

Cytotoxic evaluation of a mixture of eight pollutants at environmental relevant concentrations

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Abstract: The ubiquitous presence of pollutants and the accurate evaluation of their potential risks for environmental and human health is an area of major concern. We have simulated an *in vitro* scenario of long-term exposure to a mixture of eight pollutants at real environmental concentrations using mammalian Vero cells. Our results demonstrate that cellular proliferation rates were significantly altered, either by inhibition or stimulation, depending on the mixture composition and the exposure time. We encourage the urgency of reviewing safety levels for emerging contaminants accepted by regulatory agencies, considering that mixtures of pollutants represent a threat for environmental and human health.

Keywords: Chemical mixtures, emerging contaminants, *in vitro* studies, long-term cytotoxicity.

Resumen: Evaluación de la citotoxicidad de una mezcla de ocho contaminantes a concentraciones de relevancia ambiental. La presencia ubicua de contaminantes ambientales y la adecuada evaluación de su riesgo potencial para la salud humana y ambiental es un área de gran preocupación. En este trabajo se ha simulado un escenario *in vitro* de exposición a largo plazo de una mezcla de ocho contaminantes a concentraciones reales presentes en el medio ambiente, utilizando la línea celular de mamífero Vero. Nuestros resultados demuestran que se alteran significativamente las tasas de proliferación celular, ya sea por estimulación o inhibición, dependiendo de la composición de la mezcla y del tiempo de exposición. En vista de estos resultados, recalamos la necesidad de revisar los niveles de seguridad aceptados por las agencias reguladoras para contaminantes emergentes, teniendo en cuenta que las mezclas de contaminantes representan una amenaza para la salud humana y medioambiental.

Palabras clave: Mezclas químicas, contaminantes emergentes, estudios *in vitro*, citotoxicidad a largo plazo.

Introduction

The widespread occurrence of environmental pollutants has been an area of increasing concern for the scientific community for more than 20 years. The growing consensus connecting the exposure to chemical mixtures with relevant human diseases such as cancer, diabetes, obesity, immunosuppression, allergies and infertility has multiplied the amount of studies in this subject [1,2]. Traditional substances of concern such as polycyclic aromatic hydrocarbons, nitrates, pesticides, organochlorines and metals, among others, are being displaced by non-classical pollutants known as emerging contaminants, which include surfactants, plasticizers,

pharmaceuticals, industrial and food additives, personal care products and nanomaterials [3].

Both classical and emerging contaminants are usually detected at low levels (ng-µg/L) in the environment and, therefore, individual assessment studies have considered them as non-toxic. Although combined toxicity data at environmental relevant concentrations are scarce, the occurrence of joint effects even when all mixture components are below their individual toxic threshold has already been demonstrated [4,5]. Actually, an unknown number of substances coexist in natural matrices due to their stochastic and unpredictable releases, creating a one-time unique cocktail that interacts with biological systems. As their effects are mathematically unpredictable, the scientific community suggests experimental approaches aiming to unveil biological responses and mechanisms of toxic action [6,7]. Thereby, experimental designs reproducing real world situations such as chemical mixtures at environmental concentrations and long-term studies are toxicological priorities nowadays [8].

Our experimental approach focuses on the cytotoxic evaluation of a mixture of eight different environmental pollutants at concentrations detected in surface waters, hereafter referred as ERM (Table 1). Vero cell line, derived from mammalian kidney, was selected to conduct the toxicological studies, as we have already proved them to be very useful for the cytotoxic evaluation of environmental pollutants, including some individual components of the present mixture [9-13].

Table 1. Concentration and uses of the eight chemicals selected for the environmentally relevant mixture (ERM) employed in this study.

Chemicals	Use	µg/L*	Ref.
Carbamazepine (CBZ)	Pharmaceutical	236.3	Ternes et al. 2003 [19]
Sulfamethoxazole (SMX)	Pharmaceutical	2.0	Ferrari et al. 2004 [20]
Perfluorooctanoic acid (PFOA)	Flame retardant	67.0	Saito et al. 2004 [21]
Bis (2 ethylhexyl) phthalate (DEHP)	Plasticizer	97.8	Fromme et al. 2002 [22]
Rotenone (ROT)	Biocide	250.0	Wynne and Masser 2010 [23]
Pentachlorophenol (PCP)	Pesticide	1.5	Muir and Eduljee 1999 [24]
Butylated hydroxyanisole (BHA)	Additive	7000.0	Burse and Pellizzari 1983 [25]
Propyl paraben (PPB)	Additive	1.0	González-Mariño et al. 2009 [26]

* Maximum concentration detected in surface waters.

Experimental procedures

1. Cell culture and treatments.

Vero cell line (ATCC number CCL-81) was routinely grown at 37 °C in a 5% CO₂ humidified atmosphere, using Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. All the cell culture reagents were from Lonza

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(Switzerland). Exponentially growing cells were seeded at a density of 10^5 cells/mL in different cell culture surfaces (Falcon, Becton Dickinson, USA), depending on the experimental procedure (12 or 24 microwell plates). Following 18–20 h for properly cellular attachment, cells were exposed to the different treatments. After 24, 72, or 120 h of continuous exposure, both treated and untreated cells were gently washed with phosphate-buffered saline (PBS) and processed according to the different experimental analyses.

All drugs were purchased from Sigma (USA). Stock solutions of carbamazepine (CBZ; CAS No. 298-46-4), bis (2-ethylhexyl) phthalate (DEHP; CAS No. 117-81-7), pentachlorophenol (PCP; CAS No. 87-86-5), and butylated hydroxyanisole (BHA; CAS No. 25013-16-5) were prepared in absolute ethanol (Panreac, Spain). Otherwise, sulfamethoxazole (SMX; CAS No. 723-46-6), perfluorooctanoic acid (PFOA; CAS No. 335-67-1), rotenone (ROT; CAS No. 83-79-4), and propylparaben (PPB; CAS No. 94-13-3) were prepared in dimethylsulfoxide (DMSO, Panreac). These stock solutions were maintained in darkness at room temperature. Exposure solutions were prepared before use in DMEM with 1% serum and sterilized by filtration through a $0.22\ \mu\text{m}$ Millipore® filter. The ethanol and DMSO concentrations in all controls and exposure groups were lower than 1 and 0.2% respectively.

2. Cytotoxicity assessment.

A battery of complementary endpoints assessing cell proliferation and viability were performed in order to obtain realistic information for environmental and human health. Cell number was estimated by quantifying total protein content (TPC) according to the method of Bradford [14]. MTT assay, that involves the reduction of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) by dehydrogenases of viable cells to purple formazan, was performed according to the method of Mosmann [15]. Neutral red uptake (NRU) assay was performed following the protocol established by Borenfreund and Puerner [16]. The percentage of cells undergoing mitosis (mitotic index) was determined in cells cultured on glass coverslips into 6-well culture plates. After different exposures, cells were fixed with cold 100% methanol (v/v) during 6 min, and stained with 0.05% (w/v) toluidine blue (Sigma). Three thousand cells were scored under a Leica DMI 3000B microscope (Germany) per experimental point. Mitotic index was calculated as the ratio between the number of cells in mitosis and the total number of cells, and values were expressed as percentage of control cultures.

3. Statistical analysis.

Experiments were performed at least three times and each dose group was assayed using triplicated wells. Obtained colorimetric data were processed from absorbance values to percentage of that found in untreated cultures (percentage of control), and then represented as decreased or increased function, calculated as 100 minus the percentage of individual values. Finally, the graphical representations of toxic response were generated with individual data points of effect, and presented as the arithmetic mean \pm standard deviation using Microsoft® Office Excel® 2007. Statistical analyses, including correlations, analysis of variance (ANOVA) with the appropriate *post hoc* test (Bonferroni), and t-Student were carried out using PASW Statistic 18 (IBM-SPSS Inc., Chicago, USA). The level of statistical significance was in all cases $p \leq 0.05$. Figures were finally arranged using Adobe Photoshop® CS3.

Results and discussion

In a first set of experiments, basal cytotoxicity evaluation of ERM after 24 h exposure revealed a significant decrease in cell viability and TPC close to 60% of control untreated Vero cells (Fig. 1). The three endpoints analyzed showed a similar trend, with statistically significant correlations between TPC and both MTT ($r = 0.948$, $p \leq 0.05$) and NRU ($r = 0.926$, $p \leq 0.05$), suggesting that the effects exerted by ERM are governed by a decrease in cell number. On the contrary, cytotoxicity assays for the individual chemicals composing ERM showed no significant effects when compared with control cells, except for rotenone. Rotenone alone induced a strong and statistically significant effect upon Vero cells, equivalent to ERM treatment (t-Student, $p \leq 0.05$). Thereby, we considered this biocide as the major effector of the evaluated mixture. Nevertheless, we could not rule out the existence of other effects induced by ERM that might have been masked by the prominent influence of rotenone.

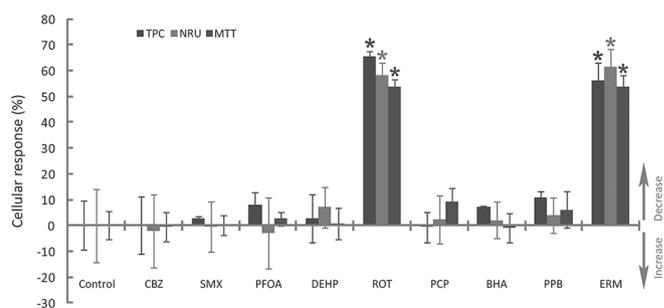


Figure 1. Cytotoxicity induced by ERM and its individual components on Vero cells after 24 h exposure. Cellular responses were classified as an increase or a decrease of the parameter analyzed, assigning zero values to untreated cultures. Asterisks indicate statistically significant differences with control cells (ANOVA, Bonferroni *post hoc* test, $p \leq 0.05$). TPC (total protein content); NRU (neutral red uptake); MTT (MTT reduction test).

In a second stage of our study, we redesigned our experimental approach evaluating new mixtures and exposure times with the same cytotoxicity endpoints. Three new mixtures based on ERM but trying to avoid the preponderant effect of rotenone were generated: ERM w/o ROT (ERM without ROT), ERM/10 (ten-fold dilution of ERM) and ERM/2 (two-fold dilution of ERM). It should be noted that, although interesting toxicological information can be acquired with short-term *in vitro* toxicity testing (24 h), a more environmental-like scenario should include longer exposure periods [17]. Thereby, the new mixtures were evaluated after 24, 72 and 120 h, allowing us to identify any possible time-dependent effect (Fig. 2).

ERM w/o ROT showed no statistically significant responses after 24 h exposure, effectively suppressing the harmful effects found with ERM. Nevertheless, unexpected increased values were detected with all the evaluated endpoints after long-term exposures, except for 72 h MTT reduction test.

A similar but even more noticeable dual response along time was observed after ERM/10 treatments. Interestingly, the highest dilution of ERM completely changed the cellular behavior with the longest exposure time (120 h). While 24 and 72 h lead to a mild decrease of the evaluated endpoint, which was statistically significant only for NRU and TPC, 120 h dramatically increased the cellular response for all the endpoints, varying the cytotoxic profile. Such a relevant rise, especially for NRU and TPC, could only be explained by a concomitant increase in the amount of cells after the 120 h treatment. To confirm whether an enhanced cell division rate was responsible

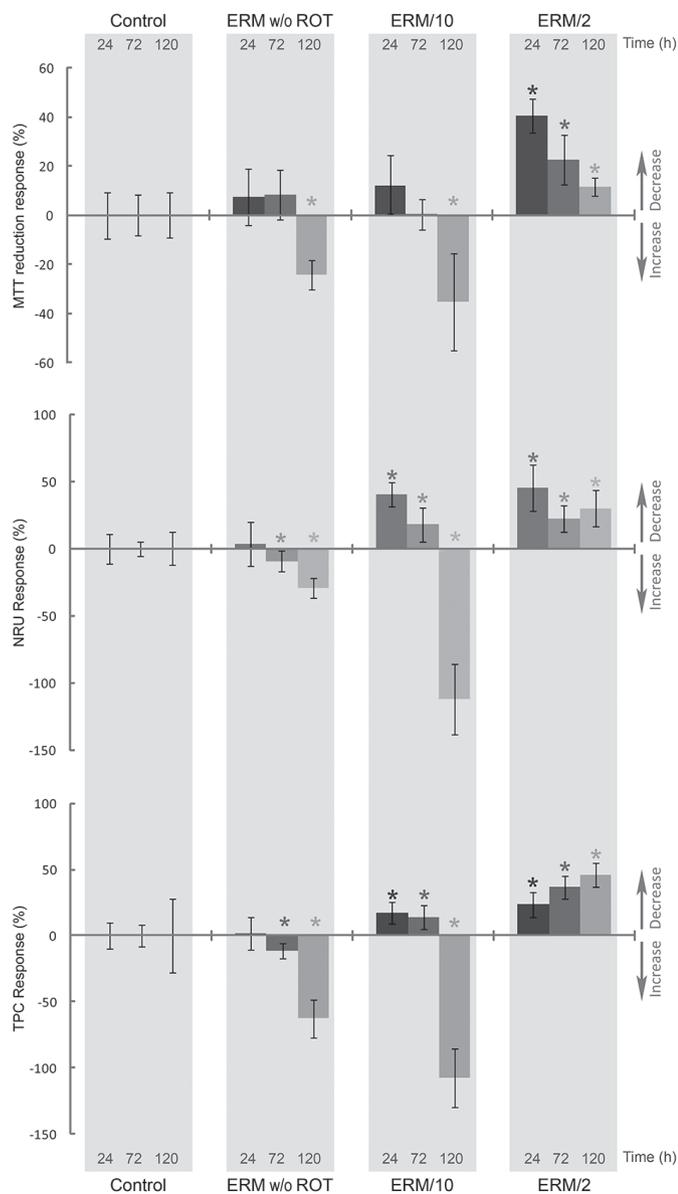


Figure 2. Cellular responses (MTT reduction, NRU and TPC) of Vero cells exposed during 24, 72 and 120 h to ERM (environmentally relevant mixture), ERM w/o ROT (ERM without rotenone), ERM/2 (ERM diluted twice) and ERM/10 (ERM diluted ten times). Asterisks indicate statistically significant differences with control cells (ANOVA, Bonferroni *post hoc* test, $p \leq 0.05$).

for those variations, mitotic index (MI) scoring after 120 h exposure to ERM/10 and ERM w/o ROT was performed. Statistically significant increases (ANOVA, *post hoc* test $p \leq 0.05$) in MI were detected for ERM/10 ($185.3 \pm 0.2\%$) and ERM w/o ROT ($147.9 \pm 18.2\%$) when compared with control untreated cells ($100.0 \pm 16.9\%$).

Finally, the ERM/2 mixture induced a significant decrease in cell response, although attenuated when compared with original ERM mixture. Remarkably, this decrease was not proportional to the dilution factor after 24 h, with values being somewhat higher than half the previously detected effect of ERM. Time-increases led to statistically significant but lower responses than those after 24 h exposure for all the endpoints. However, TPC time-dependent reduction reached a maximum response of $45.8 \pm 9.3\%$ of control

cells after treatments for 120 h. Theoretically, the dilution of a dangerous substance or preparation should decrease its detrimental effects, but the similarity between the cellular responses of ERM/2 and ERM treatments indicates that not in this case. Most probably, the toxic mechanism underlying this cytostatic response coincides with our previous results suggesting that inhibition of Vero cells proliferation caused by rotenone is due to anomalous mitotic spindle, eventually leading to mitotic catastrophe and cell death [9].

Given the strong variability of cellular responses observed even with our simple battery of endpoints when introducing slight changes in the exposure time or final concentrations, it is tempting to suggest that toxicological evaluation of chemical mixtures must be conducted considering each particular combination of chemicals as an autonomous entity.

Overall, our study encourages the urgency of reviewing the current safety levels determined for chemical substances in the environment as well as the assumed safety burdens accepted by regulatory agencies, considering that long-term exposure to chemical mixtures represent a real threat for environmental and human health. Furthermore, we support the importance of unraveling the mechanism of toxic action of chemical mixtures, in view of the new trends in toxicology claiming for the definition of adverse outcome pathways [18].

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