riferimento, quale il perossido di idrogeno, e l'idoneità del sistema di saggio adottato.

Cyto-genotoxicity studies on green carbon dots developed from biomass for biosensing applications

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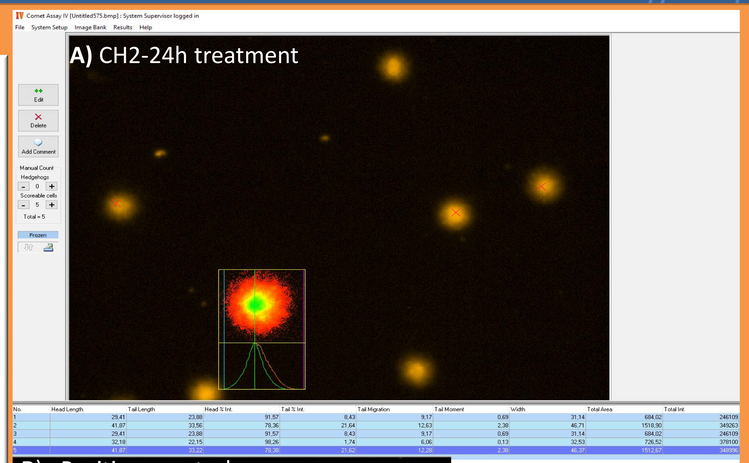
INTRODUCTION

The production of increasing amount of biomass/biowaste is a challenge faced by our society today. On the other hand, many residues of industrial processes contain valuable bio-compounds, which can be used directly or converted into them with simple and sustainable processes. Among different application fields, the production of electrochemical sensors is a recent area of research, where the utilization of biomass wastes is very promising for the creation of low-cost carbon-based nanomaterials [1]. The conversion of biomass into graphitic-based carbon (CDs) can be achieved with a hydrothermal process, using only water as an environmentally friendly solvent, i.e. without toxic solvents and with minimal environmental impact [2]. Through this conversion process, Cork powder and Fish bones were used as waste bio-nanomaterials to produce two different green Carbon Dots (see the RAD Conference poster '*Green carbon dots developed from biomass for sensing Marine Biotoxin'*, by A. Dawood, *et al*.) which will henceforth be referred to as **CH-** and **FH-**CDs, respectively.

Aim of this work is to evaluate the safety of these two green Carbon Dots both for biotechnologists, who produce disposable biosensor devices, and for environmental monitoring personnel, by analyzing their cytotoxic and genotoxic potential in Jurkat cells, a human tumor-derived lymphoblastoid cell line defective for P53 activity. According to the recommended procedure [3-5] the Trypan Blue Staining Assay and the Alkaline Comet Assay were used to assess the cytotoxic and the genotoxic effect of both compounds, respectively. The two assays complement each other since the former informs about induction of cell death, while the latter provides a quantitative estimate of DNA damage induction in (surviving) exposed cells. Scaling concentrations (up to 200 µg/ml) were selected for both compounds and prolonged exposure times were studied, up to 72h.

METHODS AND MATERIALS

Cell culture. Jurkat cells were cultured in RPMI supplemented with RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml and maintained in a humidified incubator at 37 °C and 5% CO2/air. **Treatments.** CH-CDs and FH-CDs water solutions (2mg/ml) were sterilized (0,22µm filters) and diluted in complete medium (1mg/ml) before administration. Appropriate volumes were then added to the cell suspensions to obtain the final concentrations of 0, 10, 50, 100, 200 µg/ml. Three times of prolonged exposure (24h, 48h, 72h) were studied. In addition to negative controls (NT, no treatment), hydrogen dioxide (H_2O_2) was used as positive control (150mM, 30 mins at 37°C). **Trypan Blue Staining** assay was carried out before and immediately after the end of treatments by counting viable and nonviable cells in a hemocytometer. The alkaline version of **Comet assay** was performed, according to the standard protocol [6]. Each compound was tested in a single electrophoretic run (all concentrations and incubation times run together, negative and positive controls included). Briefly, 20 µl of the cell suspension were mixed with 80 µl of 0.75% low melting-point agarose and layered onto a microscope slide pre-coated with 1% normal melting-point agarose in PBS. After incubation overnight at 4 °C in Lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA, pH 10, with 1% Triton and 10% DMSO freshly added) slides were kept in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) for 15 minutes before a 20 minutes electrophoresis, carried out at 25 V and 300 mA at 4 °C. Slides were then washed with neutralization buffer (0.4 M Tris-HCl, pH 7.5), stained with 50 µl of 10% Gel Red, (SIGMA) and analysed with a fluorescence microscope (Axio Observer 7, Zeiss) at 400X. An automatic image analyser (Comet Assay IV, Perceptive Instruments, UK) was used for comet scoring. Computer-generated Tail intensity (%TI) values were used as parameters for DNA damage, on a total of 100 cells scored from two slides per experimental point (**Figure 1, A-B**). **Statistical analysis** was performed using non-parametric tests (Kruskal-Wallis and Dunn post-hoc test for pairs, to compare each experimental point with the matched negative control.



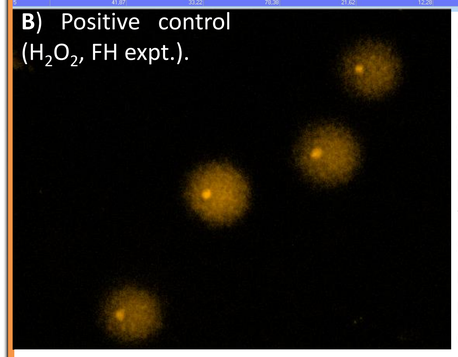


FIGURE 1. Examples of detected comets. In **A**) the Comet IV software automated analysis is shown: the yellow frame is the analysis area, where the blue and the purple straight lines are visible, represent the left and right extremes of the comet, respectively, while the green line the max fluorescence level. The curves in the diagram indicate the fluorescence intensity variation detected in the head (green) and the tail (orange). Its corresponding % values are reported in the table (blue line), together with other parameters.

RESULTS

CELL SURVIVAL. A decrease of cell viability is observed after the first 24 hours of exposure to CH, for all the tested doses (negative controls included), followed by a recovery at the subsequent exposure times (48h and 72h), except the lowest dose (CH1, 10µg/ml) where no alteration is observed, the values of which remain constant along time, if not even slightly increased at 72 hours. A progressive reduction can also be observed at increasing doses during the first 24 hours of exposure, followed by a recovery at the subsequent times, the values of which seem to remain mostly constant. In FH: no consistent decrease of cell viability is recorded in all experimental points, the mean % values ranging in a very short interval (97,52-99,72). Only for FH3 and FH4 a reduced viability is detected at 72h. **COMET ASSAY.** Results from the two CDs indicate quite different effect, as no induction of DNA damage is found for FH-CD (Figure 3-B) at all doses and exposure times (Dunn post hoc test for pairs: *p*-values range = 0,92 in FH2-24h; 0,094 in FH1-72h), while a negative result from CH-CD treatments (Figure 3-A) is found only in CH1 (10µg/ml), after 24h exposure (p=0,64), all the other ones showing significant increases of DNA damage (*p*-values ranging from 0,019 in CH2-24h, and1,15x10^{-6} in CH4-24h). However, comparing results from the two assays on FH exposures, it is possible that the negative genotoxic effect at the two highest doses could be hidden by the slight increase of cytotoxicity.

CH & FH concentrations		% CELL SURVIVAL: Cork powder, Hydrothermal (CH) 24h	48h	72h		Fish scales, Hydrothermal (FH) 24h	48h	72h
0µg/ml	NT	80,91	92,86	93,76	NT	97,52	99,41	99,77
5µg/ml	CH1	86,13	87,53	92,71	FH1	98,4	98,90	98,22
50µg/ml	CH2	74,43	92,86	96,25	FH2	98,75	98,96	99,22
100µg/ml	CH3	61,62	93	93,31	FH3	98,29	98,87	97,88
200µg/ml	CH4	67,18	92,69	90,48	FH4	99,12	98,66	97,87

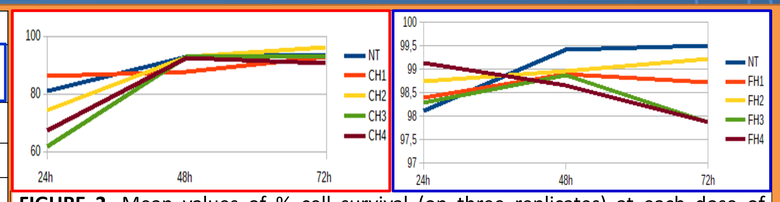


FIGURE 2. Mean values of % cell survival (on three replicates) at each dose of treatment with CH (left) or FH (right), recorded at the exposure times of 24h, 48h and 72h. The two graphs (right panels) show the variations observed for each dose (colors) as a function of exposure time. The table (left) shows the mean values calculated as the ratio: 'live cells'/ 'live + dead cells'.

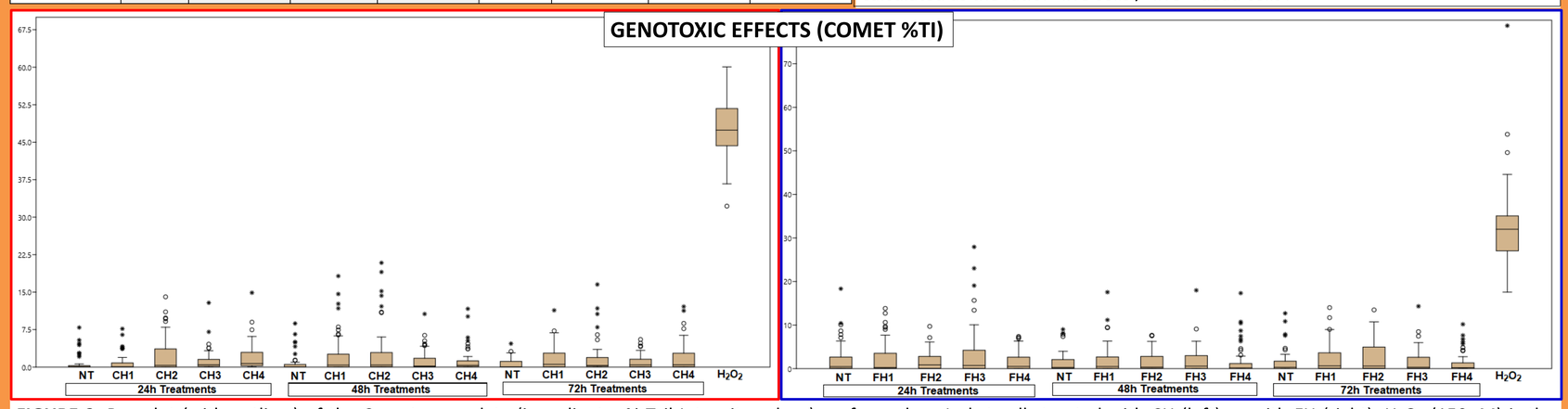


FIGURE 3. Box-plot (with outliers) of the Comet assay data (in ordinate, % Tail Intensity values) performed on Jurkat cells treated with CH (left) or with FH (right). H_2O_2 (150µM) is the positive control. The concentrations of the treatments with the two CDs are represented by the numbers following the abbreviations CH or FH (1=10 µg/ml; 2=50 µg/ml; 3=100 µg/ml; 4=200 µg/ml; NT=0 µg/ml). Bottom: duration of the treatments (24h, 48h, 72h).

CONCLUSIONS

The absence of cytotoxic effects observed for both CD compounds suggests their safety in handling and final use as biosensors for environmental monitoring, at least at concentrations below 100µg/ml. In particular, the fish-scale derived CD (FH) seems to be most promising for the purpose, due to the lack of genotoxic effect, at all doses and exposure times. Nonetheless, confirmation of the present preliminary results is needed, as well as implementation on their mutagenic potential, through the micronucleus test [4,5].

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