Study of biological effects by ultra-soft X rays on V79 cell line: preliminary results

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Abstract
Radiobiological studies essentially focuse on DNA damage, according to a dose-dependant approach. The use of ultra-soft X-rays (500 eV) allows us to determine the biological effects from the damage on peripheral and cytoplasmatic structures, such as plasmatic membrane and organelles, without DNA is involved directly by radiation. The in vitro model chosen is V79 cell line (isolated from the lung of a normal Chinese H*amster*). To reach this aim, we have developed:

- an experimental layout to obtain ultra-soft X rays from monocromatic spherical mirror (500eV) and a second apparatus based on a plane copper mirror at grazing angular incidence (300-510 eV);
- a custom biological sample holder, in order to be trasparent to X rays and visible light and realized by biocompatible materials;
- analysis methods based on mycroscopy observation with appropriate set-up and study of different end-points as viability assay and ROS detection.

In this article, we present the preliminary results and apparatus.

1. INTRODUCTION

Classical radiobiology adopted the Linear No-Threshold (LNT) paradigm, according to which the biological damage caused by radiation is directly proportional to the dose and the cancerogenic risk increases as dose increases. This model ispired the principles of radio-protection and allowed to estimate cancer risk; however, this approach can't explain the lack of linearity between doses and effects in the region of low doses.
For this reason, LNT model isn't universally accepted and many studies denied it, even though it was accepted as a reference point by the BEIR VII Committee, or the Radiation Effects Research Foundation's (RERF) Life Span Study (LSS) of Hiroshima and Nagasaki atomic bomb survivors, to evaluate consequences on humans (C.W. Pennington and J. A. Siegel, 2019).
LNT model also ignores the evolution of life on Earth 3.5 billion years ago in presence of radiation three times higher than now (Jaworowski 1997; Karam and Leslie 1999) and the development of first biological strategies of defence.
The crisis of LNT paradigm lead to new considerations and in particular to the acceptance of the hormesis model according to which low-dose radiations provide a favorable response which protects cell from stressors. Moreover, hormesis model considers the body's

response to radiation and all the cellular pathway involved.
For these reasons, ultrasoft X-rays represent the possibility to investigate the shape of the dose-response curve in the region of very low dose radiation and low energy photons. In this situation, we can determine how photons interact with cells and their structures. In fact, the damage was only evaluated in terms of DNA-targeted effects, but these effects are common in higher doses and high energy radiation: this range of energy (500-300 eV) allows us to determine consequences on peripheral structures (such as membrane) and organelles (such as mitochondria). In fact, in this range the photon energy is absorbed principally by photoelectric effect in single step with LET

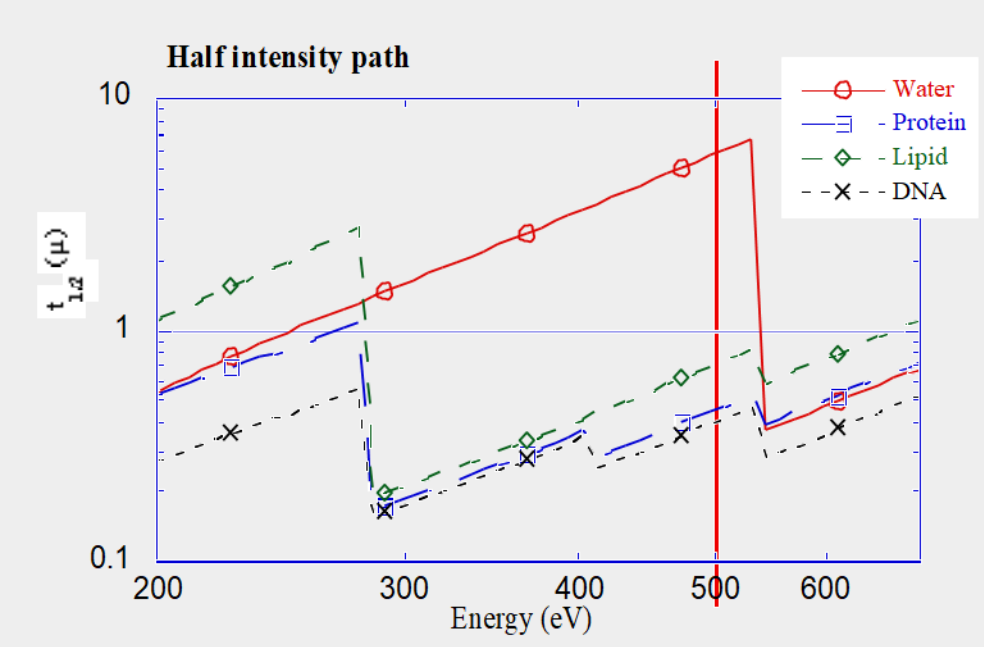


Figure 1. Water window: in the region extending from the K-absorption edge of carbon to K-edge of oxygen, water is trasparent to soft x-rays, but organic molecules (principally made of carbon) are absorbing.

The aim of the present study is to investigate the effect of ultra soft x-rays at 500eV and in the water window photon energy range (280eV-510 eV) on V79 cell line, isolated from Chinese Hamster.

2. MATERIALS AND METHODS

Cell culture.

V79 cells are isolated from Chinese Hamster lung, and they were cultured in DMEM low glucose, containing 10% of bovine serum and supplemented with penicillin and streptomycin, and were maintained in an incubator at 37°C in an atmosphere of 5% CO_2 in air. The advantages of using V79 as a model are the stability of karyotype and morphology (fibroblast).
Cells were cultured on a sample holder, made *ad hoc* for irradiation, with two fundamental characteristics: *light and X-rays transparency to* and *biocompatibility.*
A poliethilene thereftalate film (composition: $(C_{10}H_8O_4)n$; 0,5 micron of thickness) is glued

on a stainless steel ring (area: 0,785 cm²): this thickness is chosen for optimizing the transmission in our energetic range, since at 500-300eV materials are intensely absorbent. This first support is used to cultivate cells (with a density of 6000 cells in each ring), a second ring with same characteristics is put on it to realize a mini-chamber, holding culture medium, necessary to substain living cells during irradiation. Both rings are inserted in another sample holder and put on a movable system along the soft X-rays line.

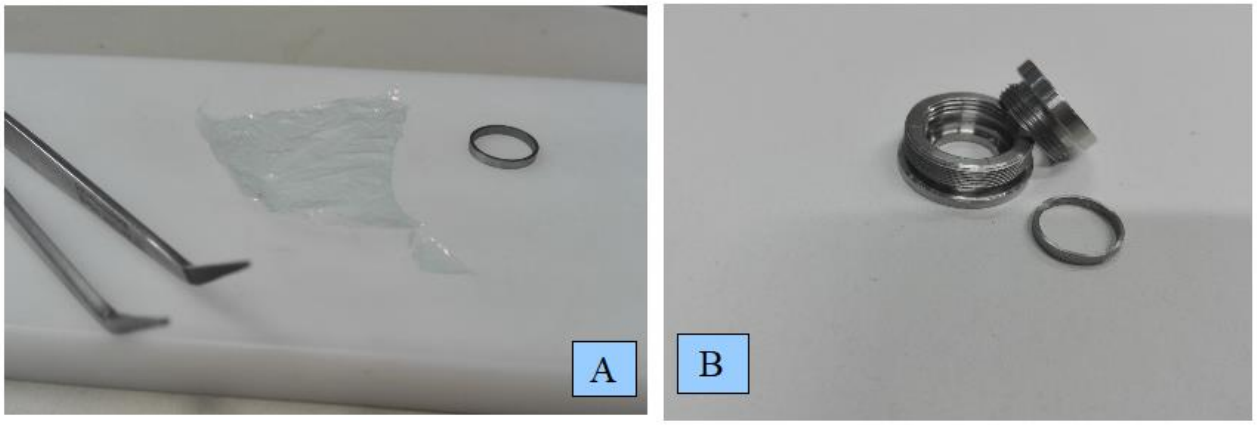


Figure 2. A- A stainless steel ring is ready to be glued on the film. On the worktable, a Polytetrafluoroethylene (PTFE) is used to minimize electrostatic forces. **B**- Stainless steel ring and the sample holder.

The film appears wrinkled as electrostatic effect. To avoid this problem and analyze the cell culture, we put the plastic film on a plexiglass cylinder-shaped support. We have chosen a plexiglass with high homogeneity and 99% transparency to light, to avoid the spurious results.

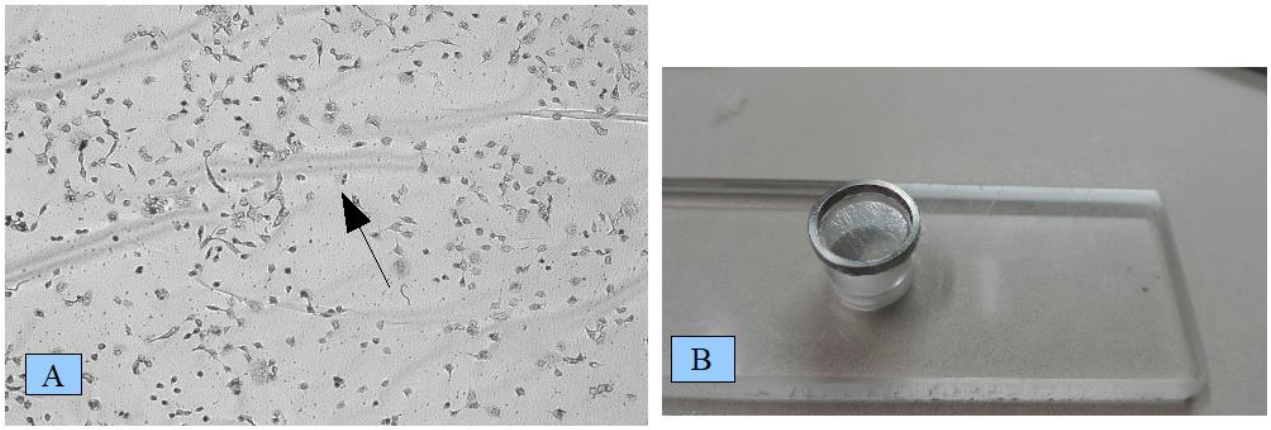


Figure 3. **A**- Cells are cultured on the film that has an irregular surface. The arrow indicates wrinkle. **B**- Plexiglass cylinder-shaped support to simplify the observation by the microscopy.

V79 cells have a doubling time of 12-14 hours, which is not really modified by the film.

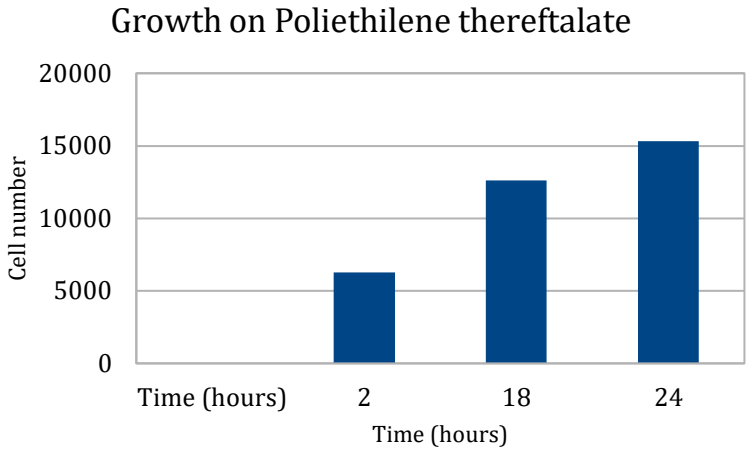


Figure 4. After seeding cells at a density of 6000 cells on each sample holder, **c**ell counting demonstrates the invariability of doubling time and a normal growth on the film.

Experimental apparatus
Ultra-soft X-rays are generated from a plasma produced by focusing a NdYag/Glass laser beam on an yttrium target. The laser beam goes into an interaction chamber at 10^{-3} mbar internal pressure, where x-rays are directly produced from plasma, in the process of hydrodynamic expansion. Outside the chamber, a vanadium filter has been positioned in order to select the beam component at 500eV (λ= 2.48 nm). The choice of the wavelenght is related to the kind of interaction made by photons with biological structures: these photons are absorbed, in particular, by Carbon (principal constituent of living matter).
Each pulse is 6 nsec duration and the dose is in the range of 0.2 and 0.002 Gy.

Irradiation
Cells were irradiated at room temperature in culture medium, which was replaced with warm medium after irradiation; then sample holders were incubated at 37°C in incubator with 5% of CO_2 before processing to analyze the effects of radiation.

Trypan Blue staining
After irradiation, we trypsinized cells and resospended them with an appropriate volume of culture medium and Trypan Blue (1:2 dilution) and counted. The number of dead cells il really low, so we concluted that there is no effect on vitality.

LIVE/DEAD® Viability Test
The LIVE/DEAD® Reduced Biohazard Viability/Cytotoxicity Kit #1 (Molecular Probes) is a two-colour fluorescence assay for cell viability. SYTO® 10 is a green fluorescent nucleic acid stain, membrane-permeant and labels all cells with intact membranes. DEAD Red™ is a cell-impermeant red fluorescent nucleic acid stain, that labels only cell with a damaged membrane.

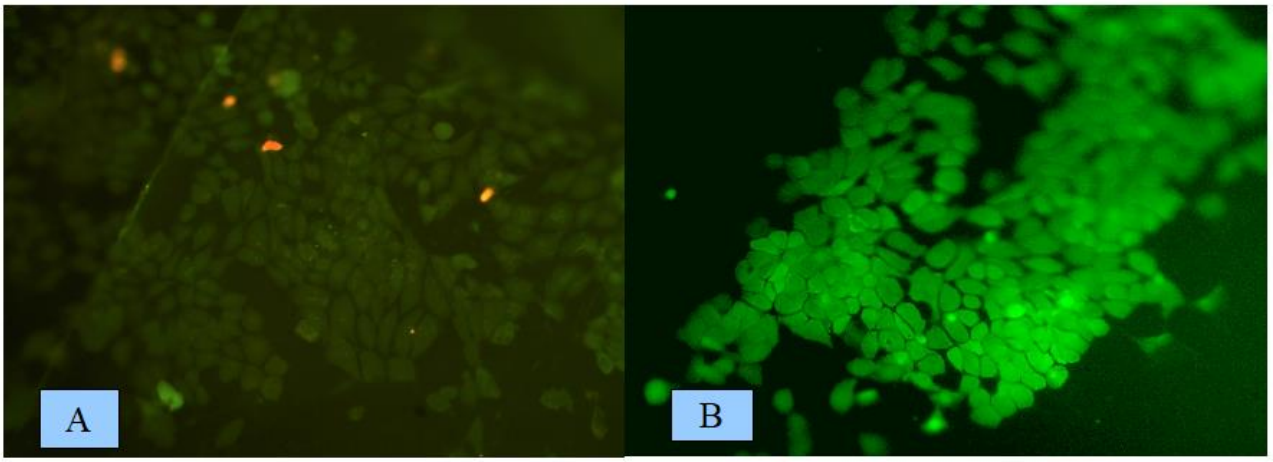


Figure 5. 3 hours after irradiation, there are no evidence about an increase of mortality. A: control and B: sample exposed to ultra soft x-rays.

Morphological tests

We investigated the presence of morphological changes in cells after irradiation (3 hours) using Phalloidin (to label cytoskeleton structures, in particular actin) and DAPI (a fluorescent stain that binds adenine-thymine rich regions in DNA). We noticed that there were no significant changes.

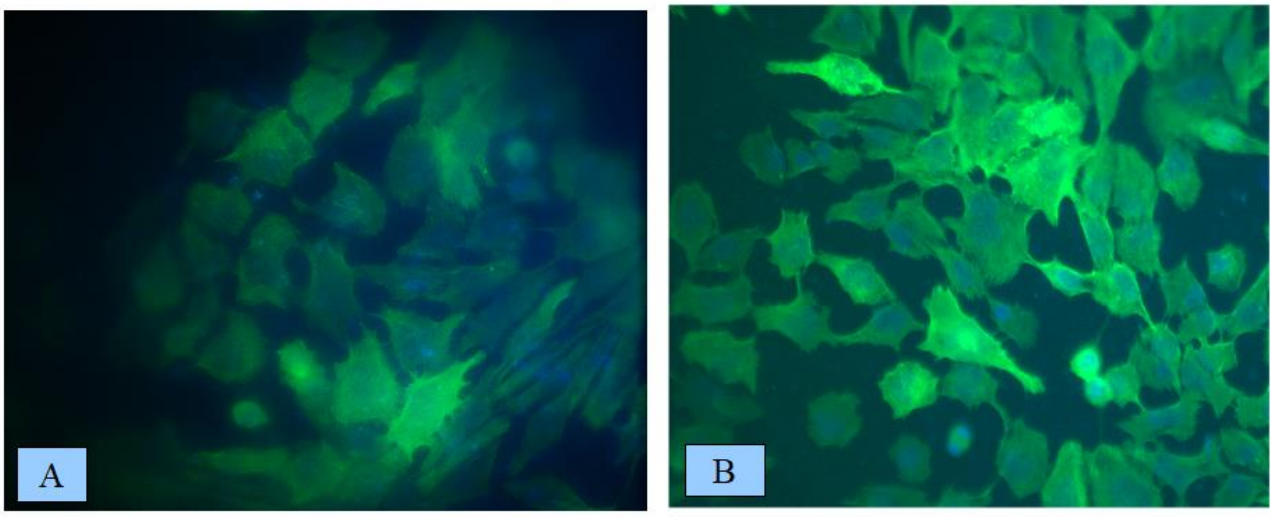


Figure 6. 3 hours after irradiation, labelling with DAPI and Phalloidin gave no significant results. A: control and B: sample exposed to ultra-soft x-rays

ROS DETECTION

Absorption of ionizing radiation directly disrupts atomic structures, producing chemical and biological changes through reactive chemical species. Ionizing radiation may also disrupt mitochondrial functions significantly contributing to persistent alterations in lipids, proteins, nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). We measured at different times (1 hour and 24 hours) ROS levels by Tali® Image-Based Cytometer,

labelling cells with 2′,7′-Dichlorofluorescin diacetate. This is a cell-permeable non-fluorescent probe which is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation. The output of the analysis is a graphic in which the Y axis is represented by the number of cell, and the X axis is represented by the intensity of fluorescence signal.
After 1 hour from irradiation ROS quantity is not significant, and the situation is similar to negative control. Only after 24 hours, there is an increase of ROS levels, probably depending on mithocondrial alterations.

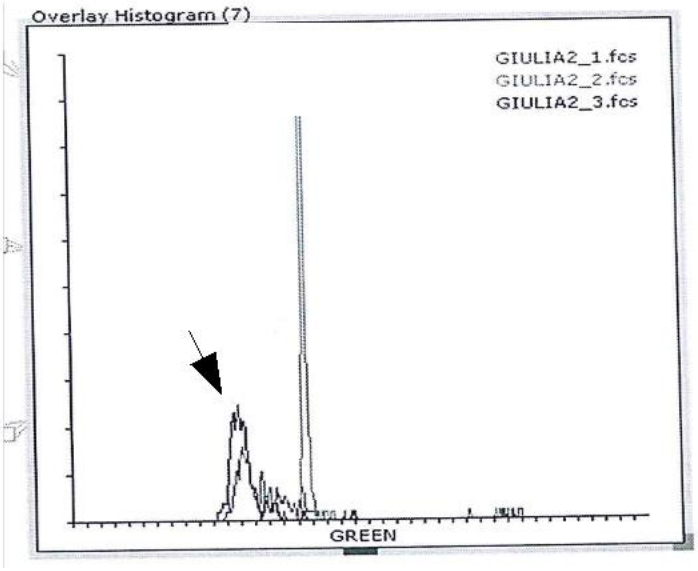
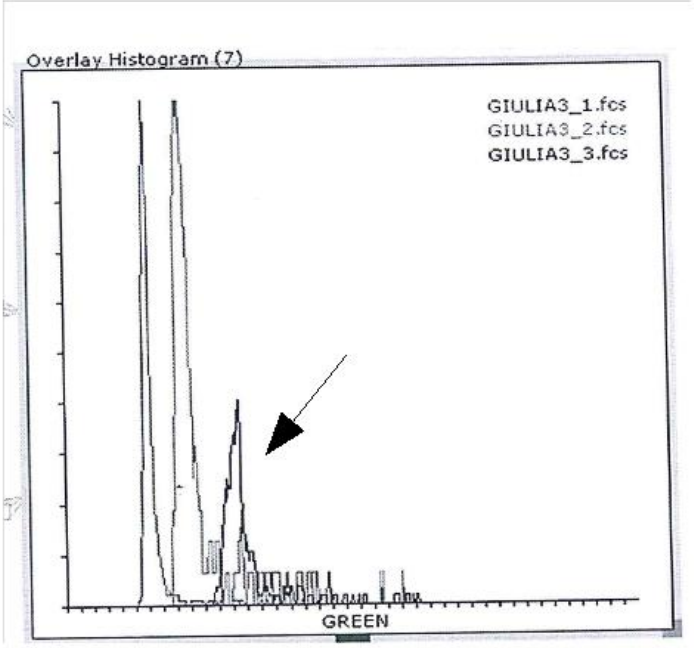


Figure 7. ROS detection after 1h from exposition. The negative control has an overlapping peak with the irradiated sample. The positive control peak, made of cells treated for 90 minutes with Hydrogen Peroxyde, is more intense.



F**igure 8**. After 24 hours, the peak of cells exposed to radiation is positioned on the right.

3. CONCLUSIONS

These preliminary results demonstrate the absence of effect of ultrasoft x-rays on vitality, and the increase in the levels of Reactive Oxygen Species, which are normally produced by the cells, even if ultrasoft X-rays should be considered an important trigger. We have to verify the kinetics of ROS and determine what happens in intermediate times and, eventually, overlap ROS levels with mitochondrial damage. The oxidative stress should be considered not only in terms of cytotoxicity or cell death, but oxidative species play an important role in signalling pathways and influence other cells which are not directly irradiated (bystander effect). As a future perspective, we are going to examine the effects of x-rays with a wavelength of 4,5 nm (276 eV).

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