

Supplemental Material

Supplemental Materials and Methods

SWCNT particles

Commercially manufactured raw SWCNT made by the Arc method in specifically designed chambers was obtained through collaboration with the National Institute of Standards and Technology (NIST, Gaithersburg, MD). The SE grade SWCNT manufactured by CarboLex was reported to have a purity of 70-90% and contained catalyst impurities encapsulated in carbon shells (Carbolex Inc., Broomall, PA). Some amorphous carbon was also found on the outer surfaces of the ropes. The SWCNT were heated by the manufacturer to 2,800° C in an argon atmosphere using a graphite furnace to improve structural integrity and to remove polyaromatic hydrocarbons and a significant amount of iron. The individual SWCNT are self-assembled into a rope bundle structure. SWCNT manufactured by this method are reported to have an average diameter of 1.4 nm with lengths of 2-5 μm .

Specific surface area measurements

An electrostatic loop discharge was used to minimize static charge in samples before weighing the SWCNT into preweighed and precalibrated glass vials. The preweighed samples were degassed for 18 h at 250° – 300° C under a vacuum pressure of ~0.15 Torr. In SWCNT samples, four adsorption/desorption isotherms were measured using Quantachrome Instruments (Boynton Beach, FL). BET equivalent surface area was calculated for 3-5 adsorption points at a relative pressure of 0.006-0.07 using pure nitrogen. An alumina reference standard (SARM 2005, Quantachrome) with a certified specific surface area of $108.1 \pm 6.6 \text{ m}^2/\text{g}$ was used as a reference. The mean equivalent specific surface area for the four SWCNT samples evaluated was $293 \pm 15 \text{ m}^2/\text{g}$.

SWCNT dispersion

For cell culture studies, SWCNT was dispersed (5 mg/ml) by an indirect ultrasonication at 4° C, at full power for 10 min in RPMI-1640 medium (American Type Culture Collection, Rockville, MD) containing 1% fetal bovine serum (FBS) as the dispersion vehicle. This was followed by a brief direct (probe) ultrasonication at 10% output for 1 min. Samples were then resonicated by a direct ultrasonication for 1 min before use.

Mesothelial cell culture

In the present study we used normal human mesothelial (NM) and malignant human mesothelial (MM) cells obtained from American Type Culture Collection (Manassas, VA), and grown in RPMI-1640 medium (American Type Culture Collection) supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin, and 5% FBS at 37° C in a humidified atmosphere (5% CO₂ plus 95% air). The medium was changed twice weekly, and cells were trypsinized and subcloned weekly. Prior to SWCNT exposure, the culture medium was replaced with the RPMI-1640 medium containing 100 µg/ml streptomycin, 100 units/ml penicillin, and 0.1% FBS.

Light microscopy, scanning and transmission electron microscopy

The SWCNT samples prepared as described above were diluted in deionized water and applied on slides. When observed under a light microscope, they showed a uniform dispersion of small agglomerates of SWCNT material (See Supplemental Material, Figure 1A). To characterize the morphological features by scanning electron microscopy (SEM) before dispersing samples, SWCNT suspensions were filtered through a 0.4 µm nucleopore filter and deposited on carbon planchettes using a small amount of water and dried. Planchettes containing

SWCNTs were then mounted with double-stick carbon tape on an aluminum stub and sputter coated with gold/palladium. Images, collected using a JEOL 6400 SEM, showed multiple nanoropes and occasional discrete single strands of nanotubes (See Supplemental Material, Figure 1B).

For transmission electron microscopy (TEM), the SWCNT samples prepared as described above in 1% FBS was diluted ten times and filtered through a 0.4 μm Nucleopore filter. Samples were deposited on to a formvar-coated copper grid, dried, and photographed using a JEOL-1220 transmission electron microscope. Discrete SWCNT having a diameter of 0.8-2.0 nm are visible (See Supplemental Material, Figure 1C).

Determination of trace metal content in the SWCNT sample

The trace metal content of the SWCNT samples used in the present study was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Samples were digested in 1 ml of concentrated HNO_3 and 5 ml HClO_4 at 150° C. An additional 5 ml HClO_4 was added and the reagent refluxed for 15 days at 180° C. The dissolved samples were then dried at 150° C, and the residue was dissolved in 4% HNO_3 and 1% HClO_4 for trace metal analysis and transferred to 10 ml volumetric flasks by rinsing with ASTM Type II water and brought up to a 10 ml volume. Aliquots were then analyzed for Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Sr, Te, Ti, V, Y, Zn, and Zr using a Thermo-Jarrel Ash ICAP-61 inductively coupled plasma emission spectrometer controlled by Thermospec software, according to NIOSH method 7500 (NIOSH NMAM 1994).

Electron spin resonance (ESR) assay

The production of reactive oxygen species (ROS) was determined using a Bruker ESP 300E EMX spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly. The spin trap, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), was purified by charcoal decolorization and vacuum distillation. The DMPO solution, thus purified, did not contain any ESR detectable impurities. The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants. Reactants (10^6 cells/ml, PBS, and 100 mM DMPO) were mixed in test tubes in a final volume of 1.0 ml with 500 μ g/ml SWCNT for 10 min, filtered and then transferred to a flat cell for ESR measurements. The ESR spectrum produced consists of a 1:2:2:1 quartet with hyperfine splitting of $a_H = a_N = 14.9$ G, where a_H and a_N denote hyperfine splittings of the nitroxyl nitrogens and α -hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was assigned the DMPO- \cdot OH adduct, which is evidence of hydroxyl radical (\cdot OH) generation (Ding et al. 2001). Experiments were performed at room temperature and under ambient air.

Intracellular detection of O_2^- and H_2O_2 in intact cells by confocal microscopy

Intracellular production of ROS generation was investigated in mesothelial cells exposed to SWCNT, crocidolite, or vehicle was assayed according to protocols described in Ding et al. 2001. Briefly, NM and MM mesothelial cells were seeded onto a glass coverslip in the wells of a 24-well plate for 24 h. The cells were exposed to SWCNT or crocidolite at 150 μ g/cm² or vehicle for one h in the presence of 25 μ M dihydroethyidium or H₂DCFDA. After the exposure the cells were washed in PBS, fixed in buffered formalin for 10 min, and mounted on glass slides using Prolong Antifade (Molecular Probes, Eugene, OR). Slides were then observed and

photographed using a Carl Zeiss LSM 510 (Carl Zeiss Inc., Thornwood, NY) laser scanning microscope.

Cell viability assay

Viability of NM and MM cells (5×10^4) was measured using an MTT assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, the cells were exposed to SWCNT or vehicle for 24 h and then incubated for 4 h in the presence of MTT reagent and lysed with lysis buffer. After 12 h for the complete solubilization of formazan crystals, absorbance was measured at A_{550} nm to A_{690} nm using a SpectraMax 250 microplate spectrophotometer reader (Molecular Devices Co., Sunnyvale, CA).

Trypan blue exclusion assay

The NM and MM cells (1×10^5) were seeded overnight in normal growth medium. After 12 h, the cells were exposed to 12.5, 25, 50, 125 $\mu\text{g}/\text{cm}^2$ of SWCNT or crocidolite in same mass concentrations in 0.1% FBS or vehicle alone for 24 h. After the incubation period, the cells were washed in Hank's buffered salt solution (HBSS), harvested with a cell scraper in HBSS, and placed on ice. Cell viability was determined immediately after the exposure using trypan blue stain (Sigma Chemical Co., St. Louis, MO). The cell suspension was mixed with an equivalent volume of 0.4% trypan blue solution, and cell counting was performed using a hemacytometer with a light microscope. The results are expressed as percent viable cells.

Lactate dehydrogenase (LDH)

LDH release from NM and MM cells exposed to SWCNT and crocidolite asbestos was measured as an index of cell membrane integrity. In these studies 1×10^5 NM or MM cells in one ml of HBSS were incubated with 25 or 50 $\mu\text{g}/\text{cm}^2$ of SWCNT or crocidolite asbestos for 24 h at 37° C. At the termination of incubation, cell suspensions were centrifuged and the supernatant collected to measure LDH activity using a Cobas Fara II Autoanalyzer using Roche LDH kits (Roche Diagnostics Systems, Monclair, NJ). Enzyme activity measured monitoring the formation of NADH by the enzyme catalyzed by the reaction of pyruvate and NAD. LDH activity is expressed as units/liter of cell supernatant.

Compared to control, crocidolite induced a significant increase in LDH release. MM cells exposed to same concentrations of asbestos induced LDH release at a greater level than the NM cells. SWCNT exposed NM and MM cells showed significantly lower levels of LDH release compared to controls. Furthermore, a higher concentration of 50 $\mu\text{g}/\text{cm}^2$ SWCNT exposure induced a lower level of LDH release than the 25 $\mu\text{g}/\text{cm}^2$ in both cell types. Crocidolite asbestos on the other hand caused a significantly greater level (1.7-fold) of LDH release in both cells types. However, exposure to higher doses of SWCNT resulted in a decrease in LDH activity in both cell types which was found to be associated with the ability of SWCNT (carbon) to interfere with the assay. Similar interference results were observed with standard known concentrations of spiked LDH in the presence of SWCNT.

DNA damage by comet assay

NM and MM cells cells (10^6) DNA damage was assessed using a commercially available comet assay according to the manufacturer's instructions (Trevigen, Gaithersburg, MD). Briefly,

slides with agarose-embedded cells were immersed in prechilled lysing solution and kept at 4° C for 1 h. The slides were then placed in a horizontal gel-electrophoresis tank, covered with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) for 20 min and electrophoresed at 300 mA for 30 min. After electrophoresis, slides fixed in 70% ethanol, dried, and stained with SYBR Green I. All procedures were conducted under dim light or in dark to prevent potential DNA damage. The slides were visualized by fluorescence microscopy, using an image capturing system (Olympus AX 70 and SamplePCI, Compix, Cranberry Township, PA). For each sample a minimum of 50 cells were scored at 400 X magnification. The length of the comet tail, which indicates DNA damage resulting in migration, was determined as the distance between edge of head and end of tail using an automated image analysis system (Optimas 6.51, Media Cybernetics Inc., Silver Spring, MD).

H2AX phosphorylation of DNA double strand breaks assay

In this chemiluminescence assay a specific mouse monoclonal antibody, anti-phospho-Histone H2AX is used for measuring DNA double strand breaks (DSBs). NM and MM cells cultured in black-wall/clear bottom microplates were exposed to SWCNT or crocidolite asbestos to induce DNA damage and H2AX phosphorylation for 24 h. Detection of H2AX phosphorylation was determined according to manufacturer's protocol (Upstate, CA). Briefly, after fixation and permeabilization of cells phosphorylated histone H2AX is detected by the sequential addition of anti-phospho-H2AX and an anti-mouse-HRP conjugate. The chemiluminescent HRP substrate LumiGLO is then added, and the signal is measured using a microplate luminometer.

Western blot analysis of cleaved PARP

NM and MM cells were subcultured in 6-well plates and maintained for 24 h in 10% FBS RPMI 1640 growth medium. The standard growth medium was then replaced with 0.1% FBS containing medium and the cells were exposed to 50 $\mu\text{g}/\text{cm}^2$ SWCNT or crocidolite for 0, 6, and 18 h. After exposure, the cells were washed once with two volumes of ice cold 1x PBS, and lysed with 1X SDS sample blue buffer supplemented with 1 $\mu\text{l}/\text{ml}$ Sigma's Protease inhibitor cocktailTM in 1 mM PMSF on ice for 10 min. Cell debris was removed by microcentrifugation for 15 min at 14,000 rpm. Twenty μg protein per sample were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Immunoblotting for cleaved PARP, full PARP, and Beta-actin was determined using the same membrane after stripping of the blot. The optical densities of bands were analyzed using a BioRad Fluor-STM MultiImager gel documentation system. Individual band intensities for cleaved PARP were expressed as fold activation after normalizing the intensities to Beta-actin and full PARP.

Protein kinase phosphorylation assay

The NM and MM cells (10^6) were treated with of SWCNT or vehicle alone containing 0.1% FBS for varying time periods up to 120 min. After treatment, the cells were lysed in a lysing buffer, Tris-Glycin SDS sample buffer supplemented with 50 mM DTT (Invitrogen, Carlsbad, CA), and left on ice for 10 min. Whole cell lysates were collected and sonicated for 8 sec, boiled at 100° C for 5 min, and centrifuged at 5500 rpm at 4° C. Protein contents in the supernatants were determined, using a BCA Protein Assay Kit (Pierce, Rockford, IL). Immunoblots for phosphorylation of ERKs, JNKs, and p38 kinase were carried out, using PhosphoPlus MAPK antibody kits as described in the protocol by manufacturer (New England

BioLabs, Boston, MA). Phosphospecific antibodies were used to detect phosphorylated sites of ERKs, JNKs, and p38 kinase as previously described (Ding et al. 2001). Nonphosphospecific antibody against p38 kinase protein was used to ensure equal loading of protein and to normalize the phosphorylation assay, using the same transferred membrane blot following a stripping procedure.

Western blot analysis of Akt

NM and MM cells (2×10^5) were cultured in 6-well plates to 90% confluence with 10% FBS RPMI 1640 medium. The cells were washed and exposed to 25, 75, 125 $\mu\text{g}/\text{cm}^2$ of SWCNT or vehicle in 0.1% FBS for 15 or 30 min. The cells were washed and lysed with SDS sample buffer, and then subjected to Western blot analysis. Phosphorylation of Akt was detected, using phospho-specific antibody against a phosphorylated site (Thr-308) of Akt. Non-phospho-specific antibodies were used to normalize the phosphorylation assay, using the same-transferred membrane blot. EGF (40 ng/ml) was used as a positive control.

References

NIOSH NMAM. 1994. NIOSH Manual of Analytical Methods, 4th edition.

Ding M, Shi X, Lu Y, Huang C, Leonard SL, et al. 2001. Induction of activator protein-1 through reactive oxygen species by crystalline silica in JB6 cells. *J Biol Chem* 276:9108–9114.

Supplemental Material, Table 1. Concentrations of major trace metal contaminations in the SWCNT ($\mu\text{g/g}$) determined by ICP-AES.

Metals	Al	Co	Cr	Fe	Mg	Mn	Ni	Y	Zn	Zr
$\mu\text{g/g}$	924	24	3,365	732	43	11	206,000	62,000	1,035	48
Percent	0.09%		0.34%	0.07%			20.6%	6.2%	0.1%	

Supplemental Figure Legends

Supplemental Material, Figure 1. Microscopic image of a random sample of the raw SWCNT.

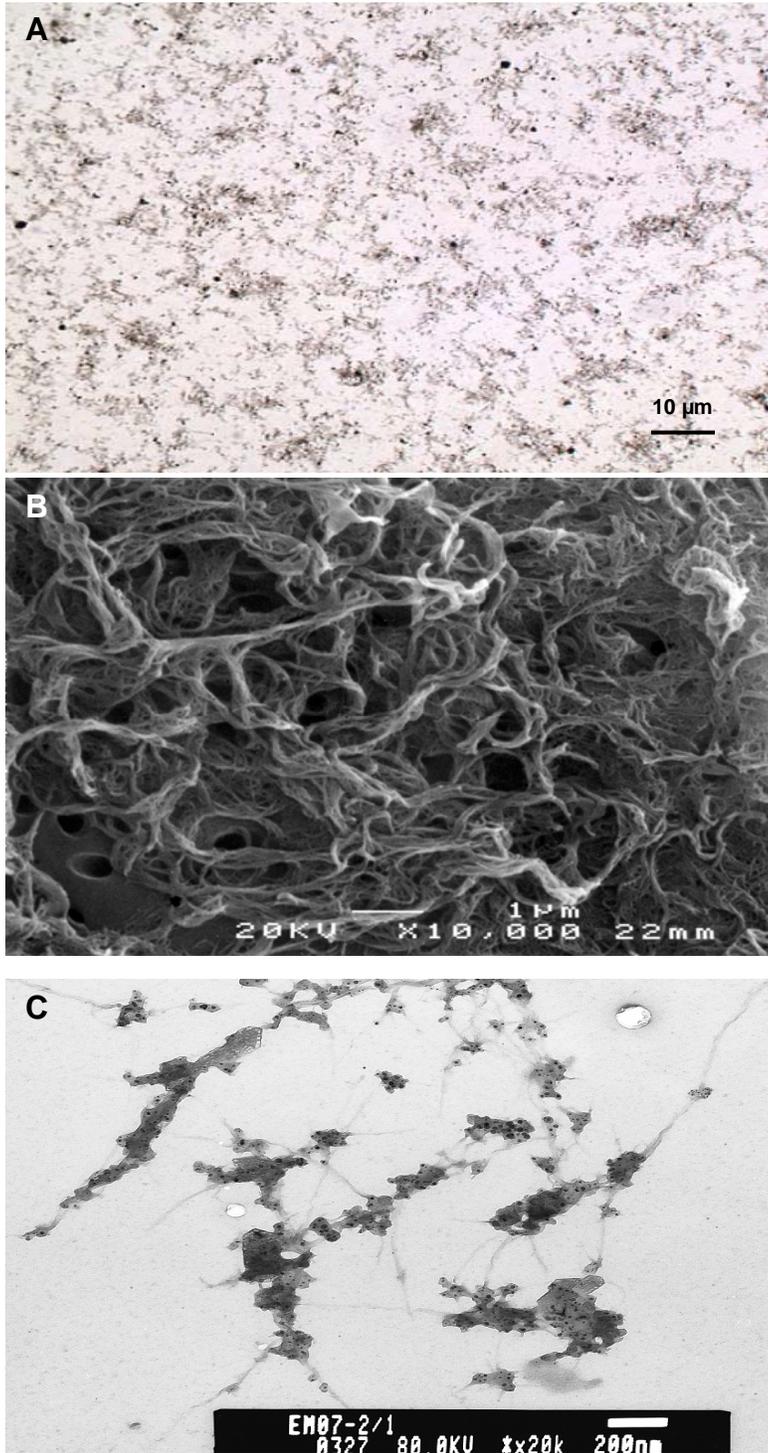
A, Light microscopic images of raw SWCNT were prepared in cell culture medium containing 0.1% FBS used in cellular studies. Note the SWCNT particles were dispersed as a uniform distribution of small agglomerates of SWCNT. B, Scanning electron micrograph of SWCNT ropes with an average diameter of < 100nm. Few amorphous carbon and catalytic impurities are present. C, Transmission electron micrograph of SWCNT as used in exposing mesothelial cells. Discrete single-wall carbon nanotubes and metal catalysts are visible.

Supplemental Material, Figure 2. Electron spin resonance analysis of NM and MM cells

exposed to SWCNT. A, ESR spectra showing signal intensity of DMPO-OH adducts generated by the interaction of NM and MM cells with 500 µg/ml SWCNT. All the spectra were recorded after suspending (10^6 cells/ml) cells for 5 min in incubation medium containing 100 mM DMPO and PBS with and without SWCNT. Co-incubation of catalase (2,000 units/ml), and deferoxamine ((2mM), not shown) were used to confirm the generation of \cdot OH radicals and investigate the role of iron in ROS generation. Instrumental conditions: microwave power, 63.96 mW; modulation amplitude, 1.0 G; time constant, 40.96ms; center field, 3480 G; sweep width, 100 G. The presented spectrum is from one experiment. B, Semiquantitative relative ESR signal intensity of DMPO-OH adducts generated

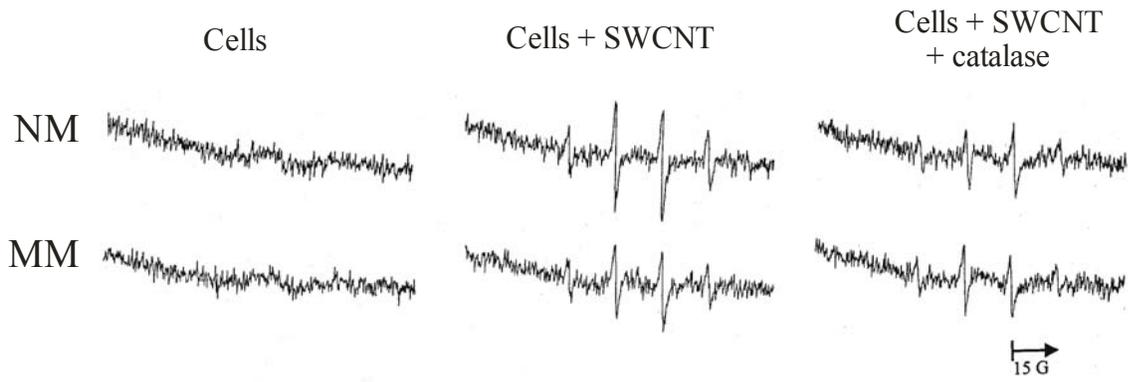
by the interaction of NM and MM cells with 500 $\mu\text{g/ml}$ SWCNT. All the spectra were recorded as indicated in Figure S2A. Co-incubation of samples with catalase (2,000 units/ml), and deferoxamine (2mM) were used to confirm the generation of $\bullet\text{OH}$ radicals, and investigate the role of iron in ROS generation. The results presented are the means \pm SEM of 3 experiments in replicates. * Indicates a significant difference between NM and MM cells. + Indicates a significant decrease from SWCNT alone ($p \leq 0.05$).

Supplemental Material Figure 3. Viability of NM and MM cells. A, Effect of SWCNT on NM and MM cell viability assayed by MTT. B, Effect of SWCNT on NM and MM cell viability assayed by trypan blue. C, Effect of crocidolite on NM and MM cell viability assayed by trypan blue. The cells were treated with dispersion vehicle (control) or 12.5, 25 or 125 $\mu\text{g/cm}^2$ of SWCNT, or crocidolite for 24 h. Cell viability was determined by MTT or by trypan blue staining assay as described in Supplemental Materials and Methods. The results presented are the means \pm SEM of 3 experiments. * Indicates a significant decrease from control ($p < 0.05$).

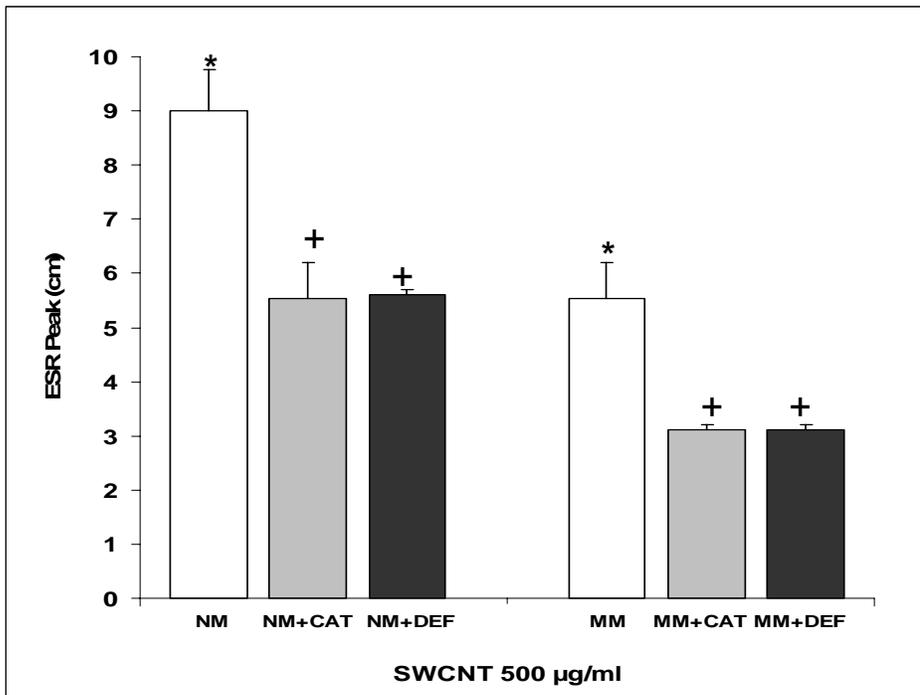


Supplemental Material, Figure 1

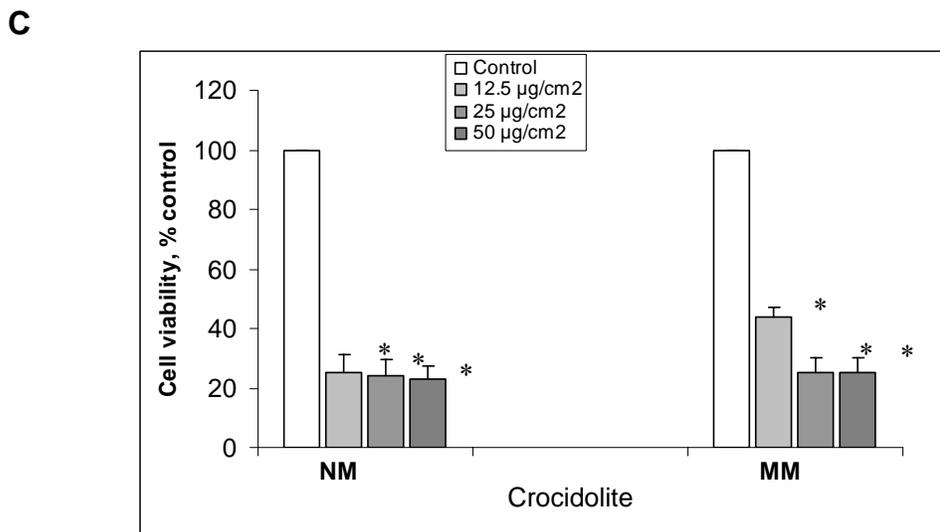
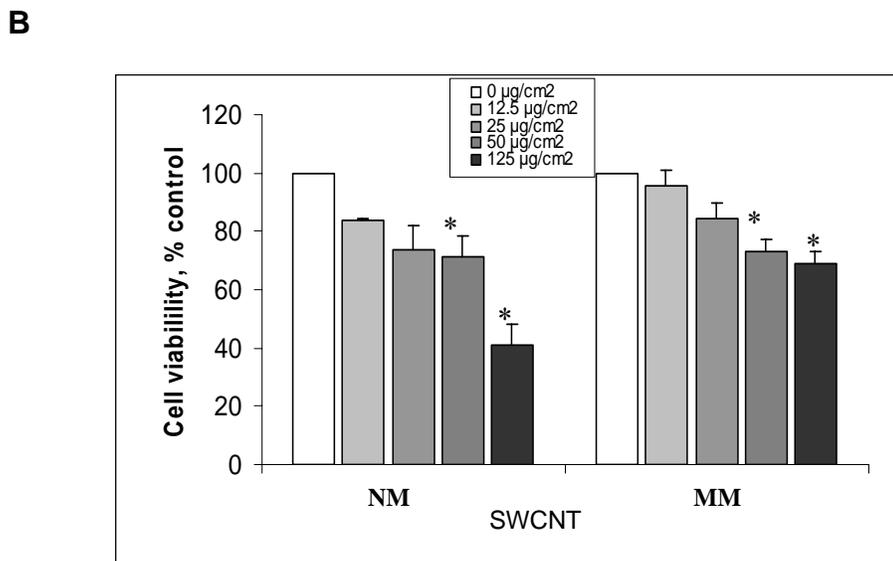
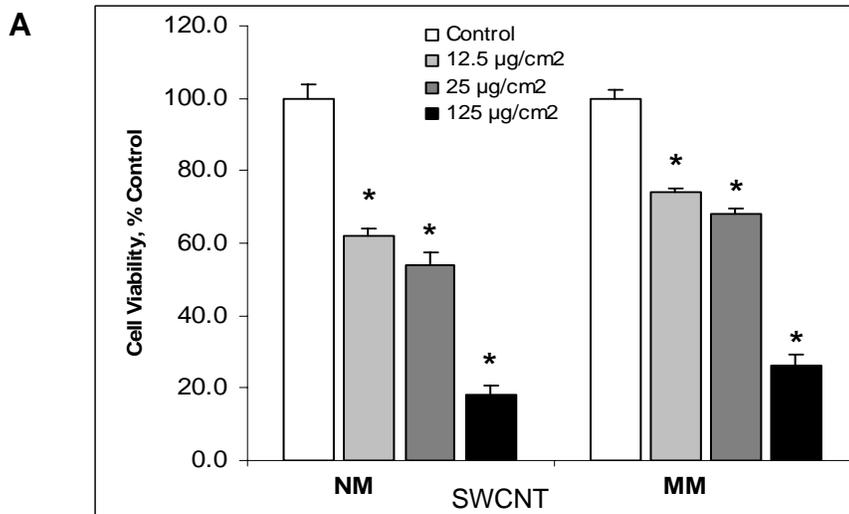
A



B



Supplemental Material, Figure 2



Supplemental Material, Figure 3