In situ detection of DNA double strand breaks by immunofluorescent γ-H2AX staining in mice exposed to multiwalled carbon nanotubes

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Content

Phosphorylation of histone H2AX (γ-H2AX) at serine 139 is an acknowledged biomarker of DNA double strand breaks in cultured cells and tissue biopsies. However, γ-H2AX *in situ* staining has rarely been used to detect genotoxic effects of nanomaterials.

The application of immunofluorescent (IF) γ-H2AX staining on tissue samples has many advantages. The same paraffin embedded tissues can be used for both histopathology and γ-H2AX analysis, which allows implementing the γ-H2AX assay also on previously conducted *in vivo* studies. The detection of γ-H2AX *in situ* enables the localization of the genotoxic effect in tissue-specific structures and even cell types. Analysis by microscopy can easily discriminate between cells with different levels of DNA damage and apoptotic cells.

The purpose of this study was to further evaluate the use of IF γ-H2AX staining for the genotoxicity assessment of nanomaterials *in vivo*. Groups of C57BL-6 female mice were exposed to three doses (10, 40 and 80 µg/mouse) of multiwalled carbon nanotubes (MWCNTs; Mitsui-7) by single pharyngeal aspiration. Lung samples for genotoxicity (Comet assay) and histopathological evaluation were collected 24 h and 28 d post-exposure and the results were compared to a negative control group. The IF γ-H2AX staining was performed on formalin-fixed paraffin-embedded lung samples after deparaffination and antigen retrieval by boiling. An autostainer was used for primary (rabbit monoclonal anti-gamma H2AX phospho-Ser139) and secondary (goat anti-rabbit IgG) antibody incubations and for tyramide amplification of the fluorescent signal (Alexa Fluor™ 488 Tyramide SuperBoost™ Kit; ThermoFisher Scientific) according to manufacturer's instructions. Samples were counterstained with 4',6-diamidino-2-phenylindole and digitized with 20x fluorescent scanning. Expression of γ-H2AX foci was analyzed using marker counter module of a digital microscope application. For each sample, all nuclei in four randomly selected annotations (200 µm x 200 µm) were classified as negative, weak positive (≤ 3 foci), positive (> 3 foci), or apoptotic (pan-stained nucleus).

The results showed a dose-dependent induction of γ-H2AX positivity 24 h post-exposure. 28 days later, the effect of the MWCNT exposure was lower, although the percentage of γ-H2AX positive nuclei remained elevated in the lungs of the exposed mice. These results were in line with comet assay data (% of DNA in tail) from the same animals. Hence, it has been shown that IF γ-H2AX staining can be used to complement the comet assay for monitoring DNA damage induced by nanomaterials *in vivo*.

[Supported by the Finnish Work Environment Fund]