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Micronucleus Induction in Erythrocytes of Tadpole *Rana saharica* (Green Frog of North Africa) Exposed to Artea 330EC.

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Abstract: Through last 30 years, there have been big declines in diverse geographic locations among amphibian populations. Multiple causes have been suggested to explain this decline, between these, environmental pollution is gaining attention. Amphibians are frequently exposed to agricultural pesticides which are known to be present in water surface. In this study, potential genotoxic of Artea 330EC, a systemic fungicide widely used to struggle against cereals parasites, was evaluated using micronucleus test (MNT). Tadpoles *R. saharica* (species widely exist in northern Africa) were exposed to different concentrations (50, 75, 100, 150 ml/l) of Artea 330EC and MMS (methyl methane sulfonate) as a positive control in a concentration of 1.56 mg/l. The Used Test procedure in this study was described in French Standard (AFNOR). The toxic conditions mitotic indices showed in erythrocytes were high, we note also few erythrocytes presented nuclear morphological aberrations, like several binucleated cells. About MN frequencies, there were significant differences between the positive control (MMS) and the groups treated with Artea 330EC concentrations whatever the time of exposure. There is a dose-effect relationship from 08 days of exposure on erythrocytes of *R.* saharica. Our results revealed a genotoxic effects of Artea 330EC on *R. saharica* tadpoles only at the highest concentrations (100 and 150µl/l) with the longest time of exposure (12 days).

Key words: Amphibians · Artea 330EC · Micronucleus test · Tadpole's erythrocytes · *R. saharica*.

INTRODUCTION

Pesticides are used extensively to improve crop yields in agriculture and as a result they are accumulated in the environment. For this reason the effects that they have on organism inhabiting adjacent small bodies of water must be determined.

In the past, organic pesticides such as dichlorodiphenyl trichloroethane (DDT) and dieldrin, which have long half-lives and readily bioaccumulated [1] have been used without knowledge about the full extent of their impact on the environment.

Several works demonstrated that amphibians are sensitive organisms, suitable for detection of genotoxic agents [2, 3]. Toxic effects, such a developmental abnormities, altered growth rates and modifications in behavior and feeding habits, in amphibians exposed to currently used pesticides were observed [4].

Also, in a genotoxicity study by (Rudek and Rozek, 1992) [5], a concentration-dependent increase in the frequency of micronuclei in Red cells was observed in *Rana temporaria and Xenopus laevis* tadpoles after 14days of exposure to relatively low levels of pesticide Fastac 10EC.

The MN assay has been used as a measure of genotoxicity in amphibians under laboratory and field conditions [6] and has shown potential for in situ monitoring of water quality [7 and 8].

The MN assay has proven most suitable for assessing genotoxic effects of environment contaminants in effluents and polluted water bodies [9]. It has been applied at different species of fish [7] and bivalves [9].

The sensitivity and reliability of the MNT to detect chromosomic and/or genomic mutations makes it a good method to analyze the potential cytogenic damage caused by pure substances [7 and 10]. This method has been standardized on *X. leavis* in French [11] and international recommendations.

MN are small fragments of intracytoplasmic chromatin which arise from chromosomes breaks or whole

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chromosomes, after the action of clastogenic substances or spindle-poisons [12].

In order to develop experimental models able to detect genotoxic effects of pollutants in aquatic organisms, the tadpoles (in premetamorphic stage) of green frog *R. saharica* were chosen in the present study to evaluate the genotoxic potential of the fungicide Artea 330EC by using the micronucleus test (MNT).

MATERIALS AND METHODS

Chemicals: The ARTEA 330EC Systemic Fungicide is a versatile family of Triazoles chemical is used in the fight against major fungal diseases of cereals that affect parts of the plant like the rust, Septoria and powdery mildew.

Propiconazole at a rate of 250g / l and Cyproconazole at a rate of 80g / l (Figure 01) are the two molecules belonging to the systemic chemical group Azoles and are responsible for the antifungal action of the product.

MMS, the methyl methane sulfonate was used as a positive control at a concentration of 1.56 mg / L put the figure at 01 which is after the bibliography.

Tadpoles: *R. saharica* tadpoles were selected to carry out the present study, this species has an extensive distribution in our area, in addition, it is relatively easy to handled and acclimated in laboratory conditions. All the tadpoles used in the experiments were collected in egg stage.

For the bioassay, premetamorphic larvae were used (from stage 26 to 36) [13] the average total size (snout-tail) was 20±2mm. Before experiment, tadpoles were acclimatized in glass tanks (12,5cm diameter and 13,5cm high); at 21±2°C.

Experimental Design: We divide samples 10 larvae per treatment group and we prepared a main solution and we prepared from it 04 concentrations; (50, 75,100,150µl/l).

Negative controls were conducted during the same period; MMS was used as positive controls at a concentration of 1.56 mg/l. The water, containing the compound and food was changed every day.

The MN frequency in each group was measured after 2, 4, 8 and 12days.

The Micronucleus Test in Tadpoles: It is important to note that red blood cells (RBCs) in amphibians are nucleated and undergo cell division in circulation, particularly during the developmental stage [14].

The test procedure used in this study was described in the French Standard [11], which is the French National Organization for quality regulation. Briefly, it consists in the evaluation of the number of micronucleated red blood cells (RBCs) in larvae exposed for 12 days at 22±0.5 under a normal light/dark cycle. The test begins are larvae at stage 26 of the development of Anurans [13]. Throughout the period of exposure, water and food are renewed daily. Positive and negative controls were included in each experiment described here.

After exposure, blood samples were taken from each larva by cardiac puncture. After fixing in methanol and staining with Hematoxylin, the smears were screened under the microscope (oil immersion lens, X1000). The number of RBCs that contained one or more micronuclei was determined in a total sample of 1000 erythrocytes per larva. For each animal, the mitotic index (number of mitotic erythrocytes per 1000) was evaluated by scoring the dividing cells of the circulating blood on the smears.For each group of animals, the results (number of micronucleated RBCs per 1000) obtained for the individual larvae were arranged in increasing order of magnitude and the medians were calculated.

Statistical Analysis: Statistical method used to compare the medians was based on the recommendations of Mac [15] and consist in determining the theoretical medians of

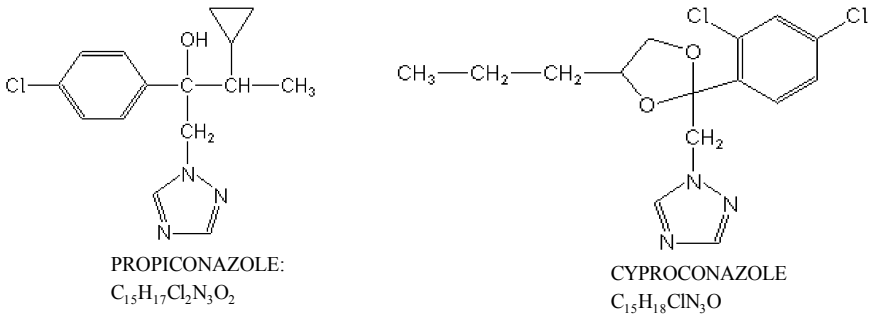

Fig. 1: Characteristics of two active molecules of the fungicide; Artea 330EC

samples of size n (where n = 7 and their 95% confidence limits expressed by (M ± 1.57 X IQR/vn), where M is the median and IQRs the Inter-Quartile Range. Under these conditions, the difference between the theoretical medians of the test groups and theoretical medians of the control groups is significant to within 95% certainty if there is no overlap. The result is then positive.

RESULTS

Nature of *Rana saharica* erythrocytes are oblong-oval shape, with centric and oval nucleus. The nucleus is clearly structured and intensively stained which facilitates identification of fragments in cytoplasm. As a result of the toxic conditions, mitotic indices scored in RBCs of tadpoles exposed to Artea 330EC were higher than those of tadpoles in negative control groups (Fig. 3).

Micro-nucleuses (MN) observed are spherical nuclear fragments separated from the principal or parent nucleus (Fig. 2 and 4). We have noted the presence of MN in erythrocytes, but we also note, few erythrocytes presented nuclear morphological aberrations, like several binucleated cells (Fig. 2)

MN frequencies by the different concentrations of Artea 330EC in erythrocytes of the tadpoles of *R. saharica* are shown in Fig. 5. There were significant differences between the positive control (MMS) and the groups treated with Artea 330EC concentrations (50, 75, 100 and 150 ml/l) whatever the time of exposure.

During two days, there was no induction of micronuclei whatever the concentration, but an effect was inducted at 4th day in highest concentrations respectively 100 and 150 µl/l.

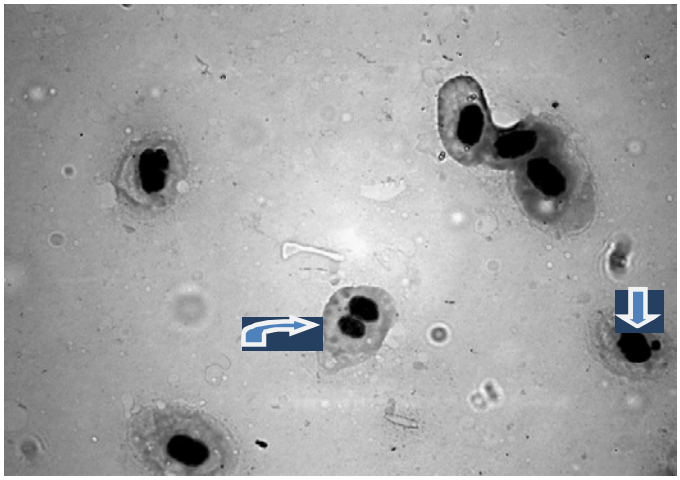

Fig. 2: Micronucleated erythrocyte (arrow) and binucleated cell in *R. saharica* tadpoles exposed to Artea 330EC; Hematoxylin stained blood (X1000)

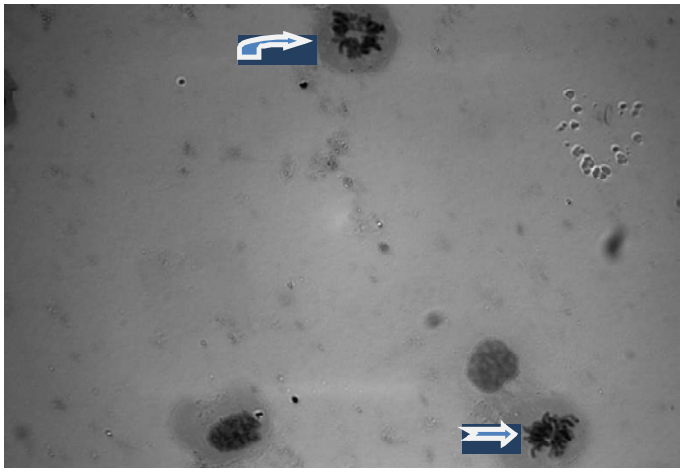

Fig. 3: Blood Smear of *R. saharica* tadpoles which shows erythrocytes in cell division (X1000)

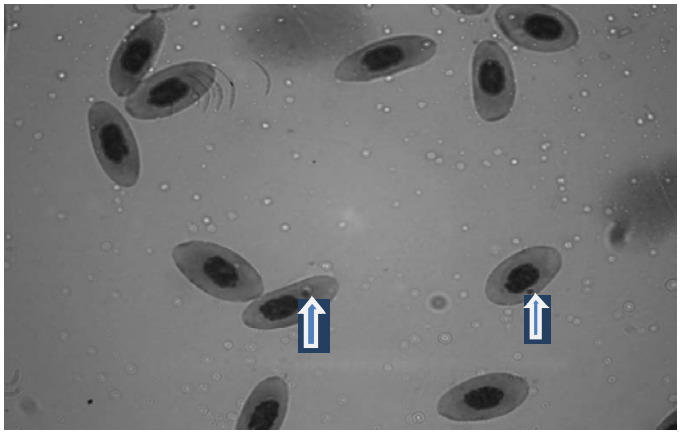

Fig. 4: Micronucleated erythrocytes of *R. saharica* tadpoles exposed to MMS (methyl methane sulfonate) at a concentration of 1.56mg/l; Hematoxylin stained blood (X1000)

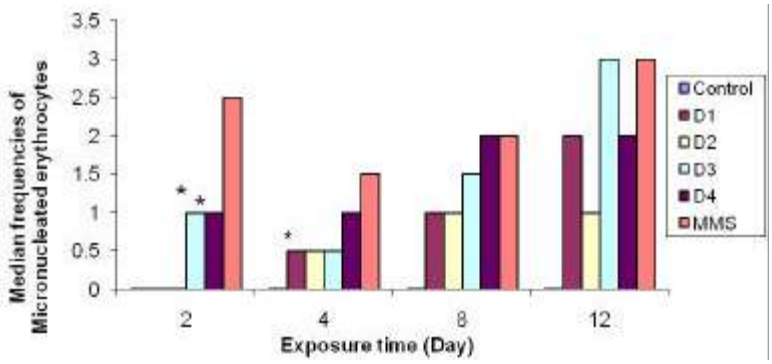

Fig. 5: Induction of erythrocytes micronucleated (per 1000 cells) in *R. saharica* tadpoles treated with different concentrations of Artea 330EC.Genotoxicity is expressed as the median values of MNE ‰ and their 95% confidence limits

From 6th day we observed a significant effect of induction of micronuclei in erythrocytes whatever the concentration: then there is a dose-effect relationship from 8th day of exposure on erythrocytes of *R. saharica.*

The lack of effect on the duration of micronuclei induction for doses 100 and 150µl/l may be due to the highest concentrations.

There is a phenomenon because of the plateau levels of damage is high (example: the rate of micronuclei D3t4 is equals to that of the positive in this case the MMS.

DISCUSSION

Over the past decades, the decline of the amphibian populations has been extensively reported [16, 17]. Amphibian are reliable indicators of environment pollution due to their biphasic life (aquatic and terrestrial) and semi-permeable skin [18]. On the other hand, since frogs are important natural enemies of many agricultural pests, they might expose to the residual pesticides in pests via food chains, as imidacloprid residues which can be present in vegetables, crops and fruits and also in pests [19, 20 and 21]. For Artea 330EC, few data are available about its residues in soil, water and pests and no studies evaluate its genotoxic effects on frogs, which makes difficult the comparison with our study.

R. saharica occurs in North Africa, in Morocco, Tunisia and Algeria [22]. It has also been reported to occur in Egypt, although it appears to be uncommon there [23]. It is distributed from around the Mediterranean Sea down to the northern edge of the Sahara desert. This fog is ecologically versatile, making use of habitants ranging from alpine to pre-desert [24]. It lives in vicinity of water bodies, both natural and manmade, ranging from lakes, ponds and puddles to flowing streams and rivers and will tolerate polluted waters [22].

The blood of amphibians is very plastic tissue. In fact, variations of several hematological parameters in

response to natural changes in the environment have been widely described by researchers [25 and 26].

As a result of the toxic conditions, mitotic indices scored in RBCs of tadpoles exposed to Artea 330EC were higher than those of tadpoles in negative control groups, also, It is important to note that the occurrence of nuclear morphological aberrations like several binucleated cells in *R. saharica*, as well as the general degenerative changes in erythrocytes during the tadpole stage studied corresponding to the period of intense hematopoietic with active cell division in circulation blood.

Jaylet *et al.* (1986) [27] first adapted the MN test to amphibians. Many MN tests on amphibians have used genotoxic agents [28, 29 and 30]. It has been used as a measure of genotoxicity in amphibians [6, 31] and has shown potential for *in situ* monitoring of water quality [8, 7]. MN derive from chromosomal fragments or whole chromosomes which are not incorporated in to main nucleus during cell division as a consequence of DNA fragmentation (clastogenic origin) or of alteration of the mitotic apparatus (an eugenic origin) [32, 33 and 34].

Studies with pesticides show differences between active ingredients and their formulation with respect of mutagenicity [35].

In our study, there were significant differences between the positive control (MMS) and the groups treated with Artea 330EC concentrations (50, 75, 100 and 150µl/l) whatever the time of exposure. MMS, methyl methane sulfonate a well known genotoxic substance, was used as a positive control for the MN test in our study. This agent caused a significant increase in frequency of micronucleated erythrocytes at almost all times observed [30] our study has confirmed this.

At 2nd day, there was no induction of micronuclei whatever the concentration, but an effect was induced at 4th day at highest concentrations respectively 100 and 150µl/l this may due to the short time of exposition.

Since 8th day there was a significant effect of induction of micronuclei in erythrocytes whatever the concentration and then there is a dose-effect relationship.

For the concentrations 50 and75µl/l, there were significant differences between groups treated after 2nd and 4th day and those treated after 8th and 12th day of exposure. However, we noted no effect of the exposure time for the highest concentrations 100 and 150µl/l.

In conclusion, the present work evaluates the genotoxicity of fungicide Artea 330EC on erythrocytes tadpoles of the green frog *Rana saharica* using micronucleus test (MNT) in controlled laboratory conditions after 12 days of exposure.

The results indicate that the Artea 330EC can be genotoxic at only high concentrations, therefore, this fungicide if inefficiently diluted or degraded after applied in the field, might reach levels that pose genotoxicity to tadpoles and frogs.

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