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Early mitochondrial fragmentation is a potential in vitro biomarker of environmental stress

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ABSTRACT

Mitochondria are essential dynamic organelles that ordinarily balance between fragmentation and fusion. Under stress conditions, a shift toward fragmentation or hyper-fusion is observed as a pro-survival reaction. Fragmentation of mitochondria occurs within minutes or hours after the beginning of the stress and occurs in response to a large number of stress stimuli, including those triggered by environmental contaminants. In this study, we tested whether the change in the mitochondrial phenotype, from tubular to fragmented, could be used as a potential environmental stress biomarker in cells and compared this response with the standard MTT-based viability assay. Firstly, we show that mitochondrial fragmentation induced by selected stressors not only increases with concentrations, but also correlates positively with the cytotoxicity. Secondly, we found that the mitochondrial fragmentation that occurs in the first hour of stress correlated with the viability measured after a 24-hour stress, allowing the establishment of a linear relation between mitochondrial fragmentation at 1 h and the predictable associated cytotoxicity of environmental contaminants alone or in mixture. In conclusion, we have succeeded in developing

a model of predictable 24 h-cytotoxicity given mitochondrial fragmentation at 1 h with a set of chemicals. This model has been successful applied to three environmental toxicants and to a set of two chemical mixtures. We thus propose that mitochondrial fragmentation is a response that could be used as an early in vitro biomarker of environmental stress for toxicants alone or in mixture.

Keywords: mitochondrial morphology, cytotoxicity, environmental toxicants

1. INTRODUCTION

Mitochondria are organelles that play major roles in cells such as the production of chemical energy in the form of ATP generated by oxidative phosphorylation, but also the control of Ca²⁺ homeostasis, the regulation of redox biology or the biosynthesis of multiple metabolites (Ahn and Metallo, 2015; Vercesi et al., 2018). In addition to these pathways, mitochondria are largely involved in cellular-stress response and are key regulators in the outcome of cellular death or life (Westermann, 2010). In living cells, mitochondria are highly dynamic organelles, which balance between large interconnected networks (tubular phenotype) under physiological conditions and hyper-fused or fragmented (due to fission) forms in non-physiological conditions (Eisner et al., 2018). Although many stressors target other cellular components than the mitochondrial network, they often converge towards modification of the mitochondria phenotype, reflecting their key role in cell survival. Stress-induced mitochondrial hyper-fusion leads to optimal mitochondrial ATP production and allows the functional complementation of damaged mitochondria conferring stress resistance (Tondera et al., 2009; Gomes et al., 2011), but increased fission also aims to eliminate portions of damaged mitochondria by mitophagy, to allow cell survival (Kim et al., 2007). An interesting feature of hyper-fusion or of increased fission is that they occur very early during stresses, within minutes or hours depending on the stress type (Benard and Karbowski, 2009; Liesa et al., 2009; Meyer et al., 2017). During the last decades, the possible effects of chronic or acute environmental exposure to chemical and physical agents on human health have been of much concern. One of the major challenges for regulatory agencies is to evaluate human health risk of

environmental exposure not only to a single toxicant but also to a “cocktail” of compounds (Bonnefoi et al., 2010). In this context, the identification and characterization of new biomarkers affected by a large variety of chemical or physical insults appears necessary. Since mitochondrial fission is implicated in human pathologies such as aging or neurological diseases (Otera et al., 2013), and since a large number of environmental chemicals have been shown recently to induce fragmentation of mitochondria (Parameyong et al., 2013; Xu et al., 2013; Aravamudan et al., 2014; Barbosa et al., 2014; Hirata et al., 2016; Bobylev et al., 2017; Yamada et al., 2017), we tested to which extent it could be a general in vitro biomarker for exposure to environmental stress and cytotoxicity measurement.

Here, we correlated mitochondria fission with cell viability after a 24-h stress, using compounds that disturb intracellular calcium homeostasis, increase reactive oxygen species production or deplete the pool of ATP. We used the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and the inhibitor of the respiratory chain rotenone (Ishihara et al., 2003; Koopman et al., 2007), but also hydrogen peroxide (H₂O₂) and sodium chloride (NaCl) both also known to induce mitochondrial fragmentation (Fan et al., 2010; Perdiz et al., 2017). We further demonstrate that the correlation we observed between mitochondrial fragmentation after 1 h and cell viability measured after a 24 h-stress can be used to predict the cytotoxicity of environmental contaminants alone or in mixture. We thus propose that mitochondrial fragmentation could be used as an early in vitro biomarker of cytotoxicity for environmental contaminants that target directly or indirectly the mitochondrial network.

2. MATERIALS AND METHODS

2.1. Chemicals and antibodies

Dimethyl sulfoxide (DMSO), CCCP (CAS number 555-60-2), rotenone (CAS number 83-79-4), H₂O₂, NaCl, methoxychlor (MTX; CAS number 72-43-5), bisphenol A (BPA; CAS number 1675-54-3), 4-nonylphenol (NP; Fluka technical mixture, CAS number 84852-15-3), staurosporin (STS) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma Aldrich. Apart of H₂O₂ and NaCl, all chemicals were diluted in DMSO that never exceeded 0.1% in culture medium. Mouse monoclonal anti-TOM20 antibodies were from BD Biosciences and secondary Alexa fluor 555-conjugated goat anti-mouse antibodies were purchased from Invitrogen.

2.2. Cell culture and stress conditions

Human malignant epithelial HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM from ATGC) containing 4.5 g/L D-Glucose, supplemented with 1% sodium pyruvate, 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) under 5% CO₂ at 37°C. For stress induction, culture medium was replaced by freshly prepared medium containing indicated concentrations of chemicals or indicated dilution of environmental contaminants mixtures.

2.3. Environmental contaminants mixture preparation

Two mixtures of environmental contaminants were extracted from River Seine (France) sediments according to Kinani et al. (2008). Briefly, 3 g of freeze-dried sediment were extracted four times with 9 mL of a hexane/acetone (2/1, v/v) mixture. For each extraction step, the sample was sonicated 10 min at room temperature and then centrifuged 10 min at 3000 rpm. The collected organic supernatants were combined, evaporated to dryness under a gentle stream of nitrogen and reconstituted into 300 µL of DMSO. HeLa cells were exposed to dilutions of 1/1000, 1/2000 and 1/4000 of each organic extract, in parallel with a 1/1000 dilution of the extraction blank.

2.4. Mitochondrial fragmentation assay

The mitochondrial fragmentation assay is based on the estimation of the mitochondrial network fragmentation by measuring lengths of mitochondria. After seeding cells on glass coverslips, cells were stressed or not, rinsed with Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 15 min at room temperature. After three washes with Ca²⁺/Mg²⁺-free PBS, cells were then permeabilized with methanol for 5 min at -20°C, next rinsed extensively with Ca²⁺/Mg²⁺-free PBS before being staining with the mitochondrial import receptor subunit antibody TOM20 (1/100) and Alexa fluor 555-conjugated goat anti-mouse (1/1500) antibodies for 1 h at 37°C in PBS supplemented with 10% bovine serum albumin. Washes between incubations were made with PBS. Images were acquired using a Scion CFW1312M CCD camera, on a Leica DMLB microscope (x40 objective). Values of mitochondrial lengths (in µm) were obtained using the Image J (NIH) 1.52g *software* and results were

presented as a population of at least 400 mitochondrial lengths from 30-50 cells per conditions performed independently in triplicate.

2.5. MTT-based viability assay

This assay is based on the ability of dehydrogenases in viable cells to cleave the tetrazolium ring of the MTT yielding purple formazan crystals. After cells were stressed or not for 24 h and consecutively rinsed with PBS, they were incubated for 2 h at 37°C in culture cell medium containing MTT at 0.5 mg/mL. Then the medium was removed, and the purple formazan product was dissolved in DMSO. Absorption was measured on a microplate reader at 540 nm (reference set to 630 nm).

2.6. Statistical analysis

Relative cell viability data were expressed as the mean of at least three independent assays \pm standard error of the mean whereas mitochondrial length was presented using a box-and-whisker plot (median with upper and lower quartile; whiskers with 10th and 90th percentile) of all mitochondrial length values extracted from all individual cells. Statistical analysis was performed using a parametric or a non-parametric analysis of variance given the Brown-Forsythe test result (GraphPad Prism 7.00 *software*). Concerning the MTT-based viability assay, linear relationship between dose of chemicals and cell survival was analyzed using a covariance analysis. Relationship between mitochondrial length and concentrations of chemicals was performed using the non-parametric Kruskal-Wallis test whereas mitochondrial length between treated and untreated cells for each treatment time with chemicals were compared using the Dunn's multiple comparisons test. Adjusted p values are given with a family-wise significance and confidence level setting at 0.05 (GraphPad Prism 7.00 *software*).

Correlation between the relative mitochondrial tubular phenotype and the relative cell viability was performed using the Pearson's-product moment correlation. The comparison between the relative cell viability obtained with the MTT-based viability assay and the predicted viability obtained from mitochondrial fragmentation assay was realized using the non-parametric Wilcoxon signed rank test. Results were considered as statistically significant when $p < 0.05$.

3. RESULTS

To correlate the MTT-based viability assay and the degree of mitochondrial fragmentation, CCCP, H₂O₂, NaCl and rotenone were selected as stressors for their toxicological properties. CCCP and rotenone are more specifically active on mitochondria whereas H₂O₂ and NaCl have a more systemic activity (Burg et al., 2007; Di Marzo et al., 2018). CCCP is a well-known chemical that targets the mitochondria as a proton ionophore and inhibits the oxidative phosphorylation owing to the loss of membrane potential. It causes a rapid fragmentation of mitochondria mostly ascribed to the proteolysis of the fusion protein OPA-1 (van der Bliek et al., 2013). More recent studies showed also that CCCP could also provoke the translocation of the major fission protein DRP1 from the cytoplasm to mitochondria, which is a prerequisite for mitochondrial fission (Park et al., 2018; Kwon et al., 2018). Rotenone inhibits the complex I of the electron transport chain that leads to electron leakage within the mitochondrial matrix and creates reactive oxygen species. This chemical has been shown to induce mitochondrial fragmentation (Koopman et al., 2007) in an AMPK-dependent manner (Toyama et al., 2016). NaCl is responsible of a hyperosmotic stress that induces numerous cellular responses (Burg et al., 2007). Among these selected chemicals, we have previously shown that NaCl is an effective mitochondrial fission inducer (Perdiz et al., 2017). H₂O₂ is an oxidizing agent, which targets cellular compounds such as proteins, lipids or DNA, when converted into hydroxyl radicals into the cell. To a lesser extent than NaCl or rotenone, H₂O₂-mediated stress deeply modified mitochondria morphology by increasing their fragmentation (Barsoum et al., 2006; Jendrach et al., 2008; Fan et al., 2010). Since the MTT-based viability assay measures mitochondrial enzyme activity, it has been intentionally used in this study to better detect cytotoxicity coupled to mitochondrial fragmentation.

3.1. MTT-based viability and mitochondrial fragmentation assays after a 24 h–cell stress by the selected CCCP, H₂O₂, NaCl and rotenone stressors.

In order to compare the cytotoxic potential of CCCP, H₂O₂, NaCl and rotenone assessed by the fragmentation approach with this measured by the MTT-based viability, HeLa cells were exposed for 24 h with a range of concentrations of these stressors before measurements of relative cell viability and mitochondrial length (Fig. 1). As expected, the relative cell viability decreased significantly in a

concentration-dependent manner ($p < 0.05$) and regardless of the chemical used (Fig. 1a). Rotenone was the most potent toxicant since the concentration required to inhibit 50% of the cell population (IC_{50}) was $0.05 \mu\text{M}$, followed by CCCP ($40 \mu\text{M}$), NaCl ($125 \mu\text{M}$) and H_2O_2 ($350 \mu\text{M}$).

Along with the viability assay, mitochondrial fragmentation was investigated after 24 h of HeLa exposure to the same concentration ranges of target stressors. The length of the mitochondria was subsequently measured and then the set of values corresponding to three independent experiments were collected and represented as a box-and-whisker plot. As for the viability assay, the length of mitochondria decreased significantly as a function of the dose ($p < 0.0001$) whatever the chemical used (Fig. 1b). Again, rotenone was the most potent chemical to induce mitochondrial fragmentation since the concentration that reduced the mitochondrial length by 50% compared to the control was $0.2 \mu\text{M}$, followed by CCCP ($60 \mu\text{M}$), NaCl ($100 \mu\text{M}$) and H_2O_2 ($400 \mu\text{M}$). When compared to the literature (Ishihara et al., 2003; Sandebring et al., 2009; Reis et al., 2012), most of the studies also described mitochondrial fragmentation using these chemicals, but only with short exposures (a few hours). In good agreement with our results, longer exposures (15 h to 72 h) of HeLa cells to FCCP (an uncoupling agent equivalent of CCCP) or to H_2O_2 also induced marked mitochondrial fission (Pletjushkina et al., 2006; Jendrach et al., 2008). This was also the case with rotenone so that 50% of the cells treated with $0.1 \mu\text{M}$ for 24 h exhibited significant fragmentation (Sandebring et al., 2009). Among the stressors initially selected for the study, only the protein kinase C (PKC) inhibitor STS has been rejected given the absence of significant mitochondrial fragmentation for a 24 h-stress duration in the dose range from 0.015 to $0.1 \mu\text{M}$ despite significant decrease of viability over this range of concentrations (Fig. S1).

3.2. Correlation between relative cell viability and relative mitochondrial tubular phenotype after a 24 h-cell stress by the selected CCCP, H_2O_2 , NaCl and rotenone stressors.

We first assumed that cell survival was proportional to the lengths of mitochondria and therefore considered that the measured population of mitochondrial lengths for the untreated cells corresponded to a mitochondrial population with a 100% tubular phenotype. By measuring the decrease of mitochondrial length when cells are stressed, any decrease in cell survival could be associated with an increased mitochondrial fragmentation. Thus, for each stressor and concentration, the mean proportion

of surviving cells was plotted against the mean proportion of mitochondrial tubular phenotypes. As shown in Fig. 2a, a linear and positive correlation between the relative cell viability and the relative mitochondrial tubular phenotype was established for each stressor, with correlation coefficient r that ranged from 0.904 for CCCP to 0.980 for NaCl. Gathering the data for all the stressors into a single figure, we observed a positive and significant ($p < 0.00009$) correlation ($r = 0.696$) between relative cell viability and relative mitochondrial tubular phenotype (Fig. 2b).

3.3. Identification of a common cell exposure time for the selected CCCP, H_2O_2 , NaCl and rotenone stressors inducing both a maximal and an early mitochondrial fragmentation.

Since the dynamic remodeling of mitochondria is known to appear within minutes under cell stress (Benard and Karbowski, 2009; Liesa et al., 2009; Meyer et al., 2017), we next asked if such a correlation between cell viability and mitochondrial fragmentation after a 24 h-cell stress could also be applied to an early mitochondrial fragmentation phenotype. Kinetics of mitochondrial fragmentation between 0 and 24 h with 40 μ M CCCP, 125 μ M NaCl, 400 μ M H_2O_2 or 0.075 μ M rotenone, *i.e.* close to their IC_{50} as established above (*see* 3.1) were therefore explored. As shown in Fig. 3a, mitochondrial fragmentation appeared very early since a significant decrease in the overall length of mitochondria was observed within the first 30 min of exposure, whatever the stressor used. Cells treated with H_2O_2 and rotenone for 24 h showed however a slight recovery from mitochondrial fragmentation relative to those treated for only few hours but still significant relative to the control (non-parametric Dunn's multiple comparisons test; $p < 0.0001$). For example, the mean mitochondrial length in cells treated with H_2O_2 for 24 h reached 3.3 μ m compared to 4.3 μ m in control cells.

Cells treated with 0.015 μ M STS close to its IC_{50} showed a significant fragmentation of their mitochondrial network within the first hour followed by a significant recovery until the end of the treatment that ultimately reached control mitochondrial lengths (Fig. S2). The reversibility of mitochondrial fission during cell stress has already been described with thapsigargin. Indeed, normal rat liver cell line treated with this chemical for 48 h revealed that mitochondria undergo fragmentation and

recovery during the first two hours of stress following by an elongation period (from 4 to 20 h), and again by a re-fragmentation (>32 h) (Hom et al., 2007).

Finally, the statistical analysis showed that the most significant difference in the mitochondrial length between control and the duration of stressed-cells was 1 h when including all the chemicals (non-parametric Dunn's multiple comparisons test; $p < 0.0001$). Fig. 3b gave an illustration of representative mitochondrial networks of stressed-cells with CCCP, H_2O_2 , NaCl and rotenone for 1 h relative to the control.

3.4. Correlation between relative cell viability after a 24 h-cell stress and relative mitochondrial tubular phenotype after a 1 h-cell stress by the selected CCCP, H_2O_2 , NaCl and rotenone stressors.

To determine if the stress-induced mitochondrial fragmentation after 1 h reflected the relative cytotoxicity after a 24 h-cell stress, the relative cell viability at 24 h was plotted for all concentrations of selected stressors against the relative mitochondrial tubular phenotypes calculated after 1 h of stress. As shown in Fig. 4a, a linear and positive correlation between these parameters was observed for each chemical used: CCCP ($r=0.997$), NaCl ($r=0.969$), H_2O_2 ($r=0.989$) and rotenone ($r=0.942$). The global data plotting from all the chemicals also showed a positive and linear ($r=0.824$) correlation (Pearson's-product moment; $p=0.0001$) (Fig. 4b). In conclusion, the level of early mitochondrial fragmentation during cell stress could be predictive of the cell survival as measured by a MTT-based viability assay according to a linear relationship (Fig. 4b).

3.5 Estimation of the relative predicted-cell viability at 24 h from relative mitochondrial tubular phenotype after a 1 h-cell stress by environmental contaminants alone or in mixture.

After its validation on the selected stressors as an early stress in vitro biomarker for cytotoxicity, the mitochondrial fragmentation assay was applied to representative anthropic environmental contaminants the organochlorine insecticide metoxychlor (MTX), the bisphenol A (BPA) used in plastics and epoxy resins and the 4-nonylphenol (NP) as metabolite of the non-ionic surfactants alkylphenol ethoxylates

used in industrial, domestic and commercial applications (Cummings, 1997; Michalowicz, 2014; Acir and Guenther, 2018). All these three chemicals are environmental endocrine disruptors (Kavlock et al., 1996; Sonnenschein and Soto, 1998) but BPA and MTX might act also as potential contributors to environmental carcinogenesis by conferring resistance to cell death (Narayanan et al., 2015).

Three concentrations inducing low, middle or high cytotoxicity levels at 24 h were selected for each chemical and used as cell stressors to measure the proportion of mitochondrial tubular phenotypes after a 1 h-cell stress from which the predicted viability at 24 h was estimated (Table 1). The latter was derived for any concentration of environmental chemicals from the linear regression parameters established during the validation step of our model of cytotoxicity based on the mitochondrial tubular phenotype (Fig. 4b). As shown in Table 1, the relative cell viability was inversely proportional to the concentrations of MTX, BPA and NP used. For example, low (94%), mild (57%) and high (9%) loss of viability were caused by 10^{-6} , 5×10^{-6} and 10^{-5} M of NP, respectively. Importantly, there was no significant difference between the experimental values of cytotoxicity from the MTT-based viability assay and the predicted ones from the relative mitochondria tubular phenotypes (Table 1; non-parametric statistical Wilcoxon signed rank test). We also quantified mitochondrial fragmentation in cells treated with STS and found that lethality calculated with the MTT-based viability assay for a 24 h-cell stress was correlated with the cytotoxicity estimations based on mitochondrial fragmentation measured at 1 h. Since recovery from mitochondrial fragmentation has been observed for long-time STS exposure (*see above*), this emphasizes the interest to explore mitochondrial fragmentation early.

Our model of cytotoxicity based on the mitochondrial tubular phenotype was finally applied to environmental river sediments that are expected to contain a mixture of contaminants from anthropic and natural sources. Indeed, among environmental compartments, the aquatic one may be considered as the final recipient of the pollutants coming from the atmosphere, storm water or treated wastewaters. A representative matrix as a fingerprint of this pollution is river sediment, since it concentrates contaminants with a wide range of polarity (Kinani et al., 2010). Three dilutions of two organic extracts from two river sediment mixtures # 1 and # 2 were used (Table 1). HeLa cells exposed for 24 h to these dilutions exhibited relative cell viability that ranged from 76% to 46% and from 84% to 52% for the mixtures # 1 and # 2, respectively. Interestingly, the corresponding relative predicted viability ranged

from 98% to 64% and from 95% to 66% for mixtures # 1 and # 2, respectively. Again, the non-parametric statistical Wilcoxon signed rank test showed no significant difference between the relative cytotoxicity obtained with the MTT-based viability assay and the predicted cytotoxicity obtained from the experimental measurement of the tubular phenotype of mitochondria.

4. DISCUSSION

In this study, we propose a new way to screen rapidly the cytotoxic potential of environmental contaminants, alone or in mixture that impact directly or indirectly the mitochondrial network. This should be the case for many chemicals since more than 40% of a set of toxicants (367/967) have been shown to act directly on this organelle while another 22% (91/967) provoked an oxidative stress known to impact on the mitochondrial functions (Shah et al., 2016). Like for the MTT-based viability assay, which consists in a quantification of the activity of the mitochondrial succinate dehydrogenase, our model reveals the impact of cell exposure to chemicals on the mitochondrial network. Of course, this is not a substitute to conventional cytotoxicity assays and one should keep in mind that our results were obtained using human carcinoma cells cultured in a medium containing high glucose (4.5 g/L) which has been shown to render cancer cells more resistant to mitochondrial toxicants (Marroquin et al., 2007). However, the most interesting finding here is that regardless of the biological outcome (death or life) and in the contrary of the MTT-based viability assay which takes as much as two days to measure an end point cytotoxicity, mitochondrial fragmentation reliably functions as an early stress-sensor, within minutes after the beginning of the stress and could be directly correlated with cytotoxicity. Stressors that are known to induce mitochondrial fragmentation are multiple and despite limited data, it is assumed that the biological role of this response may differ regarding the dose level of a given stressor (Youle and van der Bliek, 2012). Whereas irreversibly-damaged cells are eliminated at high dose levels due to the links between mitochondrial fission and apoptosis (Hoppins and Nunnari, 2012), at low dose levels, the selective degradation of damaged mitochondria may help to avoid cell death by an adaptive mechanism (Pickles et al., 2018). Overall, low level or chronic exposure would be associated to a mild increase in fission, whereas high level or acute exposure would cause a high level of mitochondrial fission (Meyer et al., 2017). Since our results showed that mitochondrial fragmentation was also

observed for low dose toxicity (*see* Table 1), our model could be relevant for low level exposure studies, thus mimicking chronic exposure, which is closer to the conditions of human exposure to environmental contaminants. In this respect, the study of the mitochondrial homeostasis as a relevant biochemical model for a better understanding of cell stress-adaptative responses in low-dose region has been proposed by Yuan et al. (2016). An extensive analysis with more chemicals would be needed to definitively confirm this hypothesis.

Furthermore, our results point out that the outcome in terms of cell death is not only a matter of dose but also of duration of cell exposure to the stressor. This has been shown previously with thapsigargin, which is able to induce a reversible mitochondrial fragmentation during the first two hours of stress followed by a second fragmentation phase initiated during apoptosis at 32 h (Hom et al., 2007). Among the chemicals we initially selected and that were known to promote mitochondrial fragmentation, only the PKC inhibitor STS had been rejected given the insufficient correlation between mitochondrial fragmentation and cytotoxicity after a 24 h-cell stress. However, the good correlation observed between the actual cytotoxicity and mitochondria fragmentation at 1 h, including with STS, overcame this problem and clearly pointed out the advantage of using the early mitochondrial phenotypes. Our validated assay was further successfully applied for predicting the cytotoxicity after only 1 h of cell exposure not only to individually environmental contaminants but also to chemical mixtures at different dilutions and representative of an environmental pollution to which human are chronically subjected. Indeed, the used chemical mixtures included two organic extracts from river sediments known to be the sink for particle-sorbed contaminants that can serve as a reservoir of a wild range of toxic pollutants (Creusot et al., 2013).

One must keep in mind that false-negative results can also occur using such an approach to measure early cytotoxicity: firstly, chemical or physical stressors that do not cause mitochondrial fragmentation or that lead to a hyper-fusion state associated with cytotoxicity could be incorrectly estimated as non-toxic using our model. Such situations have been reported in mouse embryonic fibroblasts exposed to stimuli such as UVC irradiation or actinomycin D in which mitochondria underwent hyper-fusion and formed a highly interconnected network (Tondera et al., 2009). Also, stressors that induce mitochondrial fragmentation only several hours after the exposure, like UVB-exposed normal human epidermal

keratinocytes have to be considered with great care, as dose-dependent mitochondrial fragmentation has been reported to occur only 6 h after the irradiation (Juge et al., 2016).

The linear equation ($y=1.4017x-25.053$) that links the predicted-viability value (y) to the mitochondrial tubular phenotype (x) obtained in this work yields an interpretable predicted toxicity value when the tubular phenotype is between 18% and 89%. The translation of the y values into semi quantitative information offered the possibility to classify the toxicity of chemicals into three ranges of level: low, mild or high, regarding the percentage of mitochondrial tubular phenotype. This extrapolation was obtained by calculating the average of the extreme values of viability (mean \pm 2 standard deviation) of the four-selected stressors CCCP, H₂O₂, NaCl and rotenone for each of the lowest, mildest and highest concentrations and the subsequent percentage of mitochondrial tubular phenotype. As presented in Table 2, the range between 18% and 50% of mitochondrial tubular phenotype could be associated with a high level of cytotoxicity if less than 50% survival is considered, that between 50% and 74% with a mild level of cytotoxicity (from 50% to 79% survival) and that from 75% to 89% with a low level of cytotoxicity (more than 80% survival).

In conclusion, cell stress-induced mitochondrial fragmentation could be used as a relevant in vitro biomarker for cytotoxicity upon environmental stress. Moreover, it contributes to the development of a new tool giving a fast estimation of cell survival, it may also provide mechanistic toxicity information of chemical or environmental contaminants that target directly or indirectly mitochondrial functions alone or in mixture.

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Author Contributions: D.P. conceived and performed the experiments. L.O. performed the preparation of environmental samples and participated in the manuscript. D.P. and C.P. conceived and wrote the manuscript.

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Fig. 1. MTT-based viability and mitochondrial fragmentation assays after a 24 h–cell stress by the selected CCCP, H₂O₂, NaCl and rotenone stressors. HeLa cells were exposed for 24 h to CCCP, H₂O₂, NaCl or rotenone and subsequent MTT-based cell viability assay (Fig. 1a) or mitochondrial fragmentation assay (Fig. 1b) were performed. For the relative cell viability assay, data presented are the mean ± standard error of the mean of three independent assays and relationship between dose of chemicals and cell survival was analyzed using a covariance analysis with statistical significance ($p < 0.05$). Mitochondrial length data are presented as a box-and-whisker plot (median with upper and lower quartile; whiskers with 10th and 90th percentile) corresponding to a population of at least 400 mitochondrial lengths from 30-50 cells per concentration performed independently in triplicate. The non-parametric Kruskal-Wallis test showed statistically relationship between doses and length of mitochondria ($p < 0.001$).

Fig. 2. Correlation between relative cell viability and relative mitochondrial tubular phenotype after a 24 h–cell stress by the selected CCCP, H₂O₂, NaCl and rotenone stressors. For each toxicant, means of the relative viability of HeLa cells stressed for 24 h with concentrations of CCCP, H₂O₂, NaCl or rotenone were plotted with the corresponding means of mitochondrial tubular phenotype (Fig. 2a). The set of values for all toxicants was plotted in one figure (Fig. 2b) and a global linear regression curve that showed a positive and linear relationship ($r = 0.696$) and a statistical significant correlation (Pearson's-product moment $p = 0.0001$) was deduced.

Fig. 3. Identification of a common cell exposure time for the selected CCCP, H₂O₂, NaCl and rotenone stressors inducing both a maximal and an early mitochondrial fragmentation. HeLa cells were exposed to 40 μM CCCP, 400 μM H₂O₂, 125 μM NaCl or 0.075 μM rotenone for 30 min, 1 h, 2 h, 4 h, 6 h, 16 h or 24 h before quantification of mitochondrial length (Fig. 3a). Each box-and-whisker plot (median with upper and lower quartile; whiskers with 10th and 90th percentile) was performed using at least 400 mitochondrial length from 30-50 cells per time exposure performed independently in triplicate. The non-parametric Dunn's multiple comparisons test was used for the identification of the most significant difference between untreated cells and each treatment time. Representative

mitochondrial network of cells immunolabelled with mitochondrial protein TOM20 antibody treated or not with chemicals for 1 h are presented in Fig. 3b. (*scale bare 10 μ m*).

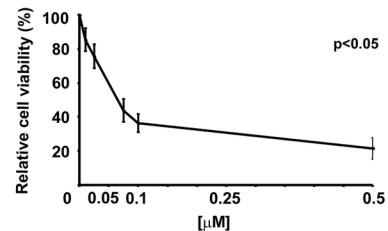
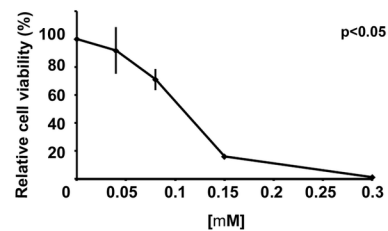
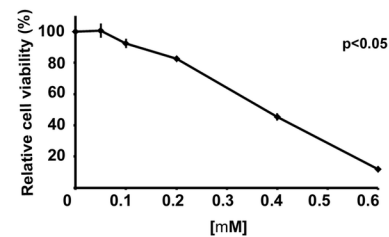
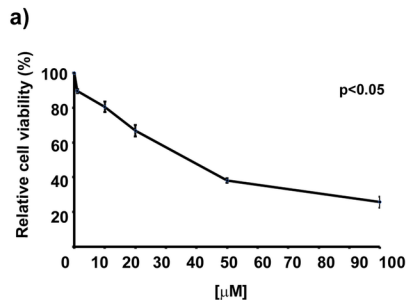
Fig. 4. Correlation between relative cell viability after a 24 h-cell stress and relative mitochondrial tubular phenotype after a 1 h-cell stress by the selected CCCP, H₂O₂, NaCl and rotenone stressors.

Mean percentages of the relative viability of HeLa cells stressed for 24 h with a range of concentrations of CCCP, H₂O₂, NaCl or rotenone were plotted with the corresponding mean percentages of mitochondrial tubular phenotype of cells treated for 1 h (Fig. 4a). The set of values for all toxics was plotted for all chemicals on a single figure (Fig. 4b) and a global linear regression curve that showed a positive and linear relationship ($r=0.82$) and statistical significant correlation (Pearson's-product moment $p=0.0001$) was deduced.

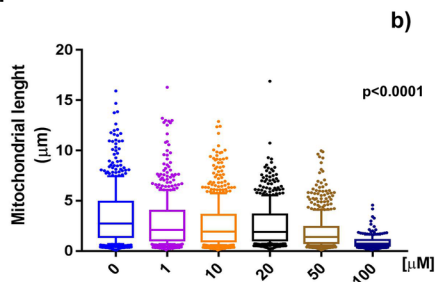
Table 1 Estimation of the relative predicted-cell viability at 24 h from relative mitochondrial tubular phenotype after a 1 h-cell stress by environmental contaminants alone or in mixture.

HeLa cells were treated with a range of concentrations of the environmental contaminants methoxychlor (MTX), bisphenol A (BPA), nonylphenol (NP), staurosporin (STS) or with dilutions of organic extract from sediment mixtures. The relative predicted-cell viability (y) was extrapolated from the relative mitochondrial tubular phenotype for a 1 h-cell stress (x) given the linear relation $y=1.4017x-25.053$. Results were statistically compared to the relative cell viability calculated from the MTT-based cell viability assay at 24 h-treatment stress (non-parametric Wilcoxon signed rank test; non-significant level when $p>0.05$).

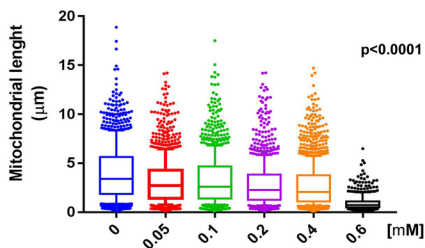
Table 2 Estimation of toxicity level thresholds for environmental contaminants alone or in mixture, expressed as ranges of relative mitochondrial tubular phenotypes (in %)



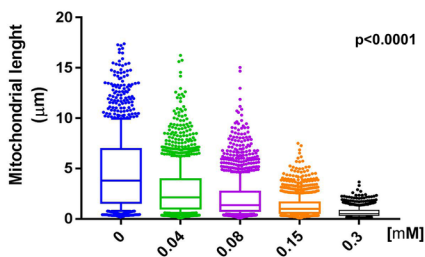
CCCP



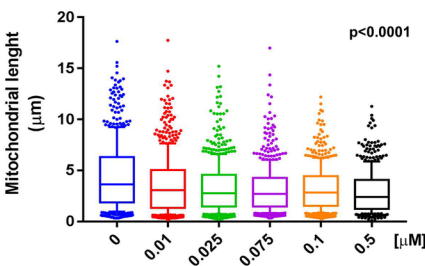
H₂O₂



NaCl

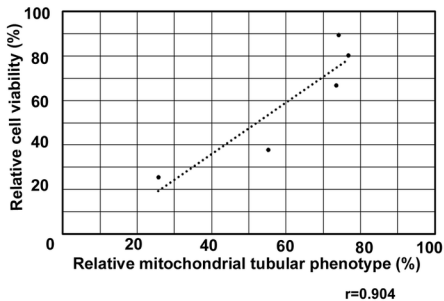


Rotenone

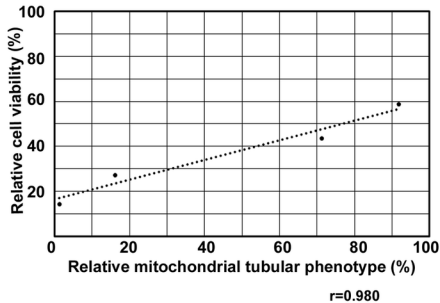
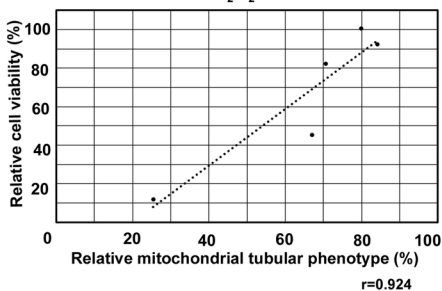


a)

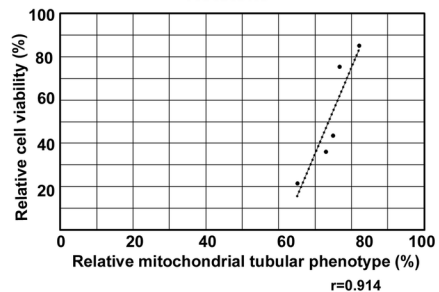
CCCP



NaCl

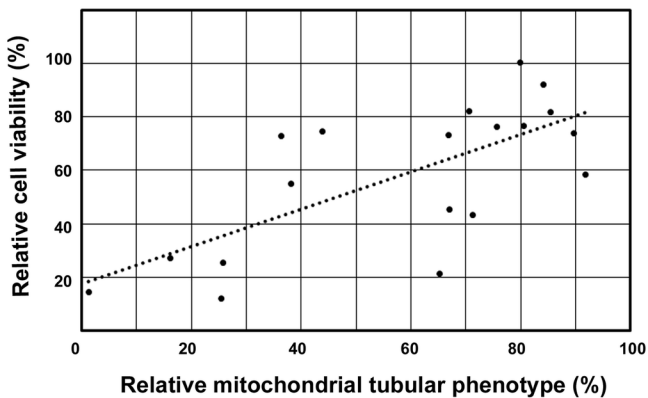
 H_2O_2 

Rotetone



b)

All stressors

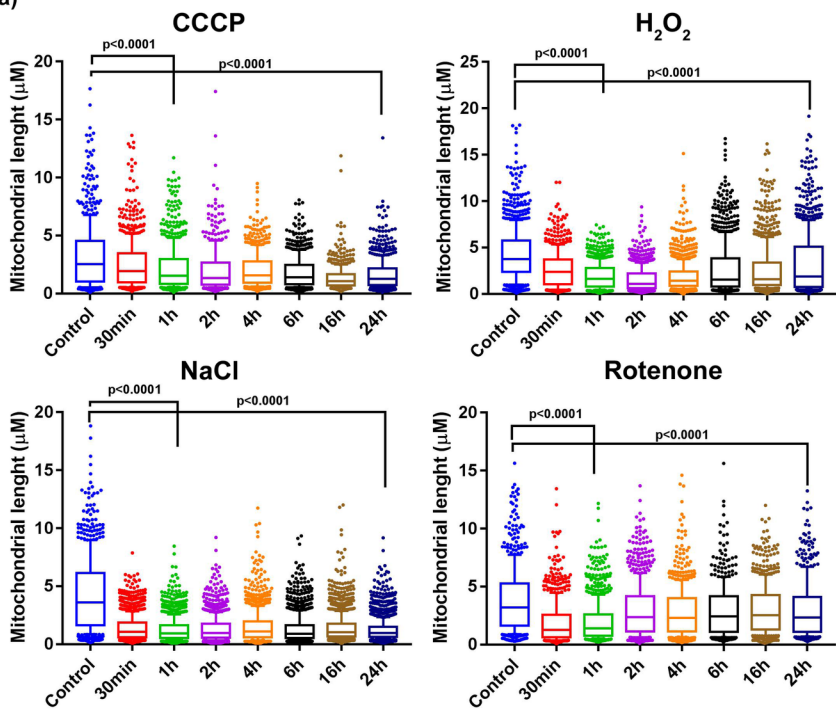


$$y=0.6967x+17.598$$

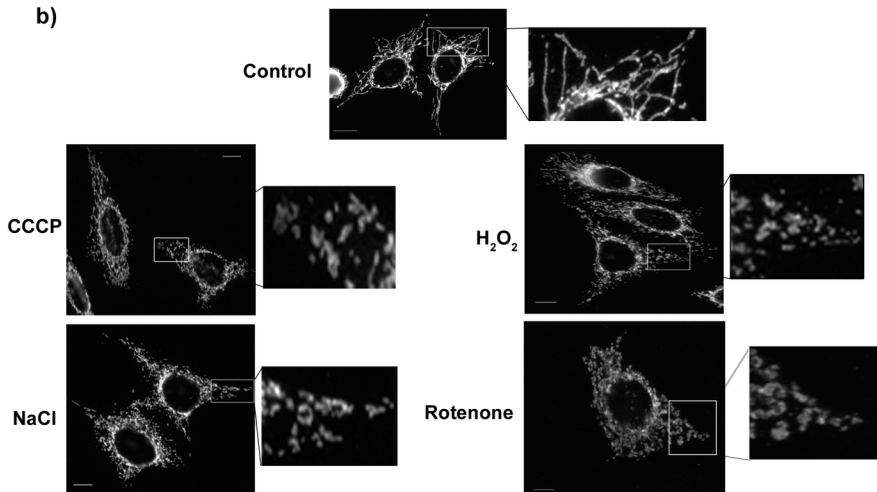
$$r=0.696$$

Pearson's-product moment
correlation $p=0.00009$

a)



b)

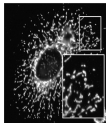
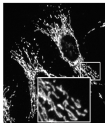
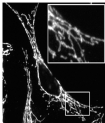
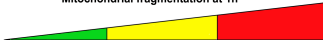


Environmental chemical (concentration)	% Relative cell viability at 24h (mean±sem)	$y=1.4017x-25.053$		Wilcoxon signed rank test
		% Relative mitochondrial tubular phenotype at 1h (mean±sem)	% Relative predicted-cell viability at 24h	
MTX (mol.l⁻¹)				
10 ⁻⁶	88±2	89±8	100	p=0.37
10 ⁻⁵	45±3	71±4	74	
10 ⁻⁴	21±6	33±5	21	
BPA (mol.l⁻¹)				
10 ⁻⁶	116±8	92±6	103.9	p=0.5
10 ⁻⁵	77±3	87±4	96	
10 ⁻⁴	28±2	51±3	46	
NP (mol.l⁻¹)				
10 ⁻⁶	94±3	84±7	92	p=0.5
5x10 ⁻⁶	57±7	67±7	68	
10 ⁻⁵	9±3	44±5	36	
STS (μmol.l⁻¹)				
0.015	50±3	71±8	74	p=0.25
0.04	32±2	54±4	50	
0.1	23±2	50±6	45	
Environmental sediment # 1 (dilution factor)				
x1/4	76±8	88±3	98	p=0.25
x1/2	57±8	77±7	83	
x1	46±4	64±4	64	
Environmental sediment # 2 (dilution factor)				
x1/4	84±3	86±2	95	p=0.25
x1/2	77±6	76±5	81	
x1	52±1	65±3	66	

% Mitochondrial tubular phenotype at 1h	% Range of viability at 24h
<18%	N/A
18-50%	High level of toxicity (<50%)
74-50%	Mild level of toxicity (50-79%)
75-89%	Low level of toxicity (>80%)
>89%	N/A



Mitochondrial fragmentation at 1h



Cytotoxicity at 24h

