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The amphibian *Pelophylax bergeri* (Günther, 1986) testis poly(ADP-ribose)polymerases: relationship to endocrine disruptors during spermatogenesis

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Abstract
In a growing deterioration of the ecosystem both in terms of biodiversity and ecological features, even more problematic is the conservation of species. The aim of this work was to study seasonal variations of biochemical and molecular DNA damage markers in *Pelophylax bergeri* exposed to potential endocrine disrupting chemicals. Frogs were collected in the main phase of the reproductive cycle: in April and May, during active spermatogenesis and the breeding season, and in October and November, in spermatogenetic regression, from a sampling site in the polluted Sarno River and from a clean reference site (Matese Lake). DNA profile and poly(ADPribose) polymerase (PARP) activity were evaluated in the testis. In the main phases of the reproductive cycle, all specimens from Sarno River showed a PARP activity higher than that measured in the same frog's testis from Matese Lake. In addition, the PARP activity in active spermatogenesis was always higher compared with the activity in the spermatogenetic regression in Sarno River frogs. PARP2, usually activated in response to exposure to heavy metals, was expressed in all testes. In the species examined from Sarno River, no evident correlation between testis DNA damage and PARP activation was found. The working hypothesis is that PARP, implicated in genome surveillance and protection, might represent in frog spermatogenesis an appealing tool for genotoxic risk assessment useful to define a warning alarm for its survival.

Keywords: *Frog conservation, spermatogenesis, oxidative stress, Sarno River, poly(ADPribose)polymerases*

Introduction

Habitat destruction or fragmentation, climate change, predators or competitors, diseases and the presence of chemical pollutants represent the main causes of species extinction risk (Kryston et al. 2011). Constant exposure to indiscriminate use of herbicides, accidental spills or contaminant discharges into natural waterways have, however, major adverse effects on populations due to endocrine chemicals' accumulation in reproductive tissues (Guillette & Edwards 2008). Their cytotoxic effects are exerted through multiple mechanisms, but most of them are involved by reactive oxygen species (ROS) and reactive nitrogen species (RNS) that potentially damage lipids, proteins and DNA, and impair cell functions and reproduction (Abele & Puntarulo 2004; Guerriero & Ciarcia 2006; Bobe & Labbé 2010; Rothfuss et al. 2010). Conservation biology studies, which include population analysis, demographic surveys and reproductive biology, allow assessment of the conservation status of the species exposed to the potential effects of endocrine disruptors. The possibility of long-term survival of populations is facilitated by the identification of strategies for their management and protection to avoid severe genetic damage which threatens the survival of the organisms, and their reproductive capacity (Wilson & Roberts 2011). Endocrine disrupting chemicals (EDCs) are of high relevance for wildlife and human health, since they control many essential physiological processes such as gametogenesis and

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gamete quality (Frye et al. 2012). Many regulatory processes that occur at genomic, transcriptional and post-transcriptional levels, translational and post-translational, mediate the cellular stress responses (Kourtis & Tavernarakis 2011).

These responses require a complex network of sensors and effectors from multiple signaling pathways, able to ensure genomic integrity, such as the abundant and ubiquitous nuclear poly(ADP-ribose) polymerases1 (PARP1) (Luo & Kraus 2012).

Among PARPs, both PARP1 (113 kDa) and its cognate poly(ADP-ribose)polymerases2 (PARP2) (62 kDa) are the sole enzymes whose catalytic activity is immediately stimulated by DNA strand-breaks (Amé et al. 2004). PARP1 catalyzes the covalent attachment of ADP-ribose units from donor NAD^+ (Nicotinamide Adenine Dinucleotide) molecules on itself and other acceptor proteins (histones, DNA repair proteins, transcription factors and chromatin modulators), altering in a reversible manner their structure, function and localization (Hassa & Hottiger 2008; Kraus 2008; Krishnakumar & Kraus 2010). PARP2 is capable of auto-poly(ADP-ribosyl)ation and is involved in DNA repair (Schreiber et al. 2002), but it cannot modify histones, which are prototypical PARP-1 substrates (Amé et al. 2004). Both PARP1 and PARP2 are involved in the regulation of several cellular functions related to the maintenance of genomic integrity (DNA repair, gene amplification, apoptosis) and to the expression and propagation of genetic information (DNA transcription and replication, differentiation, neoplastic transformation (Kim et al. 2005; Hassa & Hottiger 2008; Gagné et al. 2009; Michels et al. 2013). Exposure to different kinds of environmental stresses, such as drought and heavy metals, seems to induce the gene of the poly (ADP-ribose) polymerase family, member 2 (Amor et al. 1998). Besides the nuclear PARPs, in the cytoplasm of different eucaryotic organisms like protozoa, molluscs, cephalopods, echinoderms, fish, amphibians, avians and mammalians, a vault poly(ADPribose) polymerase (VPARP) of 193kD was found.

This enzyme is one of three protein components of the vaults, ribonucleoprotein particles, containing small untranslated RNA molecules of 88–141 bases (De Lisa et al. 2012). As in animals, PARP1 and PARP2 homologues were also isolated in plants (see Arena et al. 2011 for review). Regulation of the poly (ADPribose) turnover and variations of PARP amount, as well as changes of PARP transcription level, involve germinal cell differentiation too, being implicated in DNA replication, repair and transcription (Aoufouchi & Shall 1997; Baarends et al. 2001). During meiosis, recombination events play an important role in creating genetic diversity among individuals within a population. Recombination involves the induction of double strand breaks (DSBs), followed by crossing over between homologues and ligation of DNA molecules. A functional relationship between poly(ADP-ribosyl)ation and spermatogenesis has been widely demonstrated (Celik-Ozenci & Tasatargil 2013). Studies related to PARP expression in testicular tissue have been performed mainly in rodents, and most of these studies found high PARP activity in spermatogonia and pachytene nuclei of spermatocytes (Faraone-Mennella et al. 2000; Di Meglio et al. 2003; Tramontano et al. 2005), and on tetraploid spermatocytes which undergo meiotic division (Quesada et al. 1996, 2000).

In humans, during spermatogenesis, alterations in sperm DNA topology occur, but the persistence of DNA strand breaks during different phases of spermatogenesis can contribute to DNA damage detected in mature spermatozoa and/or in infertility (Baarends et al. 2001; Laberge & Boissonneault 2005). DNA repair mechanisms are involved not only in the repair of different types of DNA damage in developing germ line cells, but also take part in the meiotic recombination process (Guerriero & Ciarcia 2006). Based on the degree and type of the stress stimulus, PARP directs cells to specific fates (such as DNA repair *vs.* cell death).

Furthermore, the DNA repair mechanisms should tolerate mutations occurring during gametogenesis, to a limited extent (Baarends et al. 2001; Guerriero et al. 2003). Excessive activation of PARP contributes to the pathogenesis of several diseases associated with oxidative stress, which has been known to play a fundamental role in the etiology of male infertility (Celik-Ozenci & Tasatargil 2013).

The reproductive organs are highly susceptible to EDC exposure during organ development and sexual differentiation and, to date, we do not have information about the potential role of PARPs biomarkers in testes of frogs of polluted areas.

To gain new insight into the environmental impact of EDCs on species conservation, the aim of the present research was to study the ADP(ribosyl)ation reaction in the testis of frogs collected from Sarno River, a known polluted site (De Pippo et al. 2006), and from a clean reference site (Matese Lake).

Here, we proposed to localize and identify the protein responsible for this reaction on the basis of its molecular weight, DNA-dependence and cross-reactivity with anti-PARP antibodies. In addition, we have carried out the biochemical analysis using frogs in the main phase of the reproductive cycle, in order to correlate possible variations of poly(ADPribosyl)ation levels with seasonal variation of DNA damage.

As nuclear PARPs are activated following genomic material damage, we have verified their use for monitoring the reproductive health of the poikilotherm frogs, *Pelophylax bergeri* (Günther, 1986), representative from Sarno River and available in Matese Lake, and their survival.

Materials and methods

Animals

Adult male frogs were collected with the help of the 'Sarno Friends' Association from Sarno River – exposed to a wide variety of EDCs, originating from discharged industrial, agricultural and municipal effluents – and from Matese Lake in the period from April 2011 to April 2013.

Frogs representative of Sarno River and available in Matese Lake, identified as *Pelophylax bergeri* (n = 5/selected month for 3 years), were examined during the main phase of the reproductive cycle: in April and May, during active spermatogenesis and the breeding season, and in October and November, during spermatogenetic regression. Pools of testes were combined and homogenized for biochemical and molecular procedures.

DNA extraction from frog testis

The DNA extraction from the frog testis was performed as reported in Di Finizio et al. (2007) with a phenol/chloroform standard method using autoclaved glassware and equipment.

About 50 mg samples of ground freeze-dried tissues were mixed in a DNA extraction buffer (50 mM NaCl, 10 mM EDTA (Ethylene Diamine Tetracetic Acid) and 10 mM Tris base) and the cells were lysed by adding 2% sodium dodecyl sulfate. The RNA was removed by adding RNAse (10 mg/mL) followed by incubation at 37°C for 30 min. Proteinase K was added (0.5 mg/mL) to remove protein and the samples were incubated for 1 h at 37°C in a shaking water bath. The extracts were further purified by extracting twice with phenol:chloroform:isoamyl alcohol (25:24:1 volume/volume) and by centrifuging at 10,000 × g for 15 min at 4°C. The upper aqueous layer was transferred into a new micro-centrifuge tube and the DNA was precipitated by adding 1/10th volume of 3 M sodium acetate at pH 5.2 and two volumes of 100% chilled ethanol to each sample, and centrifuging at 15,000 × g for 30 min at 4°C. The pellet was washed with 70% ethanol, air dried and finally re-suspended in 50 μL of sterilized deionized water. Optical density (OD) of each sample was measured at 260 and 280 nm respectively, by UV-spectrophotometer (Biochrom Libra S12), and the purity of DNA was measured by the OD260/OD280 ratio (ideal ratio = 1.7–2.0), and the quality by electrophoresis on a 0.8% agarose gel and visualized under UV light.

Agarose gel electrophoresis and staining for DNA

The purified DNA extracts together with a marker were loaded on a non-denaturing 0.5% agarose gel in TBE buffer (45 mM boric acid, 2.5 mM EDTA, 135 mMTris base, pH 8). Bromophenol blue was added to each sample before its loading in each gel to mark the migration of these extracts. Gel electrophoresis was carried out at 70 V for around 1 h using a horizontal mini gel electrophoresis system (VWR, UK). The migrating DNA was observed by staining the gel for about 40 min in the dark in 200 ml TBE buffer and 20 μL SYBR Green l. The stained gels were captured using Gene Snap Version 4.01.00 of Syngene. The migration distance of the DNA molecules from the top of the gel was used as a measure of DNA damage and was performed as previously published in Chaudhry and Jabeen (2011). The migration of the DNA is inversely proportional to the length of the DNA molecules, and so it was used to estimate the distribution of different strand lengths (fragments) together with the relative mobility (Rf) value, where Rf = the distance from the origin to the center of each fragmented DNA band on the gel, divided by the total distance travelled by the tracking dye from the top to the bottom of that gel. It is recognized that the highly fragmented, low molecular weight DNA strands will migrate farther than undamaged high molecular weight DNA strands. The gel photographs were used to estimate the Rf values of DNA fragments of the samples. The DNA fragments and their Rf values were then compared with those of the DNA markers to determine the effect of the river water environment on the DNA of captured frogs.

Nuclei isolation

The isolation of nuclei was performed according to Arena et al. (2011). All operations were carried out on ice or at 4°C. All tissue from frogs (1 g) was collected, shredded and resuspended in 10 mM TrisHCl pH 7.0, 1 mM EDTA, 1 mM EGTA, 1 mM $PhMeSO_2F$ (Phenyl Methane Sulfonyl Fluoride), 10 mM $MgCl_2$ (Magnesium Chloride), 5 mM 2-mercaptoethanol, and 0.5% Triton X-100 [1:4 weight/volume (w/v)/buffer A]. The samples were homogenized for 30–40 s at low speed by an Ultra Turrax T8 (IKA-WERKE). The homogenates were centrifuged at 1500 × g for 30 min at 4°C. The pellets containing nuclei were suspended in buffer A

and were centrifuged as above three times. Finally, the pellets (nuclear fractions) were washed with buffer A without Triton X-100 (buffer B) and suspended in a small volume of buffer B containing 2% glycerol.

Poly-ADP-ribose polymerase standard assay

The enzymatic activity was routinely assayed as reported in Arena et al. (2011). The reaction mixture (final volume 50 µL) contained 0.5 M Tris-HCl pH 8.0, 50 mM $MgCl_2$, 10 mM DTT, 0.4 mM [^{32}P] NAD+ (10,000 cpm/nmole) and a defined amount (20 µg protein) of whole nuclear fraction from examined tissues. After incubation for 20 min at 25°C, the reaction was stopped by transfer onto ice and addition of 20% (w/v) trichloroacetic acid (final concentration). The mixture was filtered through Millipore filters (HAWPP0001, 0.45 µm) and washed with 7% trichloroacetic acid. The activity was measured as acid-insoluble radioactivity by liquid scintillation in a Beckman counter (model LS 1701).

Protein analysis and western blotting

Nuclear fractions (20 µg) from frog testes were electrophoresed onto 12% polyacrilammide mini-gel in 0.1% sodium dodecyl sulphate (SDS), according to Arena et al. (2011). Staining was in 0.1% Comassie G in 10% acetic acid/30% methanol. Western blot analysis was performed by electrotransferring proteins to a PVDF (PolyVinyl DeneFluoride) membrane using the Biorad apparatus at 200 mA for 2 h at 4°C. Filter was incubated first with anti-poly(ADP-ribose)polymerase (H-250, Santa Cruz, CA, USA 1:1000) as primary antibodies and, thereafter, with the horseradish peroxidase-conjugated goat antirabbit as secondary antibody (Pierce, 1:2000).

The stripping procedure was used to remove the anti-PARP1 antibody from the PVDF membrane, to allow the incubation with anti-PARP1 (N20) (Santa Cruz 1:1000) polyclonal primary antibodies, and, thereafter, with the horseradish peroxidase-conjugated goat antirabbit secondary antibody (Pierce, 1:2000).

The stripping buffer contained 62.5 mM Tris-HCl (pH 6.8), 2% SDS and a final concentration of 0.1 M 2-mecaptoethanol. The filter was washed in TBST (Tris Buffered Saline with Tween) (1 × 5 min) and incubated in stripping buffer for 30 min at 50–60°C (in a heating oven). Washes in TBST (2 × 5 min) followed and membrane was blocked for 1 h in TBST 3% gelatine (Arena et al. 2011).

Immunodetection by Enhanced Chemiluminescence (ECL, Celbio) was carried out by a Quantity One Program in a P-imager (Bio-rad).

Statistical analysis

The results are expressed as a mean ± standard deviation. Data were analyzed by analysis of variance (ANOVA) and considered statistically significant at $p < 0.05$ (*) and $p < 0.001$ (**).

Results

DNA analysis

DNA profiles of *Pelophylax bergeri* frog testis (Figure 1) are shown on the gel photographs from all testes examined in the different phases of the reproductive cycle, from both Sarno River and Matese Lake. DNA extracted from Sarno River frogs' testes appeared without damage, as did the same tissue from Matese Lake frogs, in both examined cycle phases.

PARP activity

PARP activity was measured in pooled testis nuclear fractions from *Pelophylax bergeri* collected from Sarno River and Matese Lake in the main phases of the reproductive cycle. In frog testis, the highest PARP activity is measured when spermatogenesis is active (Figure 2). Therefore, we found a statistically significant difference of PARP frog testis activity between Sarno River different phase samples (*$p < 0.05$, 3–4 vs 1–2) and two samples sites (**$p < 0.001$, see 5–8 vs 1–4).

PARP identification

Electrophoretic analysis of nuclear fractions from examined testes from frogs collected in April and

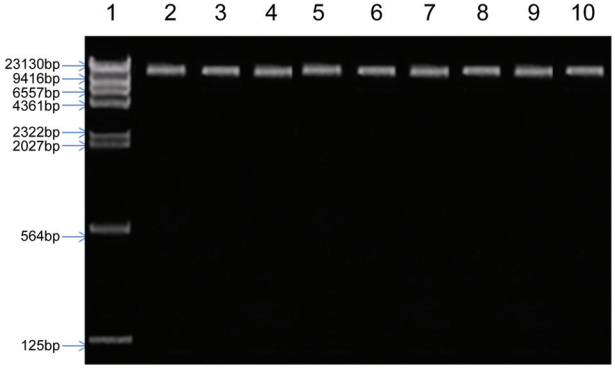

Figure 1. DNA profile in *Pelophylax bergeri* testis. (**1**) L 100 bp DNA ladder; (**2**) standard positive control; frog testis fraction **a** from Sarno River in reproductive phase: (**3**) April, (**4**) May, and in spermatogenetic regression: (**5**) October and (**6**) November; **b** from Matese Lake in reproductive phase: (**7**) April, (**8**) May, and in spermatogenetic regression: (**9**) October and (**10**) November.

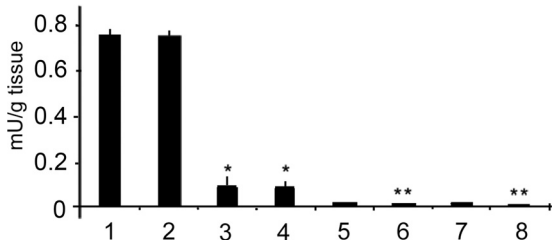

Figure 2. PARP (Poly(ADPR)polymerase) activity in *Pelophylax bergeri* testis. Frog nuclear fraction **a** from Sarno River in reproductive phase: (**1**) April, (**2**) May, and in spermatogenetic regression: (**3**) October and (**4**) November; **b** from Matese Lake in reproductive phase: (**5**) April, (**6**) May, and in spermatogenetic regression: (7) October and (**8**) November. The results (n = 5/selected month for 3 years) were analysed in triplicate and expressed as mean ± SD. *p < 0.05; **p < 0.001.

May during the reproductive cycle did not show remarkable qualitative and quantitative differences in protein patterns (Figure 3A).

Immunoblotting of all examined samples, performed with antibodies against the PARP C-terminal catalytic domain, showed a single immunoreactive band, corresponding to a protein with molecular weight of 62 kDa (Figure 3B). This molecular weight corresponds to that of mammalian PARP-2.

To confirm this hypothesis, the same samples were subjected to western blotting with anti-PARP1 (N20) antibodies able to recognize the N-Terminus zinc finger DNA-binding domain, characteristic of only PARP-1. No immunopositivity is evident in correspondence of frog samples, while in the nuclear fraction from bull testis, used as control, a net immunopositive band corresponding to PARP-1 (113 kDa) is evident (Figure 3C).

Discussion

To gain new insight into the environmental impact on species-specific conservation, this research was aimed at finding novel and specific markers to monitor DNA damage in adult male frogs, *Pelophylax bergeri*, a poikilotherm species not considered threatened by the International Union for Conservation of Nature (IUCN) and site representative of the areas selected.

As the DNA strand breaks directly and immediately activates PARP1 and PARP2 (Amé et al. 2004), in this experiment, first we proposed to identify which PARP was expressed in the testis of *Pelophylax bergeri* collected from Sarno River (polluted site) and Matese Lake (control site), during the main spermatogenesis phases.

Subsequently, in the same frogs, we verified whether variations of PARP activity occur, to correlate DNA damage to PARP activation and its repair capacity. It is known that both PARP1 and its cognate PARP2 are essential in DNA repair mechanisms, being involved in the base excision repair pathway, and exert their functions for both mild and extensive DNA damage (Schreiber et al. 2002). PARP2 is preferentially expressed and activated in response to exposure to heavy metals; for this reason, on the basis of our analysis by western blotting, we supposed that frogs collected from Sarno River are subjected to this type of pollution. The metals are reported as endocrine disruptors (Iavicoli et al. 2009) and their effects on reproduction could be varied by individual differences in metabolism, body composition and susceptibility due to genetic polymorphisms (Diamanti-Kandarakis et al. 2009, 2010).

The evidence that, in all examined tissues of frogs collected from Sarno River, the PARP activity in April and May is always higher than that measured in October and November, leads us to suppose that during active spermatogenesis the genomic material is damaged by the over-expressed free radicals inducted by the stressor insult of pollution. This appears to be in agreement with what is known from the literature (see for detail Baarends et al. 2001; Guerriero & Ciarcia 2006 for a review).

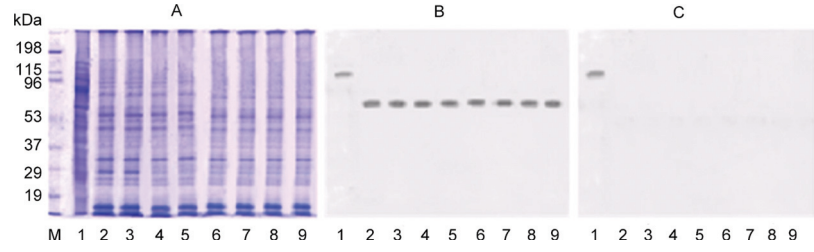

Figure 3. (**A**) SDS-PAGE of nuclear fractions from *Pelophylax bergeri* testis; (**B**) western blotting with anti PARP (H-250) on nuclear fractions from frog testis; (**C**) anti-PARP (N-20) antibodies on nuclear fractions from frog testis. (**1**) Buffalo testis nuclear fraction; frog nuclear fraction **a** from Sarno River in reproductive phase: (**2**) April, (**3**) May, and in spermatogenetic regression: (**4**) October and (**5**) November; **b** from Matese Lake in reproductive phase: (**6**) April, (**7**) May, and in spermatogenetic regression: (**8**) October and (**9**) November.

Similar reproductive problems occur in many Sarno wildlife species (data in progress).

Despite the activation of PARP, no DNA fragmentation was observed in the testis, where, probably, the DNA damage is not more extensive (Guerriero et al. 2011). The major activity of PARP in Sarno River frogs might be due to exposure to pollutants. PARP activity of Matese Lake frog testis during active reproduction represents, moreover, the poly(ADP-ribosyl)ation involved in chromatin remodeling during meiosis and spermatid nuclear condensation (see for review Celik-Ozenci & Tasatargil 2013) and necessary for maintaining normal spermatogenesis.

On the basis of the present results, we suggest PARP2 as a useful genotoxic marker to monitor the DNA damage in a one-testis gonadectomy of *Pelophylax bergeri* that lives in polluted environments. Further studies carried out on the other frog populations could contribute to extend its use.

In the testis, in which DNA fragmentation is not observable, PARP activation might represent a strategy to guarantee DNA repair, indispensable for the health of species and their reproductive capacity.

Furthermore, studies in mammalians reported by Catriona et al. (2007) seem confirm the seasonal relevance of our data in the amphibian *Pelophylax bergeri*, and highlight the importance of the differences in the timing of the vulnerability of genotoxic events.

In fact, levels of proteins involved in detection of DNA damage and in DNA repair are very high in the germ cell precursors for sperm and eggs too, and remain elevated in spermatocytes up until a transition period that precedes spermiogenesis. After meiosis is completed, haploid spermatids enter a period during which they are repair-deficient, and the condensed DNA is transcriptionally inactive, and inaccessible to DNA repair enzymes. Thus, it is critical for any DNA damage, incurred during crossing over and other phases of meiosis, to be detected and repaired prior to spermiogenesis (Marchetti & Wyrobek 2005).

Although direct causal links between exposures to EDCs, PARP activity and disease states in our biosensor *Pelophylax* are difficult to draw (we have only evidence of three gonads or damaged parts, data not shown), results from basic research and previous geomorphology studies (De Pippo et al. 2006) make it clear that more screening for exposures and targeting at risk groups is a high priority for Sarno River. The growing number of reports demonstrating that common environmental contaminants and natural factors possess endocrine disruptor activity presents the working hypothesis that the adverse trends in male reproductive health may be, at least in part, associated with exposure to estrogenic or other hormonally active (e.g., antiandrogenic) environmental chemicals (Diamanti-Kandarakis et al. 2010).

Innovative technologies by microassay and/or non-invasive tissue collection designed to improve the assessment of frog exposure and reproductive and endocrine health endpoints should be temporal applied, especially in spermatogenetic regression looking as well at poly(ADP-ribose)polymerase activity.

In conclusion, we suppose that a controlled formation of DNA strand breaks together with an efficient repair system of genomic material might represent a successful strategy to avoid detrimental damage during the spermatogenesis of frogs exposed to environmental endocrine disruptors.

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