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Novel *in vivo* experimental viability assays with high sensitivity and throughput capacity using a bdelloid rotifer

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ABSTRACT

Rotifers have been used in biological research as well-characterized models of aging. Their multi-organ characters and their sensitivity for chemicals and environmental changes make them useful as *in vivo* toxicological and lifespan models. Our aim was to create a bdelloid rotifer model to use in high-throughput viability and non-invasive assays. In order to identify our species *Philodina acuticornis odiosa* (PA), 18 S rDNA-based phylogenetic analysis was carried out and their species-specific morphological markers identified. To execute the rotifer-based experiments, we developed an oil-covered water-drop methodology adapted from human *in vitro* fertilization techniques. This enables toxicological observations of individual one-housed rotifers in a closed and controllable micro-environment for up to several weeks. Hydrogen peroxide (H_2O_2) and sodium azide (NaN_3) exposures were used as well-understood toxins. The *toxicity and survival lifespan* (TSL), the *bright light disturbance* (BLD) the *mastax contraction frequency* (MCF) and *the cellular reduction capacity* (CRC), indices were recorded. These newly developed assays were used to test the effects of lethal and sublethal doses of the toxins. The results showed the expected dose-dependent decrease in indices. These four different assays can either be used independently or as an integrated system for studying rotifers. These new indices render the PA invertebrate rotifer model a quantitative system for measuring viability, toxicity and lifespan (with TSL), systemic reaction capacity (with BLD), organic functionality (with MCF) and reductive capability of rotifers (with CRC), *in vivo*. This novel multi-level system is a reliable, sensitive and replicable screening tool with potential application in pharmaceutical science.

1. Introduction

Pharmaceutical and toxicological researches are aided by *in vivo* assays using small model organisms. Such models ideally should be multi-cellular and should meet the basic requirements of *in vitro* culturing methods. The ideal experimental methods should be non-invasive, sensitive and report on complex systems. The nematode *Caenorhabditis elegans* (CE) has been useful as a model organism for such studies despite being distant from mammals phylogenetically, with several vertebrate genes having no homolog (Snell, 2014).

Rotifers (phylum *Rotifera*) have similar advantages in culturing, anatomy, physiology and behavior similar to CE (Birky, 2004). They have the additional advantage that more than 10% of their genes have vertebrate homologs that can be found neither in nematodes nor in *Drosophila melanogaster* (Dahms et al., 2011). Rotifers are inexpensive to maintain, easy to handle, and have a relatively short lifespan; they are multicellular transparent animals with individual organs and nervous system (Snell et al., 1991). The bdelloids possess about 950–1000

Abbreviations: BLA, bright light avoidance; BLD, bright light disturbance; BLI, bright light irritation; CA, Cladophora aegagropila; CE, Caenorhabditis elegans; CRC, cellular reduction capacity; DW, distilled water; gDNA, genomic DNA; LCA, least common ancestor; MCF, mastax contraction frequency; mL, maximum likelihood; MSA, multiple sequence alignment; NJ, Neighbour-Joining; PA, Philodina acuticornis odiosa; bp, base pair; PM, Philodina megalotrocha; rDNA, ribosomal DNA; ROS, reactive oxygen species; TBE, Tris-Borate-EDTA buffer; TBS, Tris-buffered saline; TSL, toxicity and survival lifespan; XTT, (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

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somatic cells and have several well-described anatomic characteristics, such as ciliated head structure, bilateral ovaries, jaw-like mastax, ganglia, muscles, digestive- and secretory systems, photosensitive- and tactile organs (Marotta et al., 2012). They are resistant to harsh conditions (*e.g.* UV light, temperature, toxins) in their diapause stage. Only bdelloid rotifers have an uncommon degenerate tetraploid genome consisting of numerous genes acquired by horizontal gene transfer (Hagen et al., 2009). The reproduction of bdelloid rotifers is also obligatorily asexual, and males have been extinct for more than 30 million years. So, meiosis has never been documented in the bdelloid class (Gladyshev and Arkhipova, 2010).

Without any treatment or feeding, the bdelloid rotifers survive for approximately 15 days and are used as subjects of ecotoxicological research (Preston and Snell, 2001). Here, we validate novel assays to characterize *Philodina acuticornis odiosa* (PA) in terms of survival, health and behavior, named *toxicity and survival lifespan* (TSL), *bright light disturbance* (BLD), *mastax contraction frequency* (MCF) and *cellular reduction capacity* (CRC). The TSL provides mortality rate (without feeding). The BLD index measures photostimulus-triggered reflexes, individual ethology and behavioral changes (Eakin and Westfall, 1965; Snell, 2014). Bdelloids have specialized photosensitive organs ("primitive eyes"), and avoid bright light, providing information about the neurological reflexes of the subjects. CE is also sensitive to light, but its sensation is based on photochemical-triggered reactive oxygen species (ROS) production, which could be confounded by other factors of metabolism (Bhatla et al., 2015). The MCF index assays the chewing organ function, providing information about the resilience of individuals and the need for nutrition. Assaying grinder movement is difficult in CE, but, in contrast, MCF is facile for PA. CE can react to stress by entering dormancy, which can be mistaken for death (Trojanowski and Raizen, 2016). The MCF will only reach zero when the PA is dead. The CRC gives information about the degree of reduction capacity and oxidative stress triggered by treatment. These four *in vivo* screening assays give continuous and quantitative indices.

To validate the newly developed assays we tested responses to well-known toxins. Hydrogen peroxide (H_2O_2) is a biocide that can cause massive cellular damage. It removes electrons from the susceptible chemical groups, oxidizing them and overwhelming the antioxidant defense system (Russell, 2003). At low concentrations, the oxidation caused by H_2O_2 can be rescued·H_2O_2 is readily permeable through cell membranes, interacting with intracellular components. The physiological response to H_2O_2 is always similar (Yang et al., 2013). The molecule affects multiple targets, such as peroxidation or disruption of membranes, oxidation of scavengers and thiol-groups, enzymatic inhibition, oxidation of nucleosides, impairment of energy production, causing inhibition of protein synthesis (Imlay, 2003; Poeggeler et al., 2005).

The use of sodium azide (NaN_3) as an alternative insecticide, herbicide, nematocide, bactericide and fungicide has spread in the last few decades and its vasodilator effects are also well-established (Bennett et al., 1996). NaN_3 as a cytochrome oxidase inhibitor is able to inhibit the mitochondrial complex IV, therefore, it causes chemical hypoxia and lowers energy production (Ye et al., 2016). It can partially inhibit the electron transfer chain in a dose-dependent manner, leading to elevated ROS, mitochondrial membrane potential reduction and the ultra-structural changes in mitochondria (Morales-Cruz et al., 2014).

Here, we report the development and validation of novel *in vivo* experimental monitoring assays for the rotifer PA and providing reliable readout indices in four domains. The tests compile a non-invasive (TSL, BLD, MCF), invasive (CRC) and high-throughput screening index of the impact of different chemicals and environments on individual animals.

2. Materials and methods

2.1. Animal and plant

Among the bdelloid rotifers studied in our laboratory PA were considered suited for our methodological innovation because of their numerous advantages: short lifecycle, parthenogenic reproduction capability, discrete individual viability, ethological/behavioral markers and controllable culturing (Ricci, 1984).

The PA was obtained from Hungarian aquavaristique together with *Cladophora aegagropila* (CA; alternative names: *Aegogropila linnaei and/or Cladophora sauteri*) alga that is part of the environmental matrix of rotifers for living and reproduction (Hanyuda et al., 2002). This alga is found in an approximately 5–10 cm diameter spherical form in its natural habitat, but does not serve as a food source for animals, which feed off organic detritus or micro-organisms. In our experimental set-up, pasteurized *Saccharomyces cerevisiae* (yeast) homogenate was introduced as food (see in Section 2.2.). The filamentous algae were necessary for the optimal culturing to form 3D-matrix, increasing the specific surface area.

2.2. Culturing rotifers

Rotifer culturing methods were developed based on previous literature (Ricci, 1984). The animals were cultured in a supervised and semi-sterile environment. These cultures contain Jana mineral water (origin: Croatia; distributed by Fonyódi Ásványvíz Kft., Hungary), half diluted with distilled water (Millipore type ultra-pure, demineralized DW), a mixture nominated 'standard medium' (pH = 7.5). The amount of diluted cations and anions in standard medium (mg/L): Ca^{2+} 31.05; Mg^{2+} 17.6; Na^{+} 0.9; K^{+} 0.25; Fe^{2+} 0.001; HCO_3^{-} 153.097; SO_4^{-} 3; Cl^{-} 0.8; F^{-} 0.02; H_2SiO_3 3.3. Every step of the rotifer manipulation was monitored by microscope. Clear cultures of PA were kept in standardized cell culturing flasks (cat. no.: 83.3910.302, Sarstedt AG & Co., Germany) in 15 mL standard medium. The flasks contained CA alga fibers and sterile cotton wool buds in 1:1 ratio (mixed matrix) for structural stabilization. The thickness of algae/cotton wool matrix was ≈1 cm, thus significantly increasing the surface area for rotifer attachment, and so increasing the density of the culture from ≈8500 rotifers (2D; without matrix) up to ≈90,000 animals (3D; with matrix) per flask (after one month of culture, starting from one individual).

The CA alga balls were kept in standard medium, which was changed every three days. During dissection and isolation, a small part of the ball was removed using forceps and placed into a plastic petri dish (cat. no.: 430165, Corning Inc., USA). The resulting CA fragments, dispersed further into strings were washed once with 10% ethanol for 8 min and twice with standard medium for 30 min. The prepared alga strings were incubated for two days in standard medium and their quality checked daily by microscopy.

A further component of the culturing matrix was sterile cotton wool, which was dispersed with forceps into a petri dish and washed with DW thoroughly. Cotton wool fragments were then soaked in 96% ethanol for 30 min and then washed multiple times in DW. After two days the prepared pieces of cotton wool were put in a cell culture flask mixed with CA alga (1:1 vol ratio) in the medium and rotifers were then added. New cultures were started from previous ones by transferring one PA rotifer with standard medium. The flasks were kept at 25 °C and under a light/dark cycle of 12:12 h and were examined every 24 h under an inverted transparent light microscope (Leitz Labovert FS, magnification range from 32 x to 1000 x; Germany).

The culture medium was changed every two days. First, the culturing flasks were shaken carefully several times, then the old medium was decanted. As the next step, the mixed matrix was washed with abundant DW after which we supplied the standard medium again to the flask and checked the clarity of the medium under microscope. If the medium was visibly cloudy the procedure was repeated.