Micronucleus test in bivalve mollusks as an important tool for xenobiotic exposure risk assessment

Teste do micronúcleo em moluscos bivalves como uma importante ferramenta de avaliação de risco da exposição a xenobióticos

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Abstract - The interest in environmental genotoxicity studies has increased, and the micronucleus test (MN) is one of the most popular and promising tests on ecotoxicology, representing a cytogenetic indicator of DNA damage in dividing cells. Genotoxicity studies employed this methodology to evaluate possible genotoxic risk due to exposition to hazardous xenobiotics in different organisms, including aquatic sentinel organisms. In this context, bivalve mollusks stand out because of some aspects of their way of life, such as filtering food from the water. In bivalves, the MN test evaluates aneugenic and clastogenic effects preferably in hemocytes and gill cells. In the last two decades an interesting increase can be observed in the number of studies using the MN test in bivalve species, both in biomonitoring in situ and in the genotoxicity assays under controlled laboratory conditions, which are targeted to several chemicals with genotoxic and carcinogenic potential in the aquatic environment.

Keywords: bioindicator bivalves, genotoxicity, micronuclei.

Resumo - O interesse sobre estudos de genotoxicidade ambiental tem sido crescente e o teste do micronúcleo (MN) é um dos mais populares e promissores testes relacionados à ecotoxicologia, representando um indicador citogenético de danos ao DNA de células em divisão. Estudos de genotoxicidade empregam esta metodologia para avaliar possíveis riscos genotóxicos devidos à exposição a xenobióticos perigosos em diferentes organismos, incluindo organismos aquáticos sentinelas. Neste contexto, moluscos bivalves destacam-se por conta de alguns aspectos do seu modo de vida, tais como a filtração de alimentos da água. Nos bivalves, o teste do MN avalia efeitos aneugênicos e clastogênicos preferencialmente em hemócitos e células das brânquias. Nas duas últimas décadas, pode-se observar um aumento interessante no número de estudos usando o teste do MN em espécies de bivalves, incluindo tanto o biomonitoramento *in situ* quanto ensaios de genotoxicidade em condições controladas de laboratório, que são direcionados a diversas substâncias químicas com potencial genotóxico e carcinogênico no ambiente aquático.

Palavras-Chave: bivalves bioindicadores, genotoxicidade, micronúcleos.

Introduction

The micronucleus (MN) assay is a method currently used as an alternative and simple means of assessing chromosome damage *in vivo* (Carvalho Pinto-Silva, Creppy & Matias, 2005). Within genotoxicity markers, the MN test is the most widely established and relatively easy to perform. The MN formation indicates chromosomal DNA damage occurring as a result of either chromosome breakage or chromosome missegregation during mitosis (Bolognesi & Fenech, 2012).

The method consists of scoring cells with one or several cytoplasmic nuclei of reduced size separated from the main nucleus. These micronuclei are formed at the end of the cell division around chromatin elements (fragments or entire chromosome), which have not been integrated in the nucleus of the daughter cell. They provide evidence of chromatin breakage or spindle dysfunction caused by clastogenic and aneugenic poisons, respectively (Mersch & Beauvais, 1997).

The MN test has been applied extensively to test the genotoxicity of chemicals. The test has been successfully used with invertebrates, fish and amphibians, as a biological monitors of contaminated areas (*in situ* assay) (Klobuèar, Pavlica, Erben & Pape, 2003; Çavas & Ergene-Gozukara, 2005; Wirz, Saldiva & Freire-Maia, 2005) and in the screening of compounds to determine their genotoxicity, after direct or indirect exposure *in vivo* (Bolognesi et al., 2004; Rocha et al., 2009; Rocha, 2011).

A large number of chemical compounds are known to cause hazardous effects in aquatic organisms. Among those, heavy metals, oil products, chlorinated pesticides, halogenated aromatic hydrocarbons and other substances found in aquatic ecosystems have the ability to accumulate in organisms. Many contaminants exert their effects via genotoxic and metabolically toxic mechanisms, simultaneously causing carcinogenesis, embryotoxicity and inflict a long-term damage to organisms (Jha, Hagger & Hill, 2000).

Mollusks, often used as bioindicators - they are sedentary, widely distributed, very abundant, relatively sensitive to environmental contamination. They are filters (Bučinskienė, 2001) and have been considered sentinel organisms in a health assessment of an aquatic environment (Dixon, Pruski, Dixon & Jha, 2002).

Bivalves are widely distributed, sometimes sessile, filter feeders, which are good bioindicators of aquatic pollutants (Le Pennec & Le Pennec, 2001) and have often been used as test organisms in studies of aquatic pollution (Pavlica, Klobuèar, Moja, Erben, & Pape, 2001; Pruski & Dixon, 2002).

Technical Aspects and Analysis

A large number of studies using bivalve cells showed increase in MN frequency after laboratory exposure to different pollutants. The MN test is rapid, simple, and there are in the specialized literature at least two different protocols, being hemocytes and gill cells the most used.

The first protocol is applied according to the procedure proposed by UNEP (1999) and makes use of hemocytes. Hemolymph is withdrawn from the posterior adductor muscle of the bivalves in physiological saline so as to obtain a 50/50 of cell/physiological saline suspension. Suspensions are spread on slides, transferred to a lightproof humidity chamber, and allowed to attach. Cells are then fixed in

methanol:acetic acid (3:1) for 20 min, then spread on slides, air dried and stained with 3% Giemsa solution.

In the second protocol, MN test is applied on gill cells according to the procedure proposed by Baršienė et al. (2004). Bivalves are dissected, gills removed and two gill arches placed in a drop of ethanol:acetic acid or methanol:acetic acid (3:1) solution separately on two clean microscope slides and gently nipped with tweezers for 2–3 min. The produced cell suspension is softly smeared on both slides and air-dried. Dried smears are subsequently fixed in methanol for 10 min. Air-dried slides are stained with 4% Giemsa solution in phosphate buffer pH 6.8.

The stained slides should be analyzed under the light microscope at a final magnification of 1000X. Only the cells with intact cellular and nuclear membrane are scored. The MN are identified according to the following criteria: (1) diameter smaller than one-third of the main nucleus but greater than one-tenth, (2) no contact with nucleus (absence of chromatid bridge), (3) colour and texture resembling the nucleus, (4) spherical cytoplasmic inclusions with sharp contour (Countryman & Heddle, 1976). Other nuclear abnormalities like the nuclear buds and binucleated cells, may still be considered (Dailianis, Domouhtsidoub, Raftopouloub, Kaloyiannia & Dimitriadis, 2003; Bolognesi, Perrone, Roggieri, Pampanin & Sciutto, 2006; Baršienė & Andreikėnaitė, 2007; Baršienė, Andreikėnaitė & Bjornstad, 2010; Fernández, Campillo, Martínez-Gómez & Benedicto, 2011).

There is a wide divergence in the number of cells of each specimen that should be used: 250 (Eck-Varanka, Horváth, Ferincz, Paulovits & Kováts, 2014); 1,000 (Dailianis, Domouhtsidoub, Raftopouloub, Kaloyiannia & Dimitriadis, 2003; Izquierdo et al., 2003; Farhadi, Farahmand, Mirvaghefi & Khalili, 2011; Fernández, Campillo, Martínez-Gómez & Benedicto, 2011; Benitez-Trinidad et al., 2014); 2,000 (Baršienė et al., 2004; Siu et al., 2004; Baršienė, Andreikėnaitė & Bjornstad, 2010); 4,000 (Bolognesi et al., 2004; Bolognesi, Perrone, Roggieri, Pampanin & Sciutto, 2006). According to Bolognesi, Perrone, Roggieri, Pampanin & Sciutto (2006), the large interindividual variability associated with low baseline frequency of this biomarker confirms the need of assessment a consistent number of cells in adequate number of animals.

Statistical analyzes can be performed by parametric or non-parametric tests according to the distribution. Once confirmed normality of distributions and homoscedasticity of variances, a parametric ANOVA can be applied (Dailianis, Domouhtsidoub, Raftopouloub, Kaloyiannia & Dimitriadis, 2003; Siu et al., 2004; Riva, Binelli, Daniele & Provini., 2007; Villela et al., 2007; Fernández, Campillo, Martínez-Gómez & Benedicto, 2011). In cases of non-normal distributions and heteroscedasticity, non-parametric tests are indicated, such as Mann-Whitney U-test (Pavlica, Klobucar, Vetma, Erben & Papes, 2000; Bolognesi et al., 2004; Baršienė & Andreikėnaitė, 2007; Benitez-Trinidad et al., 2014) and Kruskal–Wallis test (Izquierdo et al., 2003; Carvalho Pinto-Silva, Creppy & Matias, 2005).

Ecological Biomonitoring

The need to develop and standardize tests for *in situ* biomonitoring of aquatic ecosystems has long been acknowledged (Izquierdo et al., 2003). The use of biomarkers to measure biological responses in the

exposed organisms is very useful to simplify and lower costs of biological monitoring, especially in aquatic environments (Ramsdorf, Ferraro, Oliveira Ribeiro, Costa & Cestari, 2009). In order to efficiently assess the presence of mutagens in the water, in addition to the chemical analysis, mutagenicity/genotoxicity assays should be included as additional parameters in water quality monitoring programs (Ohe, Watanabe, & Wakabayashi, 2004).

The micronucleus test, one of those most frequently applicable techniques to identify genomic alterations, has served as an index of cytogenetic damage in humans and environmental animals (Fenech et al., 2003; Bolognesi & Hayashi, 2011). Table 1 presents some *in situ* biomonitoring studies using the MN assay in bivalves.

Table 1. *In vivo* biomonitoring studies using the micronucleus test in bivalve species.

Organism	Cell type	Localization	Contaminant	Reference
Mya arenaria	Hemocytes	Southeastern Massachusetts (USA)	Polychlorinated biphenyls	Dopp, Barkerb, Schiffmann & Reinischb, 1996
Mytilus galloprovincialis	Hemocytes and Gill cells	Thermaikos gulf (Greece)	Domestic, industrial and agricultural wastes	Dailianis, Domouhtsidoub, Raftopouloub, Kaloyiannia & Dimitriadis, 2003
Mytilus edulis	Gill cells	Gijon (northern Spain) and Puerto Madryn (Argentina)	Industry and urban effluents	Izquierdo et al., 2003
Dreissena polymorpha	Gill cells	Lake Trasimeno (Italy)	Disinfectants	Baršienė et al., 2004
Mytilus spp.	Gill cells	Baltic Sea (Sweden, Lithuania, Poland and Germany)	Crude oil	Baršienė et al., 2006
Mytella falcata	Hemocytes	Santos estuary (Brazil)	Industry effluents and domestic sewage	David, 2007
Limnoperna fortunei	Hemocytes	Guaíba hydrographic region (Brazil)	Industrial, urban, and rural waste	Villela et al., 2007
Mytilus edulis	Gill cells	Göteborg harbour and nearby coastal area of the North Sea (Sweden)	Oil products	Baršienė, Rybakovas, Förlin & Šyvokienė, 2008
Macoma balthica and Mytilus edulis	Gill cells	Baltic Sea (Lithuania)	Oil products	Baršienė, Andreikėnaitė, Garnaga & Rybakovas, 2008
Mytilus edulis	Gill cells	North Sea in Førlandsfjorden (Norway)	Crude oil	Baršienė, Andreikėnaitė & Bjornstad, 2010
Soccostrea cucullata	Gill cells	Persian Gulf coast (Iran)	Oil products	Farhadi, Farahmand, Mirvaghefi & Khalili, 2011
Mytilus galloprovincialis	Gill cells	Spanish Mediterranean coast (Spain)	Polycyclic Aromatic hydrocarbons, organochlorinated compounds (PCB, DDT) Metals (Hg, Cd, Cu, Zn, As)	Fernández, Campillo, Martínez-Gómez & Benedicto, 2011
Dreissena polymorpha	Hemocytes	River Drava (Croatia)	Municipal wastewater	Thomas et al., 2014

Genotoxicity assays

The use of these bioassays allows the study of genotoxic effects of certain contaminants in isolation or associated, minimizing the influence of environmental variables. Results from bioassays cannot be transferred directly to the environment, but aid in providing a database aimed at a better understanding of the factors that interfere with the health of organisms and/or promote changes in environment conditions (Ramsdorf, 2007). Genotoxicity assays using bivalve mollusks can be performed *in vitro* and/or *in vivo*. In the *in vivo* assays, the tested agents are usually added to water. In the last years, the number of genotoxicity assays using the MN test in bivalve species has increased significantly. Some examples are presented in Table 2.

Table 2. Genotoxicity studies using the micronucleus test in bivalve species.

Organism	Cell type	Contaminant	Reference
Dreissena polymorpha	Hemocytes	Pentachlorophenol	Pavlica, Klobucar, Vetma, Erben & Papes, 2000
Dreissena polymorpha	Gill cells	Disinfectants (sodium hypochlorite, chlorine dioxide and peracetic acid)	Bolognesi et al., 2004
Mytilus edulis	Hemocytes	Tributyltin oxide (TBTO)	Hagger, Depledge & Galloway, 2005
Perna viridis	Hemocytes	Benzo(a)pyrene	Siu et al., 2004
Sinanadonta woodiana	Hemocytes	Benzo(a)pyrene	Woźnicki, Lewandowska, Brzuzan, Ziomek & Bardega, 2004
Dreissena polymorpha	Hemocytes	Decabromodiphenyl Ether	Riva, Binelli, Daniele & Provini., 2007
Mytilus edulis	Gill cells	Crude oil	Baršienė and Andreikėnaitė, 2007
Dreissena polymorpha	Hemocytes	Organochlorine insecticide	Binelli, Riva, Cogni & Provini, 2008
Meretrix ovum	Gill cells	Organophosphorous pesticide	Revankar and Shyama, 2009
Dreissena polymorpha	Gill cells and Hemocytes	Cadmium and benzo[a]pyrene	Vincent-Hubert, Arini & Gourlay-Francé, 2011
Crassostrea corteziensis	Gill cells	Organophosphorous pesticide	Benitez-Trinidad et al., 2014
Unio pictorum	Hemocytes	Benzo[a]pyrene	Eck-Varanka, Horváth, Ferincz, Paulovits & Kováts, 2014
Dreissena polymorpha	Hemocytes	Municipal wastewater	Thomas et al., 2014

Bioindicator Bivalve Species

In the present survey, the species *Dreissena polymorpha* (Pallas, 1771) and *Mytilus edulis* Linnaeus, 1758 stood out as the most used as bioindicators. The freshwater zebra mussel *D. polymorpha* is invasive and established in water bodies throughout Europe and North America and has recently colonized Ireland. As they reproduce rapidly and foul a wide range of structures, they are of serious concern to industry and environmental managers. Efficient suspension feeders, they occur in extremely high densities and cause considerable changes in ecosystem composition and function such as local extirpation of native mussel

populations (Strayer, Caraco, Cole, Findlay & Pace, 1999). While the overwhelming majority of publications on *Dreissena* concerns invasive range, information on distribution within their native range is limited, although we know that includes the Ukraine (Son, 2007).

The blue mussel *M. edulis* is widespread and found from northern parts of Spain in the south to Russia in the north. In Norway it is found along the entire coastline, but mainly up to Trondheimsfjorden, it thrives in protected waters and in fjords with brackish water. *M. edulis* is fastened to rocks or other surfaces by byssus and often found in dense populations in the tidal zone. It is also found on the west side of the Atlantic Ocean, from Carolina in the US to Newfoundland in Canada. Outside Europe, this is the only known spread of the *M. edulis* (Hovgaard, Mortensen & Strand, 2001). Blue mussel is one of the new species which have been pursued as a potential industry since the beginning of the 1970s (Ytrøy, 2008).

Chemicals Genotoxic

Many attempts to identify the chemicals responsible for the genotoxicity/mutagenicity of waters have been reported. Among these reports, researchers identified heavy metals, polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines, pesticides and so on. Chemicals with genotoxic and carcinogenic potential in the aquatic environment cause serious concern because they can bind to DNA molecules and trigger off a damaging chain of biological changes, such as an impaired enzyme function or general metabolism, cytotoxicity, immunotoxicity, reproduction disturbances, growth inhibition, or carcinogenesis (Ohe, Watanabe & Wakabayashi, 2004). Not all mutagens are carcinogens, but a wide range of scientific data reinforces the hypothesis that environmental factors are the leading cause of cancer. Table 3 presents genotoxic effects of some chemicals cited in text. The International Agency Research on Cancer's classification of these chemicals is also showed.

Table 3. Role of some genotoxic agents, and IARC classification of selected examples.

Aromatic hydrocarbons	Organochlorinated compounds	Metals
Generation of reactive oxygen species such as hydroxyl radicals and superoxide that attack DNA. Formation of epoxides, known DNA alkylating agents with high mutagenic activity. Benzene and benzo(a)pyrene, classified as group 1 (carcinogenic to humans).	Structural chromosomal rearrangements Inhibition of cell proliferation. Significant increase in the breaks in DNA strands. Tetraclorvinfós, classified as group 2B (possibly carcinogenic to humans).	Binding to DNA and proteins causing damage to DNA, altered gene expression, mutations, altered cell cycle, chromosome non-disjunction, and cytoskeleton dysfunction Arsenic and cadmium, classified as group 1 (carcinogenic to humans).

Conclusions

The MN test is quick and simple, and has been applied extensively to test the genotoxicity of chemicals, as polycyclic aromatic hydrocarbons, metals, and organochlorinated and organophosphorous compounds. On the other hand, bivalve mollusks are filters and therefore appropriate as sentinel organisms. Currently, in several countries, the MN assay has been successfully used with mollusks as biological monitors and has confirmed its usefulness as a biomarker for monitoring genotoxic pollution in aquatic environments.

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