Original Article
Toxicology International, *May-Aug 2016 / Vol-23 / Issue-2, 159-163*
ISSN (Print) : 0971-6580
ISSN (Online) : 0976-5131
DOI: 10.22506/ti/2016/v23/i2/146695

Effects of residual tobacco smoke in A549, Hep-2 and 3T3 cells

Luciana Rizzieri Figueiró[1*], Amanda Ferreira Becker[2], Ana Luiza Ziulkoski[3] and Denise Conceição Mesquita Dantas[4]

[1]Graduate Program in Pathology, Federal University of Health Sciences of Porto Alegre, UFCSPA, Brazil;
[2]Undergraduate of Biomedine, Feevale University
[3]Cytotoxicity Laboratory, Institute of Health Sciences, Feevale University
[4]Pharmacosciences Department, UFCSPA
* rizzieri@ufcspa.edu.br / lucianarizzieri@gmail.com

Abstract

The aim of this study was to assess the effects of residual tobacco smoke in the A549, Hep-2 and 3T3 cells. Cellulose papers were exposed to the smoke of 1, 3, 5 and 8 cigarettes. Cotton wipes were used to clean the chamber walls after extinction of the smoke. The paper and cotton were immersed in Dulbecco's Modified Eagle Media (DMEM) to transfer the compounds of the material to the solvent. DMEM+THS was used to expose A549, Hep-2 and 3T3 cells for 24 hours and MTT, Neutral Red Uptake (NRU) and Trypan blue exclusion assays were performed. No significantly changes were found in A549 cells. In Hep-2 cells, there was difference only in NRU assays. In 3T3 cells, changes in mitochondrial activity and proliferation rate were observed. Because of different sensibility of cells to THS, it is important to screen multiple cell types in order to better assess cytotoxicity.

Keywords: thirdhand tobacco smoke, environmental tobacco smoke, MTT assay, Neutral Red Uptake assay

Received: 19-04-2016 **Revised:** 23-06-2016 **Accepted:** 02-07-2016

1. Introduction

Thirdhand Smoke (THS) is the toxic compounds of cigarette smoke that remain in indoor environments after the cigarette is extinguished. These compounds can accumulate in the air or on surfaces or are re-emitted back into the gas phase or react with oxidants and other compounds in the environment to yield secondary pollutants[1]. THS in air, dust and on surfaces leads to multiple exposure routes such as inhalation, dermal absorption and involuntary ingestion[2].

It has been proven that THS contain several volatile organic compounds, polycyclic aromatic hydrocarbons, tobacco-specific lung carcinogen (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-4-(3-pyridyl) butanal(NNA), a nitrosamine absent in fresh smoke[3,4]. Many of these pollutants remain in the environments for months or years even if smoking does not become the environment[5-7].

The risks of THS have only recently started to be recognized. THS showed genotoxicity to human hepatocellular carcinoma cells even in acute exposure and it was sufficient to cause metabolic changes in two male reproductive murine cell lines and cancer in humans[8-10].

Nonetheless, more biological evidence of THS toxicity is needed. *In vitro* cytotoxicity assay can be useful to understand the mechanisms involved in the detrimental effects of THS. As each assay may assess different functions and different cells may have distinct sensitivity to xenobiotics, it is interesting to perform experiments with different outcomes in different targets. The aim of this study was to assess the effects of residual tobacco smoke in the A549, Hep-2 and 3T3 cell lines.

* *Author for correspondence*

2. Materials and Methods

2.1 Experimental sample collection

THS samples were generated by exposing the cellulose chromatography papers (25 cm^2) to the smoke of 1, 3, 5 and 8 cigarettes (1 cigarette = 0,9mg nicotine in mainstream smoke as reported by the manufacturer) in a 27.6L acrylic chamber. The tobacco combustion took place spontaneously and the box had an inlet where enough air had been provided. One hour after the combustion, the paper was removed and an area of equal size limited by a frame was cleaned with moistened cotton wipes (28 cm^2 cosmetic 100% cotton facial wipes). Samples were kept in a 100% smoke-free box for six hours and placed in a 50mL conical centrifuge tubes and then stored at −20°C.

2.2 Sample extraction

Each material was cut in small pieces and 25 mL Dulbecco's Modified Eagle Media (DMEM) were added. Flasks were stirred and stored overnight at 4°C. Afterwards, flasks were stirred and the supernatant was recovered by a syringe. Paper and cotton wipes without smoke exposure were processed accordingly and used as reference. The recovered medium was passed through a 0.22 µm filter and pH was adjusted at 7.4, if necessary.

2.3 Cell culture and treatment

Human alveolar type II epithelial cell line from an adenocarcinoma (A549 cells), human epithelioma cells from laryngeal carcinoma (Hep-2 cells) and mouse embryonic fibroblast cell line (3T3 cells) were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Culture was maintained at 37°C in a humid atmosphere at 5% of CO_2 and a half-open system.

For THS exposure, cells were seeded at a rate of 1 to 2 x 10^4 cells/well in a 96-well (MTT and NRU assays) or 7 to 10 x 10^4 cells/well in a 24-well polystyrene microplate (Trypan blue assay). After the cultures reached semiconfluence, culture medium was replaced with DMEM + THS obtained from different number of cigarette combustion, and cells were exposed for 24 hours to assays.

2.4 Cytotoxicity assay

Mitochondrial activity was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, which reflects the number of viable cells[11]. The assay was performed as Trintinaglia et al. (2015)[12].

The neutral red uptake (NRU) method was used to evaluate cytotoxicity through the lysosome viability. Damaged lysosomes lose the ability of incorporate the dye and lysosomal integrity is a highly sensitive indicator of cell viability[13]. The assay was performed as Trintinaglia et al. (2015)[12].

The trypan blue exclusion assay assesses cells with intact plasma membrane[14] and it was used to count the viable and nonviable cells in a Neubauer chamber and calculate the proliferation rate (number of cells after the THS exposure / the initial plating number).

3. Statistical analysis

Cytotoxicity experiments were performed in quintuplicate and repeated at least once to confirm the validity of the observation. All results are presented relative to reference (100% cellular function). The significance of differences within groups was determined by one-way ANOVA with Duncan post hoc test using IBM SPSS Statistics 19.0. Statistical significance was set at $p < 0.05$.

4. Results

The cellular effects of residual tobacco smoke are presented in Figure 1. No significantly changes were found in the A549 cells.

In Hep-2 cells, there was difference only in NRU assays. Lysosomal viability was lower when cells were exposed to eight cigarettes and higher with one cigarette in paper samples, but the latter was not different from the reference ($p<0.001$). In cotton samples, lysosomal viability with five cigarettes was higher than three, eight cigarettes and reference ($p=0.048$).

In 3T3 cells, changes in mitochondrial activity were observed. This value was increased in about 80% and 40% in the eight-cigarette exposure, both in the paper and cotton samples ($p<0.001$ and $p=0.002$, respectively). Proliferation rate was lower in both paper and cotton samples. In paper samples, the rate of the exposure to one, five and eight cigarettes was about 30% lower than the value of the reference ($p=0.017$). In cotton samples, the value with five and eight cigarettes was approximately 35% lower than the value of the reference ($p=0.038$).

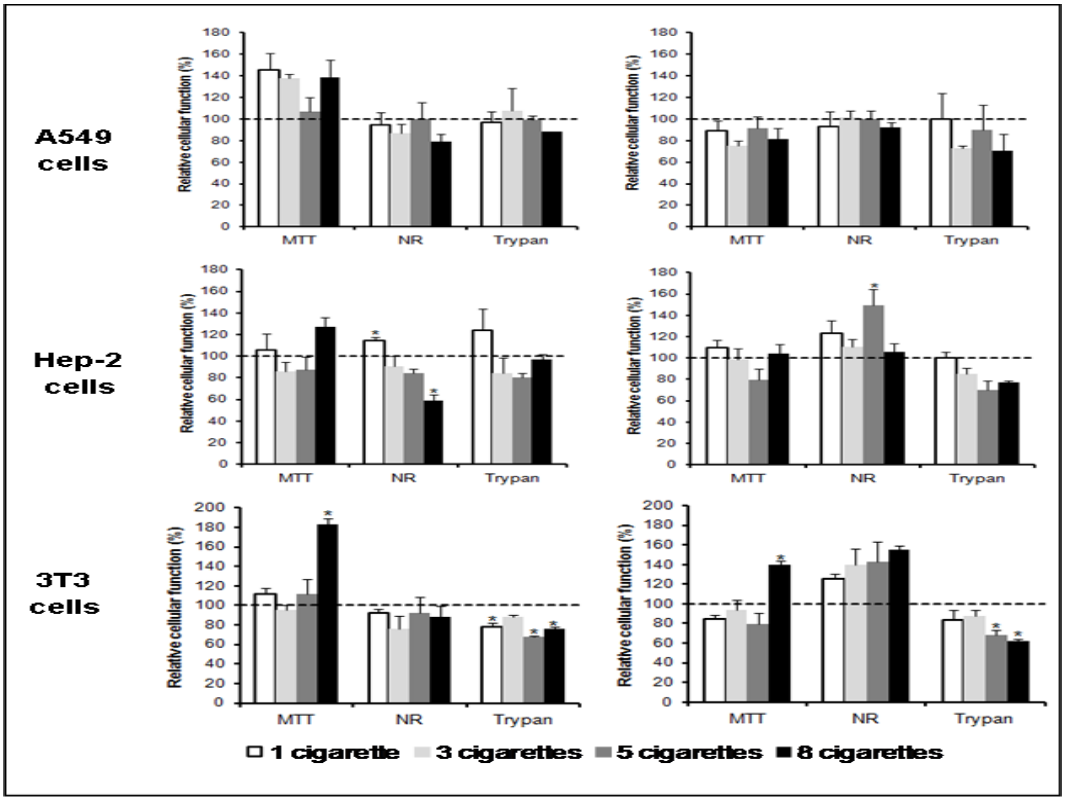


Figure 1. Cellular functions in A549 (upper), Hep-2 (middle) and 3T3 cells (bottom) of cellulose papers and cotton exposed to smoke cigarette in laboratory chamber. Data are expressed as mean + SEM. The dashed line represents the reference (paper or cotton smoke-free = cellular function 100%). Each assay was conducted at least twice in independent experiments, with five replicates for each concentration and independent assay.
*p<0.05 (One-way ANOVA)

5. Discussion

Laboratory chamber experiment was performed to generate THS samples using chromatography paper as a model surface and cotton to clean the surface. Thus, it was possible to assess the cellular effects of THS deposited on surfaces in environments where smoke occurs. Some changes in cellular functions were found in cells exposed to THS.

As the main resource of the environmental tobacco smoke is sidestream smoke, to generate smoke by spontaneous combustion is a realistic practical approach rather than using pump to smoke and an artificial source of oxygen[15,16]. Tobacco smoke toxins linger on furniture, clothes, drapes, carpets and surfaces and the collecting material may adsorb different compounds of cigarette smoke. Both chromatography papers and cotton wipes are made of cellulose and then the polar groups of nicotine form hydrogen bonds with hydroxyl groups of these materials. This chemical interaction promotes the sorption of polar compounds to polar material. Significant amounts of nicotine are retained and can be extracted with the use of aqueous or organic solvents[5]. How each of these materials incorporate and release substances is still unexplored. This may have influenced the results of the assays, since lysosomal viability and proliferation rate were different according to the collection material.

Hep-2 and A549 cells were selected because the respiratory tract is an organ directly targeted by smoke cigarettes. However, they showed different sensitivity to THS and A549 cells are not affected. Hep-2 cell line is widely used in toxicity studies and it exhibits good sensitivity to xenobiotics[17]. Even though both cells have tumor origin, Hep-2 cells seemed more sensitive than A549 cells. Generally, differentiated cells are less sensible to residual tobacco smoke[6,18]. More changes are found in 3T3 cells, which is the only non-malignant cell line in our study and one of the most commonly used fibroblast cell lines for *in vitro* assays. These cells behave differently because distinct molecular mechanisms are activated and

the effects of the cigarette smoke on cancer cell survival and response to cytotoxic agents are poorly delved[19].

Although NRU assay is considered the most sensitive assay for assessing the cytotoxic potential of cigarette smoke condensates[20], it has not been used to evaluate the THS effects. We only saw changes in Hep-2 cells, which was different depending on the sample. In 3T3 cells, increased mitochondrial activity and lower proliferation rate were seen in both paper and cotton samples, stressing there is not always association of cell viability biomarkers with the cell number[21]. Many studies interpret the MTT results as cellular viability; however it best indicates change in metabolic activity, specially the rate of glycolytic NADH production[22]. The increased mitochondrial activity may be a repercussion of an adaptive response in an attempt to handle the detrimental effects of THS. As the metabolic condition in cells is linked closely with the ability of a cell to replicate[23], an increased cellular function, especially in cancer cells, should turn on a warning sign.

In another study, cytotoxicity of THS by MTT assay in mouse neural stem cells (mNSC) was only seen when the sample extraction was made by medium containing serum. The proteins present in serum, such as albumins, may have provided the extraction of water insoluble or semi-soluble harmful THS constituents[6]. As our extraction procedure was performed with serum free medium, these harmful substances may not be present in the sample and our findings in MTT assay may be underestimating the cytotoxicity of THS.

In summary, some changes in cellular functions were found in the cells exposed to THS, however, these effects relied on the cell type. Because of different sensibility of cells to THS, it is important to screen multiple cell types in order to better assess cytotoxicity.

6. Conflict of Interest

None Declared

7. References

1. Winickoff JP, Friebely J, Tanski SE, Sherrod C, Matt GE, Hovell MF, et al. Beliefs about the health effects of "third-hand" smoke and home smoking bans. Pediatrics 2009 Jan;123(1):e74-9. doi: 10.1542/peds.2008-2184.
2. Burton, A., 2011. Burton A. Does the smoke ever really clear? Thirdhand smoke exposure raises new concerns. Environ Health Perspect 2011 Feb;119(2):A70-4.doi: 10.1289/ehp.119-a70.
3. Sleiman M, Gundel LA, Pankow JF, Jacob P 3rd, Singer BC, Destaillats H. Formation of carcinogens indoors by surface-mediated reactions of nicotine with nitrous acid, leading to potential thirdhand smoke hazards. ProcNatlAcadSci U S A 2010a;107(15):6576-81.doi: 10.1073/pnas.0912820107.
4. Hoh E, Hunt RN, Quintana PJ, Zakarian JM, Chatfield DA, Wittry BC, et al. Environmental tobacco smoke as a source of polycyclic aromatic hydrocarbons in settled household dust. Environ SciTechnol 2012 Apr 3;46(7):4174-83.doi: 10.1021/es300267g.
5. Bahl V, Jacob P 3rd, Havel C, Schick SF, Talbot P. Thirdhand cigarette smoke: factors affecting exposure and remediation. PLoS One 2014 Oct 6;9(10):e108258. doi: 10.1371/journal.pone.0108258.
6. Bahl V, Shim HJ, Jacob P 3rd, Dias K, Schick SF, Talbot P. Thirdhand smoke: Chemical dynamics, cytotoxicity, and genotoxicity in outdoor and indoor environments. ToxicolIn Vitro 2015a Dec 9. pii: S0887-2333(15)30028-X. doi: 10.1016/j.tiv.2015.12.007.
7. Matt GE, Quintana PJ, Zakarian JM, Fortmann AL, Chatfield DA, Hoh E, et al. When smokers move out and non-smokers move in: residential thirdhand smoke pollution and exposure. Tob Control 2011a Jan;20(1):e1.doi: 10.1136/tc.2010.037382.
8. Hang B, Sarker AH, Havel C, Saha S, Hazra TK, Schick S, et al. Thirdhand smoke causes DNA damage in human cells. Mutagenesis 2013 Jul;28(4):381-91.doi: 10.1093/mutage/get013.
9. Ramírez N, Ozel MZ, Lewis AC, Marcé RM, Borrull F, Hamilton JF. Exposure to nitrosamines in thirdhand tobacco smoke increases cancer risk in non-smokers. Environ Int2014;71:139-47.doi: 10.1016/j.envint.2014.06.012.
10. Xu B, Chen M, Yao M, Ji X, Mao Z, Tang W, et al. Metabolomics reveals metabolic changes in male reproductive cells exposed to thirdhand smoke. Sci Rep 2015 Oct 22;5:15512. doi: 10.1038/srep15512.
11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983 Dec 16;65(1-2):55-63.
12. Trintinaglia L, Bianchi E, Silva LB, Nascimento CA, Spilki FR, Ziulkoski AL. Cytotoxicity assays as tools to assess water quality in the Sinos river basin. Braz J Biol 2015 May;75(2 Suppl):75-80. doi: 10.1590/1519-6984.0113.
13. Borenfreund E, Puerner JA. Toxicity determined in vitro by morphological alterations and neutral red absorption. ToxicolLett 1985 Feb-Mar;24(2-3):119-24.
14. Altman SA, Randers L, Rao G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. BiotechnolProg 1993 Nov-Dec;9(6):671-4.
15. International Agency for Research on Cancer - IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Tobacco Smoke and Involuntary Smoking. IARC MonogrEvalCarcinog Risks Hum 2004;83:1-1438.
16. Hammer TR, Fischer K, Mueller M, Hoefer D. Effects of cigarette smoke residues from textiles on fibroblasts, neu-

rocytes and zebrafish embryos and nicotine permeation through human skin. Int J Hyg Environ Health 2011 Sep;214(5):384-91. doi: 10.1016/j.ijheh.2011.04.007.
17. Amaro MI, MonasteriosM, Avendaño M, Charris J. Preliminary evaluation of the toxicity of some synthetic furan derivatives in two cell lines and Artemiasalina. J ApplToxicol 2009 Jan;29(1):36-41. doi: 10.1002/jat.1379.
18. Bahl V, Weng NJ, Schick SF, Sleiman M, Whitehead J, Ibarra A, et al. Cytotoxicity of Thirdhand Smoke and Identification of Acrolein as a Volatile Thirdhand Smoke Chemical That Inhibits Cell Proliferation. ToxicolSci 2015 Dec 29. pii: kfv327.
19. Sobus SL1, Warren GW. The biologic effects of cigarette smoke on cancer cells. Cancer 2014 Dec 1;120(23):3617-26. doi: 10.1002/cncr.28904.
20. Putnam KP1, Bombick DW, Doolittle DJ. Evaluation of eight in vitro assays for assessing the cytotoxicity of cigarette smoke condensate. ToxicolIn Vitro 2002 Oct;16(5):599-607.
21. Wang H, Word B, Lyn-Cook L Jr, Yang M, Hammons G, Lyn-Cook B. Cytotoxicity of chronic exposure to 4 cigarette smoke condensates in 2 cell lines. Int J Toxicol 2015 Mar-Apr;34(2):182-94. doi: 10.1177/1091581815574349.
22. Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L. Cell Viability Assays. In: Sittampalam GS, Coussens NP, Nelson, H, et al., editors. Assay Guidance Manual [Internet]. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD): 2004. Retried: http://www.ncbi.nlm.nih.gov/books/NBK144065/. Accessed: Jan 28, 2016.
23. Fukano Y, Ogura M, Eguchi K, Shibagaki M, Suzuki M. Modified procedure of a direct in vitro exposure system for mammalian cells to whole cigarette smoke. ExpToxicolPathol 2004 Mar;55(5):317-23.