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# Cytotoxicity of aqueous cigarette smoke extract is affected by properties of pipettes used to prepare the extract

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## Abstract

Cigarette smoke contains a host of molecules including toxins and carcinogens, most of which have not been well studied. Aqueous cigarette smoke extract (CSE) is one of various cigarette smoke derivatives that can be used for *in vitro* studies, and the influence of different method parameters on CSE composition and toxicity remains incompletely understood. Herein, we prepared CSE by bubbling cigarette smoke through mammalian cell culture medium, varying the type of pipette inserted into the recipient medium. Changing this one component of the preparation apparatus had a marked effect on the toxicity of the resulting CSE. Since many other parameters can also be varied in CSE preparation, these results stress the importance of standardization within and between studies.

## Introduction

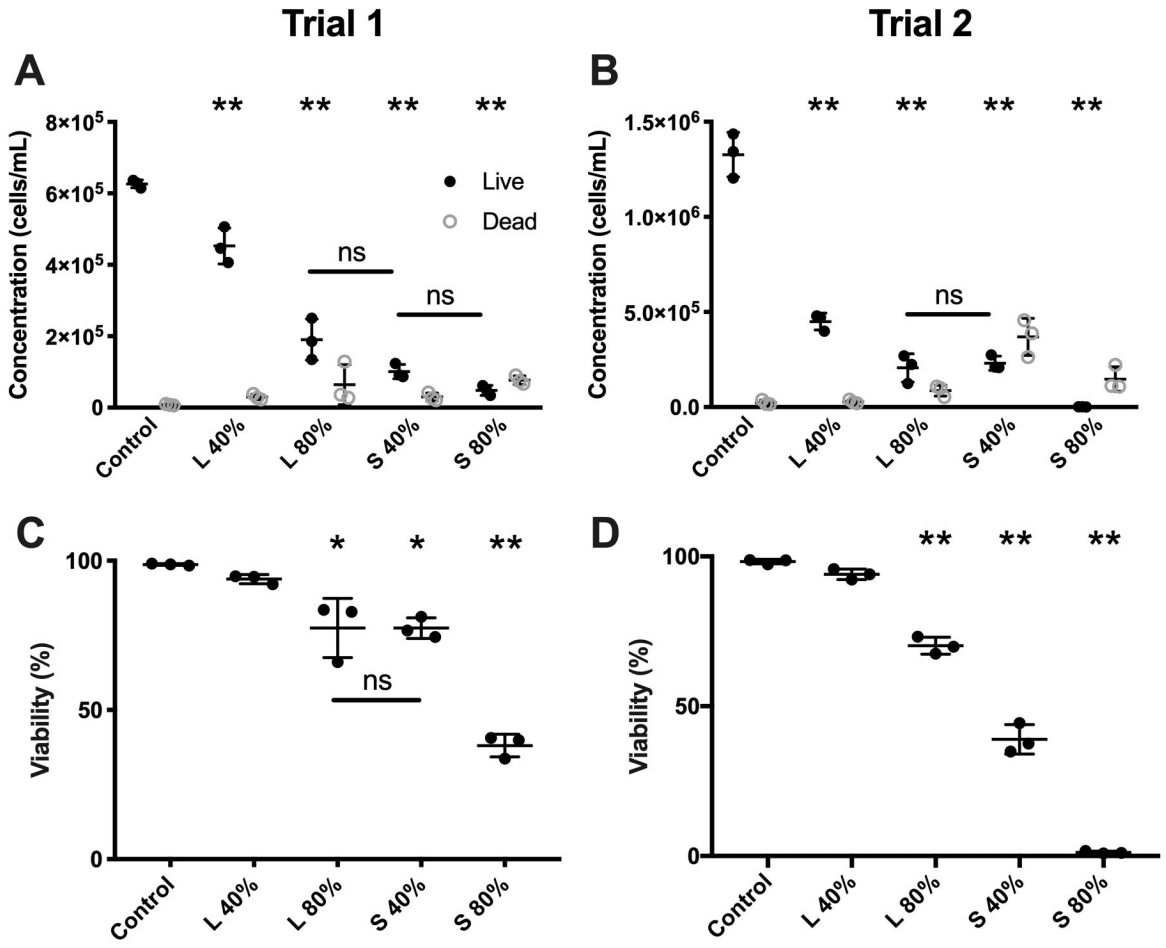
More than 7000 unique chemicals are found in cigarette smoke, of which at least 93 are considered harmful. At least 70 are thought to be carcinogenic [1]. In addition to direct cigarette smoke exposure, *in vivo* and *in vitro* studies of the effects of cigarette smoke have used several approaches to model cigarette smoking. These include exposure to: purified chemicals that may be found in cigarette smoke, such as nicotine; particulates that are liberated from smoke-exposed filters or plates [2]; extracts prepared from non-combusted tobacco; and aqueous cigarette smoke extracts (CSE) that are prepared by exposing liquid to cigarette smoke.

A wide variety of methods have been used to prepare aqueous CSE for experimental purposes, presaged by a water-based pump method to puff smoke onto eye conjunctival tissue [3]. As early as 1947, Finnegan et al. acknowledged the difficulties of working with whole cigarette smoke. and prepared saline extracts in which smoke was simply passed over the surface of a liquid column [4]. Later, cigarette smoke was bubbled through a solution using a pump/aspirator [5] or syringe [6] [7]. Various modifications of the bubbling methods have been published [8] [9] [10] [11] [12]. Parameters that might affect qualitative and quantitative features of CSE include temperature and pH of the solution; age of the extract and storage method [6]; number of cigarettes smoked/volume of liquid; cigarette composition and additives (different brands or products, water content, etc.); volume, duration, and frequency of puffs (or pressure/pump settings for continuous pumping); the presence or absence of a cigarette filter; and CSE filtration and type(s) of filter(s). However, the potential effects of these variables are seldom investigated, and sufficient methodologic details to allow complete replication of CSE production and use are not always provided.

In this study, we demonstrate that even a seemingly minor variable in the CSE preparation method—the type of pipette used to bubble cigarette smoke through the aqueous medium—can have a profound effect on the potency of the resulting CSE in a cell culture system. We used the U937 promonocytic cell line, which models exposure of monocyte-lineage cells to cigarette smoke and has been used over several decades in studies of cigarette smoke derivatives such as condensate [13], nicotine [14], and CSE [15]. Our results stress the importance of standardizing CSE preparation as much as possible within and across experiments for rigor and reproducibility.

## Objective

To ascertain the influence, if any, of pipette type on the potency of cigarette smoke extract in cell culture experiments.

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## Figure Legend

**Figure 1. The choice of the pipette for CSE generation alters the growth-suppressive and toxic properties of CSE.**

Live and dead cell counts **(A and B)** and percentage viability **(C and D)** of U937 cells were measured by the Muse cell analyzer. Dots **(A and B)** represent live (filled circles) and dead (empty circles) cell densities (concentrations) for each culture replicate. Lines indicate mean and standard deviation for each condition. Asterisks in **(A)** and **(B)** indicate significant (2-way ANOVA, Tukey's adjusted $p$ <0.0001) differences in live cell counts between control and treatment. 2-way ANOVA to assess the contribution of bore size and dose to live-cell variation for the treatment conditions revealed significant contributions for each factor and significant differences (Tukey's adjusted $p$ <0.05) between all comparisons (not shown except non-significant comparisons, indicated "ns"). Asterisks in **(C)** and **(D)** indicate significant differences (ANOVA, Dunnett multiple comparison test, *$p$ <0.01 and **$p$ <0.0001) in cell viability for treatment versus control conditions. Within the treatment groups, 2-way ANOVA showed significant contributions to variation for both dose and bore size, and all possible comparisons were significantly different (Tukey's adjusted $p$ of 0.0001 or less) except the single indicated

comparison. Two trials were conducted with two batches of CSE on separate occasions, with three culture replicates of each treatment in each. "L" is for the **L**arge-bore (1.85 mm diameter) pipette, and "S" is for the **S**mall (narrow-bore, 0.61 mm diameter) pipette. The percentage represents a dilution of CSE.

## Results & Discussion

CSE was prepared in complete cell culture medium using a pump-assisted bubbling method and either the tip of a 5 mL pipette (L=large, 1.85 mm diameter bore opening) or a narrow gel-loading pipette tip (S=small, 0.61 mm bore). Only one cigarette was used per extract to avoid potential occlusion of the orifice by tar, and no visible occlusion was observed. CSE was then added at 40% or 80% concentration to cultures of the U937 promonocytic cell line (equal starting number of cells). After 48 h, cell counts and viability were assessed. Two trials were conducted with three replicates each.

As assessed by statistical analysis of viable and dead cell densities (concentrations), treatment with CSE significantly (adjusted $p$ <0.0001) reduced cell counts compared with untreated control at each dose and with each pipette type/bore size (Fig. 1A and B). The tip type also had a significant (adjusted $p$ <0.01) effect on viable cell concentration within each dose comparison. Indeed, the only viability comparisons without significant (corrected $p$ <0.05) differences were between the two doses of CSE produced with the small-bore tip (Trial 1) and 80% large-bore versus 40% small-bore (Trial 2). A two-factor analysis of the treatment data by dose and pipette type also revealed that both variables contributed significantly to variation (adjusted $p$ of 0.0001 or less).

Dose-dependent toxicity (0%, 40%, and 80% CSE) was also observed throughout by examining the percentage of viable cells (Fig. 1C and 1D). The possible exception was 40% CSE prepared with the large-bore pipette tip, which did not demonstrate a significant difference compared to the control (0%) CSE. In this condition, viability was slightly (4-5% on average) lower than control in both trials, but the difference was not statistically significant following adjustment for multiple comparisons. Again, two-factor analysis of the treatment data by dose and bore size also revealed that both variables contributed significantly to variation (adjusted $p$ of 0.0001 or less).

These results lead us to several conclusions. First, switching from a large- to a small-bore pipette for CSE generation achieved similar (Trial 1) or greater (Trial 2) increases in toxicity and/or growth suppression compared with doubling the CSE concentration. Second, the lowest toxicity condition (40% CSE generated with a large-bore tip) appeared to suppress growth without a significant increase in toxicity, suggesting that multiple mechanisms could explain the observed results. Third, despite highly similar results between the two trials, substantial variability was also evident: viable cell percentage after the most toxic treatment (with 80% CSE generated with the small-bore tip) ranged from a negligible 1% (Trial 2) to around 39% (Trial 1). However, total cell counts in the untreated condition also differed substantially, by more than two-fold.

Several factors might contribute to these results. To us, the most obvious explanation is a gas exchange, which varies by the bore size of the pipette inserted into the aqueous medium. A narrow-bore pipette gives rise to smaller bubbles than a wider-bore pipette, achieving more gas exchange through greater surface-to-volume ratio per bubble as well as more bubbles per given volume of gas. As a result, the transfer of soluble toxins from the smoke into the medium would be expected to be more efficient for smaller bubbles, consistent with our results.

However, other explanations should be considered, as well. First, the flow rate of the apparatus might vary across pipette types despite our use of the same pump setting in all experiments. We investigated this possibility with a water displacement assay. Setting the flow rate of the apparatus without a cigarette (free intake) and with a large tip to 100.0% (standard deviation 0.5%), we found that the flow rate through the small tip was slightly decreased (94.6%+/-1.0%). Repeating these experiments with a lit cigarette did not change the flow rate: 99.9%+/-0.9% (large tip) versus 94.1%+/-1.3% (small tip;

incidentally, flow rate did not decrease over time for the small tip, suggesting that occlusion of the orifice is not significant for one cigarette). In any case, the small percent reduction in flow rate through the smaller bore tip would tend to reduce, not increase, the toxicity of CSE. A second explanation could be simple aeration; that is, greater gas exchange during small-tip bubbling would result in the medium that more closely approaches atmospheric oxygen levels (around 21%). However, we tend to discount this explanation due to the limited area of exchange and length of bubbling and, perhaps more importantly, the rapid rate of oxygen diffusion in cell culture [16] as compared with the 48 h length of our culture experiments. Finally, different materials were used for the large-bore (polystyrene) and small-bore pipettes (polypropylene). It is possible that the passage of gas through different plastics could result in different absorption or release of compounds, even though these materials are both used widely for the labware due to their relatively inert nature. Polypropylene, which can in many cases resist autoclaving, is even more resistant to high temperatures than polystyrene. In any case, all conditions included exposure to both polypropylene (gel-loading tip and/or insertion of the cigarette into a 1 mL pipette tip; 50 mL conical) and polystyrene (5 mL pipette and/or culture vessels). Thus, while we admit the possibility of other explanations and restrict our claims here to differences associated with two different pipettes, we maintain that the most likely contributor to our findings is the greater gas exchange achieved with a narrow-bore pipette, resulting in higher concentrations of cigarette smoke-derived chemicals with cytostatic and/or cytotoxic effects.

## Conclusions

Different pipettes used to prepare CSE result in large differences in CSE potency in cell culture. This study emphasizes the importance of carefully standardizing parameters of CSE preparation for cell culture experiments.

## Limitations

Our study has certain limitations. First, despite a clear dose- and bore-dependent toxicity of CSE, we do not know exactly how the chemical composition of CSE might vary. For example, while the different pipette bore sizes certainly produce bubbles with different surface area to volume ratios, they might also lead to different rates of cooling of the smoke. Some components of the extract may be more affected than others by these variables. Second, the reported experiments involve only one cell line. Other cell lines may be more or less sensitive to the cytotoxic effects of CSE. Third, as mentioned previously in the results/discussion, factors other than bore size might contribute to our results. Perhaps most plausible among these is the fact that we used pipettes made of different (albeit relatively inert) materials: polystyrene (wide-bore) and polypropylene (narrow-bore). These different plastics might differentially absorb or react with components of smoke during extract preparation. We submit, however, that these limitations do not detract from what should be the overall conclusion of this study: parameters of CSE preparation require more standardization within and across experiments.

## Alternative Explanations

## Conjectures

With over 7000 chemicals in cigarette smoke, aqueous CSE is poorly characterized. With most studies focusing primarily on nicotine, little is known about the effects of other components of the extract. Many different components could be contributing to the cytotoxicity of the extract, and differences in the method of preparation may result in different components being present in different concentrations or not at all. We will next use mass spectrometry techniques to profile the components of CSE and to determine the differences, if any, between the two methods used in this study.

This study varied only one parameter of CSE preparation, but many others that could be affecting the gas exchange or the makeup of the extract. Some examples are the volume flow rate of the pump, the ambient temperature or the temperature of the medium, the viscosity of the medium, and whether the cigarette filter is present or removed. Numerous parameters of CSE generation remain to be explored and studied more systematically.

## Additional Information

### Methods

#### Cigarette Smoke Extract Generation

25 mL of fresh R10 medium was aliquoted into a 50 mL conical tube. The cap of the tube was removed, and the tube was covered with parafilm. Either the end of a Corning 5 mL Stripette serological pipette (Cat. No. 4487, 1.85 mm diameter tip, polystyrene; pipette scored and broken to remove filter) or a Costar 1-200 µL Gel-Loading pipette tip (Cat. No. 4853, 0.61 mm diameter tip, polypropylene) was pushed through the parafilm into the tube, with the tip reaching at least one-half into the medium volume, and the hole was sealed with another piece of parafilm. The resulting unit was connected to one end of a length of Tygon tubing (Cole-Parmer, Cat. No. 06509-17) that was inserted into a Cole-Parmer Masterflex L/S peristaltic pump (speed setting 54). Into the other end of the Tygon tubing, a 1 mL pipette tip (Cole-Parmer, Cat. No. UX-25711-74, polypropylene) was inserted tightly and parafilm-sealed. One Spectrum research-grade cigarette [17] (filter intact) was lit by Bunsen burner, and the filter end was inserted tightly into the wide, protruding end of the 1 mL pipette tip. The cigarette was then smoked continuously with the peristaltic pump (and smoke thus bubbled through the medium) at pump speed setting=54 for 5 min. The resulting extract was considered a 100% cigarette smoke extract (CSE) medium.

#### Cell Culture and Treatment

U937 promonocytic cells (ATCC CRL-1593.2) were cultured in R10 medium (RPMI (Thermo Fisher 11875093) with 10% fetal bovine serum and 1% each of L-glutamine, Penicillin-Streptomycin, and HEPES buffer) in T75 flasks. Cells were incubated at 37℃ and 5% $CO_2$. The cells were passed regularly before reaching confluency. During treatment, 1E6 U937 cells were treated in 0%, 40%, or 80% CSE media from each method in triplicate. The medium was diluted from freshly prepared 100% CSE with fresh R10. Cells were grown in CSE media for 48 h at 37℃ and 5% $CO_2$. After 48 h, cells were assessed for concentration and viability.

#### Viability Assessment

Sample viability and cell concentration were assessed using the Muse Count & Viability Assay (Millipore Sigma MCH100102) on the Muse Cell Analyzer (Millipore Sigma). Cells were mixed gently to homogeneity by pipetting up and down and combined with the assay reagent per the manufacturer protocol. Size and viability thresholds were determined in relation to the samples from control conditions. Exact settings: FSC Gain 3; YEL Gain 4.6; RED Gain 2.3; Trial 1 FSC Threshold 976, Trial 2 FSC Threshold 884; YEL Threshold 0; Trial 1 RED Threshold 50, Trial 2 RED Threshold 30.

#### Pump Speed and Flow Rate: Water Displacement Assay

A 50 mL conical tube, cap removed, was filled with water by submersion in a large container of water, then positioned vertically and upside down, with the open end submerged. The same peristaltic pump and tubing apparatus was set up as described above, except that one end of the Tygon tubing was inserted with either a 5 mL pipette or a gel-loading tip into the inverted, liquid-filled conical tube. The peristaltic pump was then started at pump speed setting=54. Time to displace all water in the tube (as assessed by the first release of air from the submerged opening of the vertical conical tube) was

measured with a handheld stopwatch. Trials were done both with and without a lit cigarette. All experiments were conducted in triplicate.

### Statistical Analysis

Viable and dead cell counts (Fig. 1A and B) were compared for all conditions using 2-way ANOVA followed by Tukey's multiple comparisons tests. To assess the contribution of dose and bore size within the treatment groups, 2-way ANOVA was performed for these factors followed by Tukey's multiple comparison test for all possible comparisons. Percentage cell viability for each treatment condition (Fig. 1C and D) was assessed with one-way ANOVA followed by Dunnett multiple comparison tests to compare control with each condition. 2-way ANOVA was then done for the treatment data to assess the contribution to variation of dose and bore size, followed by Tukey's test.

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## Ethics Statement

Not applicable.

# Citations


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