Author's response to reviews

Title: Characterisation of an aerosol exposure system to evaluate the genotoxicity of whole mainstream cigarette smoke using the in vitro gammaH2AX assay by High Content Screening

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Comments from the Editor

There are some essential comments that need to be corrected, including some statistical corrections.

1.- It is reasonable to assume that there would have been negligible effects on cell viability after 3 hours. An additional recovery period is required for the manifestation of any cytotoxicity.

As we stated in the introduction (page 4 paragraph 2), we aimed to further optimised an existing γH2AX detection methodology (Garcia-Canton, C., Anadon, A., and Meredith, C., 2013. Assessment of the in vitro gammaH2AX assay by high content screening as a novel genotoxicity test. Mutat.Res. 757, 158-166) for aerosolised toxicants. The previously developed assay uses the cell viability measure named Relative Cell Counts to ensure that the DNA damage reported by the γH2AX frequency is not a by-product of a high cytotoxicity as indicated in Table 3 (False positive, cytotoxicity-driven genotoxicity). It is out of the scope of this study to report the cytotoxicity of whole mainstream cigarette smoke as this has been published previously (Phillips, J., Kluss, B., Richter, A., and Massey, E., 2005. Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses. ATLA Alternatives to Laboratory Animals 33, 239-248.)

Also, in page 13, paragraph 3 we described how pilot experiments with recovery time were not possible under the current experimental design in air and smoke exposed samples as drying was causing issues with cell proliferation. The same effect was not observed on incubator controls also at the ALI.

2.- Published literature has indicate that aerosols from flue-cured tobacco are generally more genotoxic than 3R4F counterparts in mammalian assays. There is some concern that an overall reduced assay response when evaluating M4A genotoxicity account for this apparent discrepancy with published data.

We agree with the editor that current literature seems to indicate that M4A has a higher potency than 3R4F in mammalian assays (micronucleus and mouse lymphoma assays). However, the majority of these studies have been conducted using particulate matter (PM) which contains a fraction of the total compounds present in cigarette smoke. We do not believe there is enough information in the public domain to confirm that M4A is more potent than 3R4F when whole mainstream cigarette smoke (WMCS) aerosol at the ALI is used. It is probable that the compounds driving the genotoxicity are different in PM than WMCS. More studies, using existing mammalian assays developed at the ALI could bring some light on this topic, but we think this is out of the scope of this manuscript.

Nevertheless, We have performed a 2 sample t-test statistical analysis to evaluate the concern over reduced assay response. When etoposide data from both set of experiments were analysed, statistical differences were observed (p<0.001). We then performed the same statistical analysis in untreated controls and air-treated controls to confirm if a reduction in response was also
observed at the baseline level. The Untreated controls produced a p=0.203 while air-treated controls produce a p=0.855. These results confirm that the assay performance was not reduced while testing M4A samples. Positive controls such as Etoposide produce an exaggerated response to ensure responsiveness of the \textit{in vitro} system and this could often produce more variability in the signal intensity.