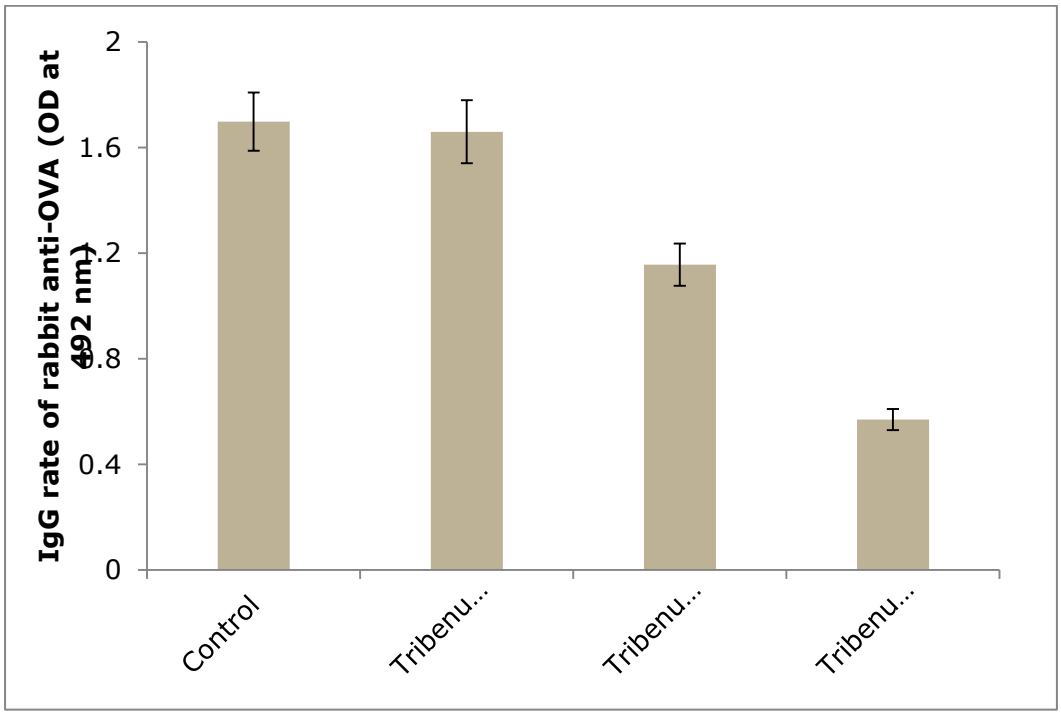

Figure 3: IgG level of rabbit anti-ovalbumin of the control group and those consuming Tribenuron-methyl at 0.1; 1 and 2 µg/l. (Values are expressed as mean ± standard deviations)

According to Bleeke et al., 1985 [47] there is no report on the effects of exposure of human beings to metribuzin. It is believed that this compound has relatively no acute toxic effects in mammals. Pesticides are often considered potentially immunotoxic products, mainly on the basis of animal data, as very little human data is currently available. Corsini et al., 2005 [57] showed that mancozeb-exposed viticulturists exhibited a significant decrease in their eosinophils and IgE serum levels, and an increase in the number of total T lymphocytes (CD3+), lymphocytes T CD4+ and T CD19+. Seth et al, 2005 [58] showed that serum levels of IgG, IgM, IgA and IgE were not affected in patients with poisoning by an organochlorine insecticide, Lindane, and that serum levels of several cytokines measured by ELISA were severely disrupted. According to these last two studies, no chronic or acute exposure to a pesticide had any clinical consequences in relation to these immunological disturbances. Immunological disturbances are meaningful only to the extent that they can be correlated with pathological events [59]. According to the same author, further studies will be needed to predict the real significance of these disturbances in terms of human health consequences. In addition, the importance of cytochrome P450 for the detoxification of metribuzin in mice has been demonstrated by Bleeke et al., 1985 [47], concerning the disturbances caused by the pesticides studied. Cyprinus carpio exposed

to metribuzin showed no change in cytochrome P450 and ethoxyresorufin-O-deethylase activity (EROD) compared to control [56]. Previous studies have shown that different herbicides in the triazine family cause disturbance of free radical processes in gills of bluegill sunfish [61] and common carp [62,63]. Husak et al., 2016 [64] showed that exposure of goldfish to metribuzin led to oxidative stress development in the gills.

Tribenuron-methyl used at doses of 1 and 2 µg/l, respectively 10 and 20 times the normal regulated dose, results in a decrease in the level of IgG in rabbits and almost similar to the effect of Metribuzin (Figure 03) . The work of Corsini et al., 2007 [65] on patients exposed for one month to an herbicide, Propanil showed the concentration-dependent effect on reduction of IL-10 and IFN-γ production and clarified that it is not possible in the light of available data to clarify the reality and magnitude of the immunotoxic risk of propanil to humans. According to Lah, 2014 [66] herbicides are much less toxic to mammals because their mechanisms of action are designed to disrupt plant metabolism. In our case, the effects of the two herbicides tested on the rabbits reveal a disturbance of the immune system, which was reflected in the decrease in serum IgG levels. The doses of pesticides used were higher than those standardized. Regulatory standards should be set based on toxicity data; This is the case for plant and animal foods, but not the case for water since the limit values of 0,1 µg/l and 0,5 µg/l which are fixed at European level according to other considerations are not specific to the pesticides studied. These limit values are maintained while the scientific data acquired since then renders them even more obsolete. The result is now a lack of clarity [67]. In Algeria, every effort should be made to intensify the cooperation of all stakeholders involved in the pesticide problem, to assess the risks to the different users and to take the necessary measures.

CONCLUSION

Tribenuron-methyl and metribuzin used at the indicated concentrations gave comparable results in weight and decreased anti-ovalbumin IgG in experimental animals. The results of the immunochemical assays show an immunomodulatory effect of the two herbicides studied. The effects observed were dose-dependent on reference to norms which today and at European level were disputed. For this purpose it is important to reconsider the validity of these standards in order to fix them according to the toxicity data. In Algeria, some producers judging the manufacturer's doses low, often have to increase or multiply the frequencies of treatments. This was problematic when crops were close to harvest. It was therefore necessary to improve the knowledge of the peasant world in the field of pesticides and to ensure the conditions of their detention. A toxicovigilance system must be established as pesticides can be incorporated into the soil and integrated into food webs and potentially biomagnified in food chains.

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