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Physiological and biochemical changes observed in alternative cellular model: *Paramecuim tetraurelia* treated with paracetamol

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Abstract

During their passage into the human or animal body, most of xenobiotics are metabolized. The metabolites formed are eliminated primarily through the kidneys and therefore are found in wastewater. Presence of synthetic molecules even at trace concentrations is an extremely pernicious pollution may lead to their accumulation in aquatic ecosystems. In this work, we focused particularly on the toxic potential of an antipyretic analgesic wide use: Paracetamol on an alternative cellular model: the protist ciliate *Paramecium tetraurelia*. The results show that the IC50 after 2h of exposure to xenobiotic is more 0.53µM; paramecia are sensitive to paracetamol and exhibit morphological and physiological changes and a strong disturbance of respiratory metabolism. In addition we have demonstrated a significant increase in GSH and MDA to confirm the toxicity of the tested compounds.

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Introduction

Medical advances and the increase in life expectancy are factors which suggest that the consumption of drugs will grow exponentially and consequently they play an increasingly important role in the contamination of the environment in aquatic systems in particular. Although pharmaceuticals have been detected frequently in aquatic ecosystems in the world, their ecological potential consequences are not yet fully understood (Binelli *et al.*, 2012). Designed to be biologically active, these molecules can interact with specific biological targets with consequent environmental and health risks associated with their presence in the environment (Coetsier, 2009).

This fact has led to questions about the possible impact of drugs on ecosystems. For this, we have chosen paracetamol, it has become one of analgesics and antipyretics commonly used in humans beings (Driad, 2009). Paracetamol (4-hydroxyacetanilide, N-acetyl-p-aminophenol, acetaminophen, APAP) is widely used on the contre-analgesic and antipyretic (McCormack, 1994). Paracetamol or acetaminophen has a mechanism of action still imperfectly known. Recently, the effects of the paracetamol were exclusively attributed to a peripheral inhibition of prostaglandin synthesis. Univocal mechanism actually seems less likely (Landis, 1995).

Currently in toxicology, the use of alternative models can help to understand the mechanisms of toxic action, at different levels of organization of ecosystems (Mohd, 2006). Algae, protozoa and bacteria form the base of the food chain and protozoan cells are used as bioindicators often of chemical pollution in particular in aqueous environment (Sako *et al.*, 1977).

Unicellular organisms offer the possibility of direct study of independent cells with specific characteristics of individual cells and whole organisms at the same time (*Epstein et al.*, 1963). This unicellular facilitates the study of physiological processes, and effects of pollutants at the cellular level, which makes it widely used to assess the toxic effects of various families of xenobiotics (Cohn and Macphail 1996 ; scherrer, 1992). If mortality is obviously not the only criterion for reliable assessment, there is a special interest biomarkers (GSH, MDA, CAT), and also in the development of behavioral markers (movement, swimming) to evaluate the toxicity sub lethal that affects behavior.

Material and methods

Biological material

The biological model used in our work is a unicellular microorganism Paramecium tetraurelia.

Chemical

We used the N-acetyl-nitrophenol chemically; it is the hydroxy-1-acetamido-4-benzene (abbreviated NAPAP).

Treatment

The cells are cultured in the laboratory, in a culture medium composed of 50 µm [Ca^{2+}], 0.4 mM [Na^{+}] and contaminated by Klebsiella pneumoniae previously at pH 6.5 and a temperature of 27 ± 3 ° C (Benbouzid *et al.*, 2012). Photoperiod is of 14 h light and 10 h of darkness. The cells were subcultured every three days to maintain a good culture and experiments are performed in logarithmic growth phase. (Azzouz *et al.*, 2011). N-(4-hydroxyphenyl) ethanamide is tested aliquots of 10 ml of culture are selected and four concentrations: 2, 4, 6 and 8 µM.

Parameters measured

Kinetics of Growth

The growth curves provide quantitative data for a reliable analysis of the toxic effect of paracetamol on paramecia. They are established by counting living cells under the microscope using a meter hand.

Percentage response (PR)

Directed by the method of (Wong *and al.,* 1999). This calculation evaluates the response of protist vis-à-vis he paracetamol taking into account the growth parameter.

It is based on the following equation

PR = [(NC - NE) / NC] x 100
NC: Number of control cells.
NE: Number of treated cells.

Positive values indicate the percentage of answers inhibition of growth, while negative values indicate a stimulation of growth (Wong *et al*., 1999).

polarographic Study

Respiratory activity of paramecia is measured using an oxygen electrode type (HANSATECH), for measuring the production or consumption of oxygen by the cells. Its Sensitivity permits the detection of concentrations of O2 under µM (Djebar, 1988).

Number of malformations

are the potential morphological changes caused by the presence of xenobiotic in Paramecium. We considered the general shape of cells and their appearance observed with an optical microscope.

Mortality rate

This rate is calculated according to the method of Abbot
Abbot's formula is

$$\frac{X - Y}{X} \times 100$$

X

X= Number of cells living witnesses
Y= Number of living cells treated

Number of vacuoles

Determining the number of digestive vacuoles was performed with neutral red staining method of Fournier and modified by (Rouabhi *and al*., 2006).

Calculation of CI50

The calculation of the CI50 is performed by probit analysis (Finney, 1971).

Determination of malondialdehyde (MDA)

This calculation is performed according to the method of Draper and Hadley (1990). This method is based on the colorimetric measurement of the reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA), giving a reddish brown product whose color intensity is measured at a wavelength of 532 nm.

Determination of glutathione (GSH)

The rate of glutathione (GSH) is quantified according to the method of Weckberker and Cory (1988) **.** The principle is based on the colorimetric measurement of the 2-nitro-5 mercapturic, resulting from the reduction of the 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) by the thiol (-SH) glutathione measured at a wavelength of 412 nm.

Results

Growth kinetics

Fig. 1. illustrates the effect of paracetamol on the evolution of the growth of paramecia. All measurements are carried out during the exponential growth phase.

Our results showed a dose-dependent inhibitory effect, between 1 and 6 hours of treatment, paramecia treated with paracetamol have a number of cells equal to that of the control. Beyond 18 h of treatment, a significant depletion in cell number was observed for those treated with the highest concentration (8µM), this depletion appears to persist over time.

Percentage response of paramecia treated with paracetamol

Fig. 2 represents the percentage of response paramecia treated with paracetamol. There is a confirmation of the inhibition after treatment. Latter is dose-dependent and proportional to the concentrations of paracetamol, there is thus a inhibition 38.28% for the concentration of 2µM and 88.26% for the concentration of 8µM. At high concentrations of paracetamol (6 8µM), growth is inhibited and slowed the mobility of paramecia.

Influence of paracetamol on the respiratory metabolism

Fig. 3 shows the effects of paracetamol on the respiratory metabolism paramecia.

Table 1. Mortality rate (%) observed in paramecia treated with paracetamol.

Concentration of Paracetamol (µM) / Time (h)	2	4	6	8
1	5±1	8±2	11±3	50.24±1.1
6	11.90±2.70	13.80±2.20	24.76 ±2.38	68.64±9.22
18	31.31±1.33	47.36±7.66	56.25±6.25	49.25±6.50
24	9.21±9.10	51.36 ±1.33	68.95±5.35	90.25±1.25
72	40.25±2.25	66.21±4.21	75.25±2.25	96.35±6.35

We note that the oxygen consumption is dose-dependent compared to concentrations of paracetamol. After a minute of treatment we noticed a respiration inhibition of treated cells, in control cells the oxygen consumption varies from 280 nmol / ml of O2 at 25 nmol / ml of O2. Finally after 1 minute of treatment, a complete inhibition of respiration is recorded. At the highest concentration (8 µM) there is a cell death because the rate of O2 in the medium is constant (280 nmol / ml). Shows that treatment with paracetamol tends to inhibit cell respiration and this effect is directly related to the concentrations of the xenobiotic.

Rates of malformation

Microscopic observations paramecia treated with paracetamol show a large number of specimens with abnormal shape, the number and severity increased in a dose-dependent.

These defects result in asymmetric shapes. In some cases, we observe the formation of large spots in the middle of paramecia, they become spherical with pointed ends and some may even burst. These effects are rapid and observable upon addition of the drug.

Rate of Mortality

Mortality is effective when the movement of paramecia is totally inhibited. We define the mortality rate as the percentage of deaths observed after 2 hours of treatment. The results obtained in our work are gathered in Table 1.

Table 1 Mortality rate (%) observed in paramecia treated with paracetamol.

We remark that the results in Table 1 showed that the mortality rate is dependent dose levels compared to paracetamol. It is 5% after 1 hour treatment in the presence of the lowest dose and increased to 40.25% after 72h. For cells treated with higher concentrations, there is a very high mortality rate and can reach 96.35%.

Number of vacuoles

Fig. 5 illustrates the effects of paracetamol on the average number of vacuoles.

Our results show a marked decrease in the number of vacuoles and this compared to increasing doses of paracetamol given to paramecia. Account on average for 7 vacuoles control cells, this number decreases to 2 poue those treated with the highest concentration 8µM. And our results show that treatment of cells with paracetamol tends to significantly reduce (75%) the average rates of vacuoles.

Calculation of LC50

The calculation of the CI50 by probit analysis shows that the average concentration that inhibits 50% growth paramecium is about 0.53 µM 2 hours after treatment.

Effect of paracetamol on the average rate of MDA

In Fig. 6 we have shown the results of changes in MDA levels obtained from paramecia treated with different concentrations of paracetamol versus time

Treatment with paracetamol tends to increase the content of MDA and this way dependent dose. MDA levels recorded in control cells and 11.5 µmol / mg protein, will increase significantly to reach 86.2 µM / mg protein in paramecia treated with 8µM after 72 h of treatment.

Effect of paracetamol on the average rate of GSH

The results are shown in Fig. 7 are the variation of GSH according to the different concentrations of paracetamol, and time (1h-72h).

After 1 hour of exposure only, the rate of GSH paramecia treated with 4 µM is 2 times higher than th at of control cells. However, and after 18 hours of treatment, stimulation of GSH activity is more visible compared to control cells. After 24 hours of treatment, depletion of GSH was observed, approaching values recorded in control cells after 72 h of treatment.

Discussion

The potential danger of drug substances on the environment lies in the fact that most of them exhibit the same behavior as the physico-chemical substances secreted by the host organism and may also have different actions within the same organization. So they can replace molecules such as enzymes, with these specific receptors and do not allow the reaction normally induced by receptor complex molecule. In addition, these substances may affect bioaccumulation after the functioning of terrestrial and aquatic ecosystems. More knowledge about the chemical nature of drugs and their metabolites are Insufficient to know the real extent of the presence of these substances in the environment.

In our work we investigated the effect of an antipyretic to wide use of paracetamol is paramecia and this by studying the physiological and biochemical changes, but also the Antioxidant response of Paramecia treated.

Paracetamol is known for its genotoxic effects and this in several in vitro and in vivo. (Hongslo *et al.*, 1990) However, the latter showed no mutagenic in bacterial mutation tests in Salmonella (Dybing *et al.*, 1984). (Barry and Bernal, 1993) Show anti-malaria drug toxicity on paramecuim calkinsi. The toxic effects of these drugs seem to have as site of action calcium channels in protists.

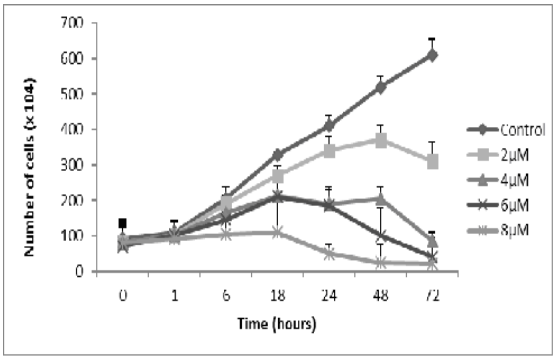

Fig. 1. Effect of Paracetamol on the growth kinetics of Paramecium tetraurelia.

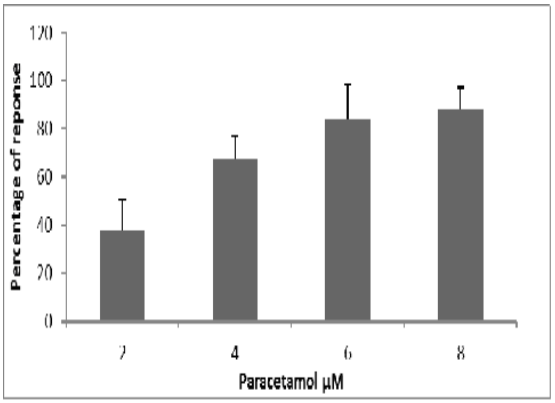

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Fig. 2. Effect of paracetamol on the percentage of cellular response (n=3).

Growth kinetics

Evaluation of cytotoxic effects of a xenobiotic can be performed using different parameters, including cell growth in microorganisms which reflects the state of the metabolite of the cell (Sauvant *et al.*, 1999) our results show a significant inhibition of growth as a function of different concentrations of paracetamol. The latter reflects the microorganisms in the state of cell metabolism (Sauvant *et al.*, 1999).

A similar result is reported in the study (Rouabhi *et al.*, 2006), who studied the effect of diflubenzuron and Flucycloxuron on the morphology and physiology of *Paramecium sp*.

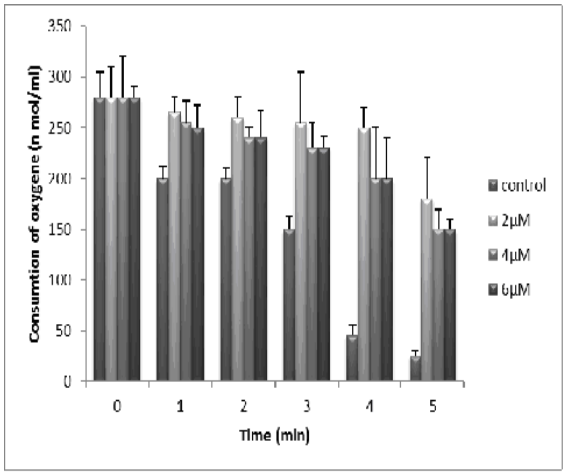

Fig. 3. Effect of Paracetamol on oxygen consumption in *Paramecium tetraurelia*.

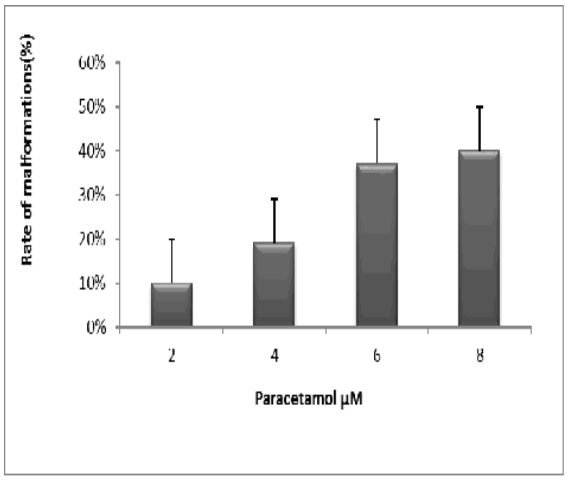

Fig. 4. Rate of malformations induced by Paracetamol in paramecia.

The percentage of responses

The evolution of the growth kinetics in the presence of paracetamol is confirmed by the percentage of responses that are reliable parameters confirming or inhibition of cell growth**.** This brings us to confirm the influx of xenobiotics within cells, despite the presence of the cell membrane forms a barrier against the entry of xenobiotics massive but is still permeable (Beaumont and Cassier, 1998)**.** These results are in agreement with those of (Einicker Lamas *and al,* 2002) who studied the toxicity of Zinc and Copper on Euglena gracilis (flagellated algae chlorophyll) (Redouane, 2004).

Mrphological changes

We also observed changes in our forms paramecia treated these defects result in budding at the plasma membrane and cytoplasm with the appearance of large bright spots causing a change in the morphology of the paramecium. These results are in agreement with those of (Azzouz *et al.*, 2011) and Venkateswara Rao *et al.* (2006, 2007 and 2008)**.** (Amanchi and Hussain, 2010) reported that treatment of paramecium caudatum by high concentrations of carbofuran, cause 47% of abnormalities affecting the macronucleus which explains malformations such as fragmentation, the unequal division and vascularization observed in Our work. Our observations highlight in this case also internal damage with the formation of large bright spots in the middle of the cell. These observations are in agreement with those reported by Venkateswara Rao *et al.* (2006)**.** These authors show that the treatment of paramecia by acephate (organophosphate insecticide) causes breaks in the membranes of vacuoles and contractile resulting mixing their contents with the protoplasm. According to the same authors, the cell volume increases due to the disintegration of protoplasm that appears condensed at the periphery of the cell.

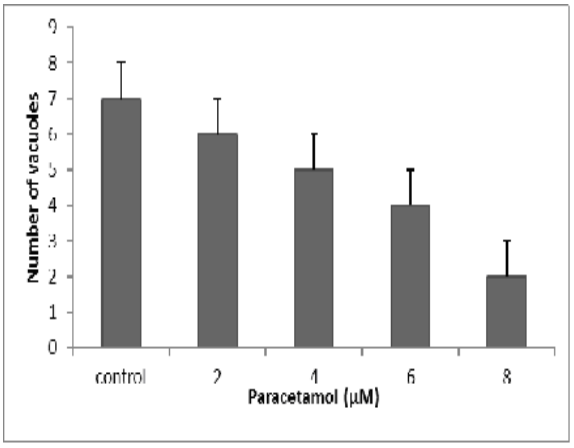

Fig. 5. Number of vacuoles from paramecia treated with paracetamol.

Our results confirm those obtained with paramecia exposed to fenthion and underwent a series of behavioral and morphological changes such as alteration of shape and budding intensive ultimately lead to the total destruction of the cells in a dose - dependent (Friedl**,** 2003; Mills, 1998). The budding process is common during apoptosis. A similar effect was observed on the cell membrane in Paramecium

with other xenobiotics and carcinogenic compounds (Guengerich, 1986).

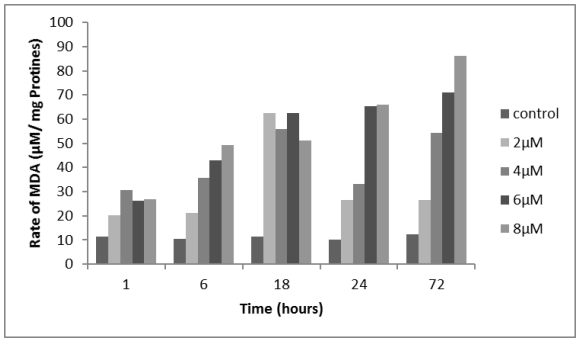

Fig. 6. Evolution of MDA levels according to increasing concentrations of paracetamol.

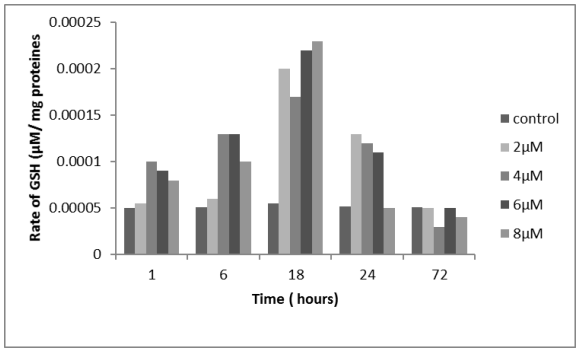

Fig. 7. Evolution of GSH according to increasing concentrations of paracetamol.

Respiratory metabolism

The other interesting aspect of our work concerns the development of respiratory metabolism of paramecia treated with paracetamol. Our results show an inhibition of respiration thus confirming the hypothesisof (Cadenasand *et al*., 1977)**.** Suggesting the presence of superoxide anion radical toxic, hence the disruption of O2 consumption observed in our work. Work on this aspect showed inhibition of cellular respiration in the presence of xenobiotics by the disruption of the mitochondrial respiratory chain. Thus (Chagra *et al*., 2009) show a disruption of oxidative photophosphoralisation in mitochondria isolated from Solanum Tuverosum treated with cadmium. On the other hand, (Sbartai *et al*, 2009) and (Benbouzide *et al*, 2012) also showed an inhibition of respiration in the presence of paramecia Bifenazate and phosphoramidate. All these studies are in agreement with our results indeed we observed an inhibition of us as respiratory metabolism paramecia especially at the highest concentration of paracetamol. These results suggest that the release of free radicals at the mitochondrial level disrupting cellular respiration.

All these observations allow us to suggest that during the metabolism of paracetamol recognized as the xenobiotic cytochrome P450 enzyme system is triggered (Guengerich, 1986). It allows you to begin the process of removing undesirable compounds. However, it produces metabolites can be toxic if they are not processed rapidly or eliminated. Indeed, it is now clearly demonstrated that free radicals are responsible for toxic processes (Lahouel *et al*., 1998) Thus paracetamol is metabolized by cytochrome P450 to give a reactive metabolite (N-acetyl-p-benzoquinone imine)(O'Grady, 1997).

The cell has for its protection of glutathione (Guengerich, 1986). Which is the main antioxidant own cell is capable of binding to SH pole by toxic metabolites. It also recognizes that today the formation of reactive metabolites of glutathione consumed when it is important and that this leads to a depletion of glutathione which main consequence: lipid peroxidation

(O'Grady, 1997) and oxidation of the thiol groups of proteins (Settaf *et al*., 2000; Bridger *et al*., 1998).

Effect of paracetamol on the average rate of GSH and MDA

Our results show a strong antioxidant enzyme activity, resulting in increased activity of Malondialdehyde (MDA) and decreases in GSH known for their role in the detoxification of free radicals. These reaction antioxidants enzymatic and no enzymatic provide the cell a state of equilibrium and protection against reactive species oxidized. (Mofredj, 1999). It should be noted that only acute observations were made in this study and the extrapolation of results obtained on a long-term toxicity on organisms from different trophic levels is important but difficult achieved Acker *et al*., 1993).

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