Developing a quick and inexpensive in vitro (non-animal) bioassay for mascara irritation

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Synopsis

OBJECTIVE: Mascara is a mild irritant that causes a range of medical problems. Animal models to predict ocular irritation have, however, been questioned at a number of levels, and there is a continued need to develop in vitro testing methods.

METHODS: We assess changes in an easily quantifiable attribute, ciliated protozoan growth rate, as a sensitive, sublethal measure. Specifically, we test six, randomly chosen, commercial mascara products against a control (as treatments) and reveal through ANOVA (n = 6, α = 0.05) significant differences in the specific growth rate to treatments (for both protozoa).

RESULTS: We provide evidence that two easily cultured protozoa (Paramecium caudatum, Blepharisma japonicum) should be considered as models to assess ocular irritancy (and possibly cosmetics in general) and establish the groundwork for such studies to be applied at a more commercial level. We do this by developing a bioassay for mascara toxicity and indicate the low cost (after equipment is purchased, on the order of $100s) and the ease of performing such tests (able to be conducted by undergraduate students), as a consideration for their future commercial application. We first examined dose dependence of responses, revealing that there was a need to conduct preliminary work to determine appropriate levels for sublethal responses. We then show that some products resulted in mortality at high concentrations, others decreased growth rate by >50% (compared with the control), whereas others had no significant effect, compared with the control.

CONCLUSION: We have provided a novel, quick and inexpensive means to assess mascara; the next step is to validate these ciliate bioassays by comparison with animal testing and epidemiological studies, which is beyond the scope of this fundamental ‘proof-of-concept’ study.

Introduction

Numerous cosmetics, including mascara, are common causes of ocular irritation, producing mild to vision-threatening conditions such as blepharitis, contact dermatitis and madarosis [1–4]. Presently though, reliance on in vivo tests to evaluate such irritation is highly controversial [5]; there remains substantial dependence on the in vivo rabbit-based Draize eye test [6, 7], which since its development 40 years ago has been increasingly criticized for its inherent subjective scoring system, questionable extrapolation to humans, time-consuming and expensive nature, ability to indicate ocular irritation and ethical acceptability [6, 8–14]. Criticism of the Draize test further extends to its poor ability to detect effects of mild ocular irritants such as mascara, where results are characterized by high variability and low reproducibility [7]. Consequently, there is continued and growing pressure to establish alternative methods.
In vitro testing provides an alternative to the Draize test, employing non-animal models, animal/human cell lines, and lower-animal developmental stages (e.g. bacteria, protozoa, human skin cells, frog embryos) to indicate toxicity [15–17]. There are substantial advantages of in vitro over in vivo techniques: a greater ability to control environmental parameters; a reduction in logistic issues, enabling increased sample sizes; the inexpensive and rapid nature; and diversion of influential animal welfare groups [18–20]. For these reasons, in this novel ‘proof-of-concept’ study, we explore a tractable and adaptable in vitro bioassay that uses two free-living, non-pathogenic model protozoa: the ‘slipper ciliate’ *Paramecium* and the ‘eyelash ciliate’ *Blepharisma* (Fig. 1).

Protozoa are ideal models [18]; being single-celled eukaryotic heterotrophs, they are physiologically animal-like, but they are not animals and, therefore, not subject to concerns associated with animal testing. Among protozoa, ciliates are the most frequently employed taxa in toxicological studies, undoubtedly as they represent good animal and human models for a range of toxins [21, 22], including those associated with skin compatibility of textiles [23]. To date, swimming patterns of the model ciliate *Tetrahymena thermophila* have been used to assess ocular irritancy [24]. However, swimming patterns are difficult to quantify, especially for non-experts, limiting their practicality. *Tetrahymena thermophila* has also been employed to examine toxicity of specific components of cosmetics [25]. Here, we focus on the potential of two other ciliates, *Paramecium caudatum* and *Blepharisma japonicum* (Fig. 1), that have long been studied as model organisms [18, 26, 27]; these large species were chosen as they are more visible than *T. thermophila*. Specifically, we assess changes in an easily quantifiable attribute, growth rate, as a sensitive, sublethal measure. Our aim is to provide clear evidence that these protozoa should continue to be considered as models to assess ocular irritancy (and possibly cosmetics in general) and establish the groundwork for such studies to be applied at a more commercial level. We do this by developing a sublethal bioassay for the assessment of mascara toxicity that, ultimately, but not here, must be compared with the Draize test and epidemiological data. Finally, we provide an indication of the low cost of such tests, as a consideration for the future commercial application and suggest directions for elaboration on our method.

**Materials and methods**

Culturing and maintenance of ciliates

*Paramecium caudatum* and *Blepharisma japonicum* (Sciенто, Manchester, U.K.) were used as bioassay organisms (Fig. 1). Both species consume bacteria. Culture medium, that would support slow but sustainable bacterial growth, was prepared by enriching it, by crushing Protozoan Pellets (Carolina Biological Supply Company, Burlington, NC, U.S.A.) into Volvic® mineral water (0.55 g L\(^{-1}\)); this was autoclaved and filtered (GF/F) using a low-vacuum system to remove sediments. The media was inoculated with the bacterium *Serratia marcescens* (cultured on LB-Agar at 20°C), which acted as food for the ciliates (at 10\(^8\)-10\(^9\) bacteria mL\(^{-1}\); to ensure ciliates were not food limited) and has long been recognized as a good food source for ciliates [28]. Stock media was stored at 3°C for up to one week and raised to 20°C before using. Stock cultures of ciliates were maintained by subculturing every 4 days. All experiments were conducted at 20°C in the dark.

**Method development**

This study examined the potential toxicity of six randomly chosen, common mascara products (referred to here as brands A to F plus a control ‘0’) by measuring ciliate growth rate. Ciliate growth rate (i.e. change in numbers, by binary fission, over time) was determined by incubating ciliates in 10-mL wells (standard 6-well tissue culture plates, Corning); each well contained 7 mL of bacterized media. To determine appropriate conditions, we examined three concentrations of each type of mascara: a thin layer of mascara was uniformly applied to one side of a standard, square 22-mm microscope cover slip using a cotton swab, in one direction to ensure even coverage, with 0% (control), 25%, 50% or 100% of the surface covered. Cover slips were placed, mascara-up, in the bottom of wells containing bacterized media, prior to adding ciliates.
Each treatment was replicated \((n = 6)\), with an initial number of 10 ciliates inoculated into a well by fine-drawn glass pasture pipette (i.e. initial counts were exact); once mastered, this is a simple and highly accurate method. Subsequent enumeration of ciliate abundance was determined visually, using a dissection microscope, daily for 2 to 4 days. Specific growth rate \((r, \text{day}^{-1})\) was determined assuming exponential growth; that is, \(N_t = N_0 e^{rt}\), where, \(N_t\) is the population size at time, \(N_0\) is the initial population size \((10)\), \(r\) is the intrinsic rate of increase \((\text{day}^{-1})\) and \(t\) is time in days; note, when numbers decrease growth rate is negative.

**Data analysis**

Differences in specific growth rate due to treatment affects (i.e. mascara types/control) were assessed by analysis of variance (ANOVA) followed by the Tukey’s multiple comparison test \((\alpha = 0.05)\). To assess if \(P. caudatum\) and \(B. japonicum\) differed in their response to treatments, a two-way ANOVA was conducted, and interaction tested. Finally, to test the overall effect of treatments, the following transformation was used to combine all the data (all concentrations for both species): for each experimental run, treatments were first standardized to the lowest treatment (i.e. the lowest treatment was subtracted from each value, including itself resulting in a relative value of zero for the lowest treatment). Then, treatment values were converted to percentages of the maximum value. All percentage data were then combined and differences assessed by ANOVA. All tests were conducted using SigmaPlot (SPSS Inc., Chicago, IL, U.S.A.).

![Graphs showing specific growth rate for Paramecium caudatum and Blepharisma japonicum](image)

**Results**

Assessment of ciliate growth rate, as a sublethal bioassay proved successful: significant differences occurred in the growth rate of both \(P. caudatum\) and \(B. japonicum\) due to exposure to mascara, and responses were dosage dependent (Fig. 2). However, for \(P. caudatum\), there were no significant responses to treatments at the lowest concentration, although there was an apparent trend (25% cover slip coverage, Fig. 2). At higher concentration, the response was not always sublethal (i.e. populations decreased, revealing mortality for some treatments).

Significant differences also occurred in response to mascara products; for example, brand-F for both species at 100 and 50% (cover slip coverage) inhibited growth rate the greatest (Fig. 2). The effect of other mascara products had on specific growth rate was less consistent across concentrations, but followed a general pattern revealed by ANOVA and post hoc tests. In addition, it appeared that the order of treatments at each concentration differs for \(P. caudatum\) and \(B. japonicum\) (Fig. 2), but overall, the effect of the treatments did not significantly differ between species (two-way ANOVA revealed no significant interaction, Fig. 3A,B). Combining species data (see Materials and methods) revealed which brands had the greatest inhibition on growth rate and which resulted in growth rates similar to those in the control, suggesting no toxic effect (Fig. 3C).

**Discussion**

This study proposes a novel method to rapidly and inexpensively assess potential ocular irritancy by mascara, through a sublethal
non-animal-based bioassay. Previous medical-based toxicological studies that have employed ciliates have used complex, relatively expensive, and potentially inaccurate turbidmetric or calorimetric methods to monitor cells [23]. Here, we use direct visual counts of large, easily recognizable protozoa to assess specific growth rate in response to treatments (mascara types). Now that we have established this bioassay as an accurate and simple method, there is a need to compare it to industry standards and epidemiological studies [e.g. 29–31], and possibly apply it to other cosmetics.

**Method developments**

For this particular case study, the optimal conditions were determined through a series of developmental stages. Firstly, we devised a simple and effective means to examine the amount of mascara to use: the response of ciliates varied to treatments in a dose-dependent manner (Fig. 2). The results, for the products examined, suggest that performing the bioassay at 100% surface area coverage (of a square 22-mm cover slip) enables subtle differences in the toxicity of treatments to be identified. This was indicated by pronounced inhibition in growth rate at 100% surface area coverage, in comparison with 50% and 25% surface area coverage. However, responses will clearly depend on the treatment (i.e. product), and preliminary tests or simultaneous analysis at a range of concentrations, following our outlined methods, are recommended for future studies; that is, the application of mascara to different amounts of the surface area of the cover slip appeared to be a simple, quick and successful method for such assessment.

No significant differences occurred in the response of species to treatments (i.e. the two-way ANOVA indicated no significant interaction). Consequently, employing either of the two model ciliates (Fig. 1) that we examined would likely suffice. However, *P. caudatum* has been used in pilot genome studies that indicate that it has higher match of relevant coding sequences to those in humans, compared with other ciliates [32]; on this basis, it may be the preferred model organism. In contrast, *B. japonicum* is the slower-moving ciliate and has a distinct pink pigment (Fig. 1, on-line color reproduction only), all of which may make it easier to observe and, therefore, easier to count. Consequently, both species have unique benefits. Furthermore, including multiple species may ensure the thorough testing of treatments, as a single species will rarely reproduce a response that signals the total effect of all ingredients [33]; for example, our data seem to suggest that there may be subtle differences in the response of the two ciliates, although our statistical analysis did not reveal these. Given the simplicity of this method, we encourage future work to include both of these species, and possibly consider others model ciliates such as *Tetrahymena* or *Euplotes* [18, 24].

Our technique was tailored to indicate mascara toxicity in a cost-efficient manner. Consumables for this type of work (bottled mineral water, nutrients, tissue culture plates, cover slips, etc.) are relatively easy to obtain and cost on the order of SUS100. Stock cultures of protozoa and bacteria range from $10 to $100, depending on the source, and then can be maintained relatively inexpensively and take up little space. A high-quality dissection microscope can cost as much $40 000, but we have used inexpensive models ($1000) that are entirely adequate for the counting of protozoa. Possibly the most expensive, regular component of the study will be labour (~3 h per day for 4 days). However, this ‘proof-of-concept’ study was stimulated by work conducted as part of an undergraduate final-year project, indicating that such efforts can be achieved by relatively inexperienced staff, once they are provided basic training (i.e. highly trained technicians are not required, and expensive equipment need not be purchased or maintained). We have not contrasted these costs against those

**Figure 3** The average scaled (see Materials and methods) relative specific growth rate (%) of *Paramaecium caudatum* (**A**) and *Blepharisma japonicum* (**B**) and the two species combined (**C**) to a randomly chosen range of brands of mascara. Underscored treatments (C) indicate no significant differences (z = 0.05). Treatments A to F refer to the brands of mascara; the control ‘0’ contained bacterized media without mascara.
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associated with animal testing (e.g. housing, highly qualified staff, security), but we expect that the difference in cost is on the order of ten- to hundred-fold higher, if not more. Therefore, from a cost-efficiency perspective alone, we encourage our proposed methodology to be pursued.

Effect of treatments

Significant differences in specific growth rate occurred in response to treatments (Figs 2 and 3). If this test is any index of ocular irritancy, our data suggest that some mascara products have a greater potential to cause problems than others, for example, brand-F exhibited the greatest inhibition on specific growth rate in both species, whereas brands B, D and E elicited specific growth rates similar to those in the control. Given these results, if the composition of the products was known, it might be possible to establish which compounds are toxic and if synergistic effects occur. Beyond the scope of this study, there is clearly now a need to compare ciliate bioassays with either animal testing or epidemiological studies, to test the former’s veracity. We see this as the next step. To this end, we encourage appropriate researchers to contact us, with a view towards collaboration.

An additional step made available by the rapidly advancing fields of ‘omic’-research (genomics, metabolomics, proteomics) will ultimately allow researchers to assess the influence of cosmetics on gene, compound, or protein expression and cellular metabolism [e.g. 18, 22]. Such approaches could be applied to Paramecium and Blepharisma, both of which are molecularly well-characterized ciliates (e.g. http://paramecium.cgm.cnrs-gif.fr/; http://www.ebi.ac.uk/QuickGO/GProtein?ac=P80738). Given the relatively close genetic affinity of ciliates to humans [22], this may aid in revealing the cause of ocular irritation by mascara, allowing the removal of toxic compounds.

Conclusion

The technique developed in this study addresses various criticisms directed at the animal testing, and specifically the Draize test including (1) the subjective visual scoring system, (2) the high variability and low reproducibility of results and (3) the time-consuming and costly nature. It further demonstrates significant advances over other in vitro techniques, being simpler and less expensive. Once our method is validated against industry standards, this should take us one a step closer to replacing the controversial Draize test and other animal-based assessments.

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References

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