ISSN : 0974 - 7532 Volume 6 Issue 11

Trade Science Inc.

Research & Reviews in

BioSciences

Regular Paper

RRBS, 6(11), 2012 [317-327]

Combination between vitamin E and calcium into the neutralization of Novaluron in a cellular model of fresh water *Paramecium sp.*

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ABSTRACT

Most of researchers in toxicology regard just toxic effects of xenobiotic, whereas the detoxication and neutralization systems didn't get yet a value as objective of experimental studies. Chitin synthesis inhibitors (CSIs), pesticides of the third generation, formed a crucial matter for recent toxicological studies especially for BPUs, whose we choose the Novaluron as representative of this class of pesticides. Our work includes two sections, the first one concern the study of toxic effects of Novaluron on a eukaryote unicellular model *Paramcium sp*. As a non-target living specimen, the second section interested in the investigation about the neutralization capacity of the mixture Calcium/Vitamin E on the harmful effects of Novaluron on *Paramcium* cells. The evaluation of toxicity and neutralization are tested by two concentrations (10 and 20µg/ml) in strictly controlled conditions. Damaged effects of Novaluron are demonstrated by perforated cell walls of *Paramecium sp.* due to the inhibition of the Chitin synthesis and justified by neutral red coloration method, also Novaluron inhibits the growth of *Paramecium sp c*ells in a dose-dependent manner, especially for the concentration (20µg/ml), in addition Novaluron increase response percentage, reduced number of generation, slow down the speed of moving with hazardous path of swimming, biochemical analyses exhibit that Novaluron exhaust the cellular supply of glutathione. Calcium and Vitamin E combined with Novaluron reduce its destructive effects on the cell wall of *Paramecium sp.*, but each of them by its mode of action, calcium as a controller agent of ciliary reversal and discharge of trichocysts by exocytosis, and Vitamin E as antioxidant agent who interferes in the detoxication system as an alternative agent of neutralization.

KEYWORDS

Chitin synthesis inhibitors;
Novaluron;
Calcium;
Vitamin E;
Paramecium sp.;
Cytotoxicity;
Detoxication;
Glutathione.

INTRODUCTION

In the last three decades, there was a big demand on the selective insecticides that spares natural enemies and non-target organisms life-history traits of bees can shows well the potential impacts of insecticides[6,31,47,65], and these is where was the invention of biorational insecticides well inserted in the integrated pest management programs, in the context new insecticides where developed as chitin synthesis inhibitors (CSIs), where the benzoylphenylurea (BPUs) as a principal group of this pesticides. BPUs interfere with the chitin biosyn-

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thesis and when the larva is preparing to molt, the available chitin is insufficient for completing the construction of the outer cuticle, and corollary the larva dies.

Zbigniew et *al*. (2009) proved that Diflubenzuron is a very toxic BPUs for micro arthropods in soil after a long term exposition, Kim et *al*. (2007) displayed that after the exposition of eggs of *Palpita indica* to Bistrifluron, hatched larva died after 24 hours after birth.

Novaluron is used in the control of mites, aleurodes, psillyds, bollworms and related insects, it has a large activity specter, it is applied on fields of fruits, cotton, maize apple trees, citrus fruits, potatoes, and ornamental plants, in some of countries (WHO, 2008). Novaluron belongs to the chitin synthesis inhibtors (CSIs), which inhibit the formation of chitin in larval stage for a multiple insects (Lepidoptera, Coléoptera, homoptéra, and diptera) , he acts via ingestion or contact causing abnormal endocuticular deposition abortive molting while the incompatibility with natural enemies has been reported , Novaluron has insecticide strong activity on important crops insects pests, and low toxicity for mammals, birds, and earth worms[44], but highly toxic for some crustacean (WHO, 2005). Consider it as a reduced risk insecticide; Novaluron was used as an alternative insecticide replacing organophosphorus. The toxicity of Novaluron was evaluated for the domestic mosquito's larva Musca domestica, also in waste water for mosquito's larva, it efficacy in the control of apple maggot fly was confirmed for Rhagoletis pomonelle.

Vitamin E is an essential vitamin for humans and animals; however, it is exclusively synthetized in photosynthetic organisms[15].

It is a lipid soluble antioxidant and in green plant tissues it is localized in the chloroplast envelope and thylakoid membranes, but also in plastoglobules, small structures within the chloroplasts attached to the thylakoid membranes and composed of lipids and proteins[28,39,72].

Spinach thylakoid membranes were supplied with exogenous tocopherol (Vitamin E) to determine if elevated tocopherol levels would protect lipids from degradation induced by UV radiation exposure, data indicate that elevated levels of tocopherol conferred antioxidant protection UV exposed thylakoid membrane lipids[16]. α-tocopherol protects biological membranes from oxidative stress[7,8]. Higher vitamin E concentration saturated the membrane architecture and quenched the propagation phase reactions that lead to extensive fatty acid destruction[16].

Ca^{2+} regulates many cellular processes, like stimulated secretion by exocytosis, gene transcription, and cell division[4,71] and ciliary activity[19,79], as in mammalian cells, these aspects also occur in ciliated protozoa including *Paramecium*[48].

Usage of non-target organisms in environmental toxicology is needed to understand the wide range of toxic effects caused by the pesticides on different organisms[74].

Fish and other aquatic biota were commonly used as bioindicators of persistent organic pollutants[70], have been replaced in recent years successfully by ciliates[53].

Protozoan cells are often used as bioindicators of chemical pollution, especially in aqueous environment, among protozoans; *Paramecium* is one of the ciliate models, most commonly used for laboratory research[41].

This unicellular ciliate facilitates the study of physiological processes and effects of pollutants on locomotory behavior; it has been widely used to evaluate the toxic effects of several food dyes, carcinogens, synthetic chemicals, carbamates pesticides, and pollutants[18,53,67,78].

In this study we uses *Paramecium sp.* As cellular model to evaluate the capacity of calcium and vitamin E in the detoxication of Novaluron.

MATERIALS AND METHODS

Material

A. Biological material

The cellular model used in this study is a ciliate Protista *Paramecium sp.* Considered as a good bio indicator for chemical pollution in aquatic environments, moreover ciliates of Paramecia represents similarly characters with those of the respiratory epithelial human cells, this protozoa facilitates the study of biological and biochemical processes, these is why a multitude of studies in toxicology took micro-organisms as cellular models.

B. Chemical material

The chemical substance that we evaluate the toxicity is Novaluron, and the chemical agents which we investigate about their detoxication effects are Calcium

and Vitamin E. two concentrations were used 10 and 20µg/ml and 3 repetitions are used for each treatment.

Methods

A. Cells culturing and treatments

Paramecium strain was used in the logarithmic phase of growth. The cells were grown at exponential phase in Proteose Peptone Yeast Medium (PPY), 2% proteose peptone and 5% yeast extract at pH 7.0-7.5, at 24±2 °C. The density of cells cultures was adjusted in fresh PPY in order to obtain at least 10^4 cells per ml. Before the experiments on respiration metabolism, the cells were washed with fresh culture medium and were resuspended at the concentration of $5x10^4$ cells ml^{-1} in 200ml flask; we take 1 ml to test in oxygraph (each time we added the appropriate concentration of DFB/ Novaluron to the reactive chamber by microsyringe). The cells were not exposed to combined molecule were used as control, the acetone is used to dissolve the pesticide so we obliged to investigate the impact of acetone on cells by addition of 5µl/ml to the medium cells (acetone-control). For the evaluation of Diflubenzuron/ Novaluron effect on *Paramecium* population growth, generation time and number, chitin integrity; we added the pesticide in culture medium before the addition of *Paramecium* cells, the used cells are starved for 96 h to become encysted. After the regeneration in culture medium that contains Novaluron, we investigate the effect on new chitin integrity.

B. Cytotoxicity tests

B-a. Mesure of growth kinetic

We estimated the optical density on 600 nm wave length on aliquots of 2,5 ml of culture, and we used distilled water as a blank.

B-b. Microscopic observations

The daily microscopic observation must take in consideration: motility, morphology, path of swimming, size, cellular division, number, speed, membrane integrity, time of coloration with neutral red.

B-b-1. Coloration with neutral red technic

The toxic effect of Novaluron on the integrity of the membrane in Paramecia, and the neutralizer effect of calcium and Vitamin E were estimated by the method of Fournier, 1993[22], modified for protozoa by Rouabhi et *al*.[51], by calculating the time of coloration of the first digestive vacuole, after 3 minutes we take photos for control and treated cells.

B-b-2. Speed of moving

With the use of graduate ocular we estimated the speed with which the cellule can cross a number of the ocular graduations (distance), the chronometer allow us to calculate the run of time when moving, the measure of the speed is done on magnifying power (×100).

B-b-3. Cellular count

The cellular number will be defined by the count of cells existing in 1 ml of culture (Gerson and Stainer, 1995), for this reason we used photonic microscope on magnifying power (×35).

B-b-3-a. Response percentage

We applied this formula

$$PR = \frac{CN - EN}{CN} \times 100$$ [76]

PR : Response percentage; CN: Number of control protozoa; EN : Number of treated protozoa

B-b-3-b. Number and time of generation

Based on the counting of the Paramecium sp. Cells, the number of generations and time required per each generation was calculated by the following formulae

$$N = \frac{\log N1 - \log N0}{\log 2}$$ [17]

N : number of generation; N1 : number of protozoa in the 10^{th} day; N0 : number of protozoa in the 9^{th} day

$$G = \frac{\text{Time of growth}}{N}$$ [17]

G : Time of generation; N : Number of generation
Time of growth : 24 hours.

B-b-4. Protein rate determination

Protein content was determined by the Bradfort[5], dye-binding procedure, using bovine seurum albumin as standard.

B-b-5. Glutathione measuring

We performed the measuring of glutathione in accordance of the protocol of Weckbecker et cory[75].

B-c. Statistical studies

All experimentations are repeated for 3 times, results were expressed by medium and standard of deviation, we used the Minitab 16.1.0 program for the

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contribution of statistical tests as variance analyses and Dunnett test.

RESULTS

Effects of Novaluron and calcium/vitamin E on the kinetic growth of *Paramecium sp.*

The inhibitor effect of Novaluron on the kinetic growth of Paramecia at the concentration of 20µg/ml is greater than the concentration of 20 µg/ml (figure 1), the analysis of variance show that there is a high significant difference between control and treated cells ($p<000.1$).

The figure 2 reveal the treated cells by Novaluron

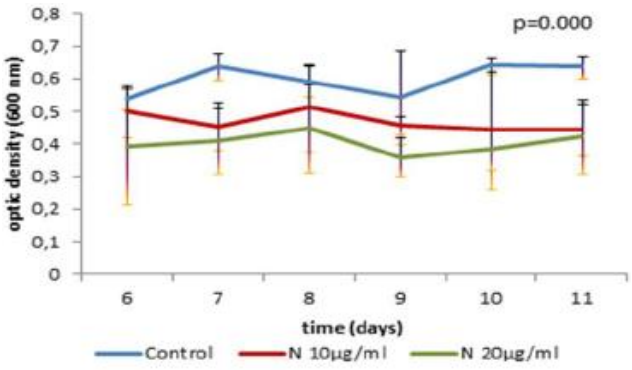

Figure 1 : Effects of Novaluron on the kinetic growth of Paramecia

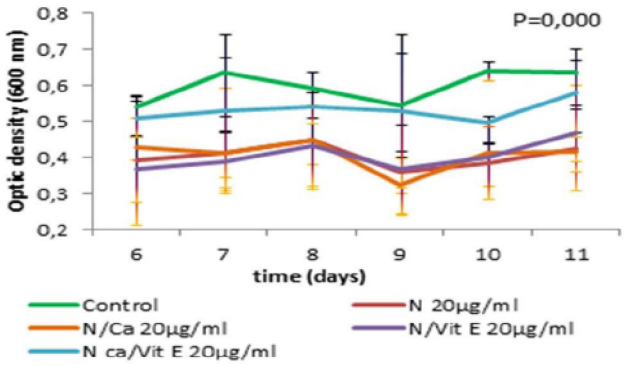

Figure 2 : Effects of Novaluron, calcium and vitamin E on the kinetic growth of Paramecia

combined with calcium/vitamin E are not inflected by any inhibition during growth in the last 6 days of the treatment and the growth reach its peak in the day 11 with an O. D. of 0.58 very close to control cells optic density (0.63) and lower than the cells treated only with Novaluron (0.42).

Dunnett test confirms that the medium of treated cells by Novaluron-Calcium/Vitamin E (0.53) is approximated to control's cells (0.59), comparatively with Novaluron's only treated cells (0.40)

Effects of Novaluron and calcium/vitamin E on the morphology and the wall integrity of *Paramecium sp.*

Red neutral test

A. Cells appearance

Paramecia swimming pathway is disrupted in the presence of Novaluron (figure 3a, b, c), especially for the 20µg/ml concentration with a pathway, and a massif entry of neutral red.

In the presence of Calcium (figure 4a, b), Paramecia swim in a reversal way differently than control cells, and lower entry of neutral red coloration, whereas in the presence of Vitamin E (figure 5a, b) the speed is higher and with a regulated pathway swimming and a lower entry of neutral red.

B. Coloration time

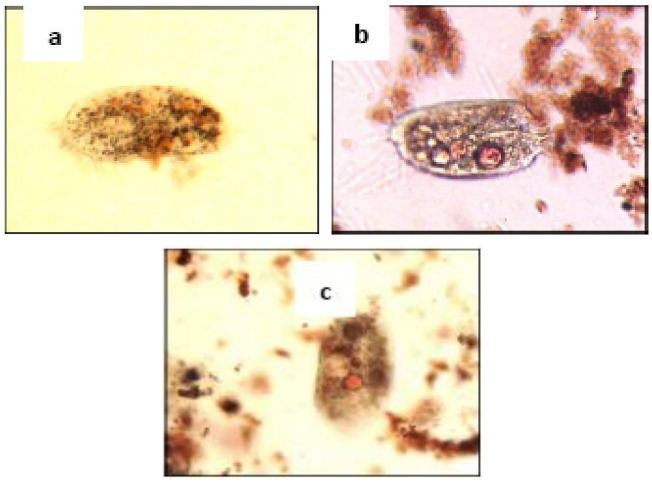

Figure 3 : Microscopic vu of Paramecia (×100). a: Control, b: Novaluron 10µg/ml, c: Novaluron 20µg/ml

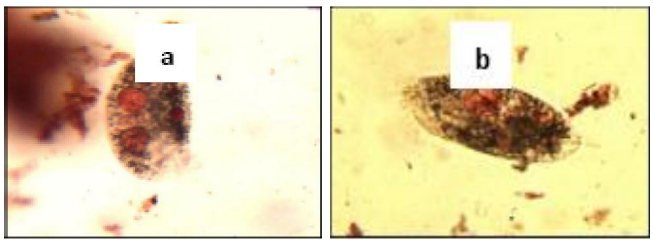

Figure 4 : Microscopic vu of Paramecia (×100). a: Novaluron/Ca^{2+} 10µg/ml, b: Novaluron/Ca^{2+} 20µg/ml

We noticed according to the (figure 7) that cell walls treated only by Novaluron (20µg/ml) are the most affected by a minor time of coloration (1mn 2s in the day 9), whereas, the cells treated by Novaluron beside the

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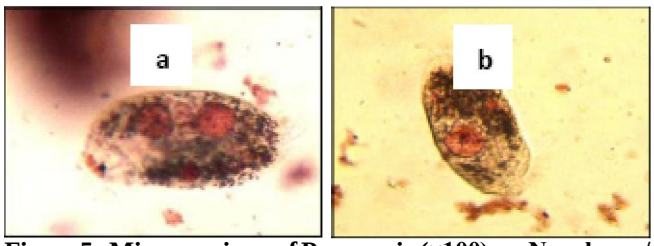

Figure 5 : Microscopic vu of Paramecia (×100). a : Novaluron/ Vit E 10µg/ml, b : Novaluron/Vit E 20µg/ml

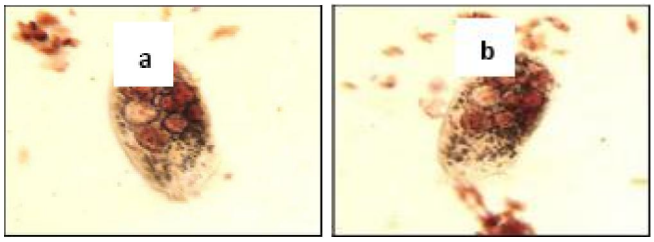

Figure 6. Microscopic vu of Paramecia (×100). a : Novaluron-Ca^{2+}/Vit E 10µg/ml, b : Novaluron-Ca^{2+}/Vit E 20µg/ml

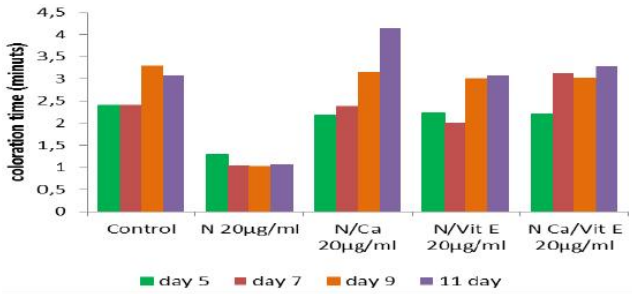

Figure 7 : Coloration by neutral red time variation; N: nuvaluron

combined treatment calcium /vitamin E shows a higher time of coloration (3mn 28 s in the day 11), which mean less affected cell wall.

Effects of Novaluron and calcium/vitamin E on moving speed of *Paramecium sp.*

The effect of Novaluron on the moving speed of Paramecia start being seen from the first day of the treatment, with a dose dependent slow-moving (figure 8)

The analysis of variance proves that there is a high

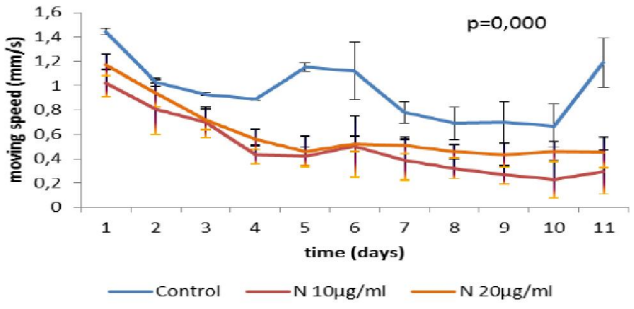

Figure 8 : Effect of Novaluron on the moving speed of *Paramecium sp.*

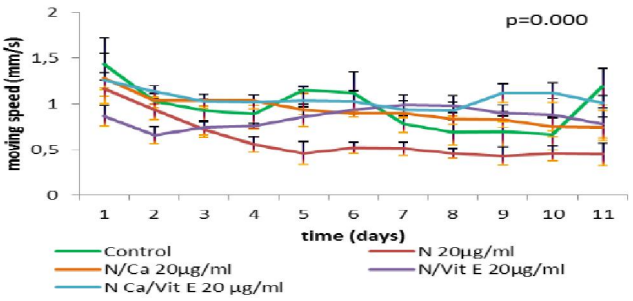

Figure 9 : Effects of Novaluron, calcium and vitamin E on the moving speed of *Paramecium sp.*

significant difference between control and treated cells ($p<000.1$).

At the concentration of 20µg/ml calcium/vitamin E neutralize the effect of Novaluron on the moving speed of Paramecia (figure 9), the value of speed can reach 1,12 mm/s in the 9th and the 10th day of treatment, whiles the speed of the cells treated only with Novaluron don't exceed 0.45 mm/s.

Dunnett test shows that the medium of treated cells by novaluron-calcium/vitamin E is adjacent to control cells.

Effects of Novaluron and calcium/vitamin E on the response percentage of *Paramecium sp.*

Novaluron accentuate the percentage response of Paramecia (figure 10) for the two concentrations (10 and 20µg/ml), whiles the presence of the combined mixture calcium/vitamin E decrease it where we saw the negatives values.

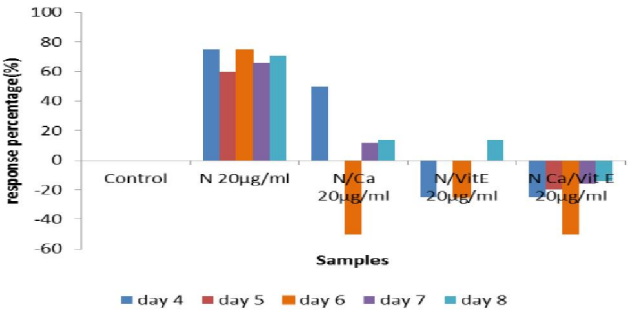

Figure 10 : Effects of Novaluron, calcium and vitamin E on the response percentage in *Paramecium sp.*

Effects of Novaluron and calcium/vitamin E on generation number and time of *Paramecium sp.*

The number of generation is non-existent for the cells treated only by Novaluron for the two concentrations (10 and 20µg/ml) whiles the presence of Calcium/ Vitamin E influence positively the number of generation comparatively with control cells (figure 11).

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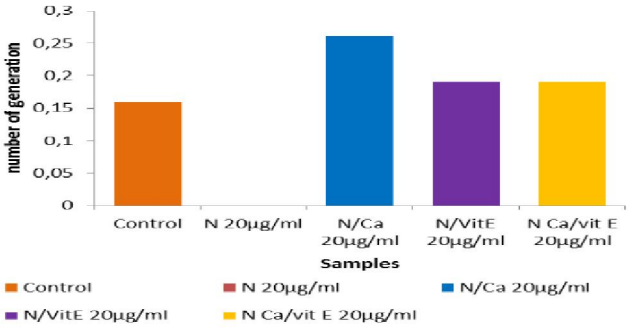

Figure 11 : Effects of Novaluron, calcium and vitamin E on the number of generation in *Paramecium sp.*

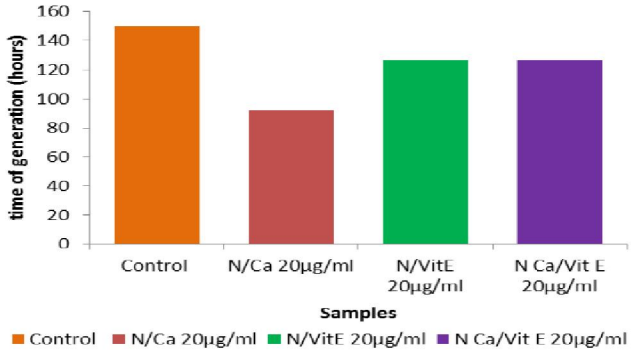

Figure 12 : Effects of Novaluron, calcium and vitamin E on the time of generation in *Paramecium sp.*

Generation time of cells treated by calcium/vitamin E beside Novaluron is approximated to control's cells time (figure 12).

Effects of Novaluron and calcium/vitamin E on the rate of proteins

The figure 13 elucidate that proteins rate in cells treated by combined treatment Calcium/Vitamin E in the presence of Novaluron are higher than the control cells and the cells treated only by Novaluron.

We can notice according to figure 14 that the highest amounts of GSH are localized in the cells treated by calcium, and vitamin E in the presence of Novaluron,

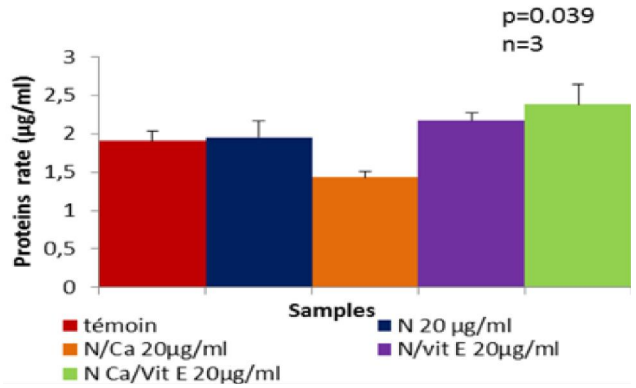

Figure 13 : Fluctuation of protein rate according to treatments at the concentration of 20µg/ml

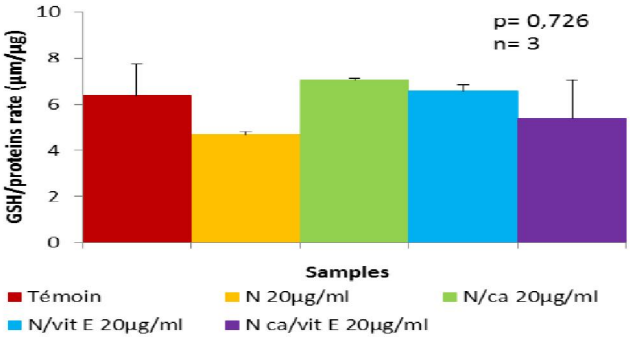

Figure 14 : Fluctuation of GSH rate according to treatments at the concentration of 20µg/ml

whereas the cells treated by Novaluron only possessed a minor amount of GSH.

DISCUSSION

Protozoa are real eukaryotic cells and ubiquitous in the aquatic and terrestrial environment, their normal behavior in nature may be related to the presence of pollutants and to air, soil and water quality. This fact has led toxicologists and ecotoxicologists to use protozoa as test systems for studies on xenobiotics and health risk assessments[24], in our study we choose *Paramecium sp*. As an alternative cellular model to evaluate toxic effects of Novaluron and the detoxication effects of calcium and Vitamin E.

Novaluron as a representative of the BPUs insecticides group has inhibitor effect on growth rate of Paramecia, revealing by our results and confirmed by[51,52], especially for the higher concentration 20µg/ml and from the 6th day of treatment as a starting time of inhibition. This inhibition is due principally for the mode of action of Novaluron, which is typically the same for the CSIs, having the cuticle composition in Paramecia as a target, specifically Chitin synthesis, leading to the formation of abnormal cuticle, fragile and permeable which is confirmed by the test of neutral red, where a massive entry of coloration in a reduced time was shown, also the presence of rounded cells (encysted form) with the disappear of lashes and mouth, the body is surrounded by a protector membrane.

Other modes of action have been suggested for BPUs, they can: (1) inhibit the transport of UDP-GlcNAc across biomembranes; (2) block the binding of chitin to cuticular proteins resulting in inhibition of cuticle deposition and fibrillogenesis; (3) inhibit the formation of chitin due to an inhibition of the protease that

activates chitin synthase, and activation of chitinases and phenoloxidases, which are both connected with chitin catabolism; (4) affect ecdysone metabolism, resulting in ecdysone accumulation that stimulates chitinase, which in turn digests nascent chitin; (5) block the conversion of glucose to fructose- 6-phosphate; and (6) inhibit the DNA synthesis[42,44,45,50] revealed that BPUs integrate on the N-glucosamine, so the perturbation of the chitin synthesis. Other studies insinuate that BPUs inhibit the growth of protozoa by the reduction of the thickness of chitin due to the incorporation of BPUs on protista ARN[32].

The production of free radicals and various reactive oxygen species (ROS), derived exogenously from oxidative injuries related to pollution, radiation[21]. Pesticides are recently known to be able to induce in vitro and in vivo generation of ROS[2]. In our case Novaluron is responsible for the production of ROS by its own metabolism or by acceleration respiratory metabolism. Which explain the higher response percentage of cells treated only by Novaluron. The antioxidant property of vitamin E, as a chain-breaking donor molecule, is excreted through the phenolic hydroxyl group, which readily donates its hydrogen (H) to the lipid peroxyl radical, resulting in the formation of a stable species, in donating the H, vitamin E becomes a relatively unreactive free radical, as the unpaired electron becomes delocalized into the aromatic ring[11,37,40,66,69]. The role of Vitamin E as an antioxidant agent can explain its capacity to neutralize deleterious effects of Novaluron with a rate of growth approximate to the control cells a lower response percentage, a number and time of generation imminent to control cells. Calcium neutralize Hazardous effects of Novaluron by its proper mode of action, and specifically in Paramecium sp. Cells, because Paramecia are able to regulate exocytosis of secretion vesicles (trichocysts) as a response for the presence of calcium, discharge of trichocysts is an efficient defense of Paramecia against pollutants, the possible defense function is the reversal swimming, after trichocysts discharge when the meeting of pollutant and Paramecia[26,33]. a fast augmentation of intracellular calcium launch directly regulated exocytosis, proved by Jeff et *al*. (2005). The test of neutral red coloration we precede confirmed that calcium and vitamin E preserve cell wall of Paramecia with a higher time of coloration.

Many emphasis studies shows the role of vitamin E in the neutralization of ROS derived from exposition to pesticides; supplementation of α-tocopherol to PCB (polychlorobiphenyl) exposed rats showed a protective role on free radical-induced toxicity in rat testicular Sertoli and Leydig cells and brain regions[59,64] and ventral prostatic dysfunction[58]. Vitamin E and selenium were effective in partially alleviating degenerative changes induced by Malathion in the liver of chicks by attenuating process leading to lipid peroxidation. Dipel, a *Bacillus thuringiensis*-based bioinsecticide, induced oxidative stress in rat liver that has been protected by prior administration of α tocopherol[60]. Tocopherol modulates liver toxicity of the synthetic pyrethroid cypermethrin[1]. Application of tocopherol resulted in a lower sensitivity of *Phaseolus vulgaris* and *Malus Domestica* leaves to paraquat induced stress, as compared to treated with paraquat only[57]. *In vivo*, tocopherol has protective role in organophosphate toxicity[10]. Naphtalene induces oxidative stress and tissue damage, and that vitamin E provides significant protection[73].

Behavior is considered as a promising tool in ecotoxicology[12,56], and these studies are becoming prominent in toxicity assessments in unicellular organisms[68], locomotion has been found to be concisely sensitive measure of toxic stress for wide range of environmental contamination[35].

In our study of the locomotion of Paramecia we saw that Novaluron provoke the slowing of moving speed of Paramecia. At the concentration of 10µg/ml, Novaluron accelerate cellular respiratory[52] therefore a big production of free radicals per mitochondria, those lasts provoke oxidative degradation to mitochondria, a deficiency of oxidative phosphorylation, wish inflict a decline of ATP production[9,25] where the perturbation of the swimming path as a consequence because of the depletion in ATP produced by mitochondria needed in the moving of Paramecia lashes. But in the presence of Vitamin E, moving speed of Paremcia increase and with a similar pathway of swimming as control cells, because vitamin E provoke the opening of potassic channels, hyperpolarization of the plasmin membrane, the activation of adenylcyclase and the production of AMPc, AMPc induce phosphorylation of anoxemic proteins and release the augmentation of ciliary beats frequency, this results are proved by[13,14,38].

Our results shows that the presence of calcium in-

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crease the moving speed of Paramecia, and in a reversal pathway of swimming, because Calcium regulates ciliary beat[54], it is relevant for normal beat activity, not only in ciliated epithelia[19,34,54] but also in Paramecium[36,34]. Beyond that a [Ca^{2+}] increase can induce a ciliary or flagellar beat reversal in lower eukaryots like the green alga Chlamydomonas[3,62] ciliates[48,49]. The frequency of ciliary beats in the reversal direction was significantly higher than the normal direction, this results indicate that the contraction of the cellular body is regulated by calcium in Paramecia in a dose-dependent manner, in the other side, ciliary reversal and the rising of ciliary beats frequency where launched by calcium, these results are confirmed by[27,29,48]. The relationship between ciliary beats and the presence of calcium was proved on the lashes of mammalian epithelial cells[30]. Many experimentations confirms the role of calcium in exocytosis.

Vitamin E neutralizes toxic effects of Novaluron in a synergy with GSH, because it captivates free radicals, giving them to GSH system in order to neutralize it, and go for his turn for the searching of other free radicals[46].

Proteins rate in cells treated by Nvaluron combined with calcium/vitamin E is higher that the cells treated only by Novaluron, which explain the capacity of Vitamine E in the reduction of protein oxidation, the rise of the activity of the detoxication system[61].

GSH acts as a cellular reducing reagent and protects against the cellular redox cycling changes induced by toxic substances, in cells treated only by Novaluron, we observe that there were a decline of GSH rate because of the depletion of cellular GSH stock, whereas cells treated by Vitamin E the rate of GSH is higher, because Vitamin E cooperate with GSH in detoxication, so no exhausted stock of GSH. The role of GSH in detoxication was demonstrated in many studies[20].

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