Hirudo medicinalis AS ALTERNATIVE MODEL FOR *in vivo* AND *in vitro* STUDIES ON NANOMATERIALS TOXICITY

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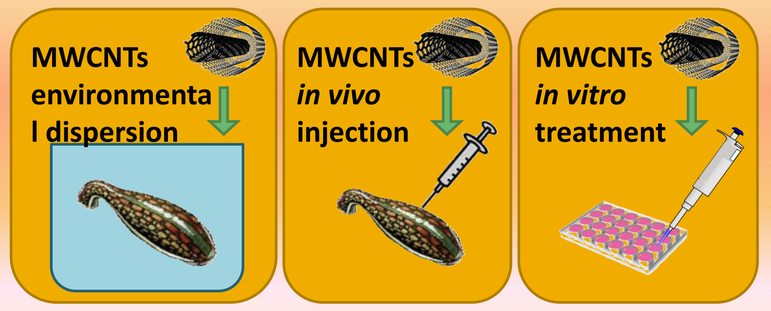
INTRODUCTION: Nanomaterials (NMs) are widely used in industry. In particular multiwall carbon nanotubes (MWCNTs), consisting of several concentric graphene tubes with diameters of up to 100 nm, are employed for many applications (i.e. biomedicine, nanoelectronics, mechanical engineering), however they do not degrade and persist in biological systems for months or even years. For these reasons the risk assessment for this NM is becoming essential.

The immune system of organisms is one of the first frontiers affected by NMs and represents a sensitive physiological indicator which is affected even at low concentrations of NMs exposure. Here we propose the leech *Hirudo medicinalis* as new sentinel model for studying MWCNTs effects since its simple anatomy and its immune response processes are clear and easily interpretable.

AIMS: Develop and optimize approaches to: 1) investigate the mode of action of MWCNTs on different levels of biological organisation from cells/tissue to individuals and the effects of this nanomaterial as stressor on organisms; 2) to give rapid and sensitive responses upon the presence of MWCNTs even if at low concentration.

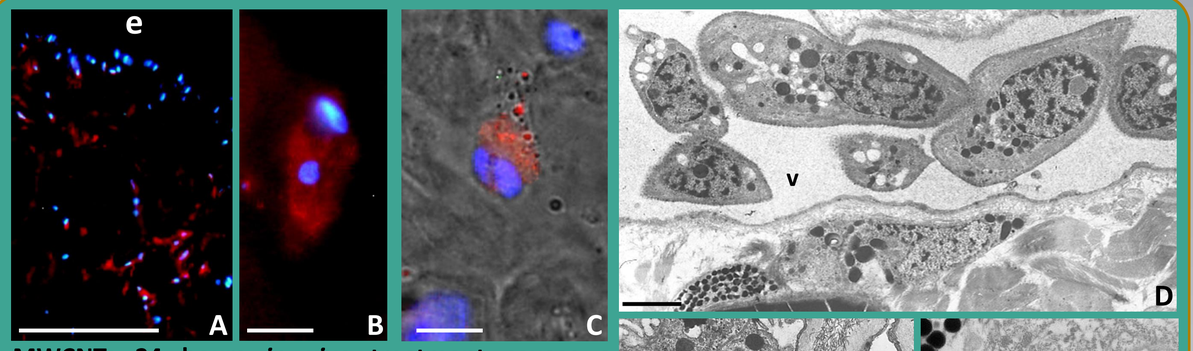
METHODS:



1) ***in vivo*** **exposure**: MWCNTs were dispersed in leeches' water.
2) ***In vivo*** **injection**: MWCNTs supplemented biomatrice (MG) was injected in the leech body wall.
3) ***in vitro*** **exposure**: MWCNTs were dispersed in the culture medium of macrophages.

MWCNTs INDUCED IMMUNE RESPONSE

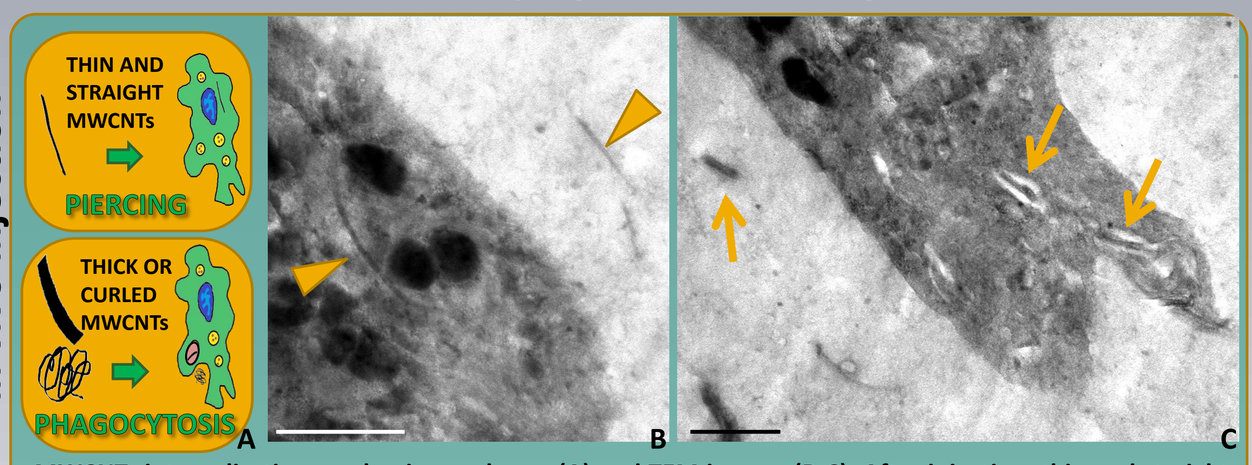
in vivo **exposure**



MWCNTs 24 hours *in vivo* treatment causes a massive migration of $CD68^+$ macrophage cells (red) in the leech body wall (A, B). These cells derive from $CD45^+$ circulating precursors (C). TEM details showing circulating monocytes (D) in a blood vessel (v), during diapedesis (E) and a mature macrophage (F) in the extracellular matrix (ECM). Nuclei are counterstained with DAPI (blue). Bar in A: 100 µm; bars in B, C: 10 µm; bars in D, F: 1µm; bar in E: 500nm.

MWCNTs INTERNALIZATION

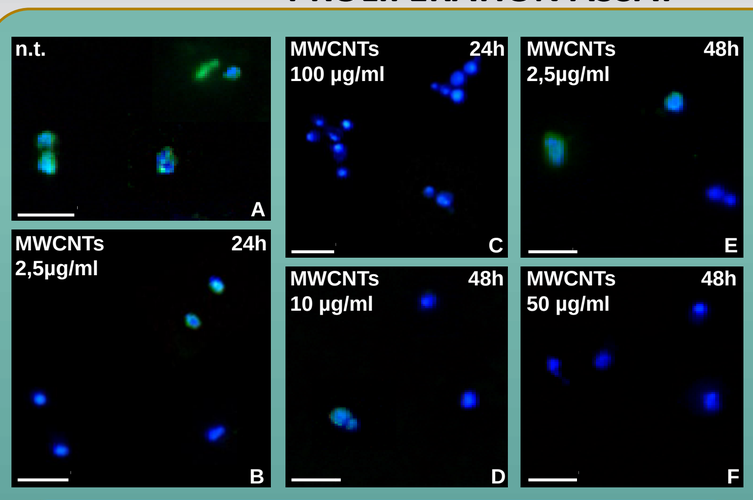
in vivo **injection**

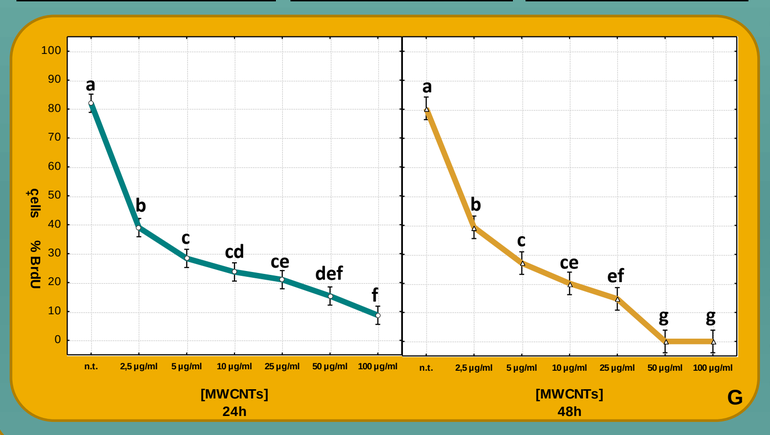


MWCNTs internalization mechanisms scheme (A) and TEM images (B-C). After injection, thin and straight MWCNTs are observed in the MG and freely dispersed in citosol (arrowheads in B), while thick or curled MWCNTs (arrows in C) are phagocytized by macrophages migrated in the MG. Bars in C, D: 1µm.

PROLIFERATION ASSAY

in vitro **exposure**



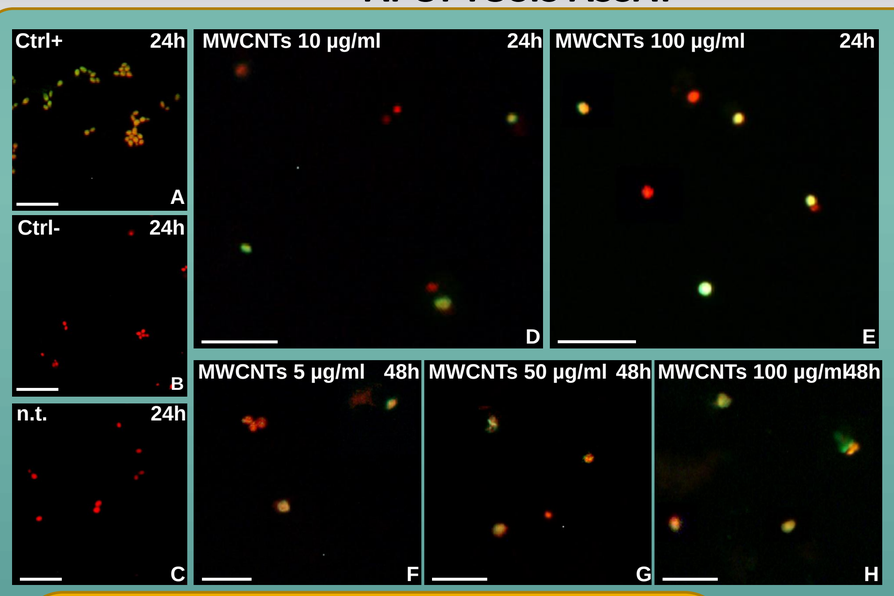


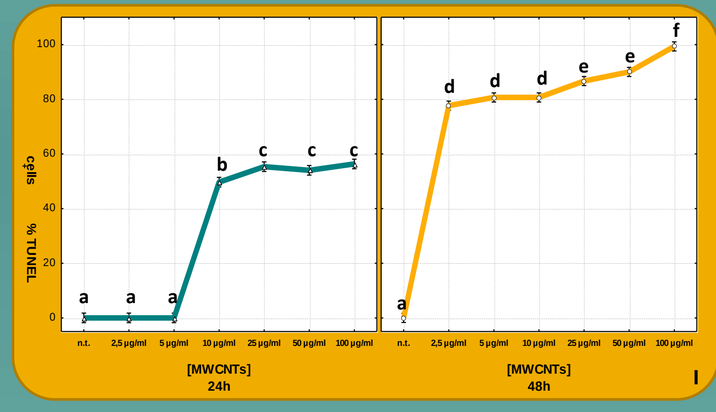
Compared to not treated samples (A), in which the major part of cells is $BrdU^+$ (green), in 24 (B) and 48h (D-F) MWCNTs treated samples, the number of positive cells decreases. Nuclei were counterstained with DAPI (blue). Bars in A-F: 10 µm.

The graph (G) shows the percentage of $BrdU^+$ cells after MWCNTs treatment showing a dose and time-dependent reduction of proliferative rate. Different letters indicate statistically significant differences (Factorial ANOVA followed by Tukey's post-hoc test, vertical bars: 0,95 confidence intervals, $p<0.01$).

APOPTOSIS ASSAY

in vitro **exposure**



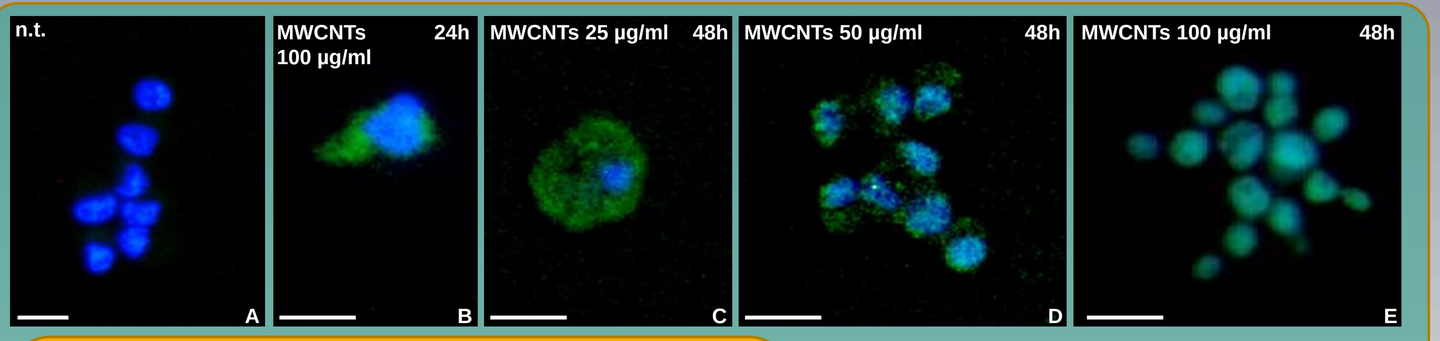


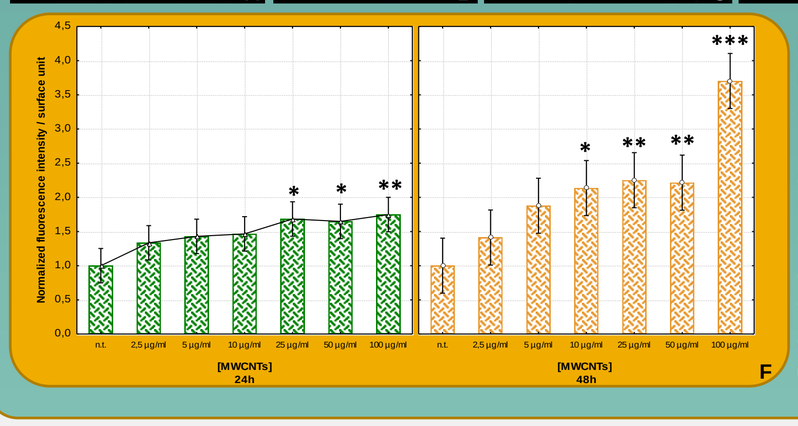
TUNEL assay for apoptosis after 24h (A-I) and 48h (J-O) *in vitro* MWCNTs treatment. Nuclei were counterstained with propidium iodide (red). TUNEL positivity is visible in green/yellow. Bars in A-O: 20 µm.

The percentages of $TUNEL^+$ cells after *in vitro* MWCNTs treatment are illustrated in the graphs (I). Statistical differences were calculated by Factorial ANOVA followed by Tukey's post-hoc test; vertical bars denote 0,95 confidence intervals, different letters indicate statistically significant differences ($p<0.01$).

REACTIVE OXYGEN SPECIES

in vitro **exposure**





H_2DCFH-DA positivity (green) is not visible in n.t. Macrophages (A). After 24h *in vitro* MWCNTs treatment positivity is detectable from 25 up to 100µg/ml (B). A dose-dependent ROS production is appreciable also after 48h treatment (B-E). Bars in A-E: 5 µm. Histogram showing fluorescence intensity for surface unit after H_2DCFH-DA assay (F). Statistical differences were calculated by Factorial ANOVA followed by Tukey's post-hoc test, vertical bars denote 0,95 confidence intervals, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (between n.t. and treatments).

RESULTS:

Low concentration of MWCNTs dispersed in water evoke, in a short period (24h) a strong inflammatory response in the leech body wall involving monocyte-macrophages cells activation and migration. TEM analysis revealed that MWCNTs can enter cells both by diffusing through cell membranes (membrane piercing) and active uptake (phagocytosis). Within cells, MWCNTs accumulate and causes the decrease of cell proliferation rate and the increase of apoptosis and ROS production.

CONCLUSIONS: Our combined experimental approaches, not only attest the ability of MWCNTs in inducing a potent inflammatory response, but also confirm *H. medicinalis* as a good alternative model that can be successfully used to study, both *in vivo* and *in vitro*, the possible harmful effects of any nanomaterial.