Evaluation of *Demodex* mite viability using motility and scattered light intensity

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ABSTRACT

Demodex mites have been suggested to have a role in various cutaneous and ocular disorders pathogenesis, such as rosacea or blepharitis. Evaluation of potential treatments with anti-*Demodex* effects is difficult because the viability of living mites needs to be evaluated during their exposure to the agent being tested. Mite viability is currently based solely on their observed movement. However, this method of assessing viability has significant limitations as mites may be resting, immobile or paralysed at any given observation point giving the observer a false impression of the organism's death. To overcome this limitation we evaluated a new quantitative method of evaluating the viability of *Demodex* mites by using scattered light intensity (SLI). We demonstrated that when combined with observation of mite motility, SLI provided increased accuracy of the evaluation of viability of mites being studied. This new viability assay will help address the technical challenges of mite viability

experiments. Accurate evaluation of mite viability will enhance mite biology research and allow for more accurate in vitro toxicity assays of proposed anti-mite agents.

Keywords: *Demodex*, viability assay, scaterred light intensity, rosacea, demodicosis, blepharitis

INTRODUCTION

Demodex mites are follicular mites, between 100 and 400 μm in length, that reside in the pilosebaceous unit in the skin of humans and numerous mammals such as dogs, mice, goats and fruit bats. *Demodex* mites are worm-like arthropods and possess a chitinous exoskeleton with a body that is divided into anatomically distinct features such as a gnathosoma (mouthparts), podosoma (with four pairs of short legs) and an annulated tubular-shaped opisthosoma (Lacey et al. 2016).

Demodex mites are the most complex of the resident human skin microflora. However, little is known of their biology or pathogenic potential mainly because of the challenges of performing *in vitro* experiments (Zhao et al. 2009; Clanner-Engelshofen et al. 2018; Shiels et al. 2018). Increased numbers of mites are present in the facial skin of patients with the skin disorder rosacea and may be related to its pathogenesis (Bonnar et al. 1993; Chang and Huang 2017). In humans, a high density of mites is also observed in blepharitis, pityriasis folliculorum and in immunosuppressed hosts (Chen and Plewig 2014). In veterinary medicine, demodicosis can lead to severe cutaneous disruption with secondary infection leading to the animals death (Ferrer et al. 2014).

Currently, evaluation of anti-*Demodex* agents is based either on the decrease of *Demodex* density on skin surface biopsy (Forton et al. 1998; Schaller et al. 2017) or on observed movement of the mites in culture medium while they are exposed to the agent being tested (Gao et al. 2005; Tighe et al. 2013). We have found the latter method to have significant limitations, such as the intermittence of mite movement and the subjectivity of the observer. The aim of the study is to evaluate a new quantitative parameter, scattered light intensity (SLI), as method of evaluating *Demodex* mite viability.

MATERIALS AND METHODS

Extraction of mites from human skin

Live *Demodex* mites were isolated from human skin of healthy volunteers after informed consent, using the 'modified standardized skin surface biopsy' (MSSSB) technique as previously described (Lacey et al. 2016). Briefly, a 1-cm^2 grid on a glass slide was covered by cyanoacrylate glue and applied on the donor's forehead for 1 min. The slide when gently removed leading to the extraction of the follicular contents. Three consecutive MSSSBs were taken from the same facial site. Extracted mites were then transferred to 0, 5, 10, 20 and 50% of dimethyl sulfoxide (DMSO), a compound known to be non-toxic at low concentrations (1-5%) and rapidly toxic to mites at 50%, in KGM-Gold Keratinocyte medium for 72 h (n = 10/group) at 28 °C.

Mite movement assessment

Movement of the legs and, to a lesser extent, the mouthparts of mites was assessed after 0, 2, 6, 24, 48 and 72 h under a stereomicroscope (SZX16, Olympus) using light field (Fig 1a). At each time point, mites were observed for 3 min.

Scattered light intensity (SLI) evaluation

Pictures were taken for each mite at each observation time point under the dark field of a stereomicroscope. The SLI was then calculated by subtracting the light intensity of the background from the light intensity generated by each individual mite, using ImageJ software with the following formula (Fig 1b):

$$SLI = integrated\ density - (area\ of\ selected\ mite \times mean\ intensity\ of\ background).$$

The percentage of change of SLI at a specific time (tx) from a baseline (t0) was determined as: [(SLI tx-SLI t0)/(SLI t0)] × 100%.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software. Fisher's exact test was used for contingency table and the Mantel-Cox test was used for survival analyses ($\alpha = 0.05$).

RESULTS

Scattered light intensity (SLI) as a quantitative marker of viability

Mite motility was recorded, and SLI was measured after 0, 2, 6, 24, 48 and 72 h. When mites are incubated in DMSO-free medium, their brightness did not change over time whereas their

brightness faded rapidly in 50% DMSO (Figure 2a). The SLI value at time point of 0 h was taken as baseline for each mite and was used to calculate the percentage of change of SLI at each subsequent time point. SLI decreased by at least 25% (-25%) from the baseline after 2 h corresponding with the death of all the mites. In contrast, the SLI of most of the mites incubated in 0% DMSO (non-toxic) did not decrease more than 25% over a prolonged time period (up to 72 h) indicating continuous viability of the mites. (Figure 2b). Moreover, we found that a decrease greater than 25% of SLI from the baseline is correlated with lack of mite motility (Figure 2c) ($p < 0.0001$) with a sensitivity and a specificity of 0.84 and 0.94, respectively. Thus, we therefore propose that a threshold of -25% SLI be included with the evaluation of mite motility as a quantitative marker for mite viability.

Motility and SLI for evaluating viability

To evaluate whether motility combined with SLI offers an improvement over motility alone as a method of scoring viability, mites were then incubated at varying concentrations of DMSO (0, 5, 10, 20%) for 0, 2, 6, 24, 48 and 72 h. The flowchart in Figure 3a outlines the process of determining viability in mites using a combination of movement and SLI. Briefly, if the mite moves it is considered as alive. If no movement is detected, the percentage of change to the SLI baseline is calculated. If this percentage is less than -25%, the mite is considered as alive and if the threshold of -25% is reached and no movement is observed then the mite is considered as non-viable. The combined method shows significantly higher viability for all DMSO concentrations, 0% ($p < 0.05$), 5% ($p < 0.05$) 10% ($p < 0.01$) and 20% ($p < 0.001$) with a survival after 3 days of 100, 87.5, 72.7 and 50%, respectively, compared to 55, 60, 10 and 0% when evaluated by movement alone (Figure 3b).

DISCUSSION

Currently, the toxicity of compounds, thought to be toxic to *Demodex*, is either evaluated indirectly by the reduction of the mite population or the microscopic cessation of movement of observed mites exposed to the compound being evaluated (Forton et al. 1998; Gao et al. 2005; Tighe et al. 2013; Schaller et al. 2017). A recent publication suggests that a staining with propidium iodide could be also used for viability testing (Clanner-Engelshofen et al. 2018). However, none of those methods are a direct objective evaluation of whether mites are alive or dead after exposure to a compound.

In this study, we combined evaluation of live mite motility with a new marker, SLI, to

evaluate the viability of *Demodex* mites. Movement of the legs and, to a lesser extent, the mouthparts of mites were observed. Although the presence of movement proves that a mite is alive, the absence of observed movement at a particular time cannot be taken as absolute proof that a mite is dead. Indeed, we have observed mites not moving at one time point but clearly moving at a later time point. Thus false negatives (live mites considered dead) may occur by assessing viability based on movement at any given time point. Previous observations in our laboratory and by others showed that mites appeared to 'fluoresce' under darkfield illumination and this seemed to be related to their viability (Aytekin et al. 2016; Lacey et al. 2016).

Darkfield illumination on a stereomicroscope blocks out the central light rays, and if no specimen is present, the oblique rays do not enter the objective lens because of their angle. SLI is the term used for the phenomenon whereby, on darkfield illumination using a stereomicroscope, oblique rays which strike a specimen are diffracted, reflected, and/or refracted and enter the objective lens. The specimen being examined is thus seen as a bright object on an otherwise black background. This principle has been used in the past to observe cell membranes (Srinivas et al. 2003).

In order to evaluate SLI as an effective viability marker, we incubated *Demodex* mites in medium (0% DMSO) and 50% DMSO, concentrations known to be non-toxic and lethal, respectively (Shiels et al. 2018). We showed that a decrease of more than 25% from their baseline is correlated to the mite mortality. The reason for this is unclear but might reflect a change in their chitinous exoskeleton after death.

These results lead us to propose a flowchart with motility and SLI as marker to improve toxicity assays. In addition to evaluating mite viability, the measurement of motility and SLI could be useful to evaluate the potential paralyzing effect of drugs on *Demodex*, a suggested mechanism of action of anti-parasitic drugs such as ivermectin (Abokwidir and Fleischer 2015).

The method described here, for evaluation of mite viability by motility combined with SLI, will facilitate future research on mite biology and will allow for more accurate *in vitro* toxicity assays of anti-*Demodex* agents proposed for the treatment of demodicosis. However, further studies using other compounds known to be toxic to mites (such as tea tree oil) must be carried out to confirm the validity of those results.

CONFLICT OF INTEREST: The authors state no conflict of interest

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FIGURE LEGENDS

Figure 1 Assessment of motility and scattered light intensity (SLI). (**a)** Motility: leg movement (arrow) of a *Demodex* mite observed using stereomicroscope light field. (**b**) SLI: mites were photographed using stereomicroscope darkfield conditions and SLI was measured and calculated with the following formula: SLI = integrated density – (area of selected mite × mean intensity of background).

Figure 2 Scattered light intensity (SLI) as quantitative marker of mite viability. (**a**) Representative images of *Demodex* mites incubated in 0 and 50% DMSO for 0, 6 and 72 h. (**b**) Percentage of change of SLI from the baseline (t = 0 h) in 0 and 50% DMSO (n = 10 per group). The red dashed line represents a threshold of -25%. (c) Histogram representing the percentage of change of the SLI compared to the motility of the *Demodex* mite (n = 56 observations).

Figure 3 Comparison of motility vs. motility + scattered light intensity (SLI). (**a**) Flow chart using motility and the percentage of change of SLI for the evaluation of *Demodex* mite viability. (**b**) Kaplan-Meier curve for DMSO (0, 5, 10 and 20%, n = 10 per group) comparing motility only and motility + SLI as markers of viability.