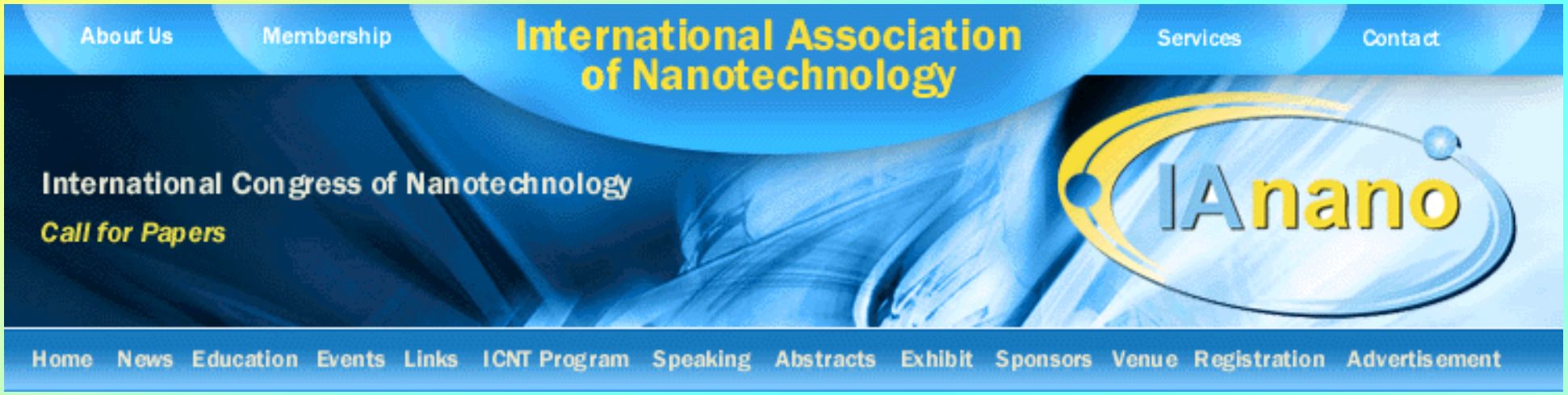
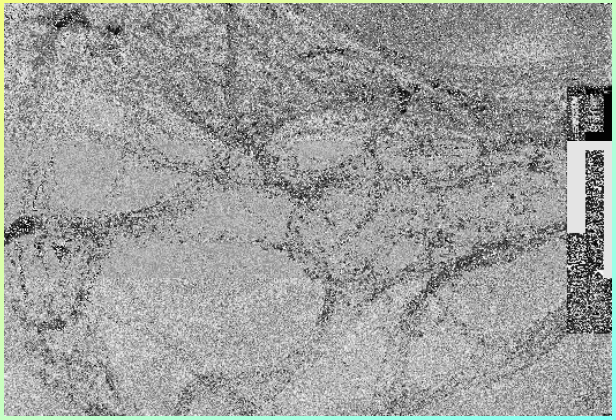


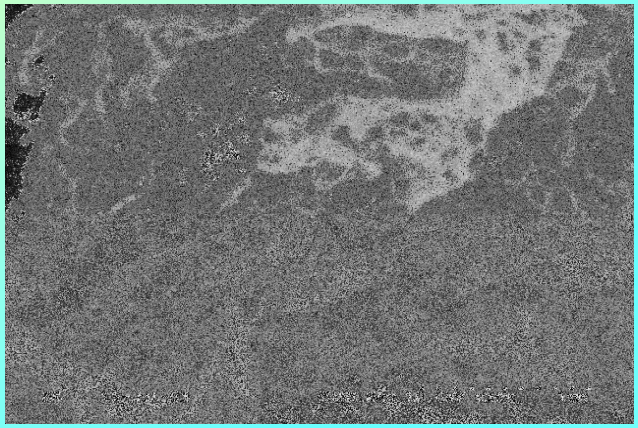
Valerian E. Kagan

Macrophage Response to Single Walled Carbon Nanotubes: Oxidative Stress and Inflammatory Consequences.

Center For Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, University of Pittsburgh,

Single Walled Carbon Nanotubes

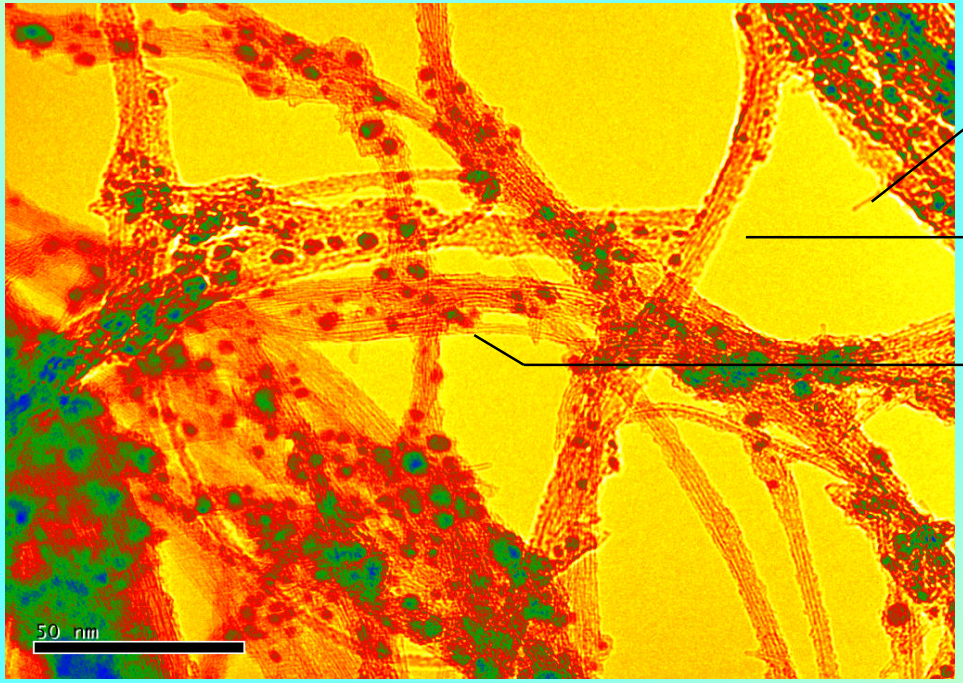




‘Tangles’ of nanotubes and nanoropes

Handling nanotube materia

Raw SWCNT material



Nanotubes

Nanoropes

Catalyst particles

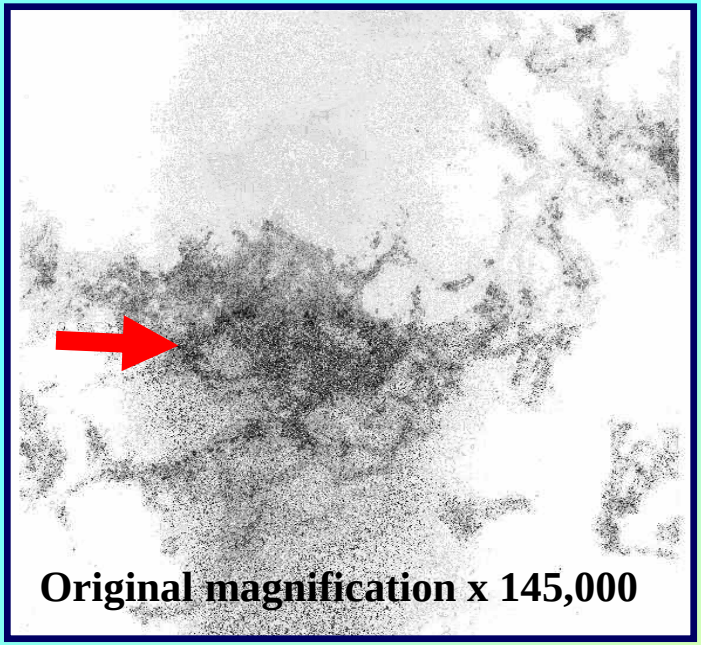
Raw single walled carbon nanotube material.

Courtesy of Andrew Maynard

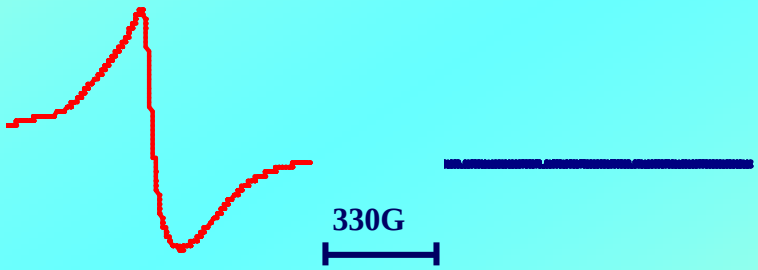
Metal Components of SWCNT

Component	(μg/gram)	Component	(μg/gram)
Aluminum	233	Molybdenum	1070
Calcium	164	Sodium	8750
Cadmium	23.4	Nickel	8,750
Chromium	13.1	Palladium	28
Copper	2,530	Selenium	<2.001
Iron	239, 000	Titanium	6.92
		Zinc	85.9

Transmission Electron Microscopy of SWCNT



Original magnification x 145,000



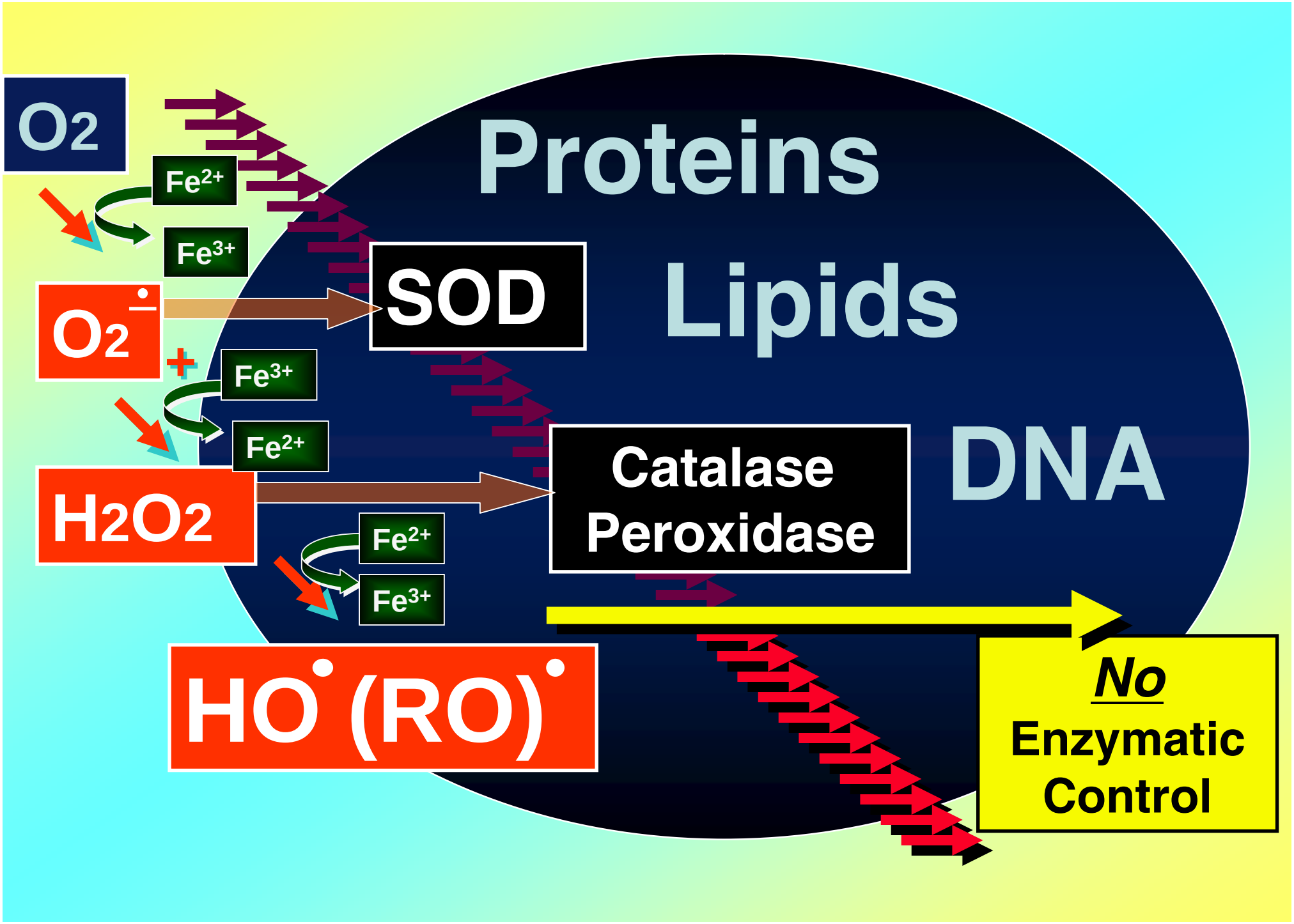
Partially-Purified SWCNT

Purified SWCNT+DFO

EPR spectra of partially-purified SWCNT manufactured by high-pressure CO conversion (HiPco™) technology as compared to purified SWCNT additionally treated with an iron chelator, deferoxamine (DFO).

Note that partially-purified SWCNT displayed a broad signal with g value 2.0 and half-width of 640G, the signal was not detectable in purified DFO-treated SWCNT.

Raw Samples of Carbon Nanotubes Contain Redox-Active Iron


O2
Fe2+
Fe3+
O2 -
+
Fe3+
Fe2+
H2O2
Fe2+
Fe3+
HO (RO)
SOD
Proteins
Lipids
DNA
Catalase
Peroxidase
No
Enzymatic
Control

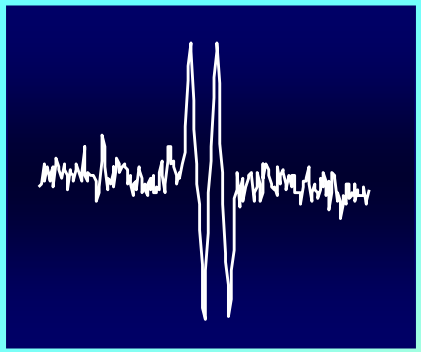
Iron-Catalyzed Decomposition of H_2O_2 Forms A Potent Oxidant – Hydroxyl Radical

H_2O_2

Fe^{2+}

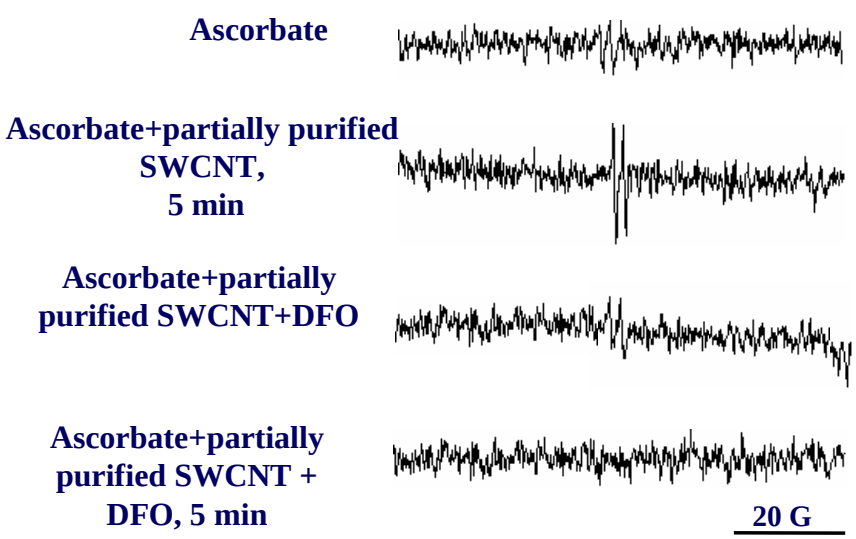
Fe^{3+}

$OH^{\bullet}$ + Ascorbate



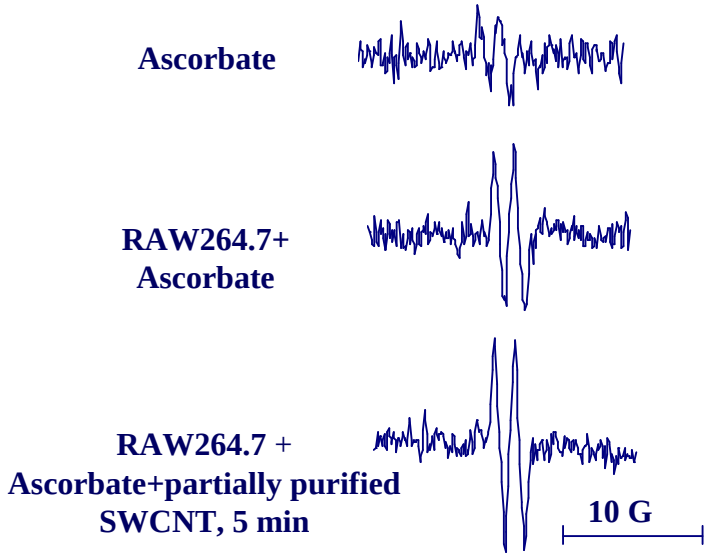
$Asc^{\bullet}$ + HOH

EPR spectra of ascorbate radicals generated by partially purified SWCNT.



Model system

Conditions: Ascorbate (10 mM) in PBS (pH 7.4); partially purified SWCNT (0.12 mg/ml, 2.5 wt% of iron); desferioxamine, DFO (0.2 mM);



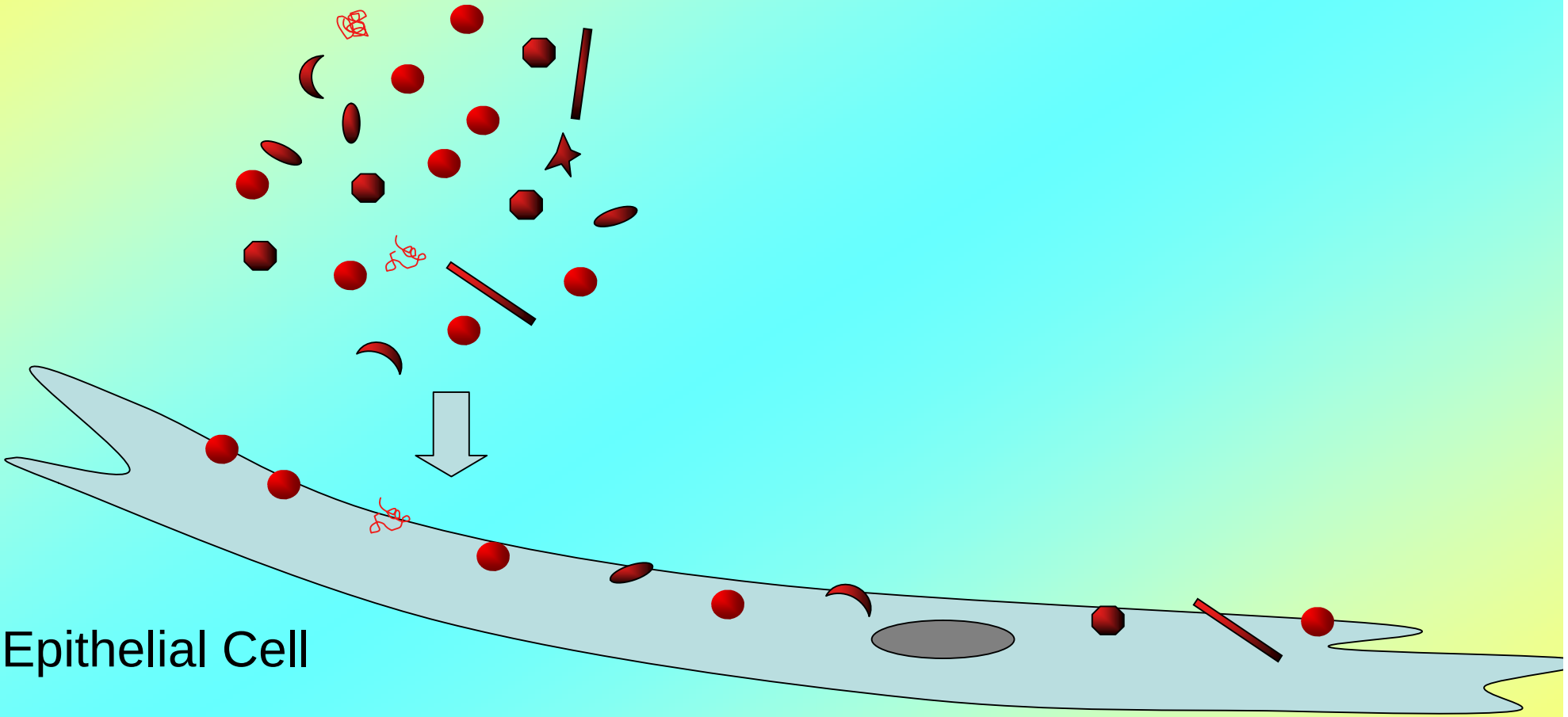
RAW264.7 macrophages

Conditions: Zymosan (2.5 mg/ml)-stimulated RAW264.7 macrophages (20x106 cells/ml); partially purified SWCNT (2.5 wt% of iron, 0.12 mg/ml)

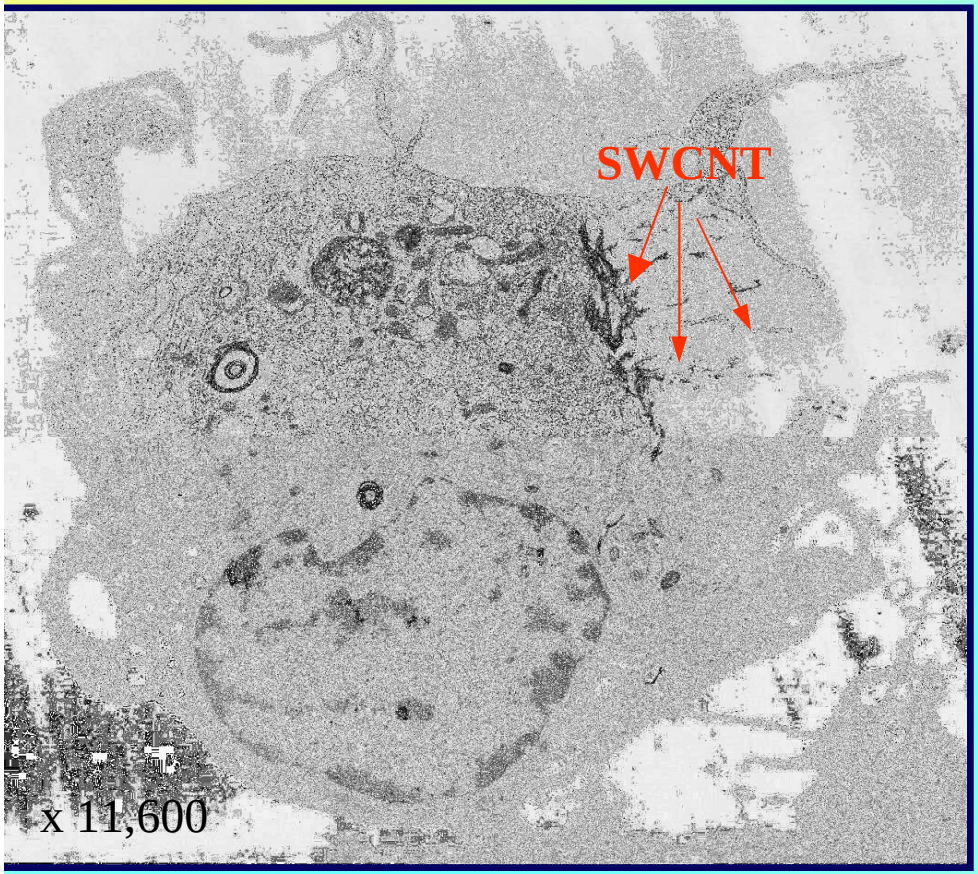
EPR conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 sec; conversion time, 0.6 sec.

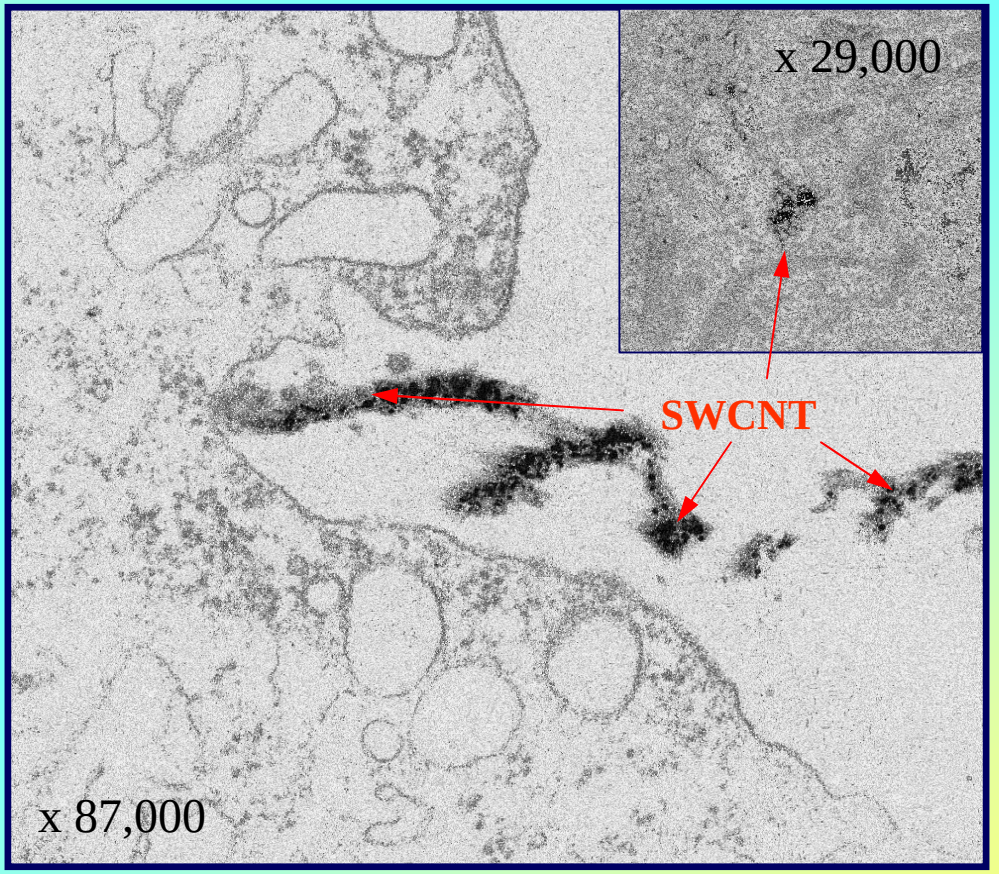
Carbon Nanotubes Directly Damage Broncho-Epithelial Cells

Particles, Nanotubes



Engulfment of SWCNT by BEAS-2B Cells

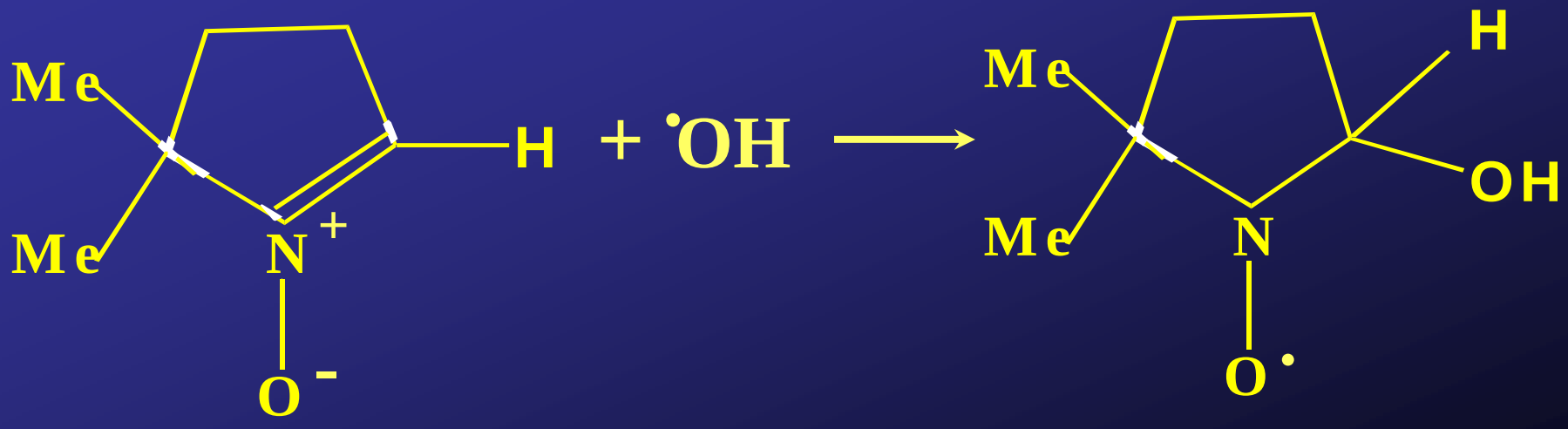




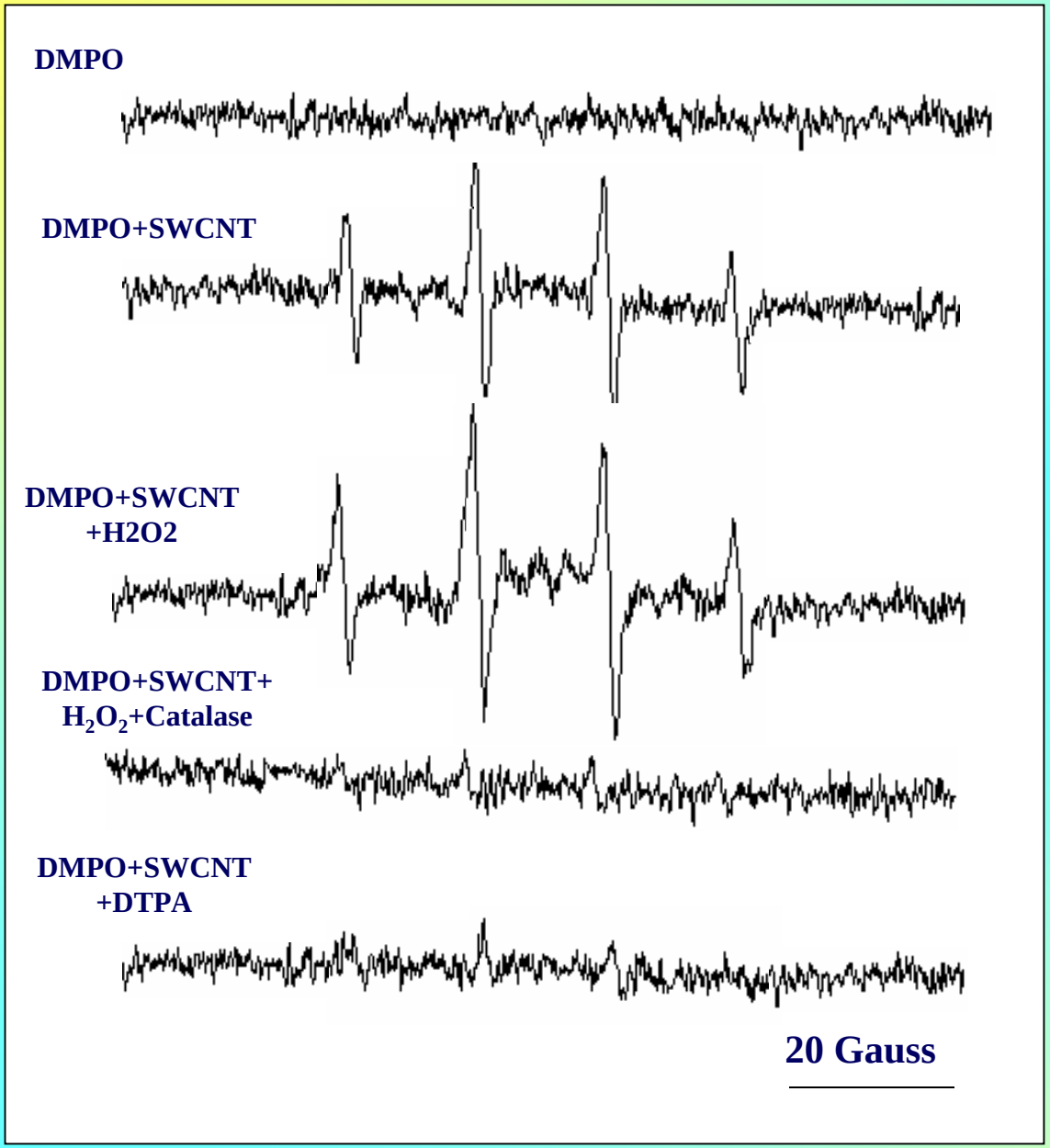
Fenton Reaction

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + {}^{\bullet}OH$$

DMPO Adduct Formation



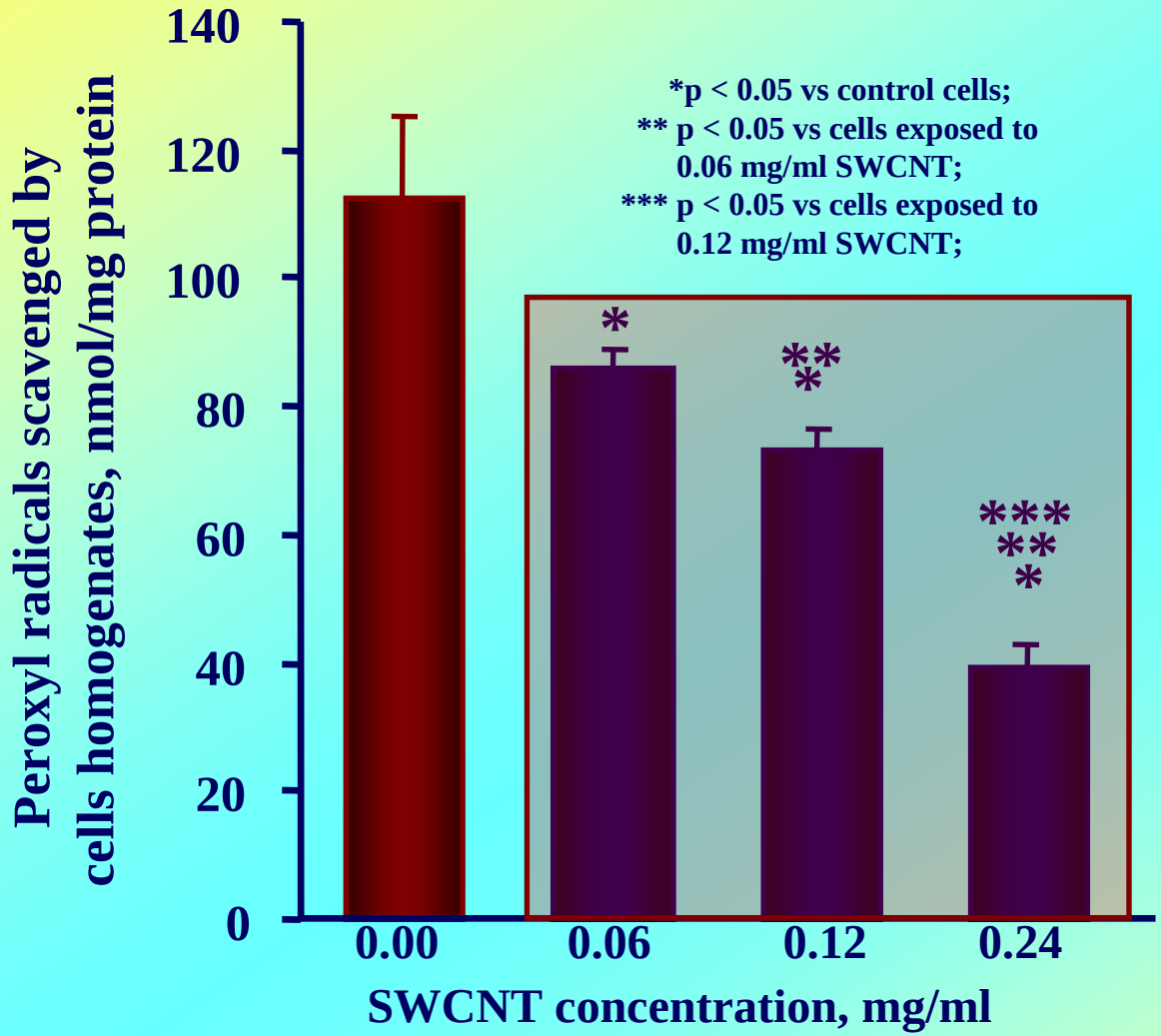
5,5-Dimethyl-1-Pyrroline-N-Oxide
(DMPO)



ESR spectra of DMPO adducts of free radicals formed by partially purified SWCNT in the presence of BEAS-2B cells.

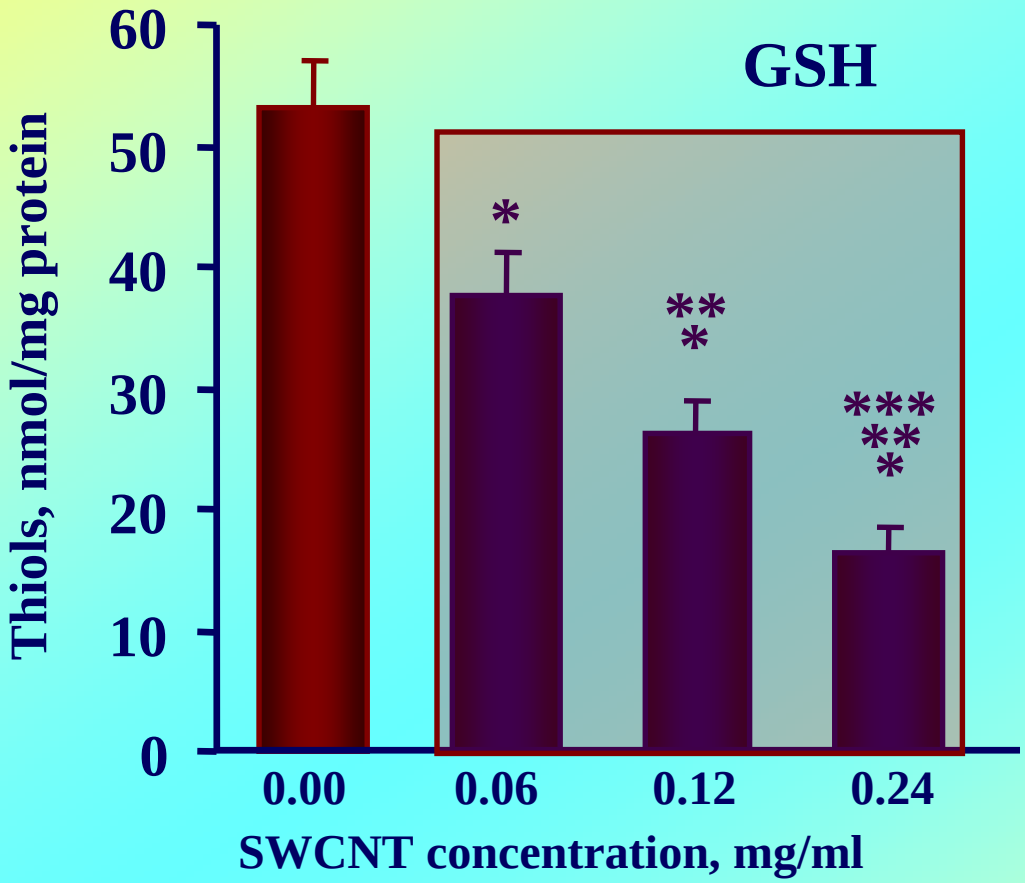
Conditions: BEAS-2B (2x105 cells/ml) in PBS (pH 7.4); 100 mM DMPO; partially purified SWCNT (2.5 wt% of iron, 0.12 mg/ml) H_2O_2 (1 mM); catalase (20 U/ml); DTPA (0.2 mM).

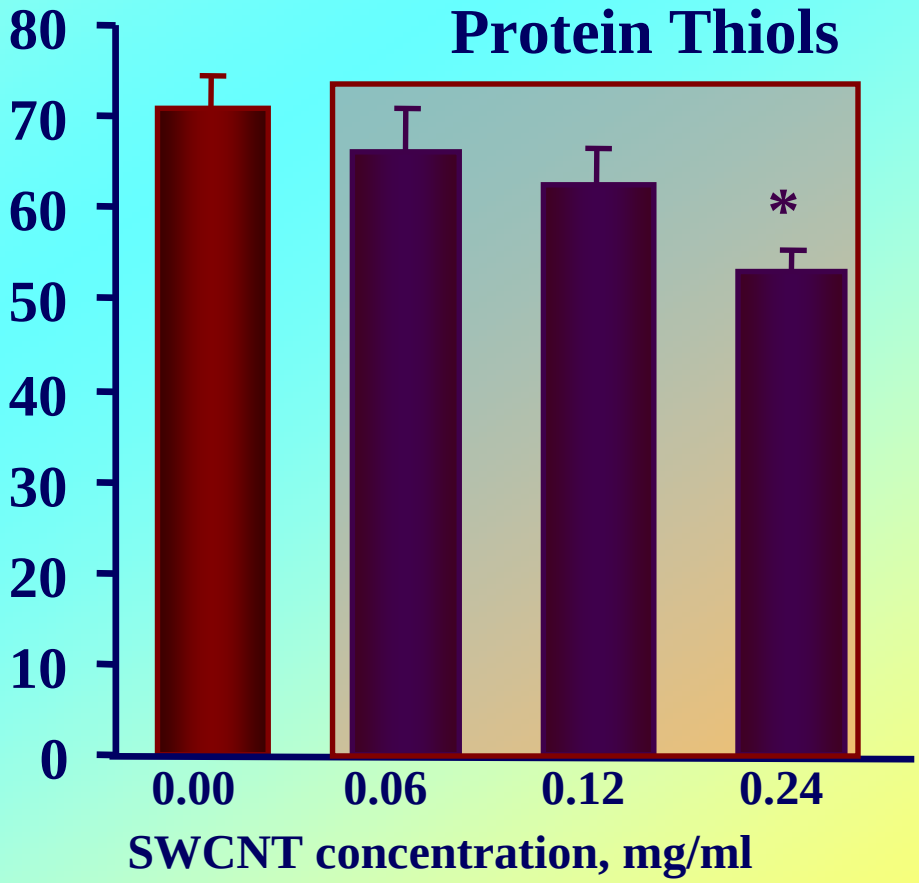
ESR conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 sec; conversion time, 0.6 sec.



Total Antioxidant Reserve in BEAS-2B Cells Following Exposure to SWCNT

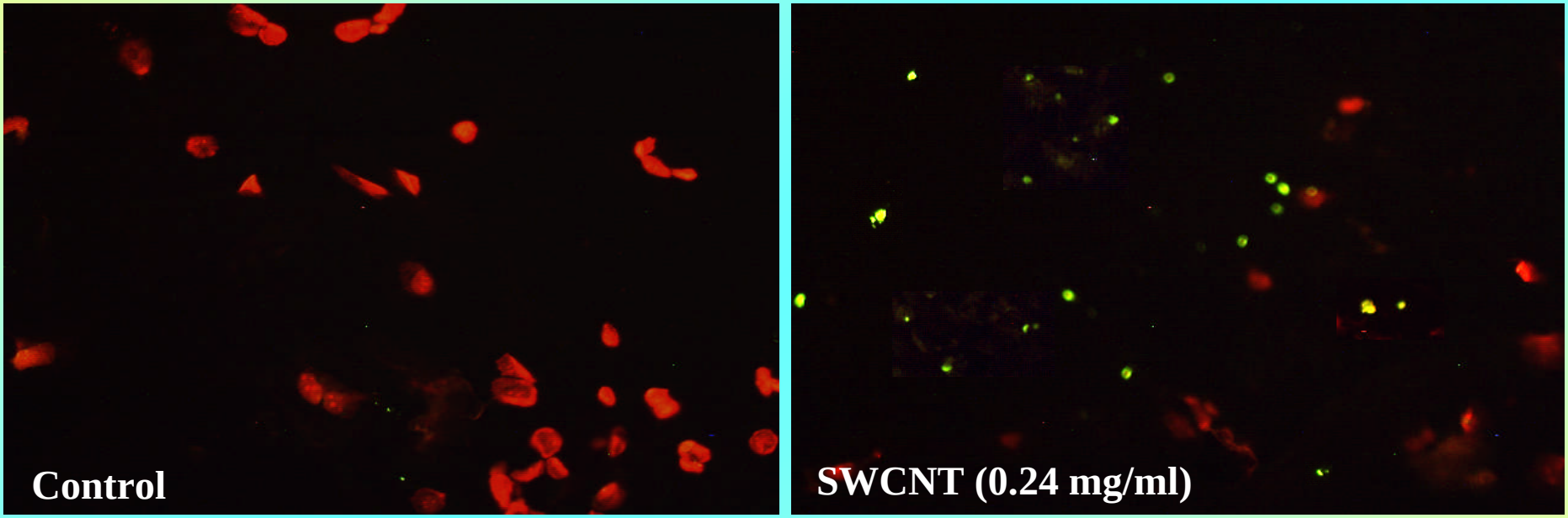
GSH and Protein Thiols in BEAS-2B Cells Following Exposure to SWCNT



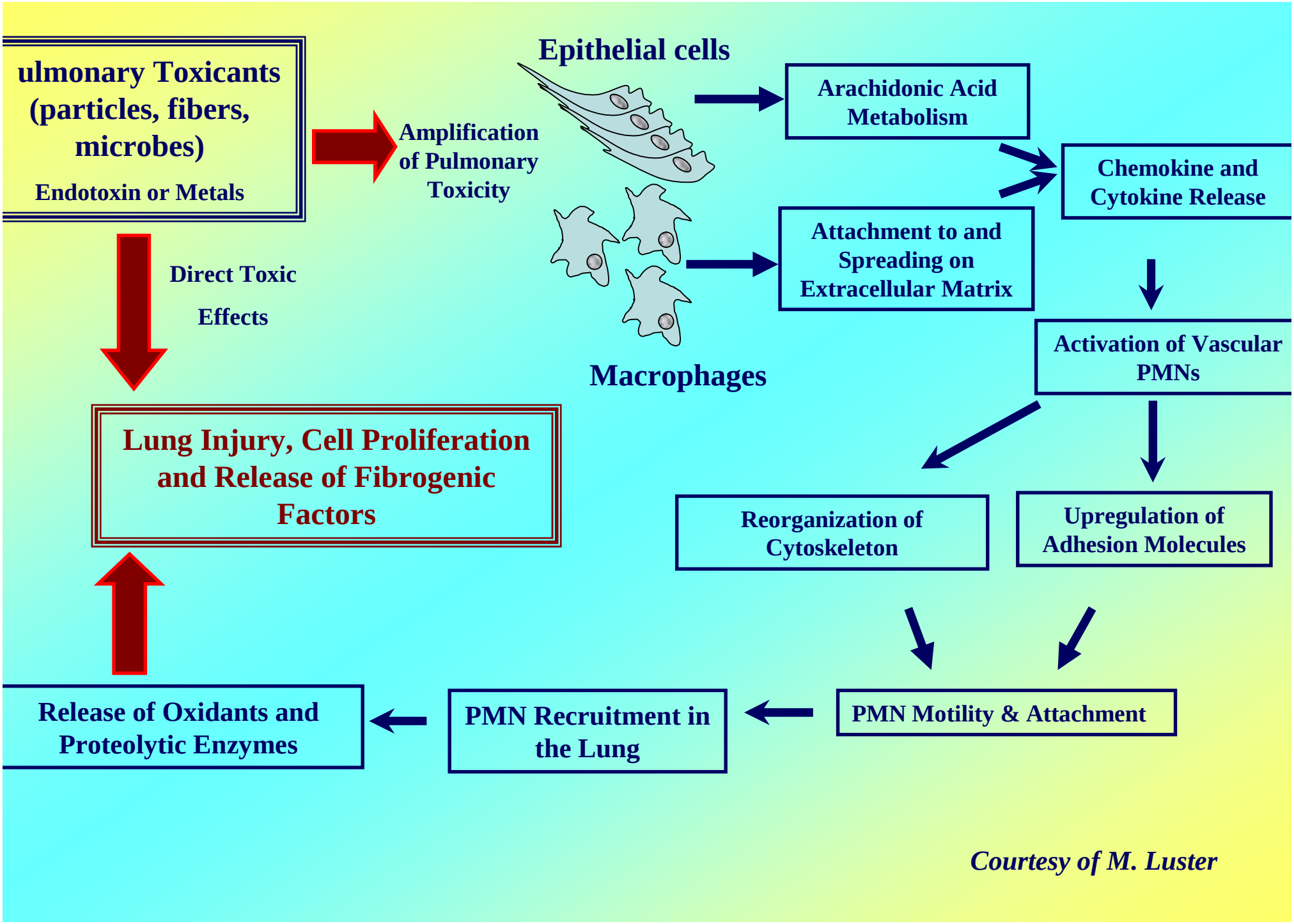


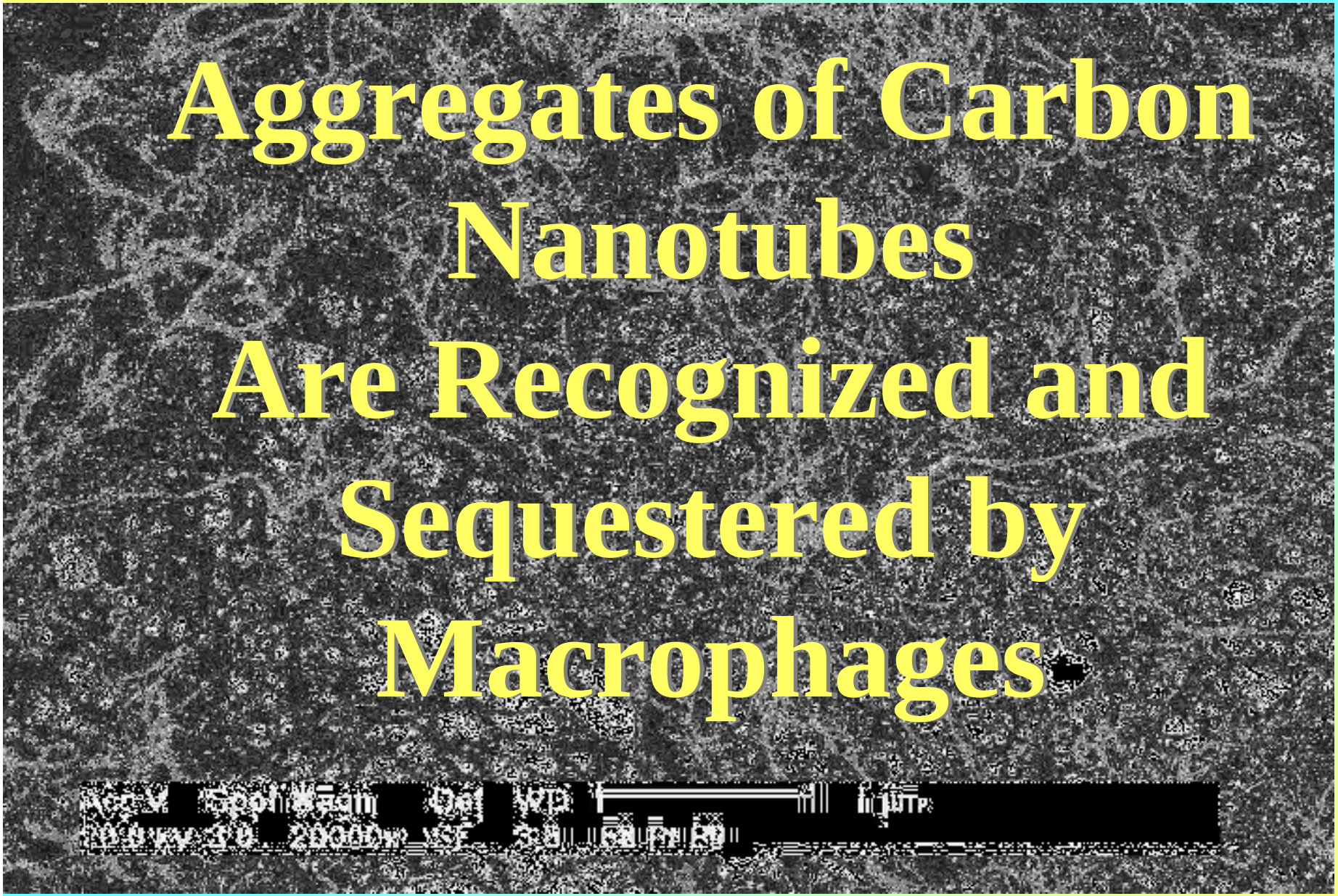
*p < 0.05 vs control cells; ** p < 0.05 vs cells exposed to 0.06 mg/ml SWCNT; *** p < 0.05 vs cells exposed to 0.12 mg/ml SWCNT;

Exposure to SWCNT Induced Apoptotic Cell Death

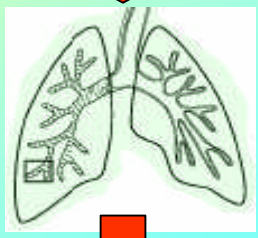


BEAS-2B cells were stained with TUNEL reagent. Apoptotic cells exhibited yellow-green fluorescence, while normal cells counter-stained with propidium iodide fluoresced red. Original magnification x 40.


ulmonary Toxicants (particles, fibers, microbes)
Endotoxin or Metals
Amplification of Pulmonary Toxicity
Epithelial cells
Arachidonic Acid Metabolism
Chemokine and Cytokine Release
Attachment to and Spreading on Extracellular Matrix
Macrophages
Direct Toxic Effects
Activation of Vascular PMNs
Lung Injury, Cell Proliferation and Release of Fibrogenic Factors
Reorganization of Cytoskeleton
Upregulation of Adhesion Molecules
Release of Oxidants and Proteolytic Enzymes
PMN Recruitment in the Lung
PMN Motility & Attachment
Courtesy of M. Luster


Aggregates of Carbon Nanotubes
Are Recognized and Sequestered by Macrophages

Day 0



Aspiration with:

CNT (10, 20, 40 μg/mouse)
UFCB (40 μg/mouse)
Silica (40 μg/mouse & 2.5 mg/mouse)

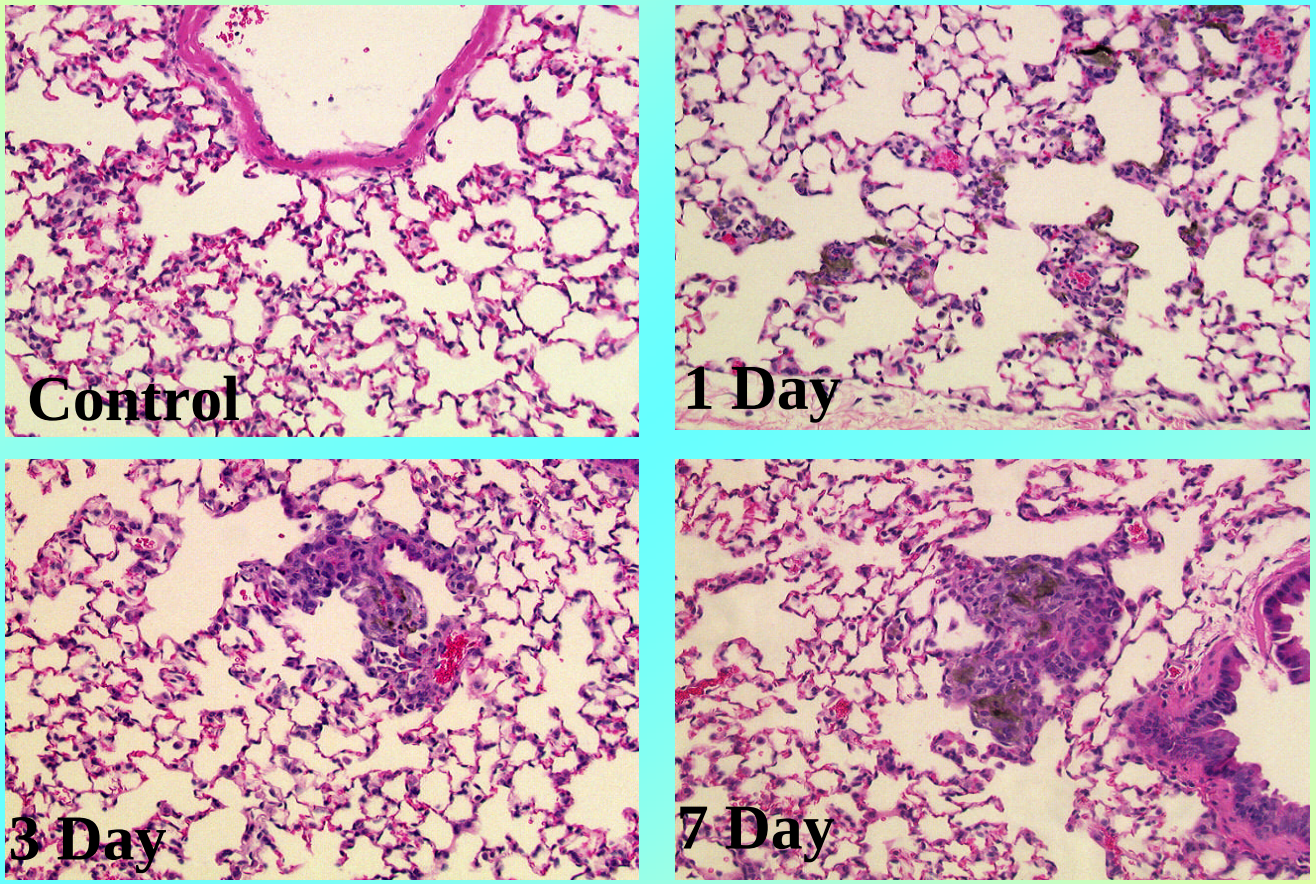
Days post Exposure

1 3 7 28 60

- Histopathology
- Pulmonary injury
- Lung functions
- Collagen morphometry
- Oxidative stress
- Cytokines

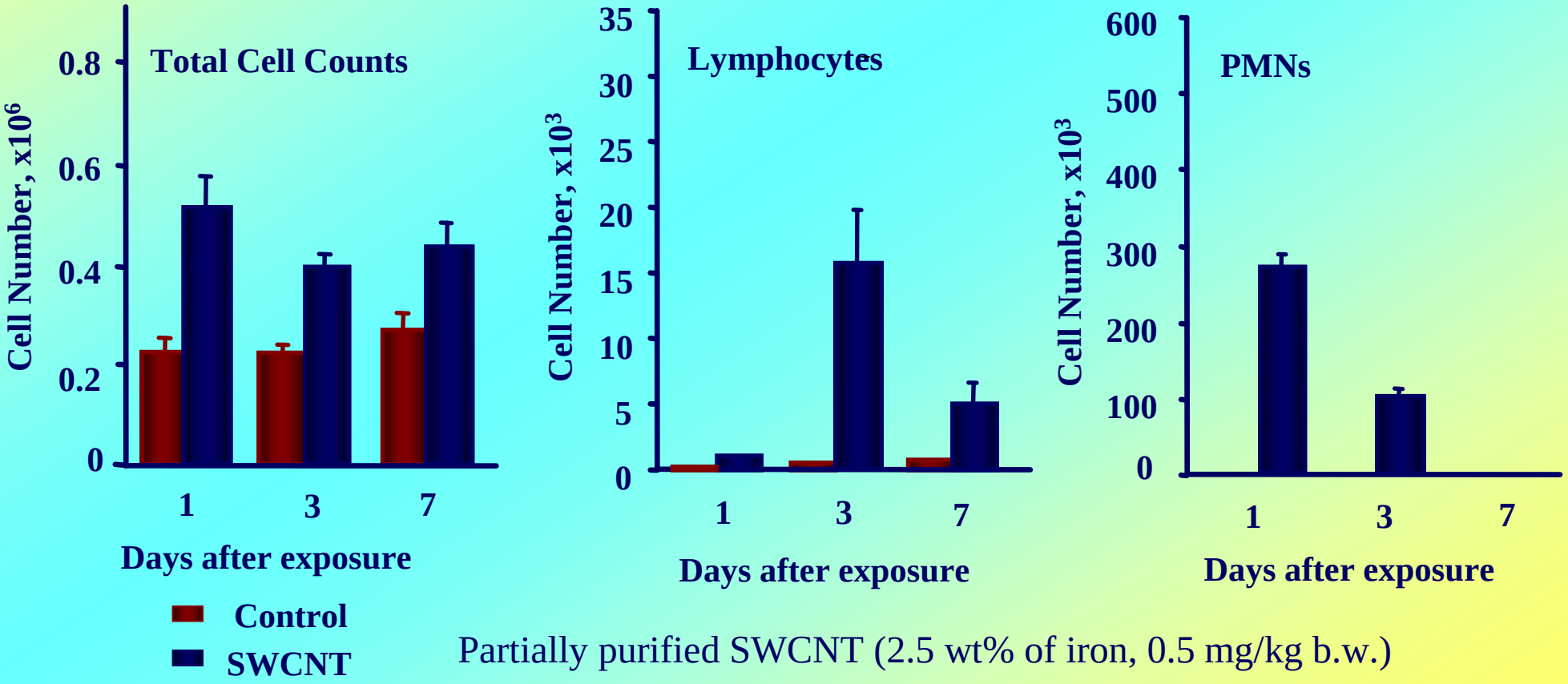
Single Walled Carbon Nanotube Toxicity - purified SWCNT

Histopathology of lung of C57BL/6J mice after pharyngeal aspiration of partially purified SWCNT



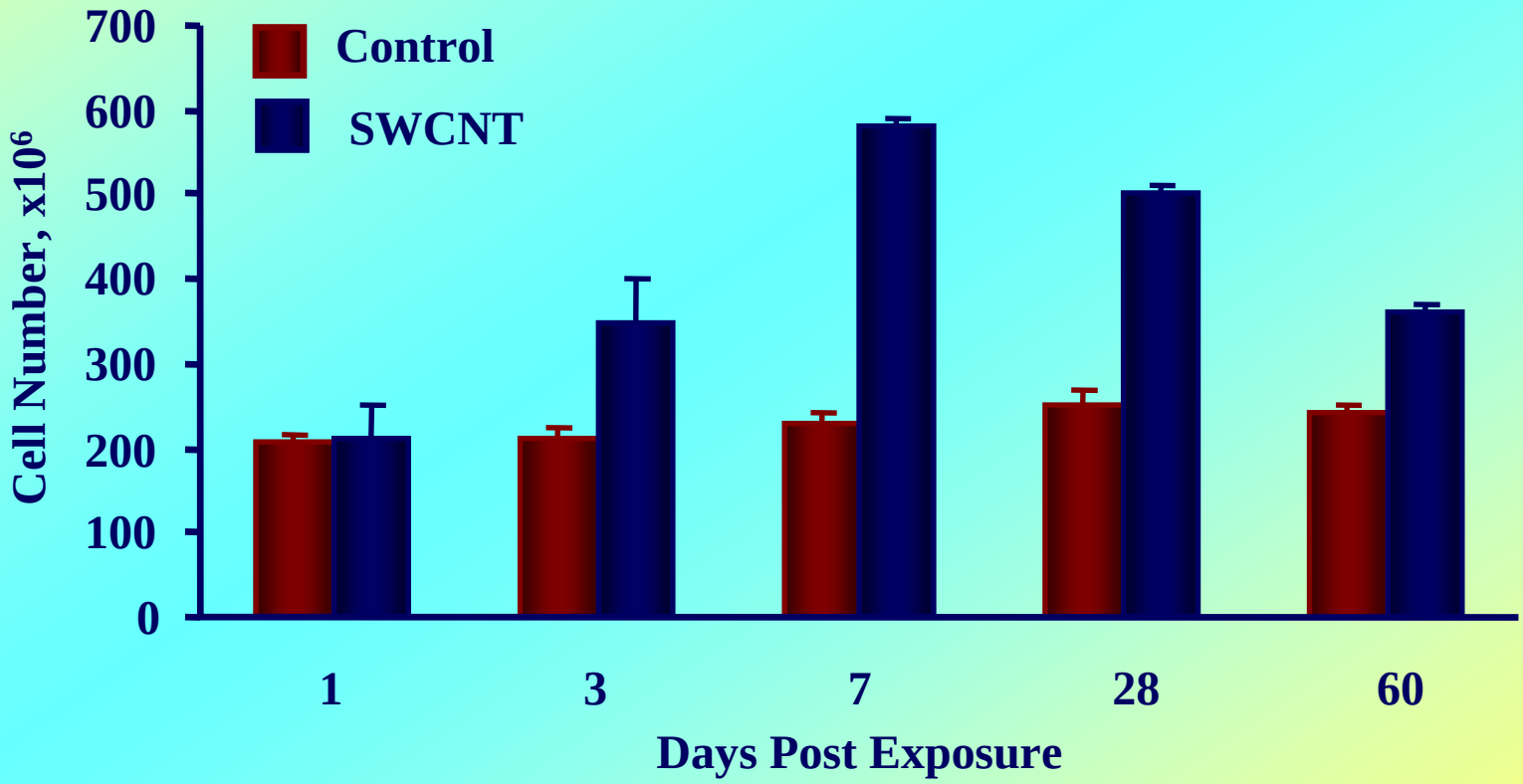
Partially purified SWCNT (2.5 wt% of iron, 0.5 mg/kg b. w.).

Cell differential in BAL fluid of C57BL/6J mice after treatment with partially purified SWCNT.

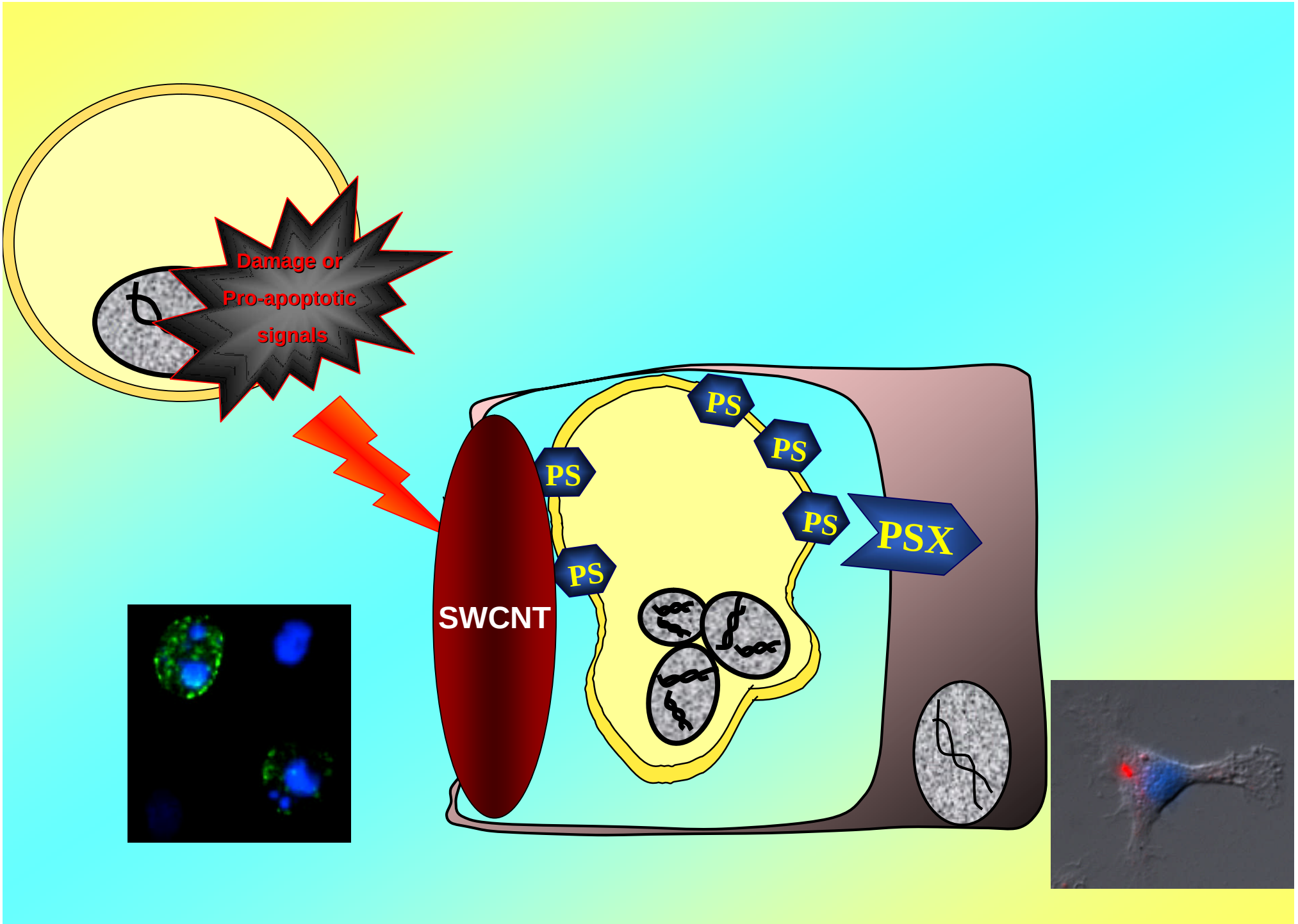


Partially purified SWCNT (2.5 wt% of iron, 0.5 mg/kg b.w.)

Macrophages in BAL fluid of C57BL/6J mice after treatment with partially purified SWCNT.



Partially purified SWCNT (40 μg per mouse)

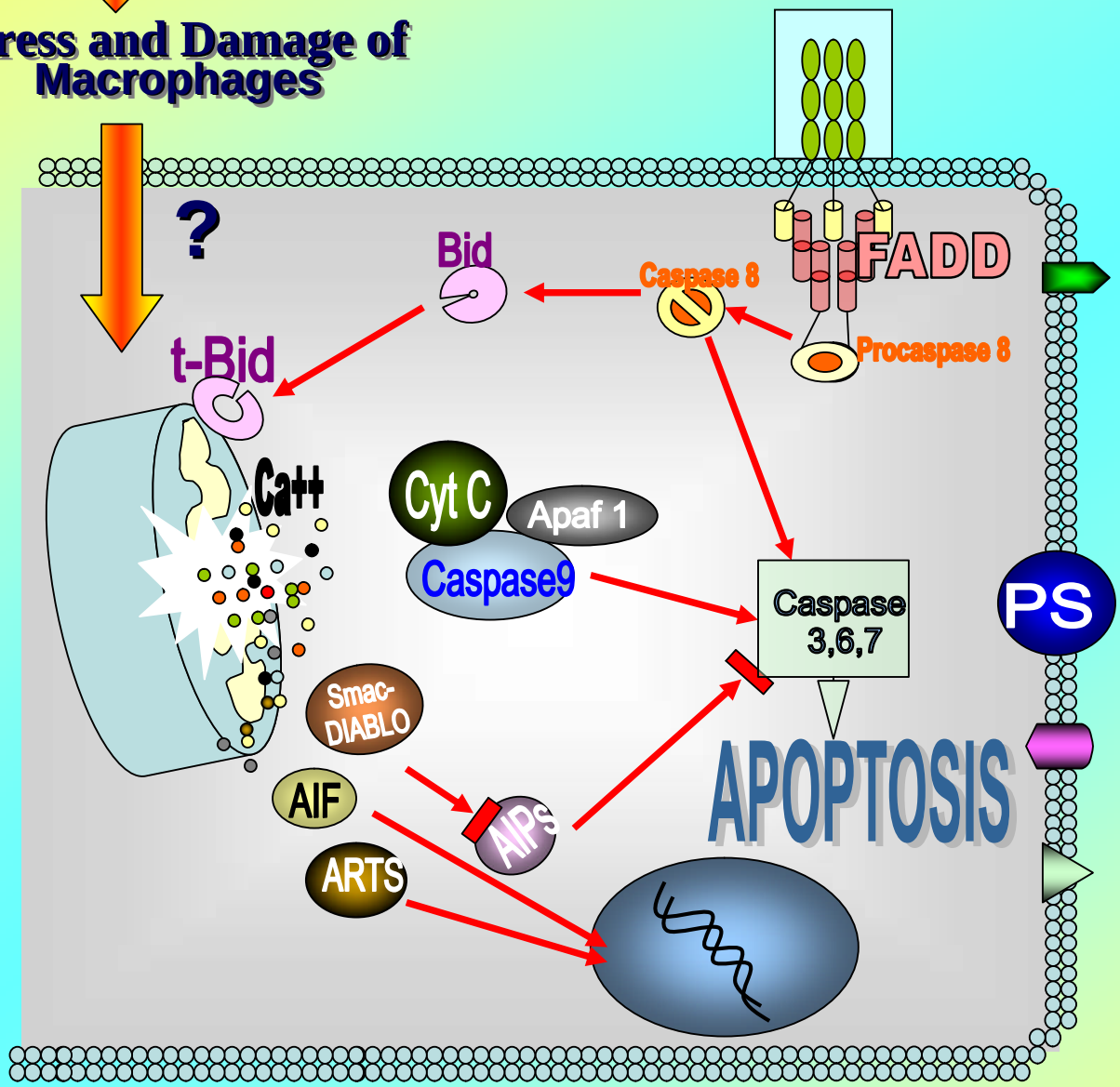

Damage or
Pro-apoptotic
signals
PS
PS
PS
PS
PS
PSX
SWCNT

Non-Aggregated Carbon Nanotubes Do NOT Significantly Damage Macrophages


ΑΠΟΠΤΟΣΙΣ

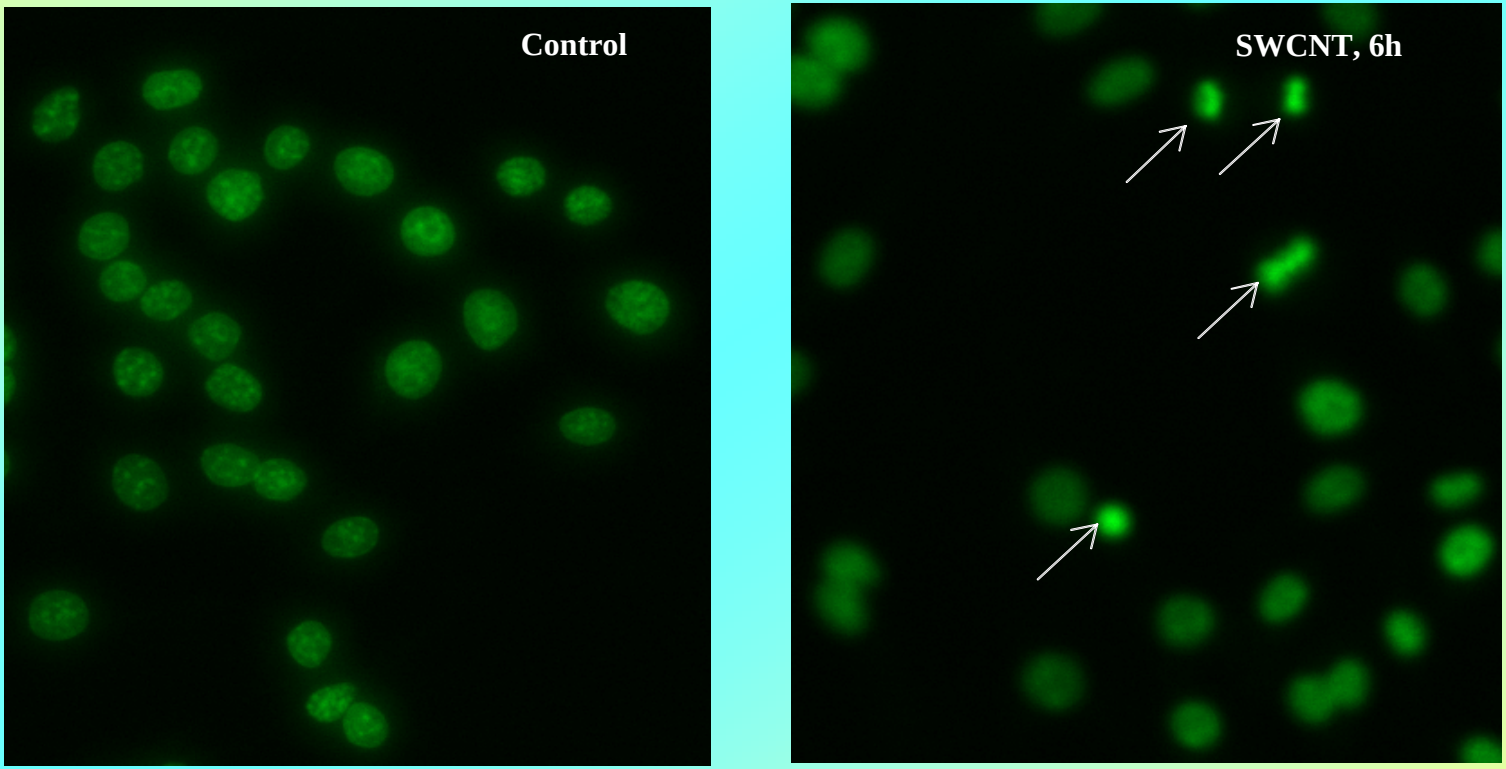
Carbon Nanotubes

Stress and Damage of Macrophages



Do Carbon Nanotubes Induce Apoptosis in Macrophages?

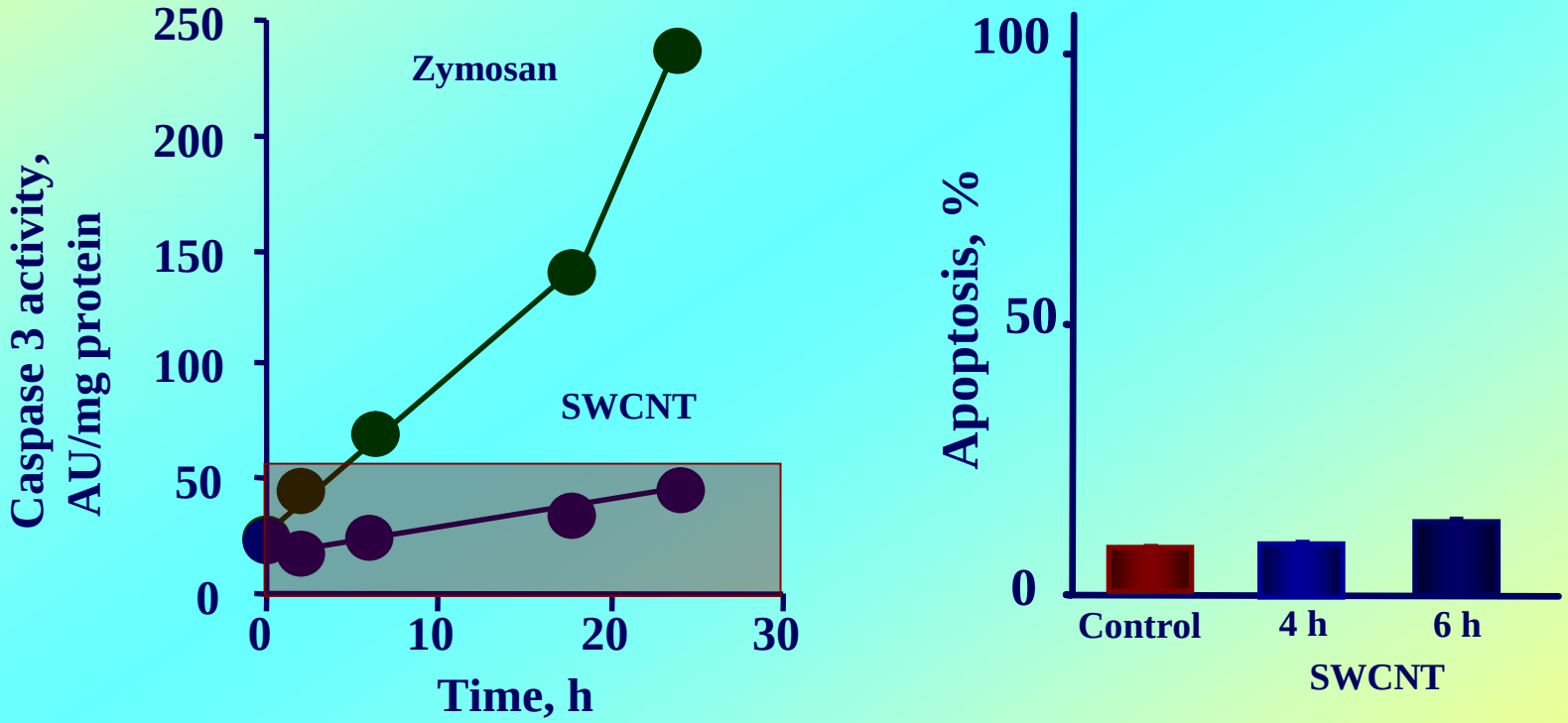
Fluorescent micrographs of RAW 264.7 macrophages incubated in the presence of partially purified SWCNT.



Note nuclear condensation and fragmentation as revealed by Hoechst 33342 staining (shown for clarity in green pseudo-color).

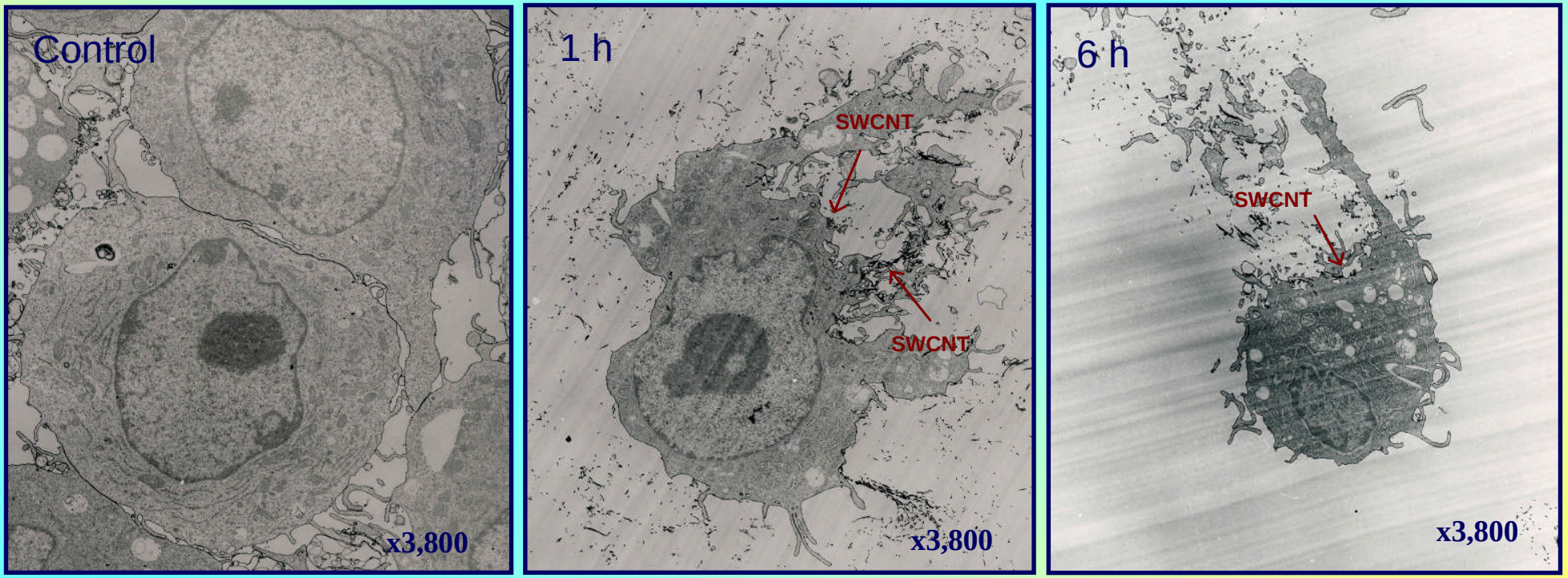
Partially purified SWCNT (2.5 wt% of iron, 0.12 mg/ml).

Apoptosis induced by partially purified SWCNT in RAW 264.7 macrophages .



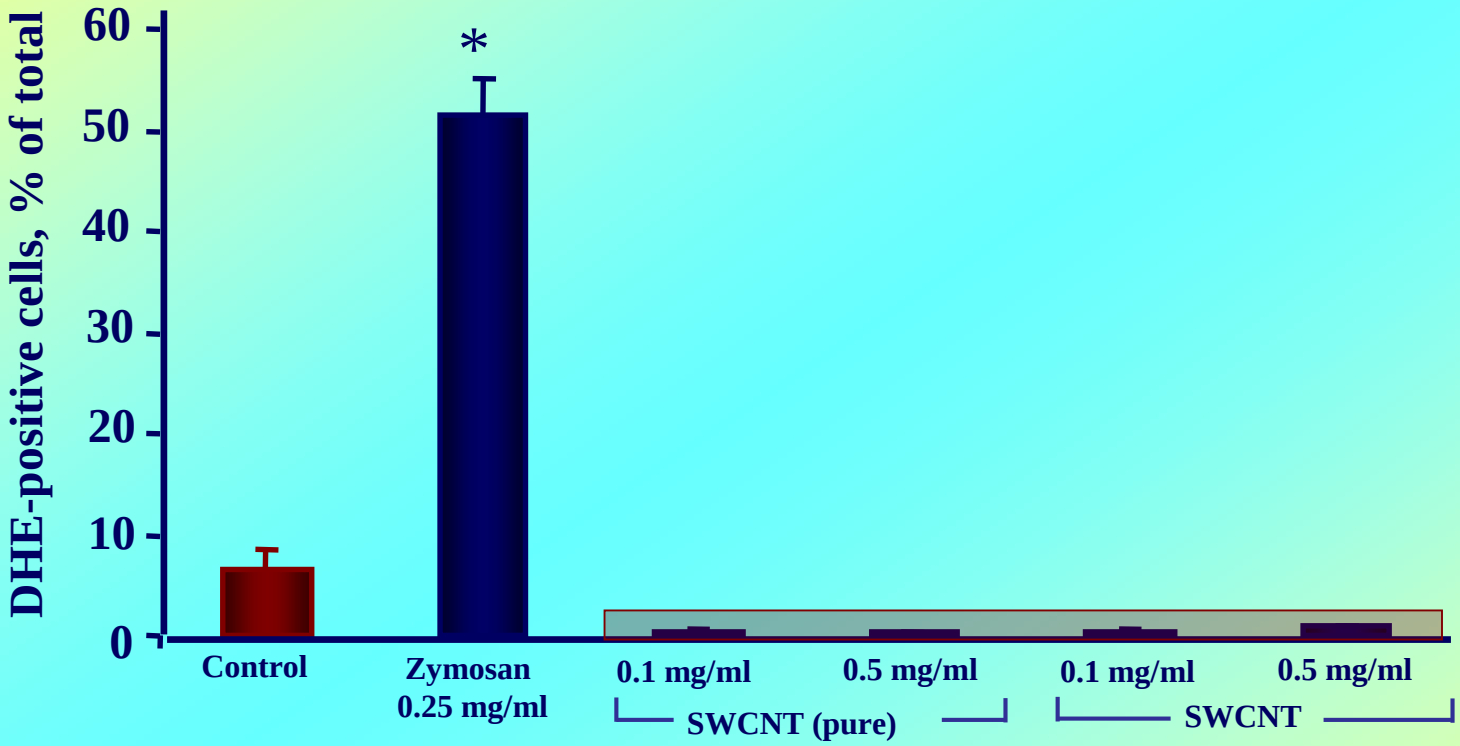
Partially purified SWCNT (2.5 wt% of iron, 0.1 mg/ml).

Electron micrographs illustrating effects of partially purified SWCNT exposure on RAW 264.7 macrophages.



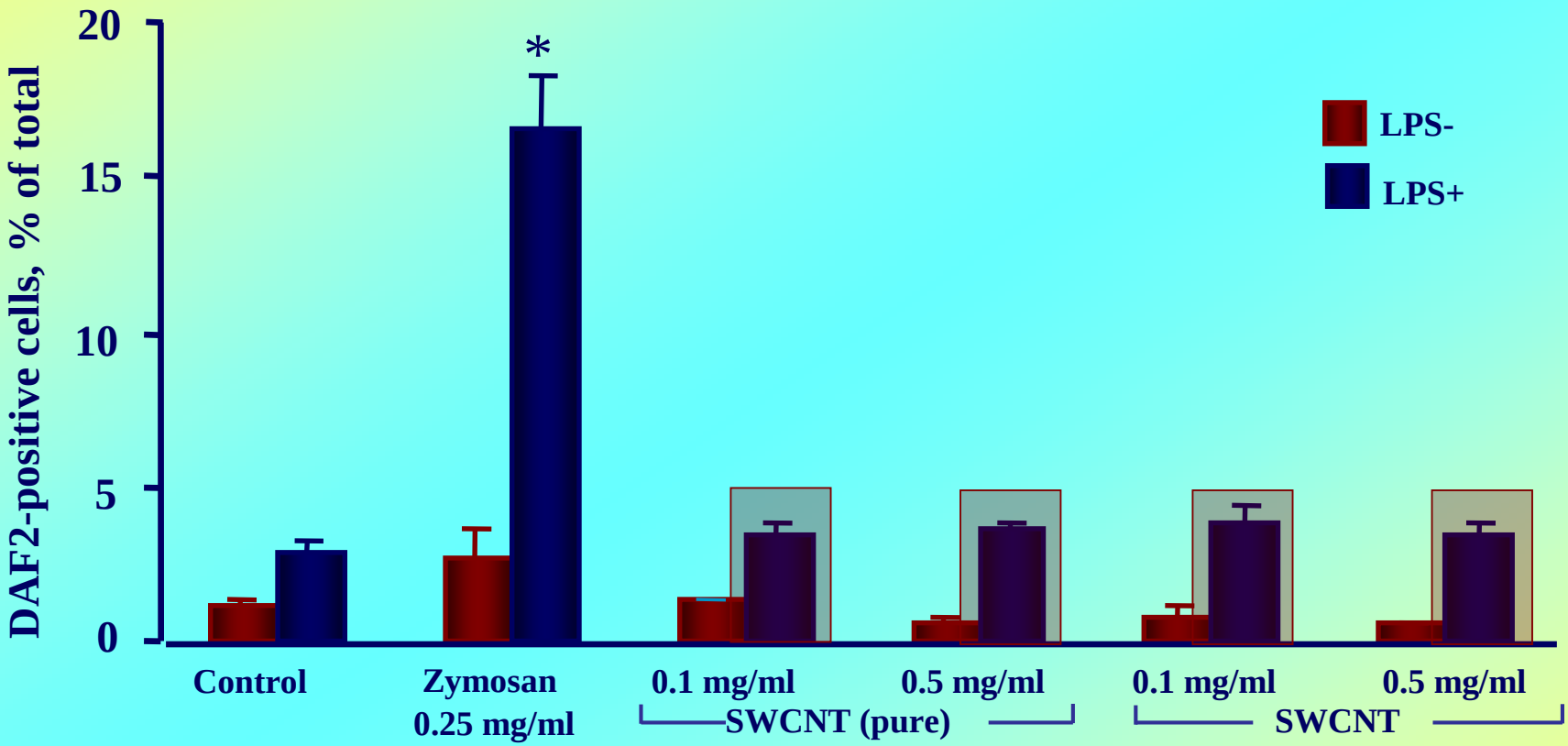
Partially purified SWCNT exposure (2.5 wt% of iron, 0.12 mg/ml)

DHE-positive RAW 264.7 macrophages: zymosan and SWCNT.

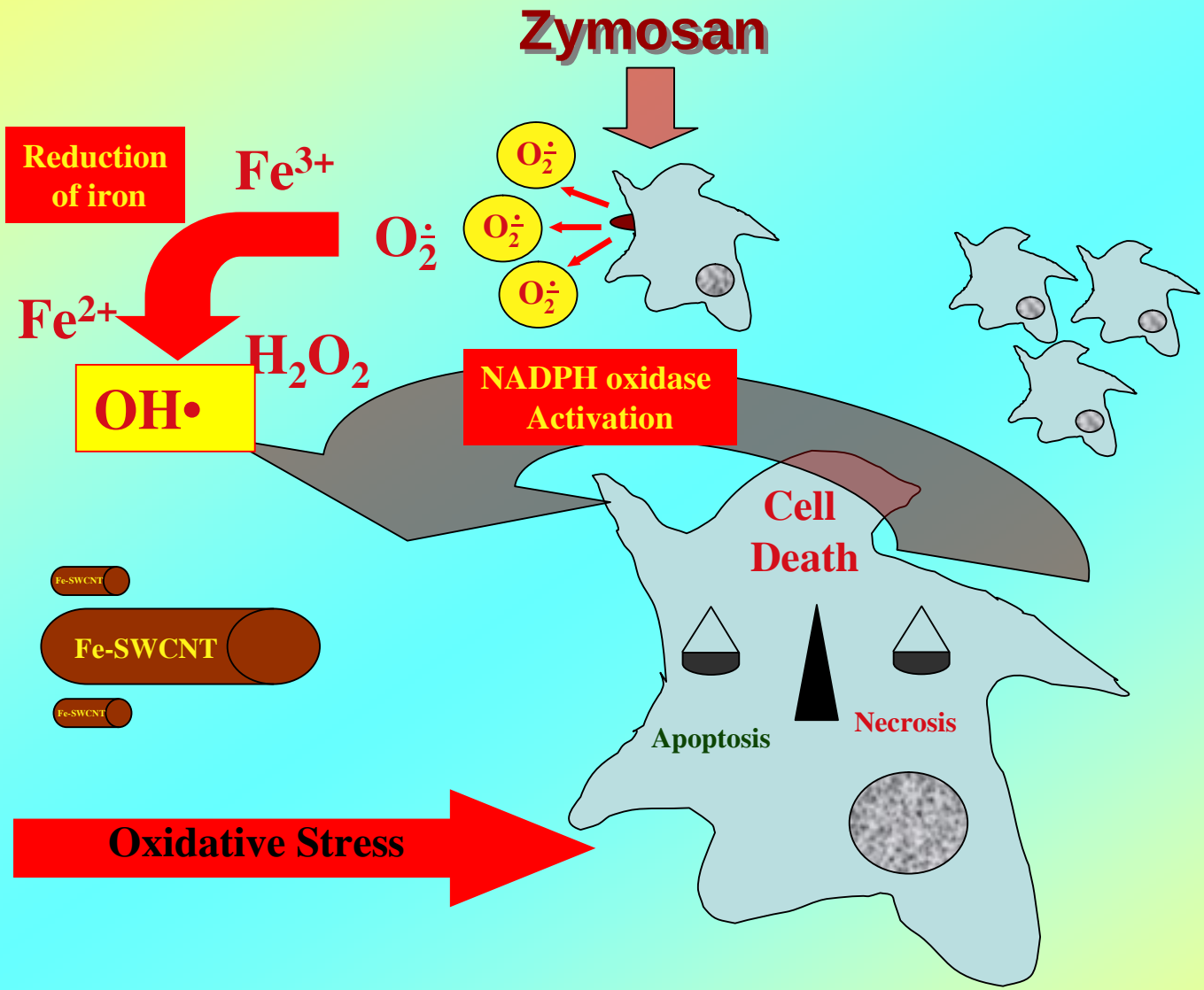


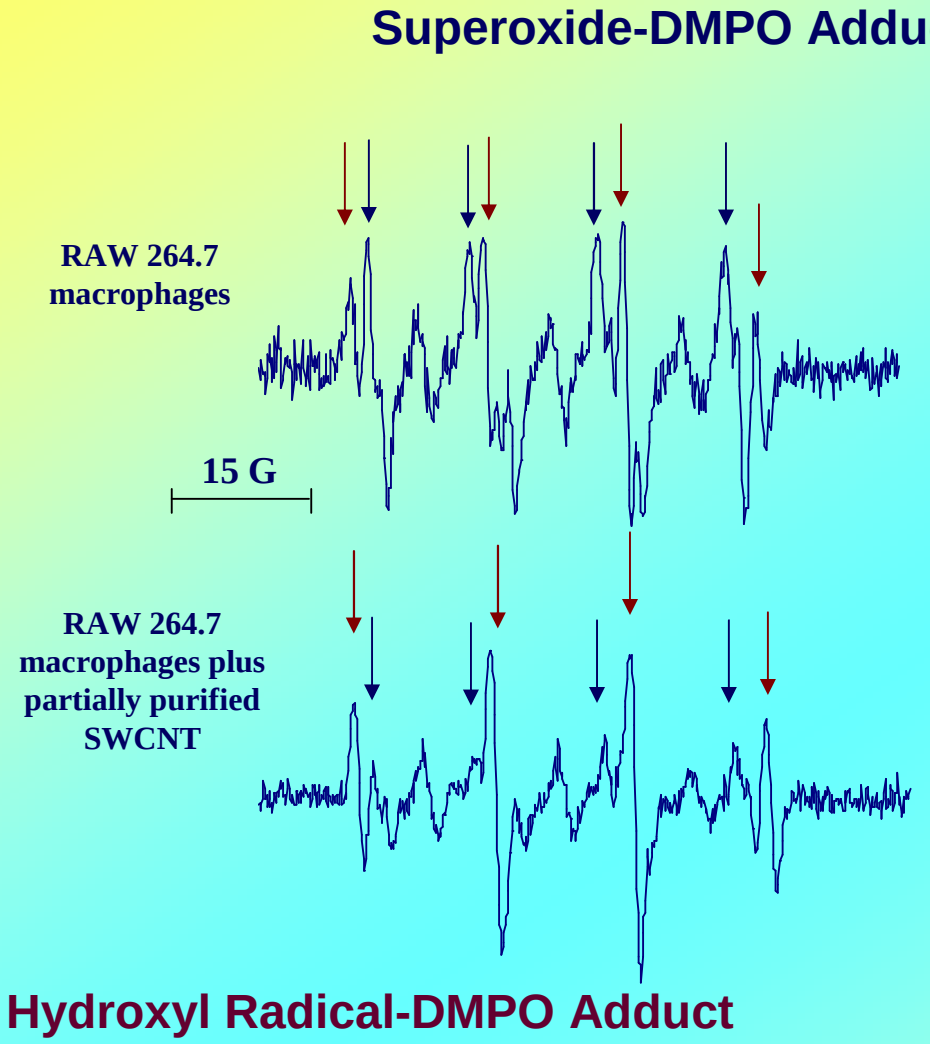
Macrophages 0.3 x 10^6/well) were pre-incubated with DHE (10 mM for 10 min at 37oC). Then RAW 264.7 macrophages were stimulated by zymosan or SWCNT (for 30 min at 37oC). * - $p<0.05$ vs. control cells

DAF2-positive RAW 264.7 macrophages: zymosan and SWCNT.



Naïve macrophages (0.3 x 10^6/well) and macrophages stimulated by LPS (0.1 mg/ml for 6 h at 37°C) macrophages were pre-incubated with DAF-2DA (2 mM for 1 h at 37°C). Then RAW 264.7 macrophages were stimulated by zymosan or SWCNT (for 2h at 37°C). * - $p<0.05$ vs. control cells

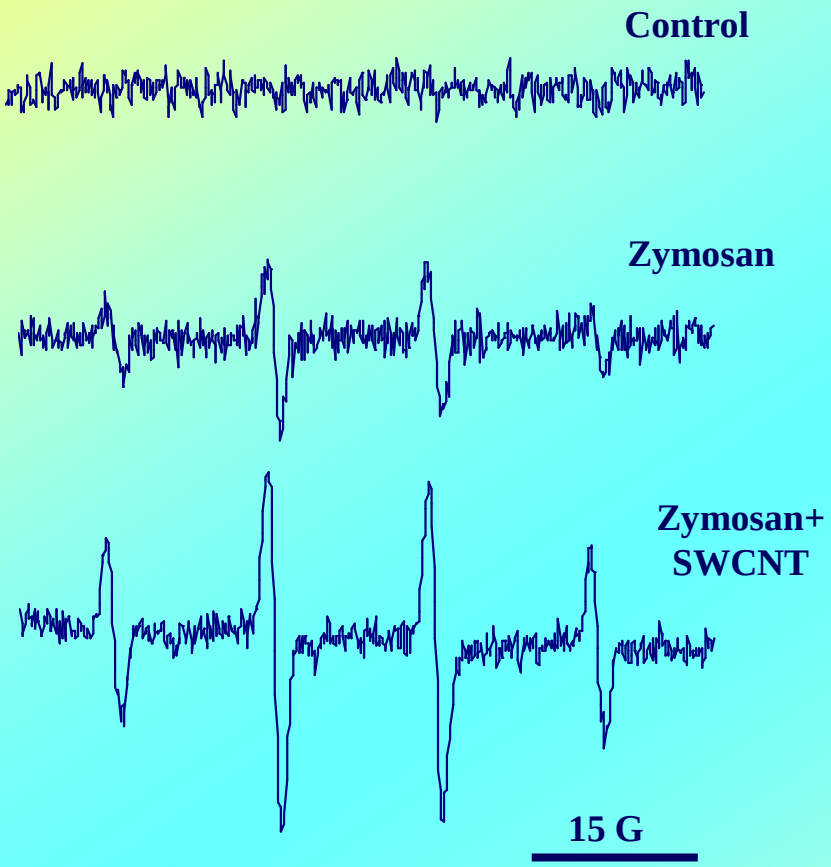

Zymosan
Reduction of iron
Fe^{3+}
$O_2^{\cdot-}$
Fe^{2+}
H_2O_2
OH•
NADPH oxidase Activation
Cell Death
Fe-SWCNT
Fe-SWCNT
Fe-SWCNT
Apoptosis
Necrosis
Oxidative Stress



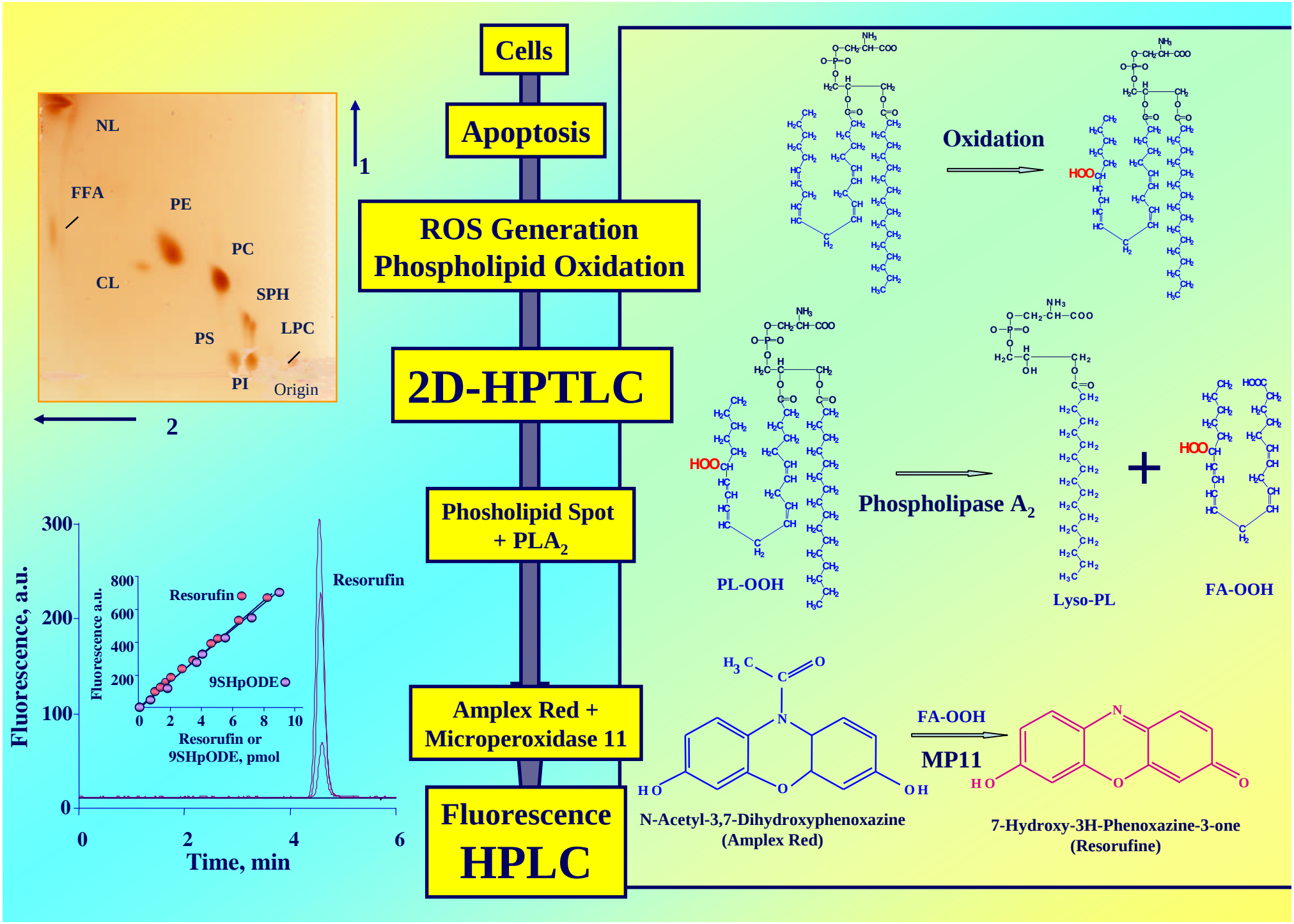
EPR spectra of DMPO radical adducts generated during incubation of xanthine oxidase/xanthine and zymosan-stimulated RAW 264.7 macrophages by partially purified SWCNT in the absence or presence of partially purified SWCNT.

Incubation system contained: xanthine oxidase (0.1U/mL), xanthine (1 mM), zymosan (2.5 mg/mL)-stimulated RAW264.7 macrophages ($20x10^6$ cells/ml) in PBS (pH 7.4) plus 100 mM DMPO; partially purified SWCNT (2.5 wt% of iron, 0.12 mg/ml); EPR conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 sec; conversion time, 0.6 sec.

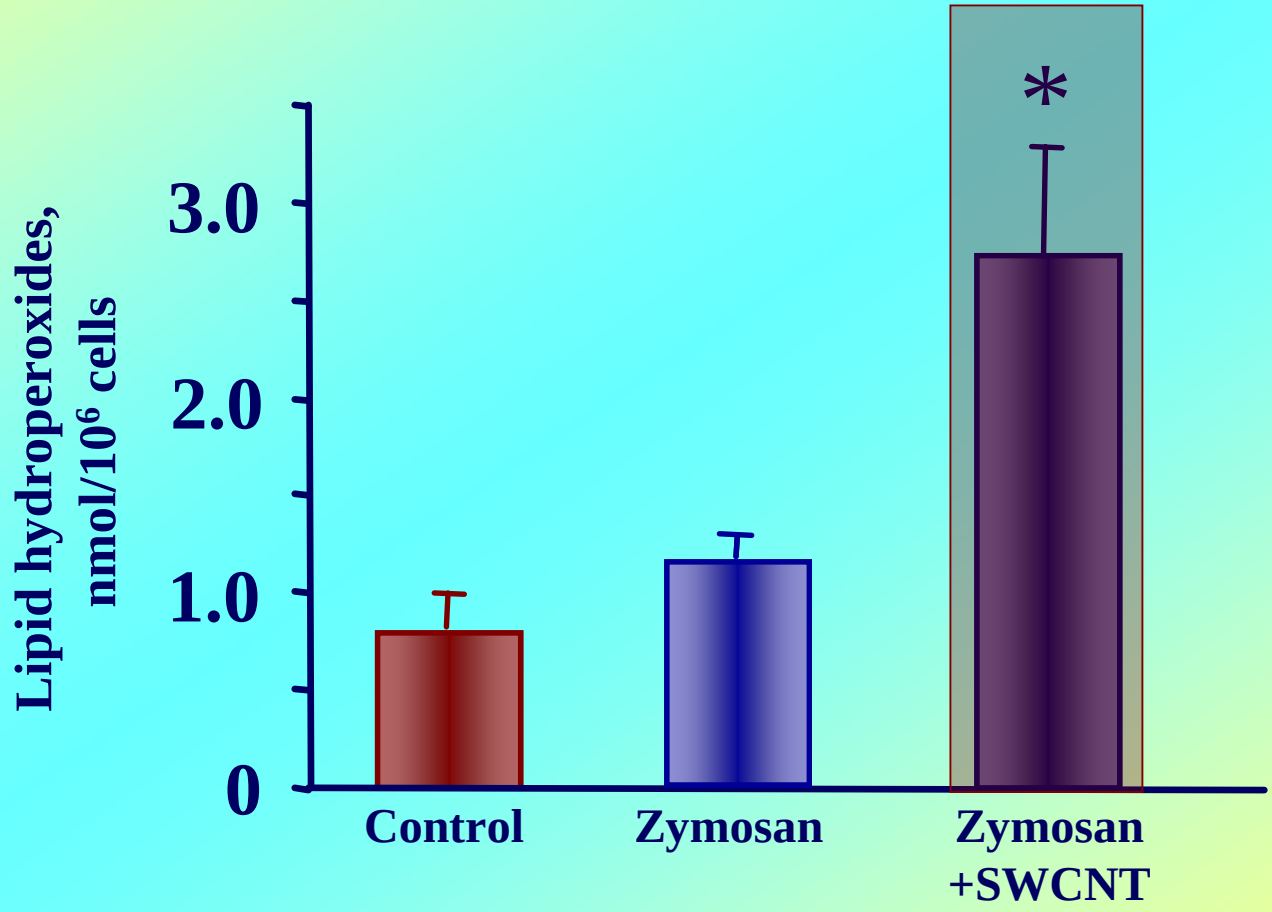
Hydroxyl Radical-DMPO Adduct



ESR spectra of DMPO adducts of free radicals formed by partially purified SWCNT in the presence of RAW 264.7 macrophages

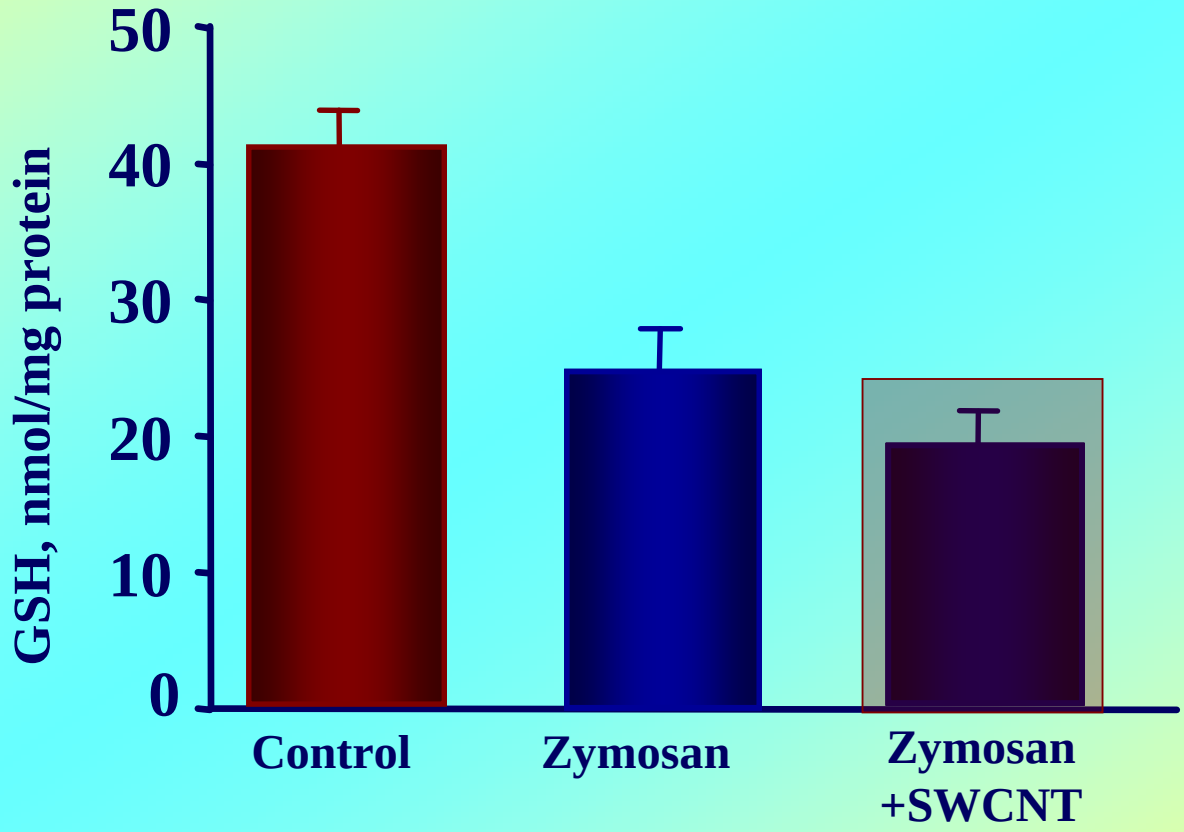

Cells
Apoptosis
ROS Generation
Phospholipid Oxidation
2D-HPTLC
Phosholipid Spot
+ PLA2
Amplex Red +
Microperoxidase 11
Fluorescence
HPLC
NL
FFA
PE
PC
CL
SPH
PS
LPC
PI
Origin
1
2
Oxidation
PL-OOH
Phospholipase A2
Lyso-PL
FA-OOH
Fluorescence, a.u.
Time, min
Resorufin
9SHpODE
Resorufin or
9SHpODE, pmol
Fluorescence a.u.
N-Acetyl-3,7-Dihydroxyphenoxazine
(Amplex Red)
FA-OOH
MP11
7-Hydroxy-3H-Phenoxazine-3-one
(Resorufine)

Partially purified SWCNT induce lipid peroxidation in RAW 264.7 macrophages .

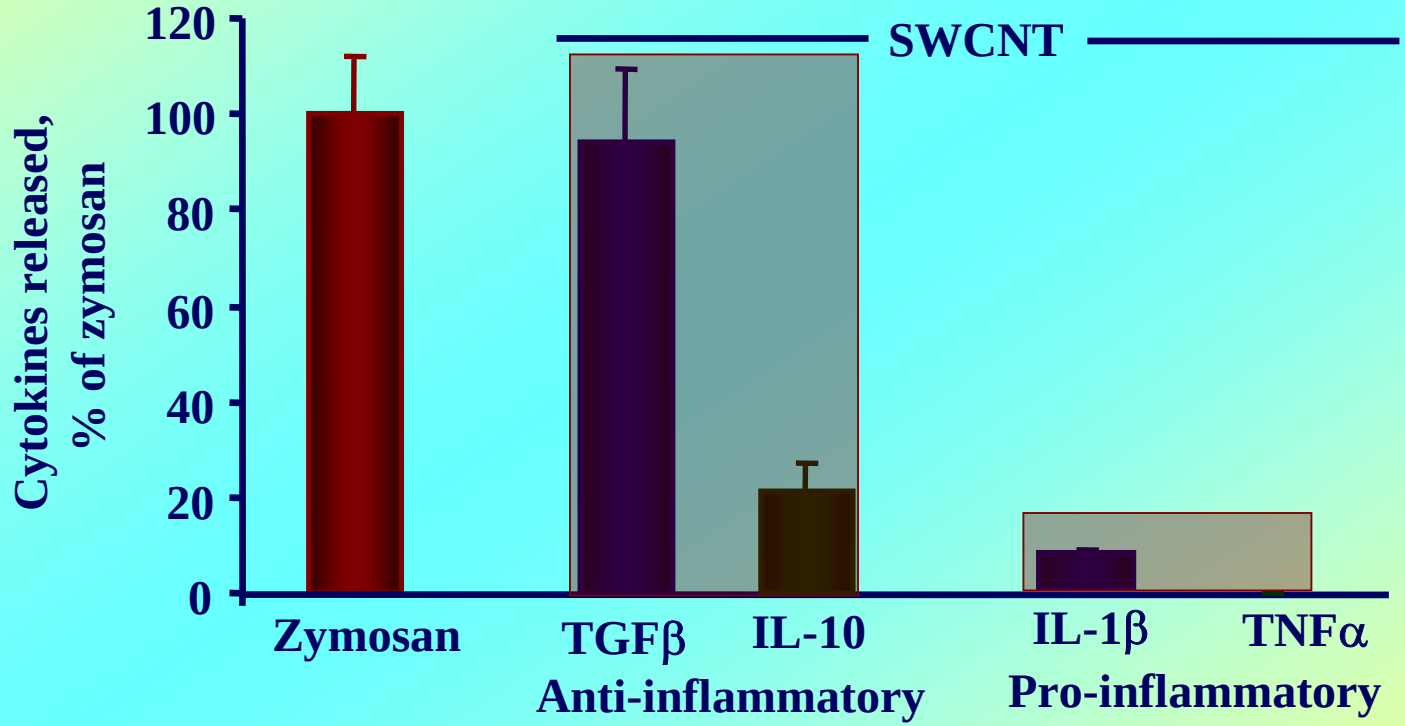


Stimulation of macrophages with zymosan (0.25 mg/ml) cause a slight increase in lipid peroxidationm further enhanced by partially purified iron-containing SCWNT (0.1 mg/ml). Lipid peroxidation was assessed by our newly developed fluorescence HPLC-based protocol for lipid hydroperoxides

Effect of partially purified SWCNT exposure on GSH content in RAW 264.7 macrophages.



Production of cytokines by zymosan-stimulated RAW 264.7 macrophages and SWCNT treated RAW 264.7 macrophages.



Zymosan- 0.25 mg/ml; SWCNT 0.1 mg/ml.

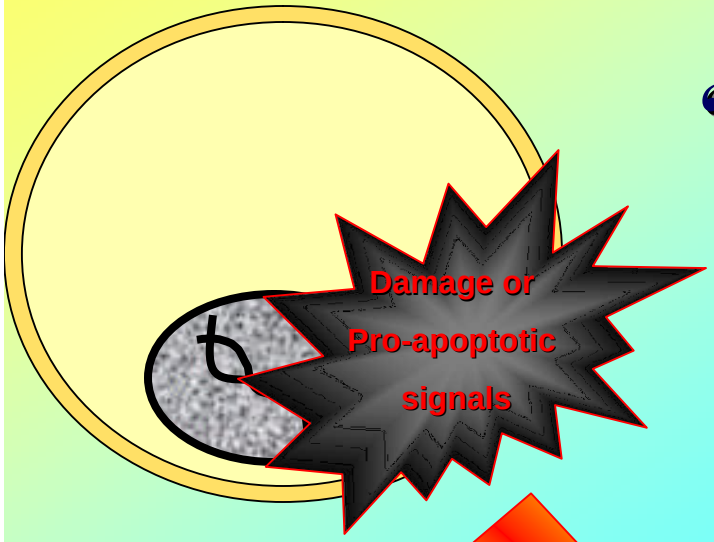

Clearance,
Phagocytosis

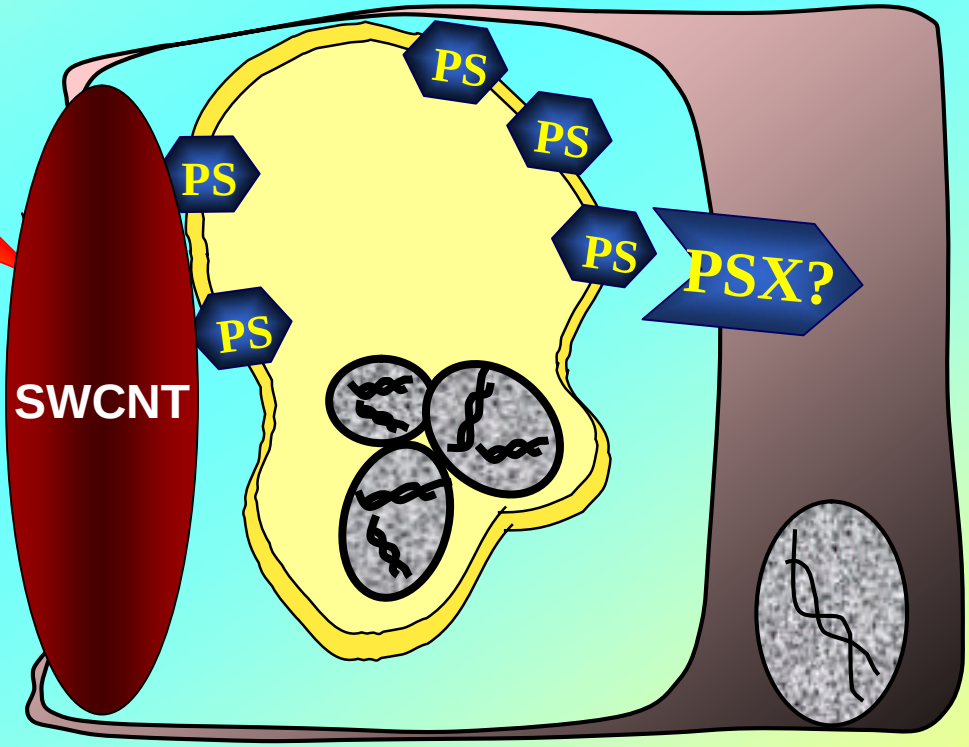
Recognitio and digestion of Cells

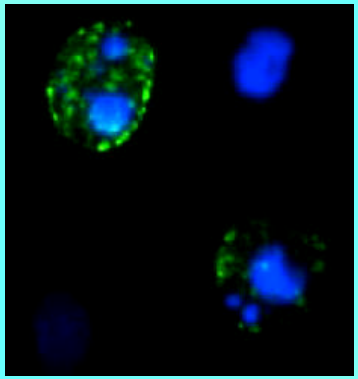
B16 melanoma cells (green) and dendritic cells

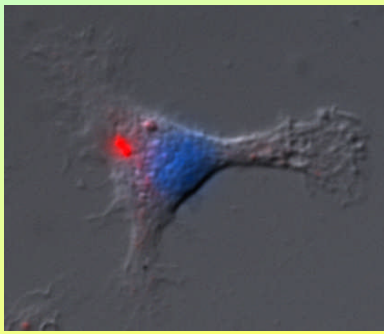
Can Macrophages Be Forced to Recognize and Digest Carbon Nanotubes

Phosphatidylserine (PS) as an "eat-me" signal in phagocytosis of apoptotic cells

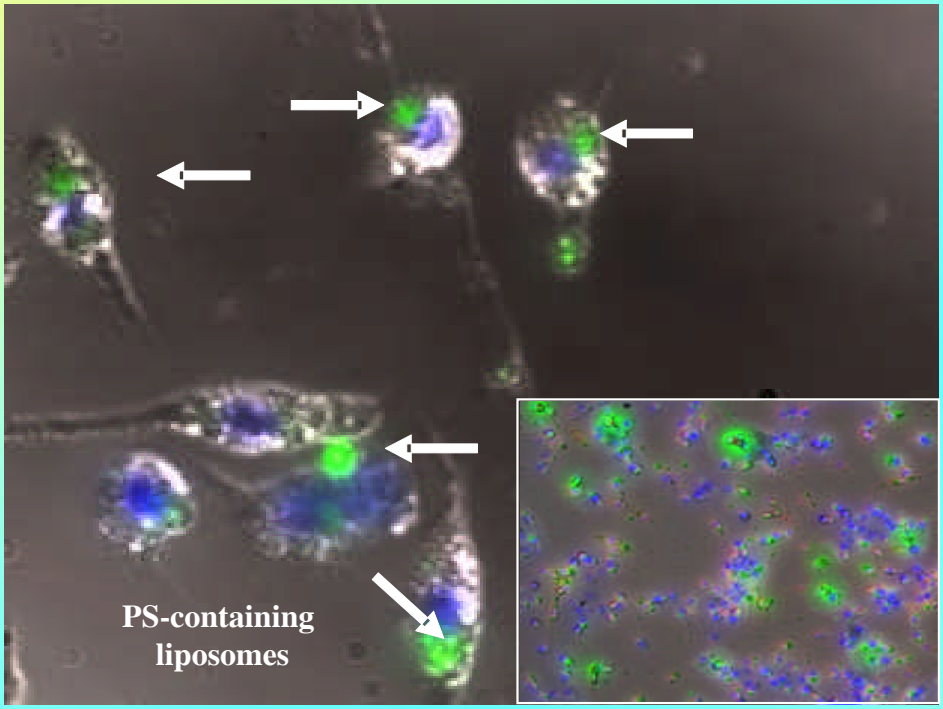


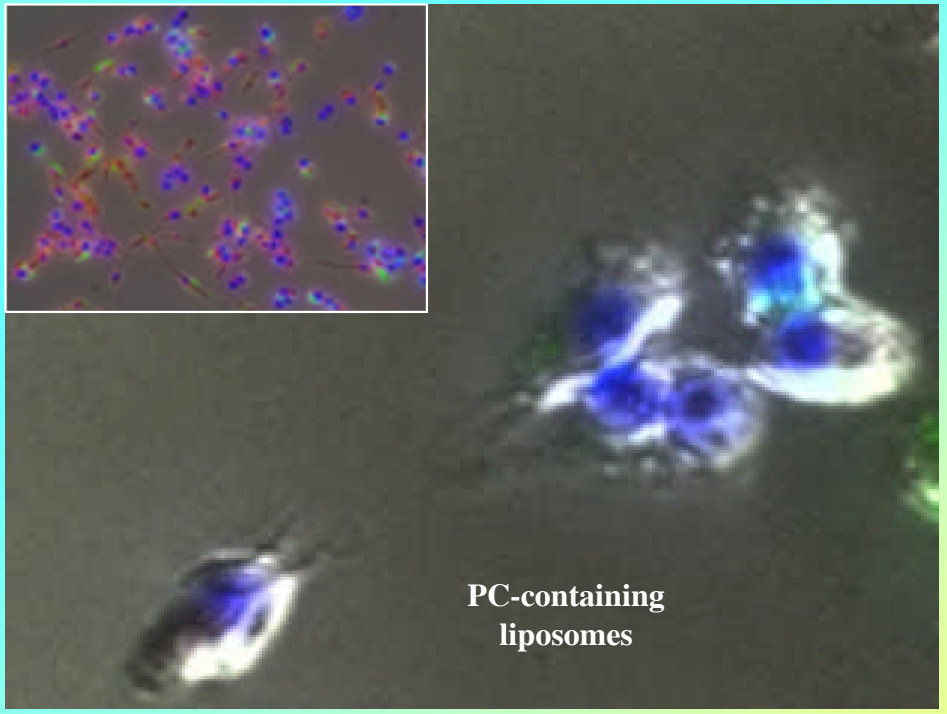




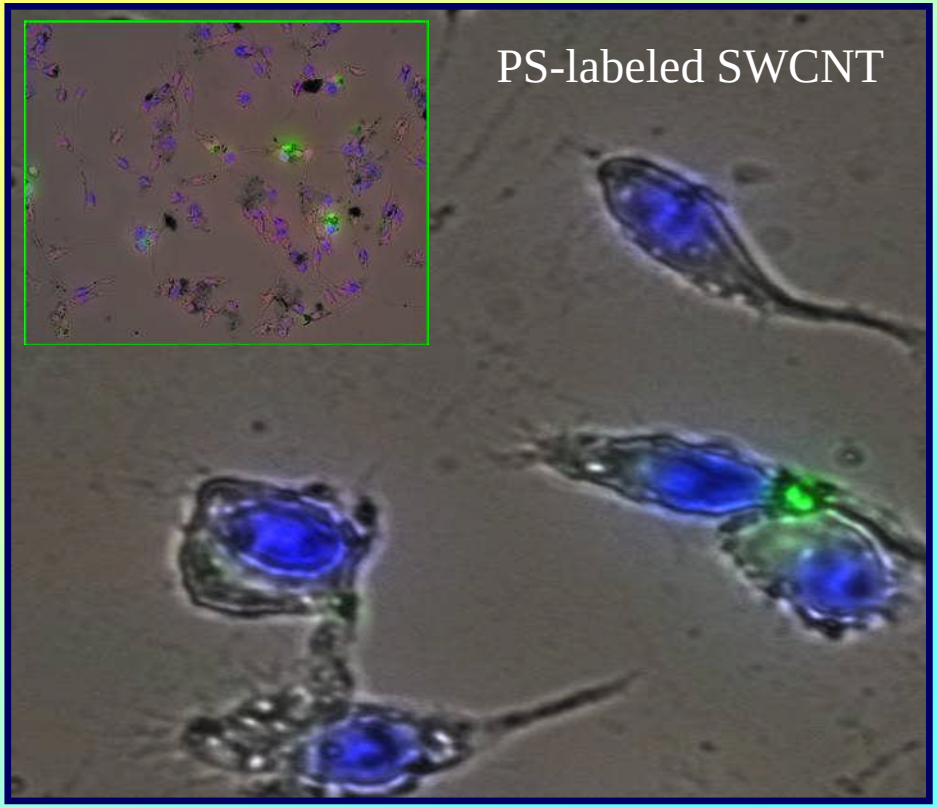


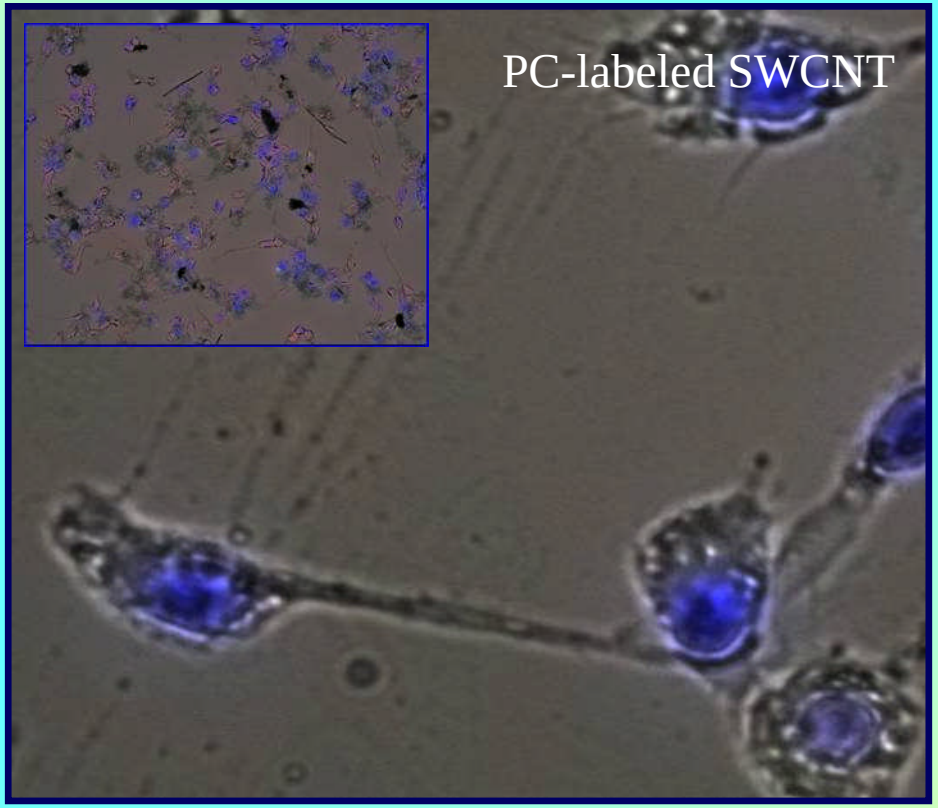
RAW 264.7 macrophages effectively phagocytose PS-containing liposomes but not PC-containing liposomes.





RAW 264.7 macrophages (10^5 cells/ml) were incubated for 6 h with liposomes (0.33 mM) composed of a mixture of PC:PS (with fluorescently labeled PS) or PC (with fluorescently labeled PC). After incubation, macrophages were fluorescently labeled with Hoechst 3343 (nuclei, blue fluorescence), and Cell Tracker Orange (cytosol, red fluorescence). Liposomes fluorescently labeled with with NBD-phospholipids (green fluorescence) PS-containing liposomes were prepared by sonication of a mixture of PC:PS 1:1 with the addition of 10 mol% of NBD-PS). PC-containing liposomes were prepared by sonication of a mixture of PC with the addition of 10 mol% of NBD-PC.





RAW 264.7 macrophages effectively phagocytose PS-labeled SWCNT but not PC labeled SWCNT.

RAW264.7 macrophages (10^5 cells/ml) were incubated for 6h with fluorescently labeled SWCNT (0.1 mg/ml).

Thanks To My Collaborators:

Kagan's Lab:

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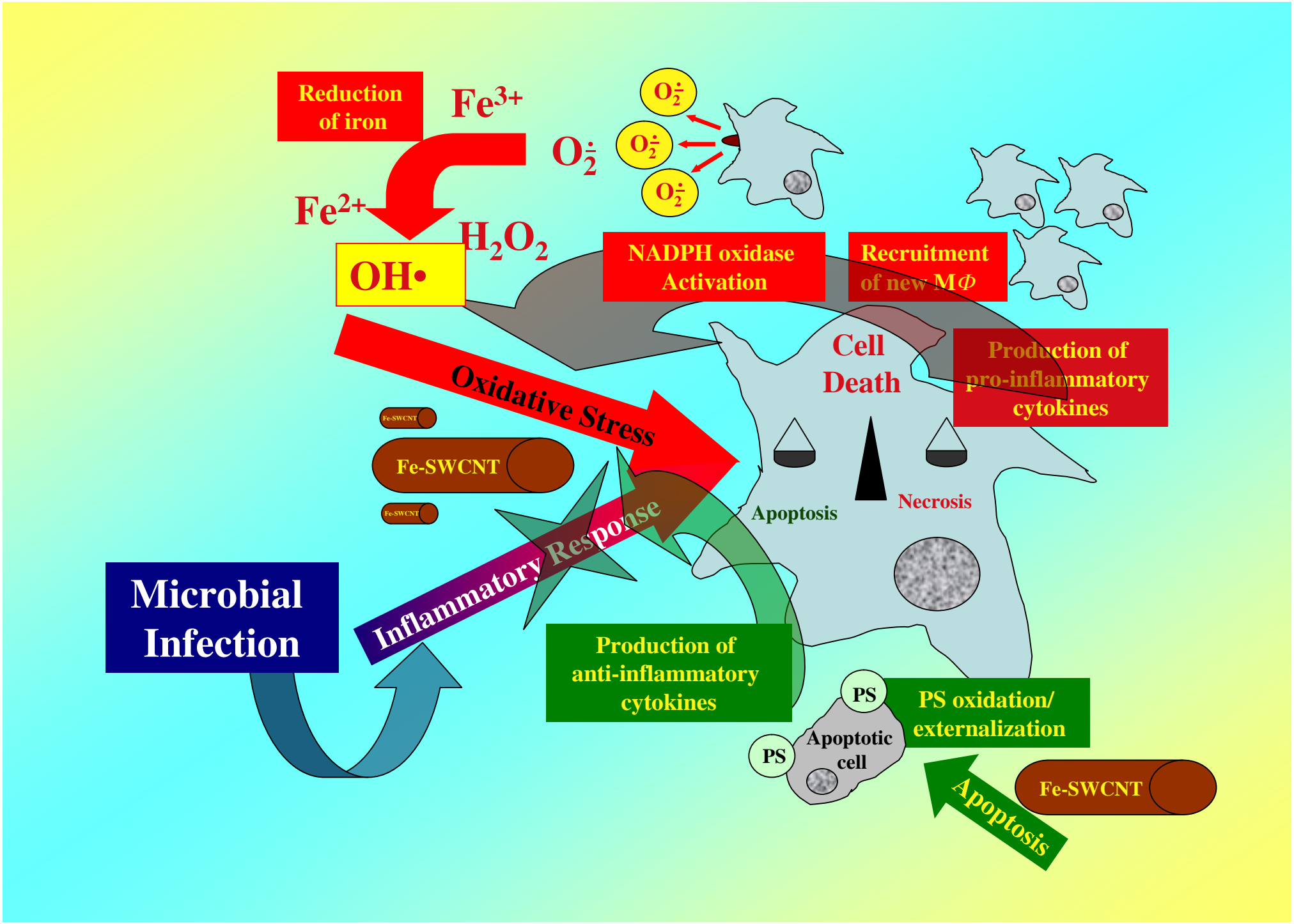
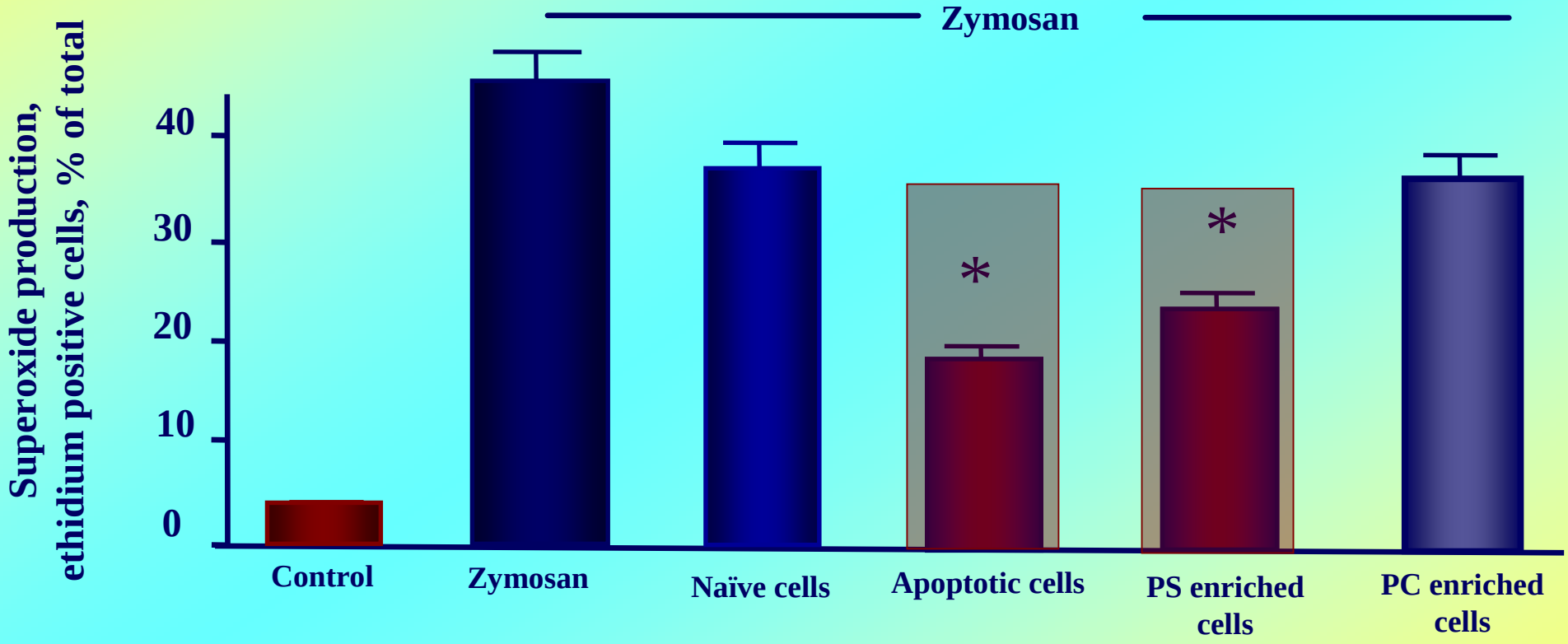
V. Castranova (Morgantown)
R. Mercer (Morgantown)
E. Kisin (Morgantown)
A. Maynard (Cincinnati)

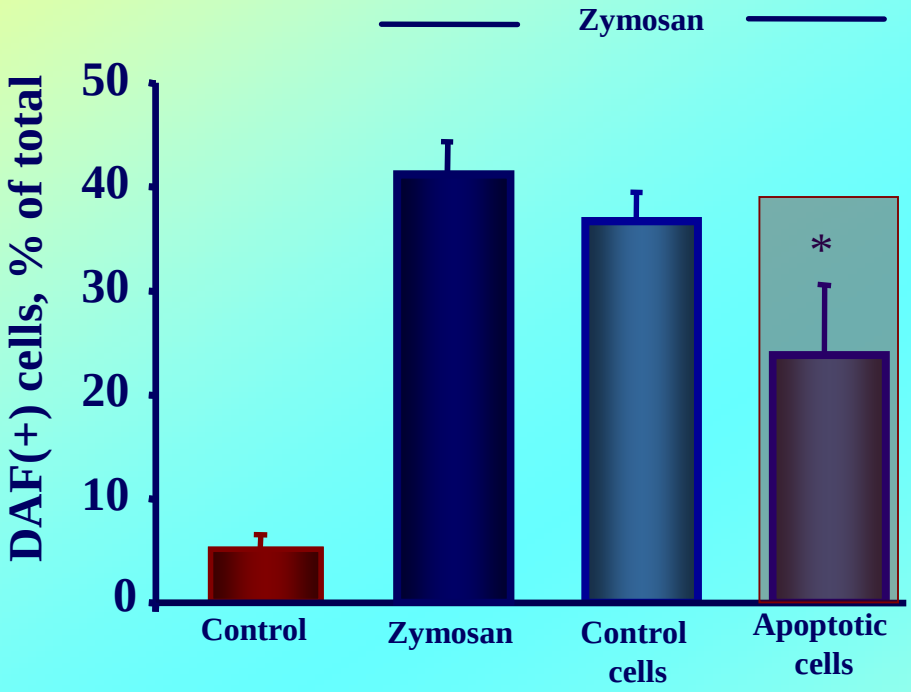

Reduction of iron
Fe^{3+}
Fe^{2+}
$O_2^{\cdot-}$
H_2O_2
OH•
NADPH oxidase Activation
Recruitment of new MΦ
Cell Death
Production of pro-inflammatory cytokines
Oxidative Stress
Fe-SWCNT
Fe-SWCNT
Fe-SWCNT
Apoptosis
Necrosis
Inflammatory Response
Microbial Infection
Production of anti-inflammatory cytokines
PS
PS
Apoptotic cell
PS oxidation/ externalization
Fe-SWCNT
Apoptosis

Effect of apoptotic cells and PS on superoxide generation in zymosan-stimulated RAW 264.7 macrophages.

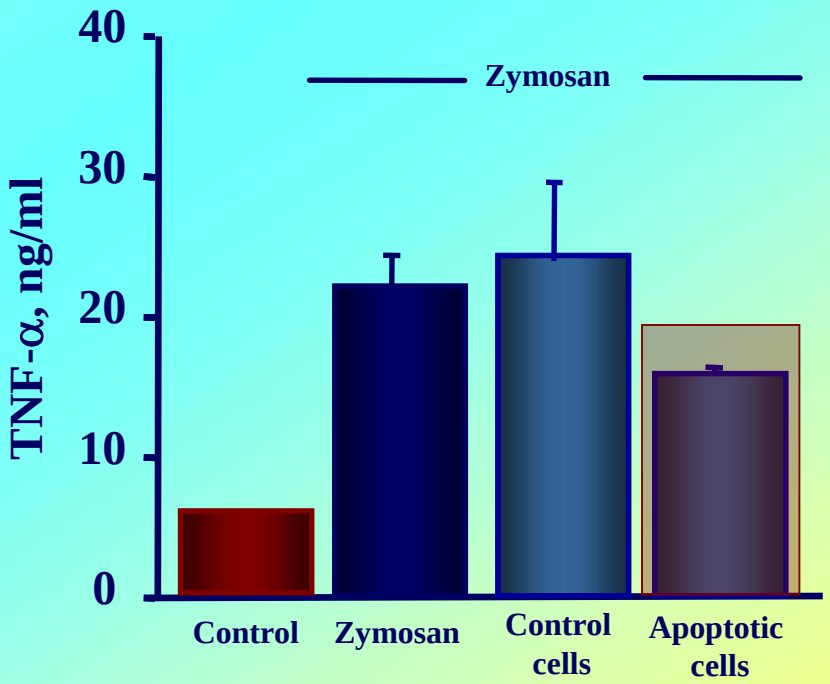


PS - 150 nmol/10^6 cells (30 min at 37oC); PC - 150 nmol/10^6 cells (30 min at 37°C); Zymosan - 0.25 mg/ml (1h at 37°C); DHE - 10 µM.
Apoptosis in Jurkat cells was induced by anti-FAS (250ng/10^6 cells, 4h at 37°C.)

Effect of apoptotic cells on formation of NO• in LPS-induced zymosan-stimulated RAW 264.7 macrophages.

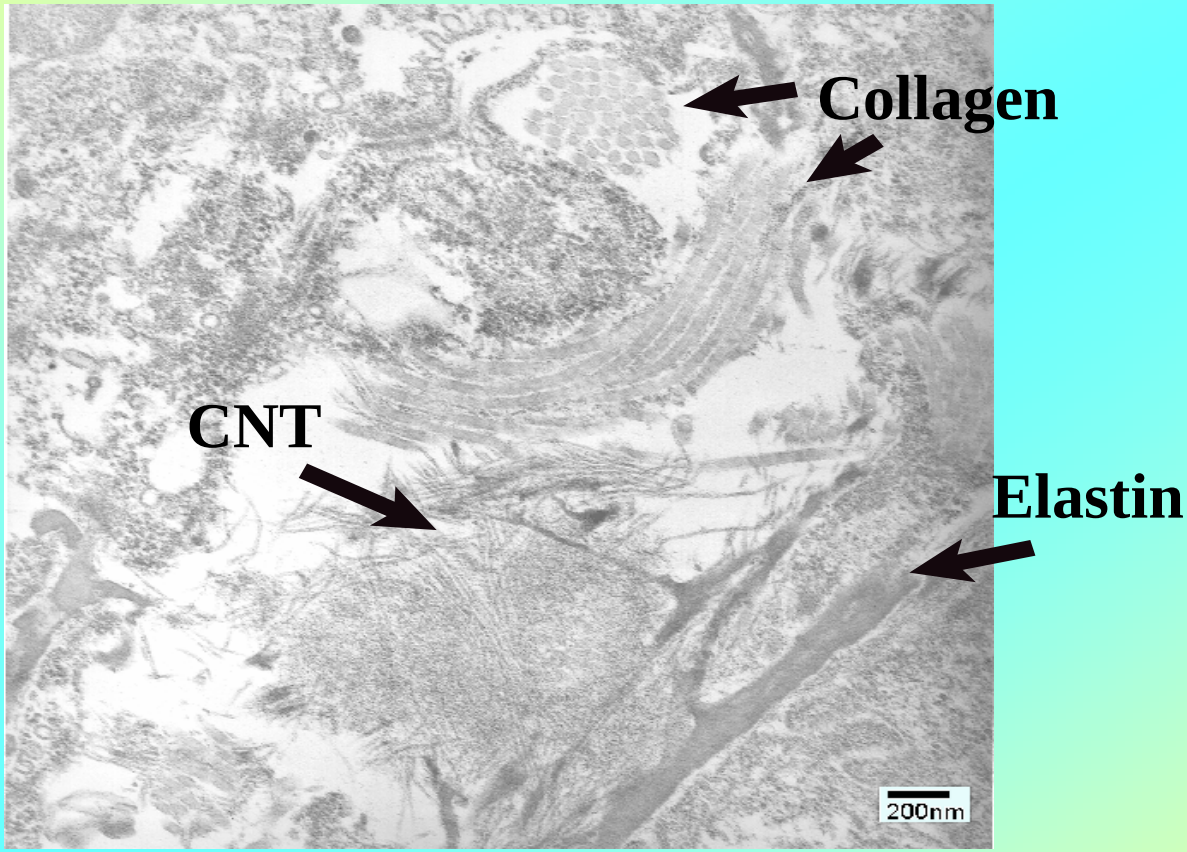


Production of TNFa by zymosan-stimulated (0.25mg/ml) RAW 264.7 macrophages.



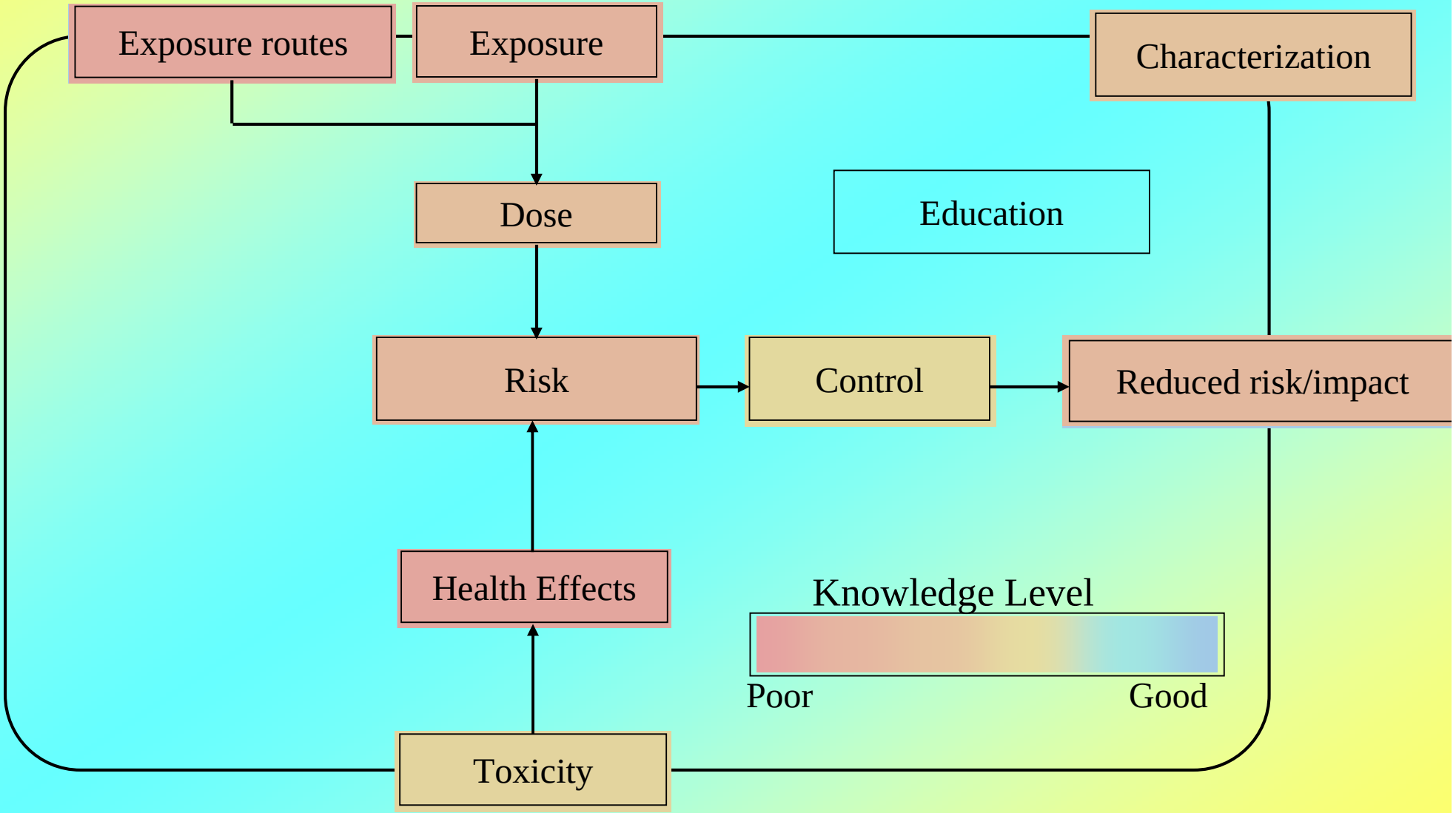
Zymosan - 0.25 mg/ml (1h at 37ºC). Apoptosis in Jurkat cells was induced by anti-FAS (250ng/106 cells, 4h at 37ºC.)

Carbon Nanotubes in Interstitial Space



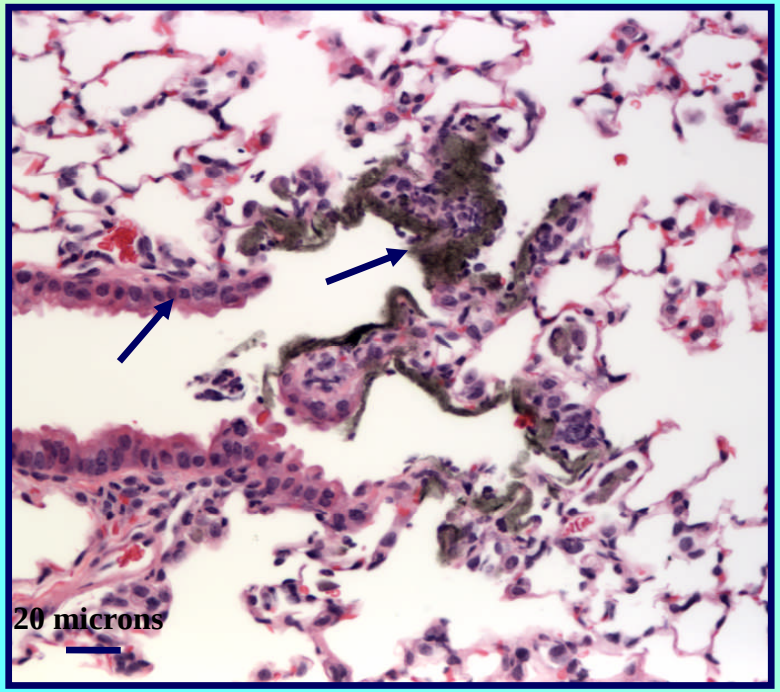
TEM of carbon nanotubes in interstitial space. Micrograph shows carbon nanotubes intermixed with normal connective tissue matrix of the lungs.

Addressing occupational impact

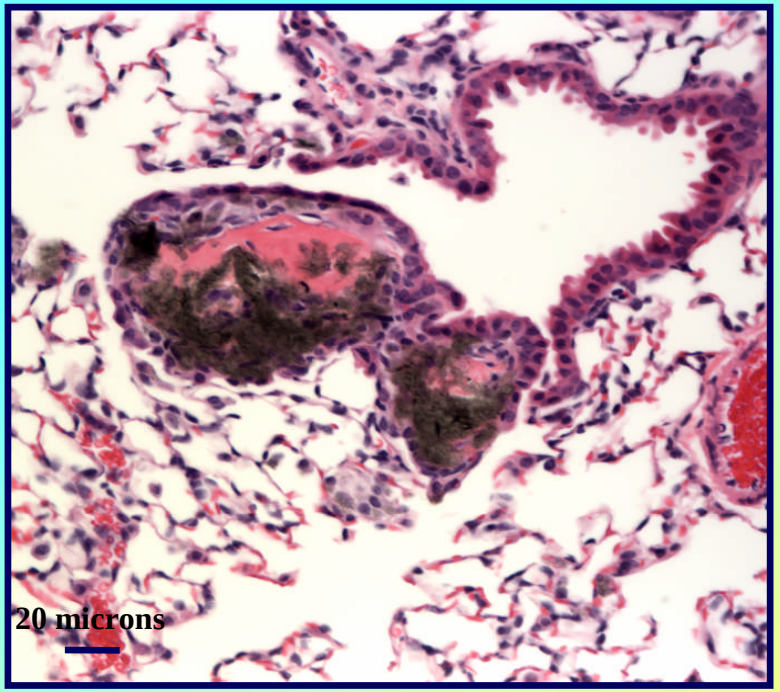


Pharyngeal Aspiration of CNT in Mice Caused:

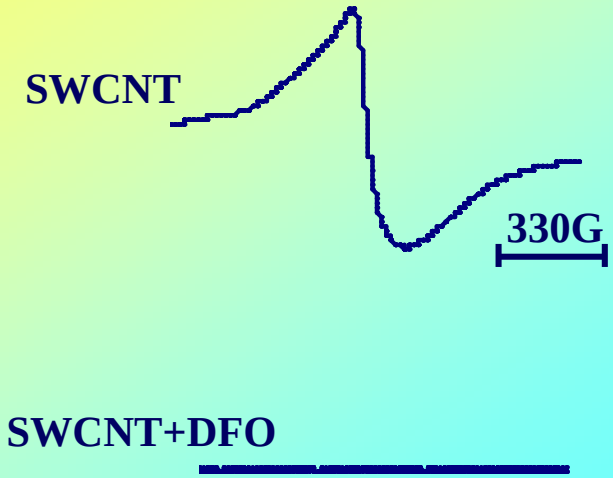
- Rapid development of granulomatous bronchointerstitial pneumonia
- Inflammation evolving with time from neutrophilic to granulomatous
- Morphologic alterations localized to the interstitial of the bronchiolar walls, alveolar ducts and adjacent alveoli
- Hypocellular eosinophilic material consistent with fibrous connective tissue observed within granulomas



1 day post exposure 40 μg/mouse CNT

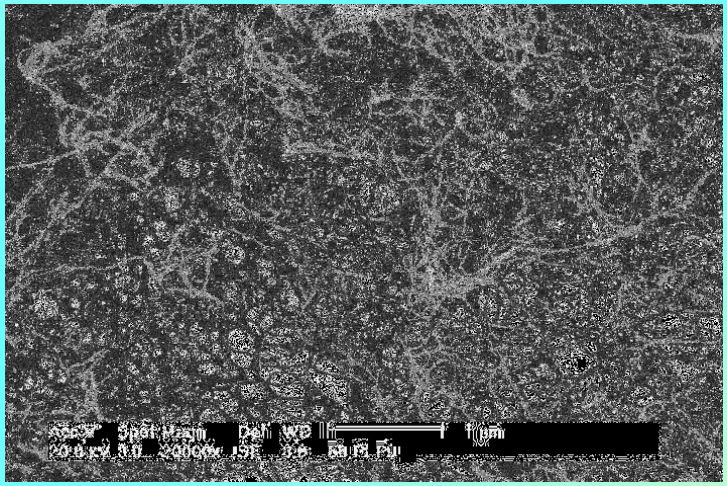


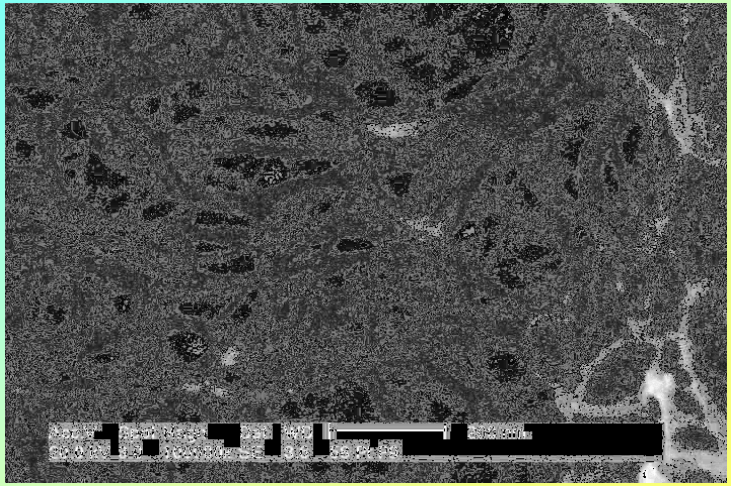
28 days post exposure 40 μg/mouse CNT



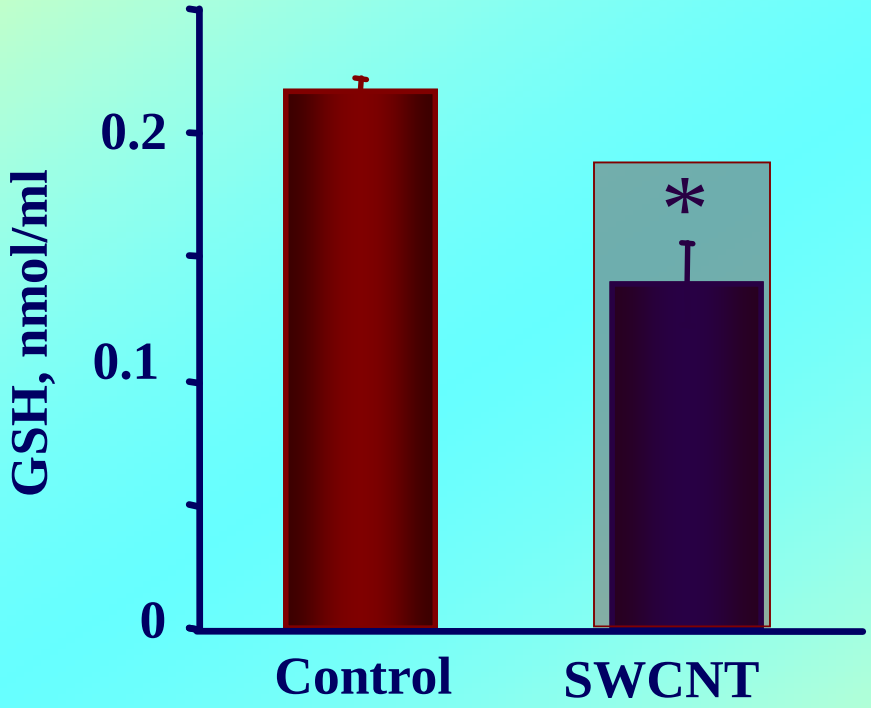
EPR spectra of partially-purified SWCNT (0.5 mg/ml, 2.5wt% iron) manufactured by high-pressure CO conversion (HiPco™) technology as compared to purified SWCNT additionally treated with an iron chelator, deferoxamine (DFO).

Note that partially-purified SWCNT displayed a broad signal with g value 2.0 and half-width of 640G, the signal was not detectable in purified DFO-treated SWCNT.



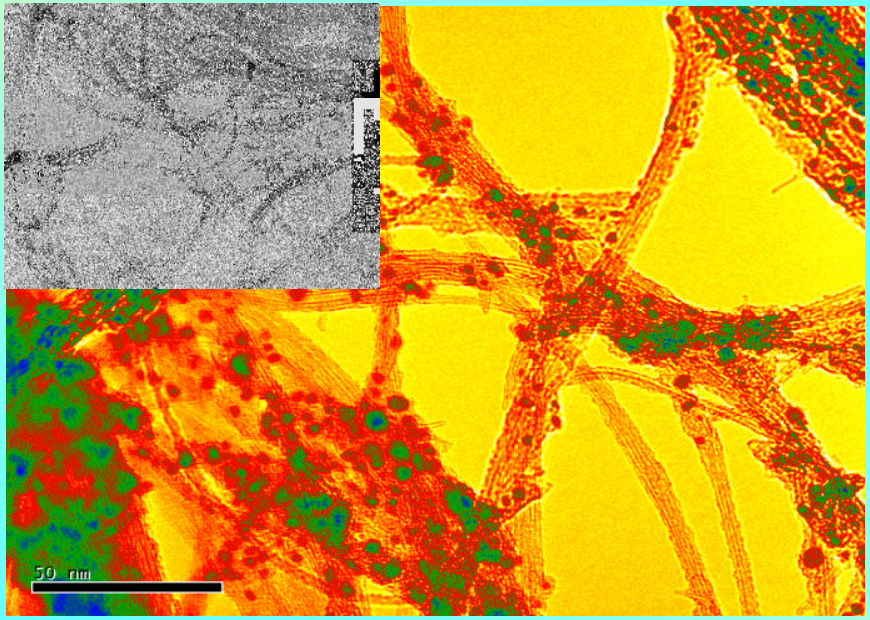


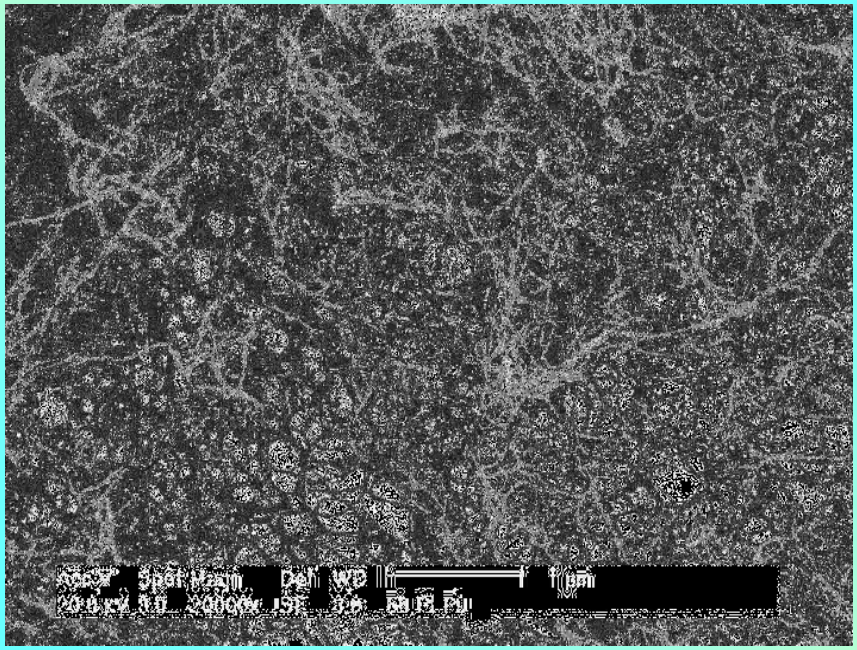
Levels of GSH in BAL of C57BL/6J mice 7 days after exposure to partially purified SWCNT.

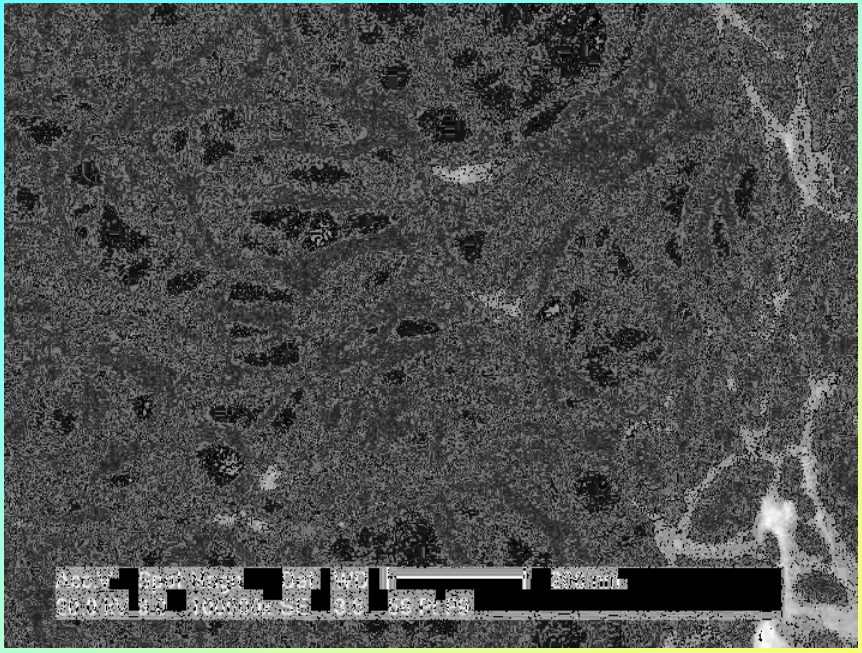


Partially purified SWCNT (2.0 mg/kg b.w.). *p<0.05 vs. control

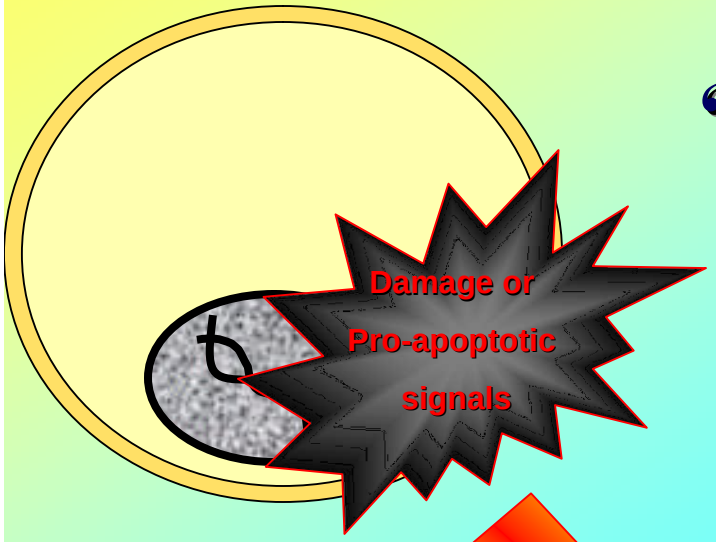
Single walled carbon nanotubes (SWCNT) aggregate and form ropes, bundles, and bird's nests

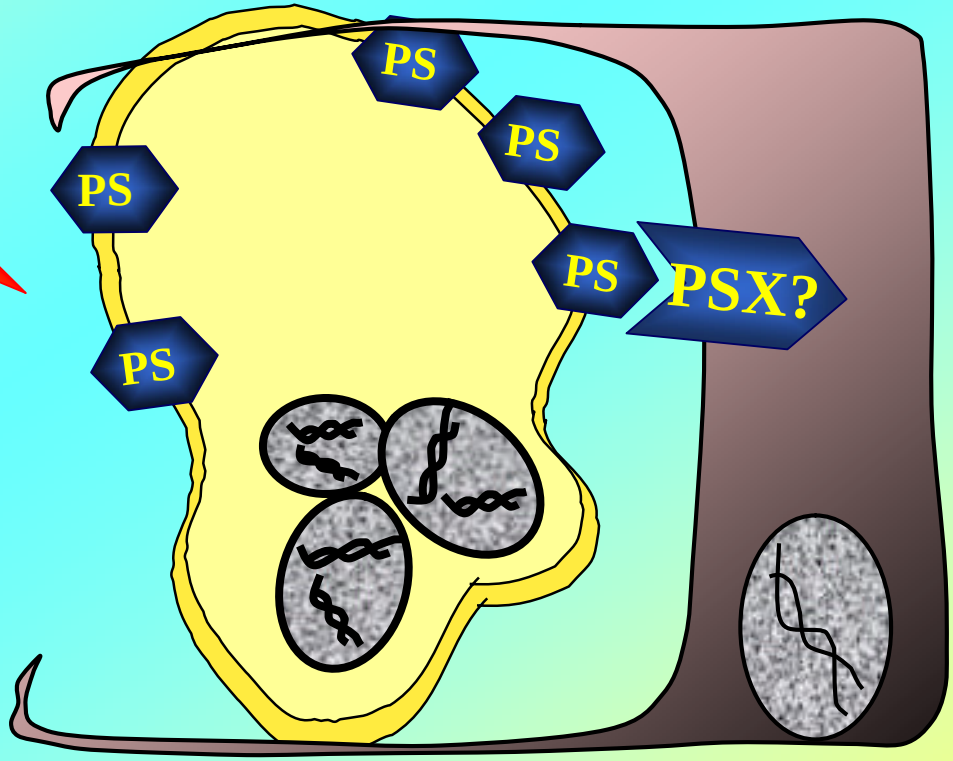


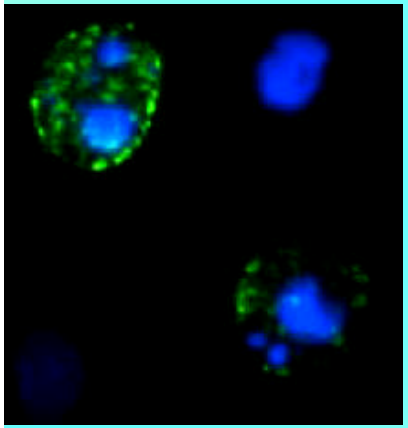


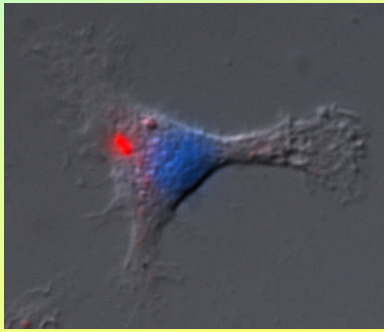


Phosphatidylserine (PS) as an "eat-me" signal in phagocytosis of apoptotic cells

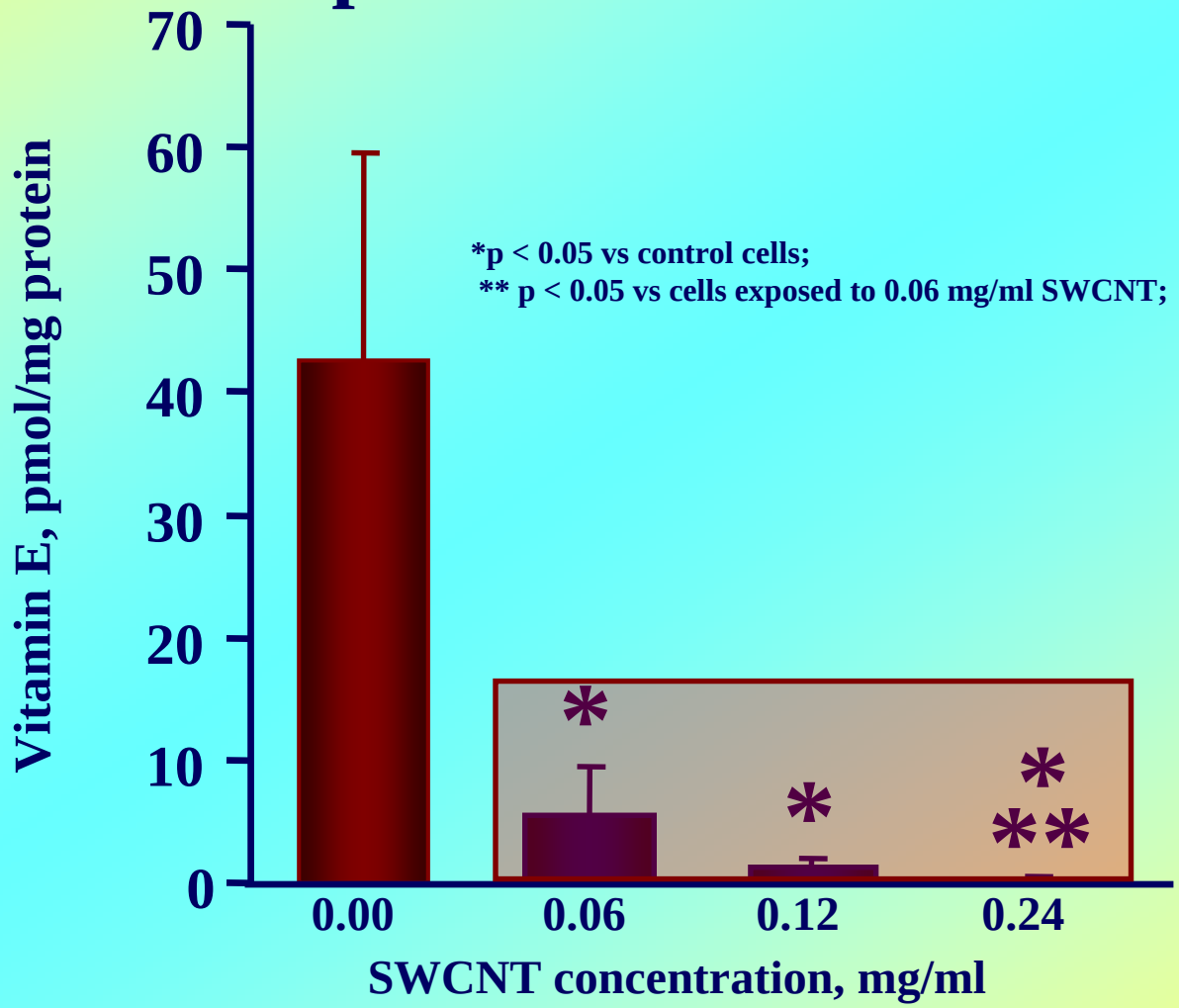




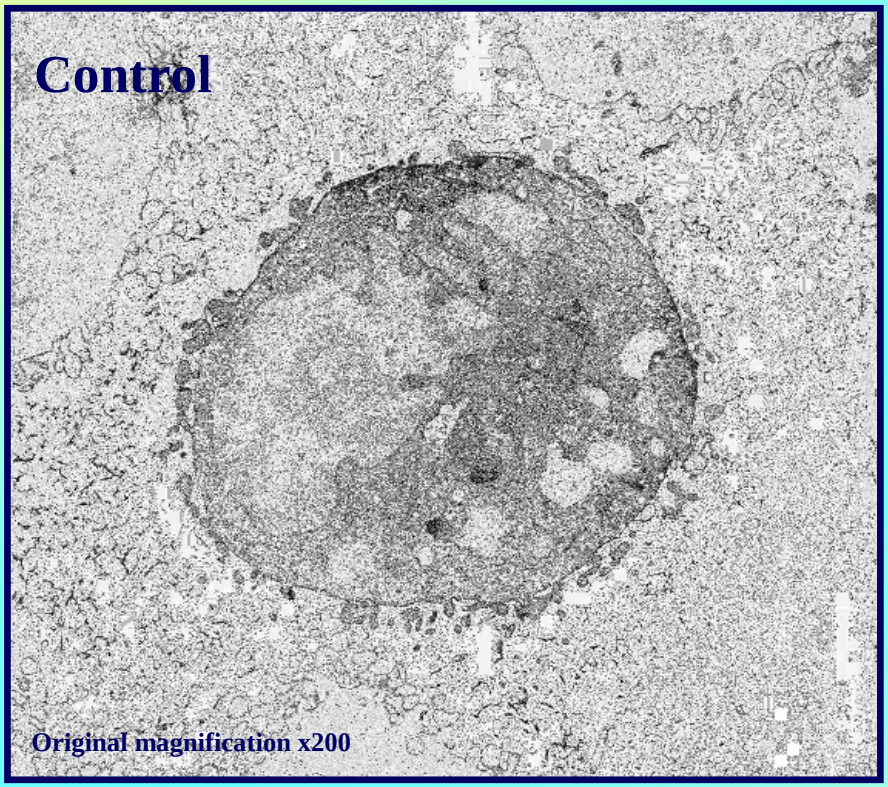


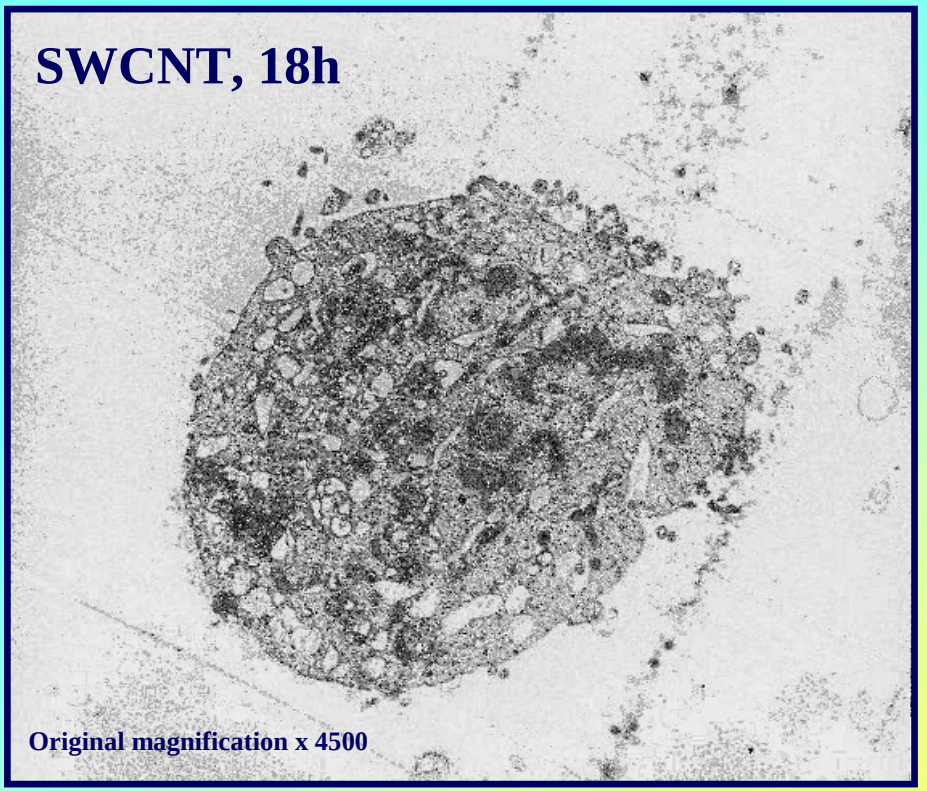


Vitamin E Level in BEAS-2B Cells Following Exposure to SWCNT



Electron Microscopy of BEAS-2B Cells Exposed to SWCNT





SWCNT, 0.24 mg/ml