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(RESEARCH ARTICLE)

Effects of sublethal exposure of fungal xenoestrogen on oxidative stress and hepatic histology of tadpoles *Rana saharica*

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Abstract

Xenoestrogens are artificial products that can be of industrial, agricultural or pharmaceutical origin. We have undertaken work to examine the possible effects of a stressor; a systemic fungicide considered to be an estrogen mimetic, Mancozebe, on tadpoles of an amphibian species; the green frog *Rana saharica*. After treatment for 5 weeks with increasing concentrations of Mancozeb (0.25, 0.50, 0.75 and 1 mg/l). In order to evaluate the biochemical and metabolic aspect and to know the details of the oxidative stress, we performed some assays. A disturbance of the anti-oxidant detoxification systems (Glutathion-S-Transferase GST and Catalase CAT) which are involved in the defense mechanisms against stress caused by Mancozeb was highlighted. We also found an increase in Malondialdehyde (MDA) levels due to lipid peroxidation and neurotoxicity confirmed by inhibition of Acetylcholinesterase (AchE) activity. The induction of oxidative stress prompted us to explore, among other things, the histological side and tissue damage caused by Mancozeb in tadpoles. Our results revealed hepatocyte necrosis and cytoplasmic vacuolation.

Keywords: Oxidative stress; Xenoestrogen; Fungicide; Mancozeb; Tadpoles; Histology**.**

1. Introduction

Xenoestrogens are artificial products that can be of industrial, agricultural or pharmaceutical origin. Most of the work on the toxicological impact of xenoestrogens focuses on their pharmaceutical origin. Toxicological studies of xeno-hormones of agricultural origin are much neglected.

These compounds such as pesticides or plastics products are suspected of being endocrine disruptors. Of natural or synthetic origin, endocrine disruptors can alter the functioning of the hormonal system. These substances could therefore induce adverse effects on the health of an organism, its offspring, or a population, and therefore constitute a potential health hazard not yet assessed [1]**.**

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Several pesticides are on the list of endocrine disrupters, some of whose active molecules are now banned in Europe, but the majority is still used in third world countries [2].

Our problem consists in developing and analysing many aspects related to the toxicity of mimetic-oestrogens of agricultural origin: "Mancozeb" on pollution bioindicators, representative of the Amphibian group, the green frog *Rana saharica*, in the tadpole phase.

There is a need for indicators of environmental disturbance; organisms or a set of organisms that are used as sentinels by studying the physiological, biochemical and ecological changes that affect them. These living beings, which are highly sensitive to contaminants, have the advantage that they are more easily adapted than humans to the study of the effects of pollutants and make it possible to identify chronic or sudden pollution [3].

Our objective is to characterize the sensitivity of these organisms to Mancozeb from two aspects: the antioxidant response of these organisms and the endocrine disruption of this xenobiotic.

2. Material and methods

2.1. Biological material

The experiments were carried out on a freshwater species; the green frog: *Rana saharica*[4], which occurs in most of the humid regions of North Africa, also in northern Western Sahara, Tripoli and the oases of the Algerian Sahara [5].

2.2. Chemical material

We chose Mancozebe (Manganese Zinc Ethylene Zinc Bis Dithiocarbamate), a non-systemic fungicide belonging to the carbamate family. It is a dithiocarbamate, which includes Maneb and Metiram as active ingredients [6].

2.3. Processing mode

We have created an artificial milieu in the laboratory, with pH (between 7 and 9) and temperature (between 16 and 20°C) of the medium to guarantee favourable conditions for the development of the tadpoles. The maintenance or monitoring of the parameters of comfort and breeding of the tadpoles is essential throughout the experiment because these amphibians are very sensitive to variations of pH and T° [7].

Experiments are carried out on tadpoles in the early stages of larval development and end with the pre-metamorphosis stages [8], which impose a limited exposure period of 5 weeks.

We determined 04 concentrations of Mancozebe (C1, C2, C3, and C4) corresponding respectively to 0.25; 0.5; 0.75 and 1mg/l and a control sample (T) as well as an acetone control sample. Each batch is composed of 10 tadpoles.

These concentrations were selected from an LC50 from an acute toxicity test in fish and aquatic invertebrates 1mg/l < LC50 <10mg/l. (Product safety data sheet).

2.4. Evaluation of oxidative stress

For the assays to be carried out in the present study, the decapitated bodies were used for the determinations of the specific antioxidant activity of Catalase (CAT) and other biomarkers such as Glutathione S-transferase GST) and Malondialdehyde MDA. The heads of the tadpoles from the control series and treated with different concentrations of the estrogen mimetic (Mancozeb) were assayed for Acetylcholinesterase (AChE) activity. In parallel, and for the enzyme assays, the total protein concentration of the samples was previously determined according to the method of Bradford [9].

Assays were conducted on pre-metamorphosing tadpoles having approximately the same weight and linear characteristics.

2.4.1. Measurement of Glutathione S-Transferase (GST) activity

The measurement of GST activity is carried out according to the method of Habig et al.[10] based on the photometric measurement of the conjugation kinetics of the product formed with a substrate: 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of a cofactor, Glutathione (GSH).

2.4.2. Catalase Activity Measurement (CAT)

The Catalase activity (CAT) is determined according to the method of Regoli and Principato [11], which is based on the change in optical density resulting from the dismutation of hydrogen peroxide (H_2O_2) at a wavelength of 240 nm.

2.4.3. Determination of Malondialdehyde (MDA)

Lipid peroxidation is estimated by quantification of MDA using the method of Draper and Hadley [12]. This method is based on the colorimetric measurement of the reaction between Thiobarbituric acid (TBA) and Malondialdehyde (MDA), resulting in a reddish-brown product whose colour intensity is measured at a wavelength of 532 nm.

2.4.4. Measurement of Acetylcholinesterase (AchE) activity

Our chosen method for the determination of Acetylcholinesterase (AChE) is that of Ellman et al. [13], in which the enzyme is provided with a substrate, Acetylthiocholine (ASCh), the hydrolysis of which releases Thiocholine (SCh) and acetic acid.

2.5. Statistical study

Statistical analysis of the data is performed by the Student's t-test. This test is performed using data analysis software: Minitab (Version 14.0) [14]. The results are represented by the mean ± standard deviation.

2.6. Histological study

A histological study has been carried out to characterise tissue alterations and lesions according to the method of Martoja and Martoja [15].

3. Results

3.1. Evolution of the Glutathione-S-Transferase activity

According to Figure (1) we see that treatment of tadpoles with increasing concentrations of estrogen mimetic induces a significant increase (p= 0.045< 0.05) in GST enzyme activity compared to controls.

Indeed, the GST activity is on the order of 0.015 nM/ mg protein in control individuals and increases by about 63% (0.032 nM/ mg) in tadpoles treated with the lowest concentration (0.25 mg/l), while this increase is 84% (0.093 nM/ mg) in tadpoles treated with the highest concentration (1 mg/l).

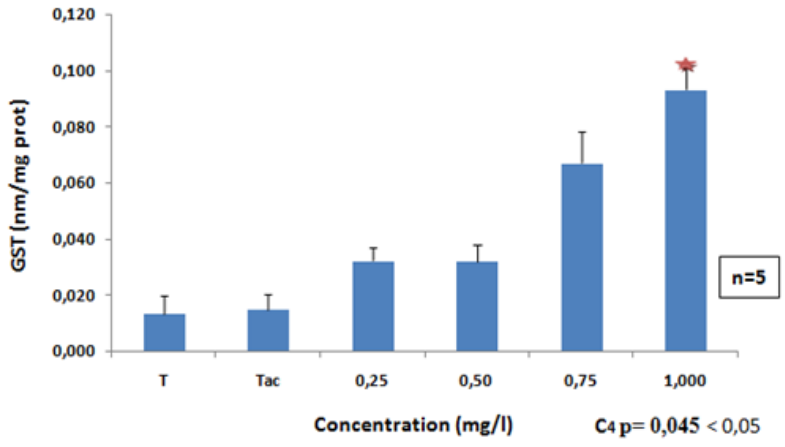

Figure 1 Effect of increasing Mancozeb concentrations on the evolution of GST activity in tadpoles (*Rana Saharica*).

3.2. Evolution of the Catalase activity

Figure (2) illustrates the evolution of CAT activity in tadpoles of *Rana saharica* treated with increasing concentrations of estrogen mimetic. This activity tends to increase in a dose-dependent and significant manner (p= 0.038 < 0.05) in tadpoles treated with the lowest concentration (0.25 mg/l) and highly significant (p < 0.001) in tadpoles treated with the highest concentrations compared to controls.

Indeed, for the lowest concentration (0.25 mg/l of Mancozeb) Catalase activity increases by 78% while in the highest concentration treated tadpoles Catalase activity increases by 80% to 85% compared to controls.

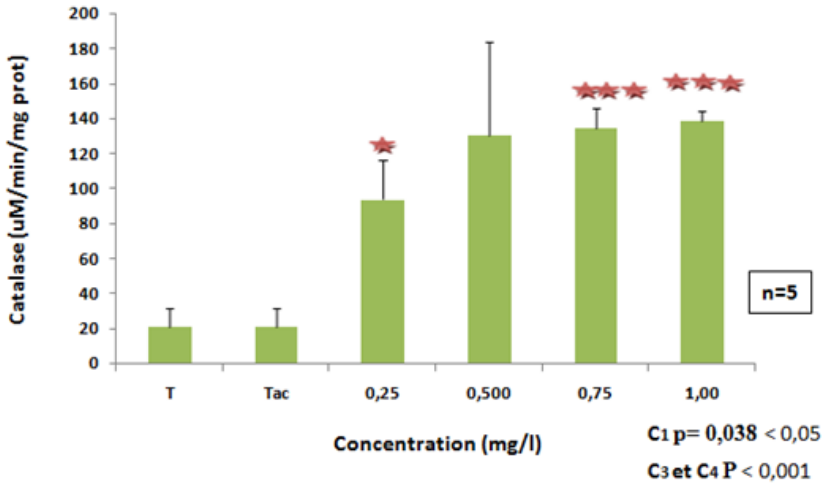

Figure 2 Effects of increasing Mancozeb concentrations on the evolution of CAT activity in tadpoles (*Rana Saharica*).

3.3. Evolution of the level of Malondialdehyde

Figure (3) shows the evolution of MDA content in tadpoles treated with increasing concentrations of fungicide. We see that the MDA level tends to increase in a dose-dependent manner. This increase is significant ($p=0.039 < 0.05$) and is of the order of 53% for the 0.75 mg/l Mancozeb concentration and highly significant ($p=0.019 < 0.01$) for the highest concentration (1 mg/l) of which it increases by 63% compared to controls.

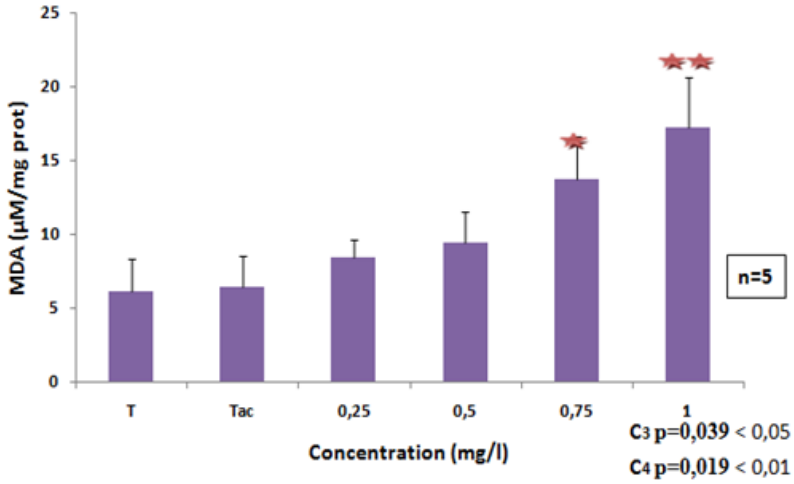

Figure 3 Effects of increasing Mancozeb concentrations on the variation of the mean MDA level in tadpoles (*Rana Saharica*).

3.4. Evolution of Acetylcholinesterase activity

Figure (4) illustrates the effect of Mancozeb on cholinesterase activity in tadpoles (*Rana saharica*). We see a significant decrease ($p=0.05$) in AChE enzyme activity after a 4-week exposure period (before metamorphosis) from 1.55 nM/min/mg protein. 105 in controls to 0.44 nM/min/mg protein .105 in the highest concentration (1 mg/l) treated. Thus, this activity decreases by 72% in the highest concentration of Mancozeb treated patients compared to controls.

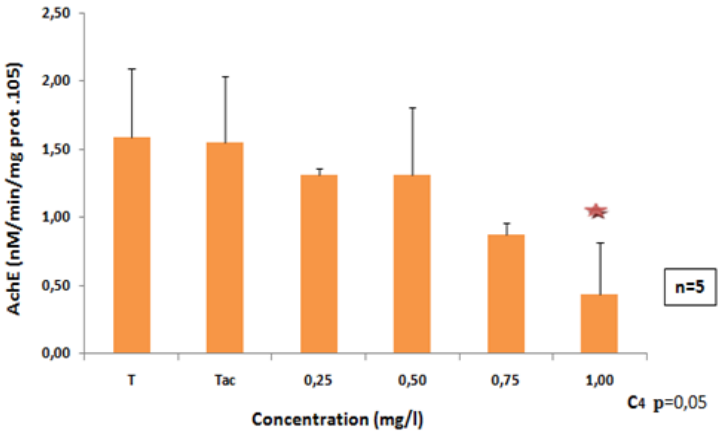

Figure 4 Effects of increasing Mancozeb concentrations on the evolution of AchE activity in tadpoles (*Rana Saharica).*

3.5. Histological study

Figure (5) shows the aspect of the liver tissue of the tadpoles. Histological examination has shown that the liver of tadpoles is not divided into distinct lobules as in most mammals. However, the periphery of the lobules is delimited by the portal spaces and their centre is occupied by a centrolobular vein (the VP portal vein) towards which the sinusoids (S) converge. The portal spaces, together with the portal vein (VP), include lymphatic channels (→) as well as the constituents of the portal triad, that is, the portal vein, a hepatic artery (A) and bile ducts (CB). We also notice rows of hepatocytes with bile ducts (→) running between these hepatocytes separated by sinusoids (S) (Figure 5 a and b).

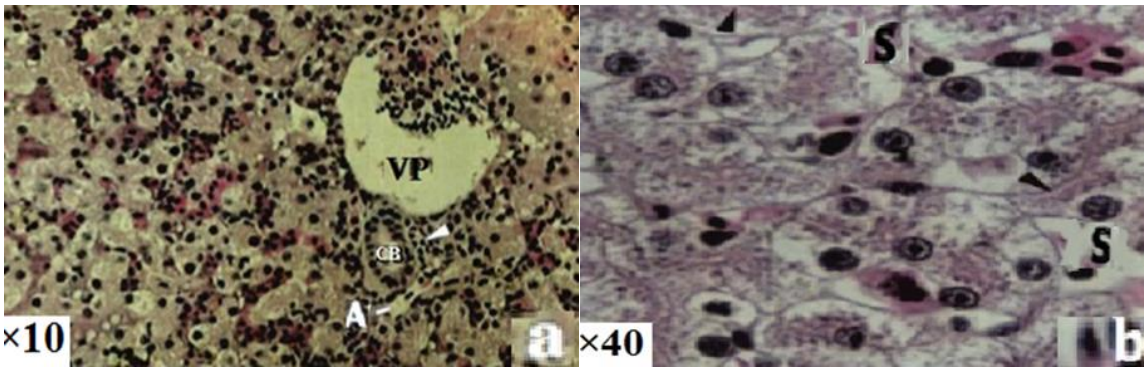

Figure 5 Histological section of the liver of a control tadpole (*Rana saharica*).

In the treated animals, we note that the hepatocytes of the tadpoles exposed to Mancozeb (1mg/l) are deformed with a disturbance of the tissue architecture (Figure 6 a). The cytoplasm of the hepatocytes is more aerated than normal; the cellular organelles (→) are clustered near the bile ducticles. Areas of parenchyma in which the high number of necrotic cells gives a moth-like appearance are observed in these individuals (Figure 6 b).

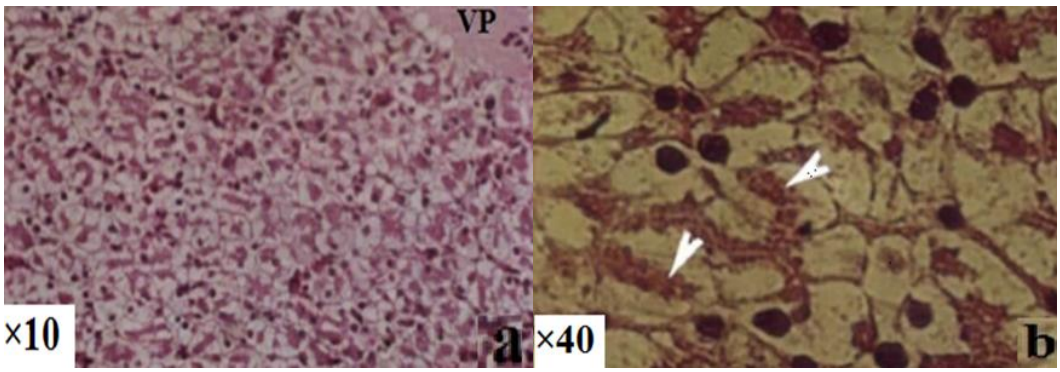

Figure 6 Histological section of the liver of a tadpole (*Rana saharica*) exposed to 1 mg/l of Mancozeb.

4. Discussion

The green frog is one of the bio-indicator species of pollution that are very sensitive to the slightest variations in the ecosystem, and any change in their environment could have consequences on their embryonic development and physiology [16].

A wide range of contaminants and pollutants affect amphibian populations in terms of their lifespan and diversity: pesticides, fungicides, herbicides and fertilizers and many other molecules [17]. All of these xenobiotics are toxic directly by affecting the immediate environment of the animals or indirectly by reducing their growth through damage to their endocrine system or by inducing immunesuppression [17]. Furthermore, the nature and intensity of the toxic effects of a fungicide on an organism depend on its concentration in the target organs. This concentration is related to the administered dose, its distribution and metabolism [18].

Indeed, work by Benosmane et al. [7] carried out before this one concerning the survival of amphibians under the fungal xenoestrogen stress (Mancozeb), shows that in the presence of the latter, the growth of the tadpoles is inhibited; the toxicity of this xenobiotic in tadpoles manifests itself primarily through a delay in weight and height growth at all the concentrations tested (0.25, 0.50, 0.75 and 1 mg/l). However, at the highest concentration, we recorded a mortality rate of more than 80% after 5 weeks of treatment.

This left us oriented towards the biochemical and metabolic aspect and to know the details of oxidative stress.

According to Lagadic et al. [19] and Nzengue [20], when the change (stress) is not intense and xenobiotic concentrations in the organism are still low, tadpoles deploy a battery of responses through the activation of their detoxification mechanisms in order to fight, survive and acclimatize to this new parameter. This is the case for the induction of metabolism/detoxification enzymes for xenobiotic management as long as the xenobiotic/enzyme balance is tilted towards the second parameter.

Oxidative stress is the consequence of an imbalance between oxidants and antioxidants in which the activity of the oxidant is greater than the capacity to neutralize the antioxidants [21]. Thus, there must be a balance between the formation and elimination of reactive oxygen species, in the normal case and the case of oxidative stress.

Among the enzymes involved in the protection of the organism against oxidative stress, we mainly distinguish: Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX), glutathione S-transferase (GST) or Catalase (CAT). These enzymes are also inducible under chemical stress [22, 23]. As a result, these enzyme systems are considered excellent biomarkers.

Indeed, our results showed a progressive increase in GST activity in tadpoles treated with the fungal xenoestrogen (Mancozeb) compared to controls. At the same time, results from the same study; monitoring the evolution of glutathione levels, which represents one of the first barriers against xenobiotic toxicity, revealed a decrease due to the establishment of detoxification mechanisms [1]; this decrease is due, on the one hand, to its spontaneous conjugation to the ultimate toxicants and, on the other hand, to its binding to GST.

Glutathione S-transferase (GST) is a family of specific enzymes that catalyze the conjugation of GSH with xenobiotics in phase II of metabolization, thereby favouring their elimination from the body [24, 25]. It plays a key role in the mechanism of detoxification of reactive oxygen ("ROS") species and the regulation of redox balance [26].

Catalase is also a defence system involved in detoxification mechanisms. We have shown an increase in Catalase activity in tadpoles treated with Mancozeb, probably for the degradation of oxygen radicals and the conversion of hydrogen peroxide (toxic to cells) into oxygen gas and water.

When antioxidant defense systems are saturated, ROS become abundant and their toxicity is manifested in particular by the phenomenon of lipid peroxidation itself, which causes an increase in the level of MDA, the final product of peroxidation. Malondialdehydes are considered as end products of the oxidation of polyunsaturated fatty acids and are specific biomarkers [27].

Many studies have shown the exacerbation of lipid peroxidation in aquatic organisms exposed to high concentrations of toxic substances, including endocrine disruptors [28]. Our results showed an increase in MDA levels in *Rana saharica* tadpoles. This increase in MDA level confirms the saturation of antioxidant systems and supports the increase in the ROS level in the blood of tadpoles treated with the same concentrations of our study [1].

Radical membranes attack causes membrane permeability perturbations related to the formation of lipid peroxides [29]; all components can be affected: lipids, proteins and therefore the membrane as a whole [30]. The same applies to DNA, the attack of which results in an increase in chronic pathologies [31]. Amamra et al. [32] stipulate that the specific effects of a radical attack are manifested at the cellular level by lipid peroxidation inducing pronounced disturbances in cellular functioning.

Apart from ROS, inhibition of acetylcholinesterase AchE is considered to be one of the major biomarkers of pesticide toxicity. Indeed, many toxicants, particularly insecticides, cause the chemical mediator to accumulate in synaptic spaces, resulting in tetany [33, 34]. Inhibition of this enzyme is the direct reaction of the body to most neurotoxic insecticides.

In this work, we focused on the neurotoxicity of Mancozeb primarily due to its high lipophilicity, and measurement of AChE activity revealed a neurotoxic effect of Mancozeb.

The other aspect that we have addressed in this work, concerns the possible disturbances to tissue that may be generated by the presence of Mancozeb in the tadpole milieu. We focused on the liver because of its strong involvement in the metabolism phenomenon and because this organ is the obligatory passage for most xenobiotics.

The normal cell undergoes mechanical, chemical and other lesions during a chemical aggression, this provokes changes such modifications which result in a rupture of the plasma membrane and a release of the intracellular contents which causes an inflammatory reaction which is a major response of the body's defense against external aggressions which explains the induced cellular necrosis.

5. Conclusion

All observations converge on an induction of oxidative stress; the higher the concentration of Moncozeb, the greater the production of reactive oxygenated metabolite derivatives. Therefore, an enzymatic and non-enzymatic antioxidant system intervenes for the detoxification of the cell and protection against superoxide ions and hydrogen peroxides prevent the formation of the hydroxyl radical OH° (the most reactive species which is not detoxifiable by enzymes). As a result, the oxidative stress induced induces lipid peroxidation at the level of cell membranes or membranes of other organelles as well as neurotoxicity. All these alterations cause cellular dysfunction. If these alterations are not repaired, this leads to inflammation, malformations or cell cycle arrest, or in high doses it causes death of the organism.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The author declares no conflict of interest.

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