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Introduction and objectives

Carbon nanofilaments have been extensively studied in terms of safety and toxicity. The main conclusion of these studies is that toxicological aspects cannot be generalized, since they depend on surface and structural properties of the considered type of carbon nanofilament. Therefore, the toxicological profile that CNF could have on biological systems and the potential adverse health effects remains unclear. Besides due to the high aspect ratio of the CNF (HARN) they can be considered as potentially hazardous.

In this study we attempt to evaluate those effects for assessing the occupational risk of the CNF production process before and after Safe by Design measures have been implemented. For this purpose we have analysed several *in-vitro* toxicological parameters that were determined as gaps in a preliminary risk assessment as: cytotoxicity, ROS production, secretion of inflammatory cytokines, phagocytosis, macrophage clearance as well as ocular and dermal irritation. We have compared three CNF's that differs in their C, O and impurities content in order to understand better how these differences may affect to the outcome. In the same way, the possible impact that different dispersion protocols could have on the analysis has also been evaluated.

Results

1 Effect of CNFs on cell viability

The viability of 3T3 and A549 cell lines exposed 24 hours to three different CNFs in two distinct dispersion protocols was measured by using MTS assay. The results showed that the dispersion protocol and the CNFs tested do not affect the cell viability under these conditions.

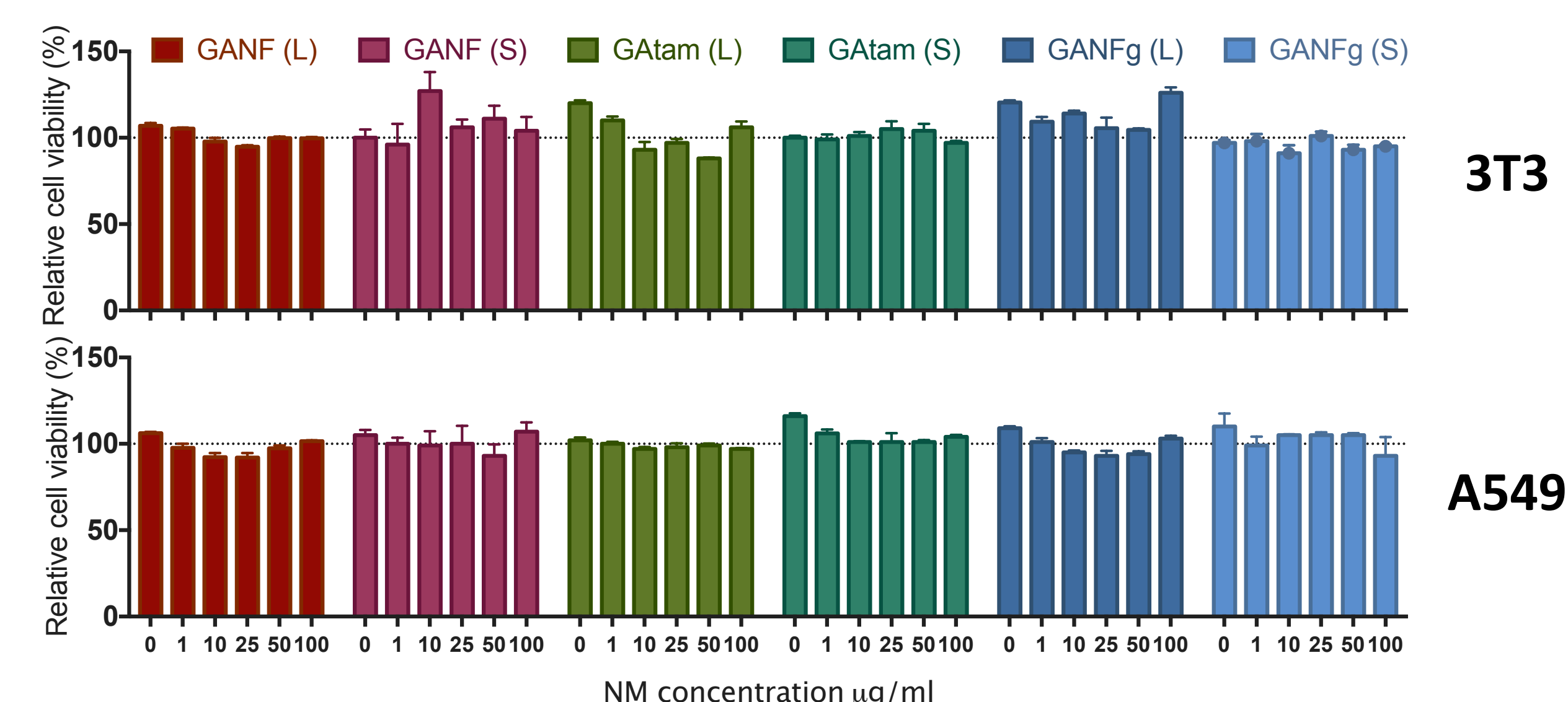


Figure 1: MTS assay. Cytotoxicity of three different CNFs in two different dispersion protocols.

2 Induction of oxidative stress

Oxidative stress is well known to play a major role in NM induced toxicity. We observed that CNFs exposure induced a significant increase in reactive oxygen species (ROS) production in a dose-response manner. Moreover, the absence of BSA in the dispersion protocol, (L), potentiated the ROS production in both cell lines.

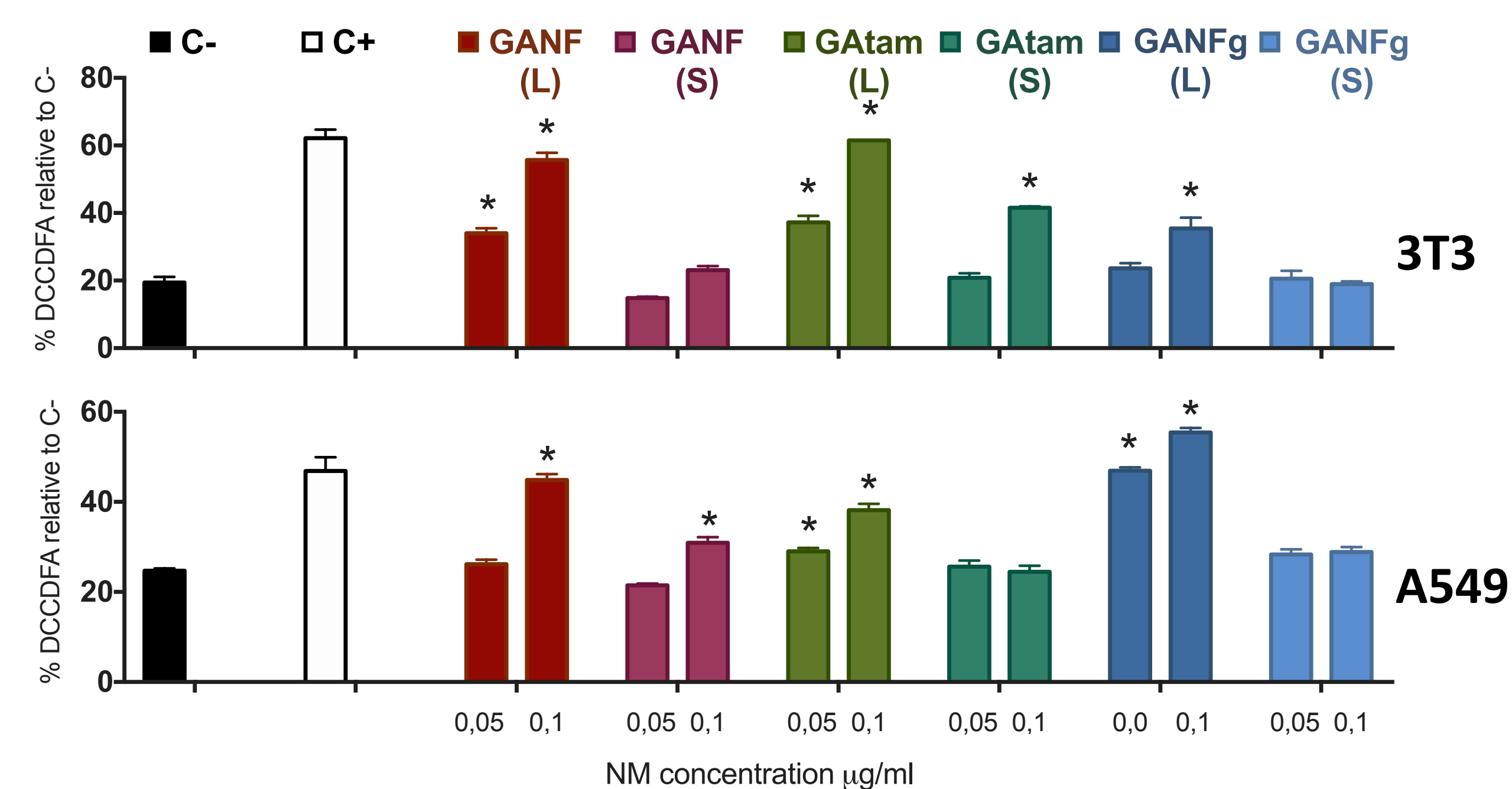


Figure 2: CNFs induce ROS production by 3T3 and A549 after 24 hours exposure.

3 CNF GANFg, induce IL-1 β secretion

Production of pro-inflammatory cytokines under NP exposure is usually measured to predict the immunomodulatory effect and possible inflammation-mediated-toxicity. We measured by ELISA the levels of IL-1 β , TNF- α and IL-8 secretion in differentiated THP-1-derived macrophages under 24 hours exposure to different CNFs concentrations. We only observe that GANFg significantly increased IL-1 β secretion. We did not observe pro-inflammatory cytokine production for GANF or GATam.

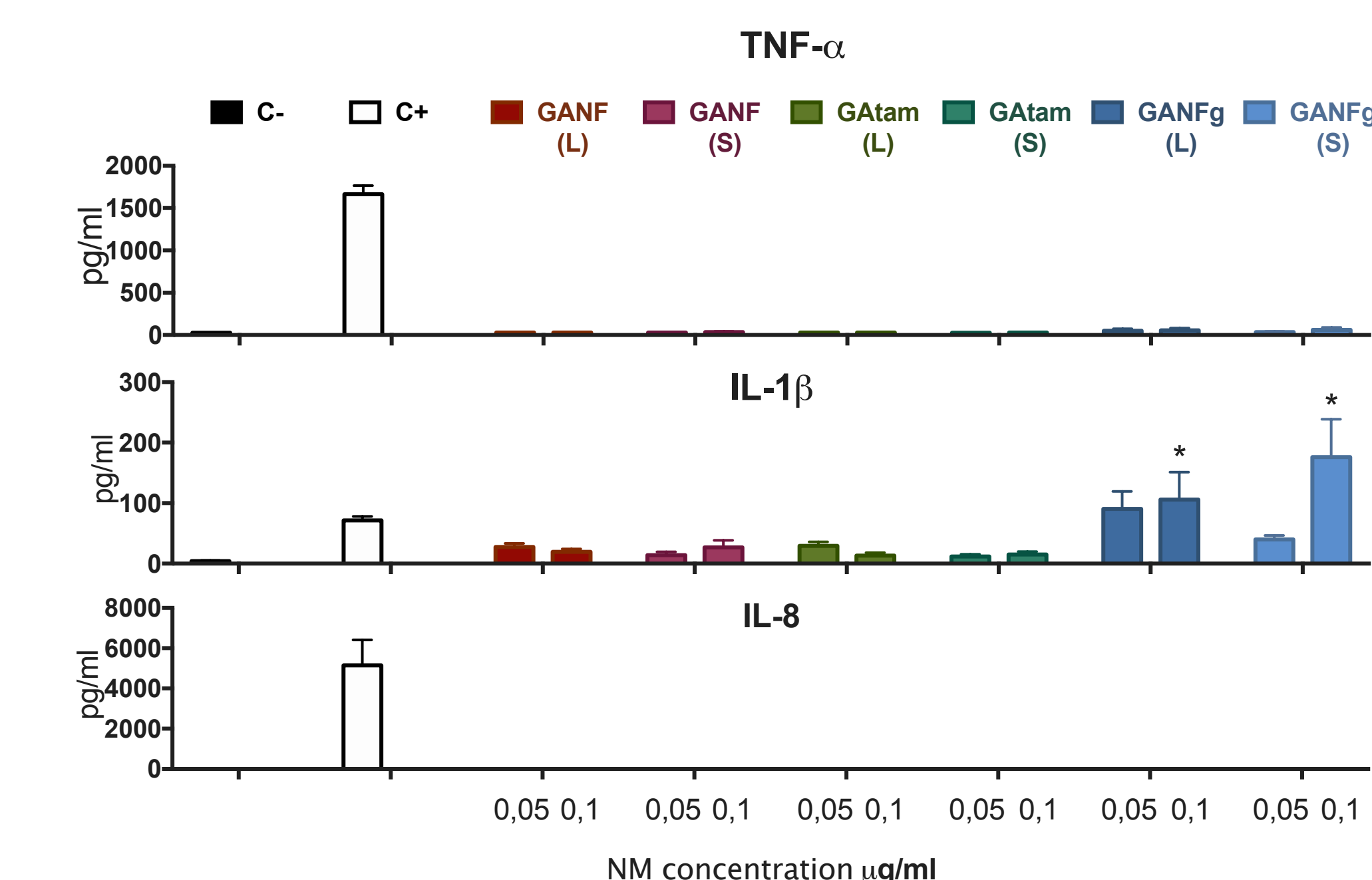


Figure 3: GANF induce IL-1 β secretion in THP-1 derived macrophages

4 CNFs do not generate ocular or skin irritation

The irritation studies were carried out in accordance to the OECD guidelines 439 and 492, using human epidermis and cornea epithelium, respectively.

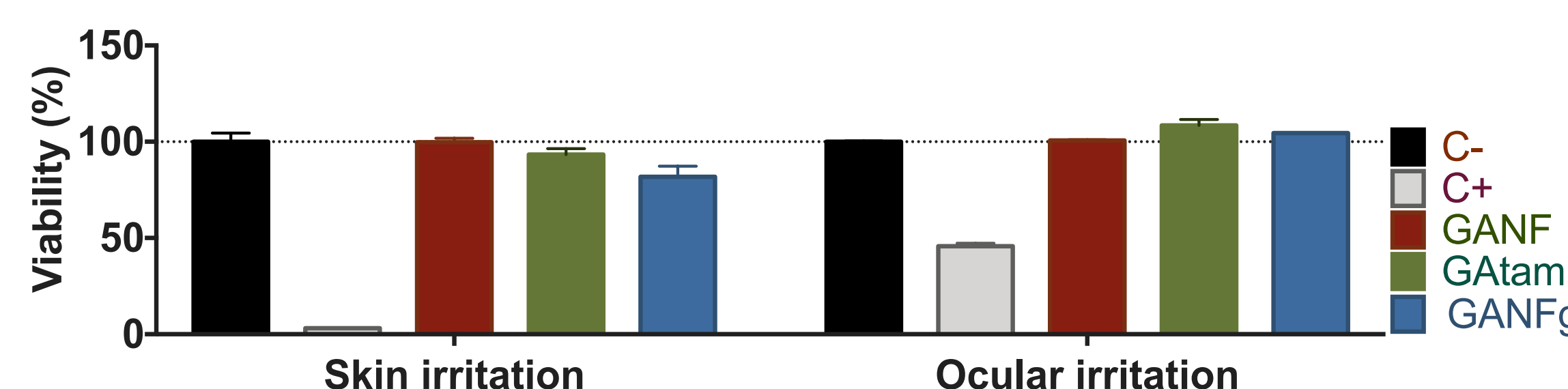


Figure 4: CNFs do not induce skin or ocular irritation.

Materials and methods

Grupo Antolin-CNf's

Properties	Unit	GANF	GATam	GANFg
Fibre diameter	nm	20-80	20-80	20-80
Carbon purity (TGA)	%	>85	>80	>99
Apparent density	g/cc	~0.06	~0.08	~0.08
Specific surface area (BET N ₂)	m ² /g	100-170	70-140	70-90
Graphitization degree	%	~70	~60	~90
Electrical resistivity	Ω .m	1.10 ⁻³	1.10 ⁻³	1.10 ⁻⁴

ASSAYS

1. Cytotoxicity: 3T3 (mouse fibroblast) and A549 (human lung epithelial) cell lines.
2. Dermal and ocular irritation in human epidermis and cornea epithelium.
3. Inflammation generated in THP-1-derived macrophages.
4. Reactive oxidative stress production by 3T3 and A549 cell lines.
5. Epithelium integrity (trans-epithelial electrical resistance, TEER), cell viability (LDH), metabolic activity (Resazurin assay) and NM uptake by an *in-vitro* 3D Air-Liquid Interphase (ALI) cell model of human airway epithelium (MucilAir™).

DISPERSION PROTOCOL

(L) Dispersion in ultrapure H₂O

(S) Dispersion in BSA- H₂O (NanoGenotox)

5 GANF and GATam CNFs affect human airway epithelium

One concentration of CNFs (32 μ g/cm²) was delivered to the ALI culture system for a 24 hours-length period. After that time the NM was removed. Four administrations were performed at the indicated days (red arrows). Different parameters were measured to evaluate the status of the epithelium including LDH, resazurin assay, TEER and NM uptake.

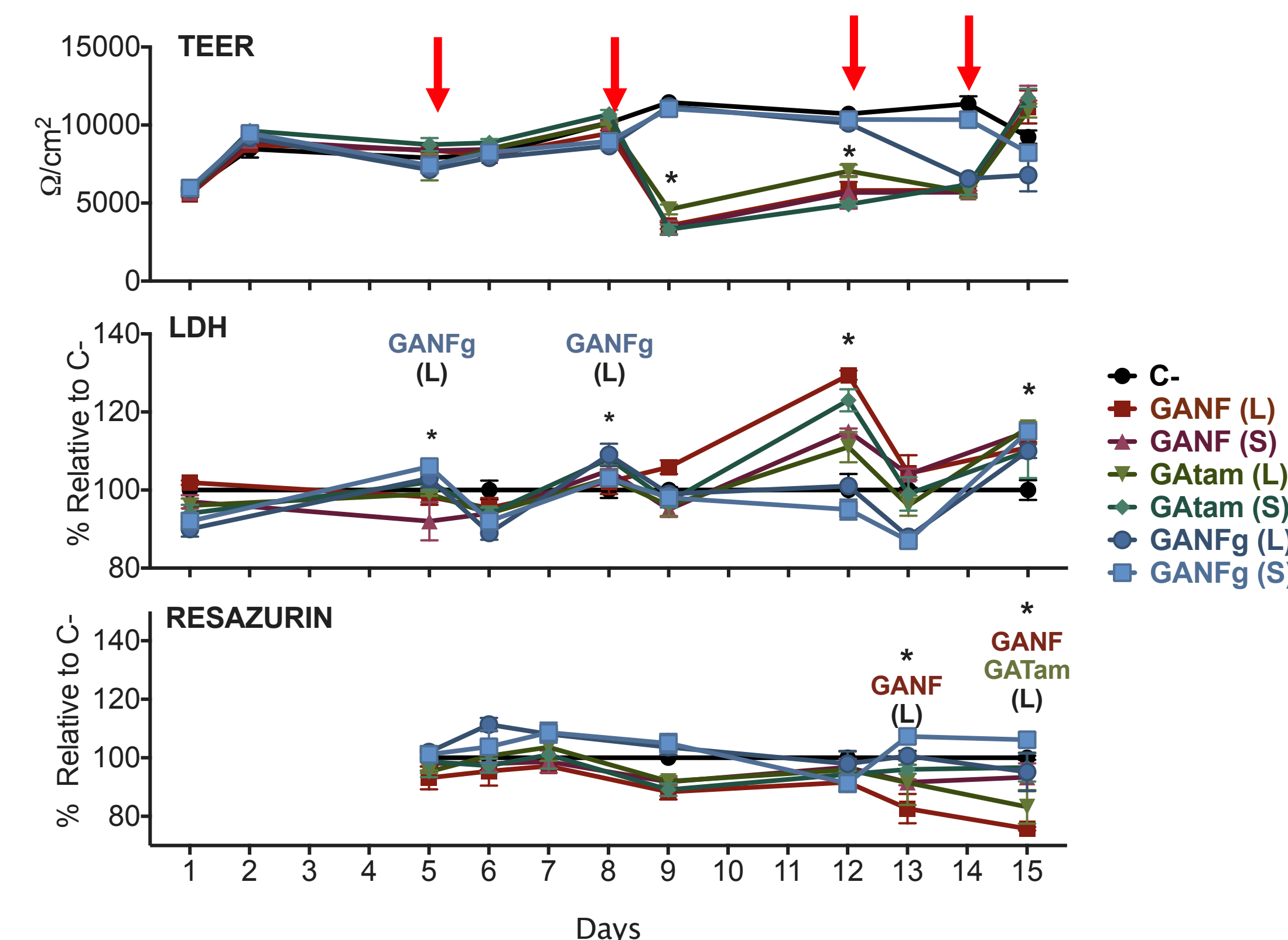


Figure 5: GANF and GATam affect integrity and viability of airway epithelium.

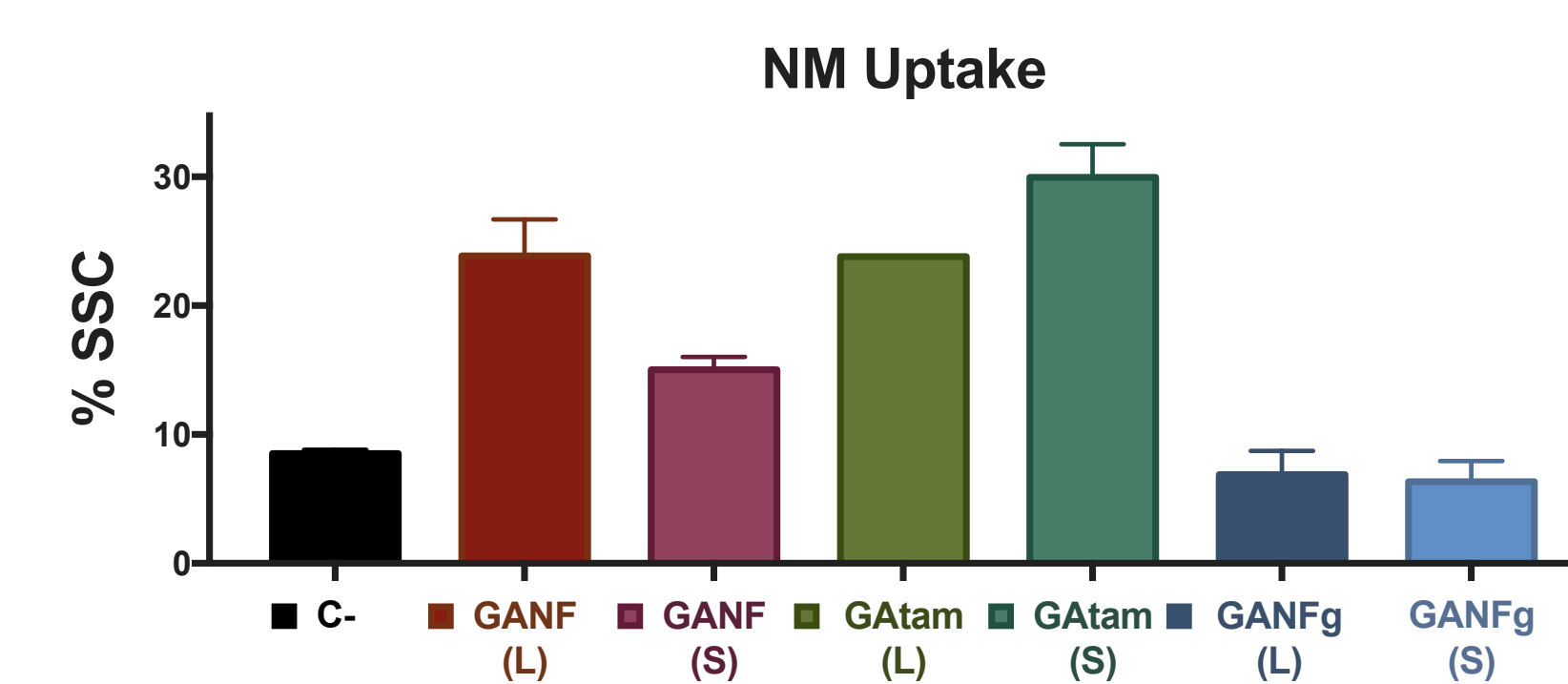


Figure 6: GANF and GATam CNFs are internalized by airway epithelium cells.

Conclusions

- Different dispersion protocols affect results for a given endpoint.
- The presence of BSA in the dispersion protocol prevents cells from cytotoxicity
- CNFs do not affect cell viability of A549 or 3T3 cell lines.
- Among the three CNFs tested, only GANFg increases significantly the secretion of IL-1 β
- CNFs do not generate ocular or skin irritation
- GANF and GATam compromise human airway epithelium in acute response of NFs