

Scanning electron microscopic evaluation of the successful sterilization of *Lucilia cuprina* (Wiedemann) utilized in maggot debridement therapy (mdt)

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Abstract. In Malaysia, maggot debridement therapy (MDT) utilizes maggots of *Lucilia cuprina* (Wiedemann) to debride necrotic tissue from wound surface, reduce bacterial infection and therefore, enhance wound healing process. To evaluate the sterility of the sterile maggots produced after sterilization process before delivering onto patient wounds. Sterility of sterile maggots is crucial in ensuring the safe usage of MDT and patient's health. Eggs of *L. cuprina* collected from a laboratory colony were divided into treated group (sterilized) and control group (non-sterilized). Treated group underwent sterilization while eggs from control group were allowed to hatch without sterilization. Sodium hypochlorite and formaldehyde were the main disinfectants used in this sterilization process. Scanning electron microscope (SEM) was used to examine and ascertain the sterility of sterile maggots. SEM results showed that all sterilized *L. cuprina* eggs and maggots achieved sterility and all were cleared from bacterial contamination. In contrast, all non-sterilized eggs and maggots were found to be colonized by microorganisms. Sterilization method employed to sterilize eggs and maggots used in Malaysia MDT was proven successful and MDT is safe to be used as wound management tools.

INTRODUCTION

Maggot debridement therapy (MDT) is a form of therapeutic wound treatment utilizing maggots of certain blowfly species to remove non-vitalized tissue, pus, slough and metabolic wastes on the wound (Hebda & Lo, 2001). Maggot debridement therapy hastens healing time and therefore decreases hospitalization duration. Wound healed by MDT appeared to have smaller scar because of the rapid proliferation of healthy granulation tissue (Sherman & Pechter, 1988).

In temperate countries such as United States of America, United Kingdom and Europe, *Lucilia sericata* is being employed as a debriding agent in MDT (Geary & Russel,

2004). In Malaysia, the tropical bronze blowfly, *Lucilia cuprina* is utilized. MDT was long established and widely used in the USA since 1930. Malaysia is the first to conduct MDT research (Mohd Masri *et al.*, 2005) in Southeast Asia region and this treatment is gradually being adopted by local hospitals and private clinics to benefit patients suffering from non-healing wounds (Massari, *et al.*, 2008).

In Malaysia, the first MDT clinical trial dated back to 2003 with 12 patients suffering from diabetic ulcers. Ten out of 12 patients healed satisfactory after MDT treatment (Rajoo, 2004). Malaysia's first MDT case-control clinical study was conducted in 2008 and MDT was proven to be as efficient as conventional treatment (Rajoo, 2004; Aaron

et al., 2009). Thus far, no complication and detrimental effects are reported from physicians, nurses and patients who employ or utilizing MDT.

Sterilization is the process of sterilizing that completely destroys or removes all living microorganisms (Rutala & Weber, 1999). The Council on Pharmacy and Chemistry of U.S in 1936 noted that sterilization means the absence or total destruction of all microorganisms (American Medical Association, 1936). Sterilization of the eggs of *L. cuprina* for use in MDT is vital and is a pre-requisite before using them for MDT application. This step is crucial to ensure the safe usage of sterile maggots on patients' wounds and their health.

The present paper reports the first attempt to utilize scanning electron microscope (SEM) to evaluate the sterility of sterile maggots produced and effectiveness of the sterilization process used in maggot debridement therapy utilizing the Malaysian blowfly, *L. cuprina*.

MATERIALS AND METHODS

Flies

Fly colony of *L. cuprina* used in this study was bred and maintained in the insectarium of Institute for Medical Research (IMR), Kuala Lumpur. They were kept at 27-29°C with relative humidity of about 70%. They were fed with water and castor sugar as routine supplement. Pieces of cow liver were given to flies as protein source to induce oviposition as well as an oviposition medium.

Lucilia cuprina maggots

Eggs were removed from the cow liver and divided into treated and control group. The treated group was surface sterilized with 0.05% sodium hypochlorite followed by 5% formaldehyde and then washed with sterile distilled water. The eggs were then transferred onto agar plates and incubated at 28°C for 24 hours (Wolff & Hasson, 2005). The control group was allowed to hatch without undergoing sterilization and were transferred onto sheep blood agar and kept separately inside an incubator. After 24 hours, maggots

that hatched from treated eggs and control eggs were fixed with 2.5% glutaraldehyde in 0.1 normal phosphate buffer solution and were immediately sent to Electron Microscopy Unit of Institute for Medical Research for SEM evaluation. They were then processed by dehydrating using a graded series of ethanol solutions, coated with gold and viewed using SEM.

RESULTS

The scanning electron microscope (SEM) results on both treated (sterilized) and control (non-sterilize) eggs showed clear and significant differences in term of sterility. Significant differences between non-sterilized egg and sterilized egg were observed under SEM. Contaminants were seen adhering to the external surface of non-sterilized egg (Figure 1), whereas sterilized egg appeared to be smooth and clean and clear of any external contaminants (Figure 2). Vastly different SEM view of the non-sterilized and sterilized egg surfaces was shown on Figures 3 and 4. Non-sterilized eggs exhibited clusters of microbial flora on its external surface (Figure 3), while external surface of sterilized egg was clean under the same magnification (Figure 4). Figure 5 clearly showed colonization of microbial flora on the outer surface of non-sterilized egg, while Figure 6 indicated the structure of plastron in an egg at the magnification of 10748X. The plastron is a widely spaced meshwork of the eggshell that allows air to trap within it for ensuring respiratory exchange during period of immersion (Bursell, 1970). No bacterial contaminants were trapped within this fine mesh of plastron (Figure 6).

After 24 hours of incubation period, eggs were hatched into first instar maggots. Maggots that hatched from sterilized and non-sterilized eggs were fixed with glutaraldehyde and sent to Electron Microscopic Unit of IMR for evaluation. Figure 7, clearly showed the colonization of bacteria on the anterior head of non-sterilized *L. cuprina* maggot, whereas Figure 8 showed clean and clear anterior head of



Figure 1. Contaminated outer surface of non-sterilized *L. cuprina* egg. M: 196X

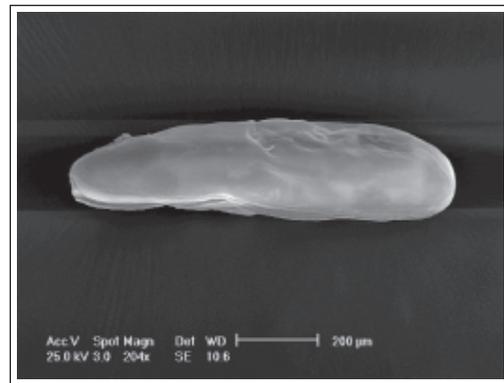


Figure 2. Clean and clear outer surface of sterilized *L. cuprina* egg. M: 204X

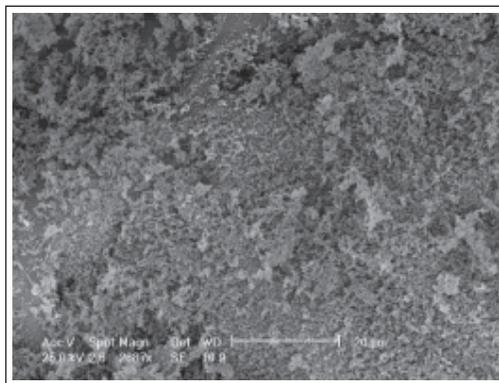


Figure 3. Clumps of microorganisms on the outer surface of non-sterilized *L. cuprina* egg. M: 2687X

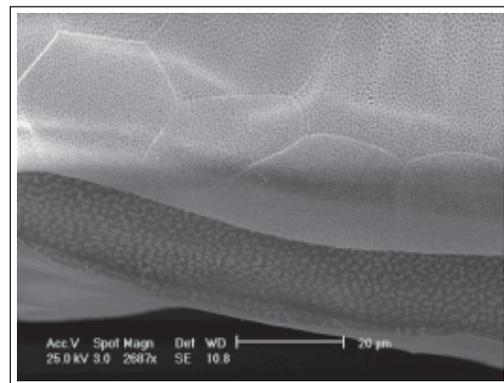


Figure 4. Clear and smooth outer surface of sterilized *L. cuprina* egg. M: 2687X

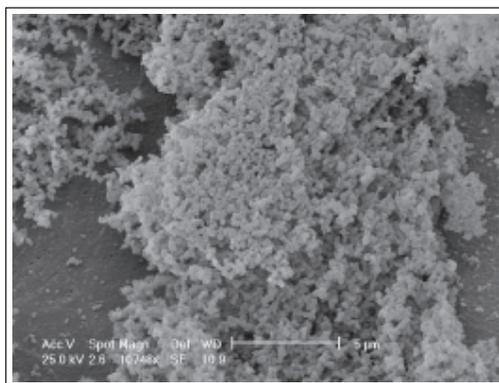


Figure 5. Clusters of cocci-like bacteria on the outer surface of non-sterilized *L. cuprina* egg. M: 10748X

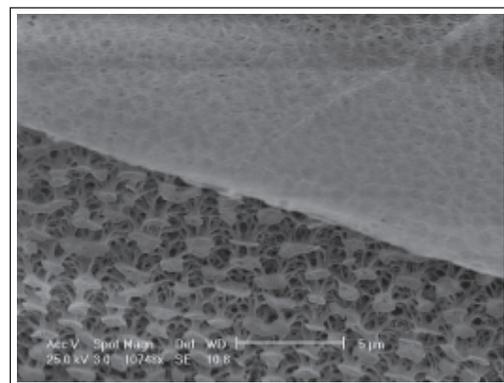


Figure 6. Plastrons of *L. cuprina* sterilized egg can be observed free of microorganism. M: 10478X

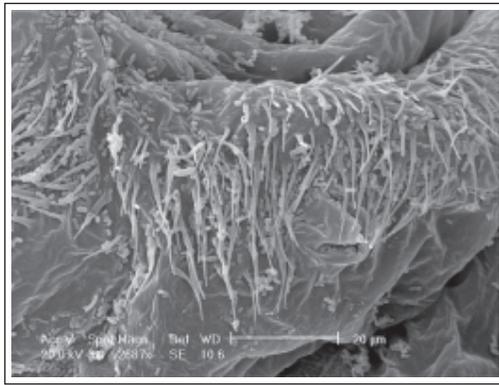


Figure 7. Colonization of bacteria on the anterior head of non-sterilized *L. cuprina* maggot. M: 2687X

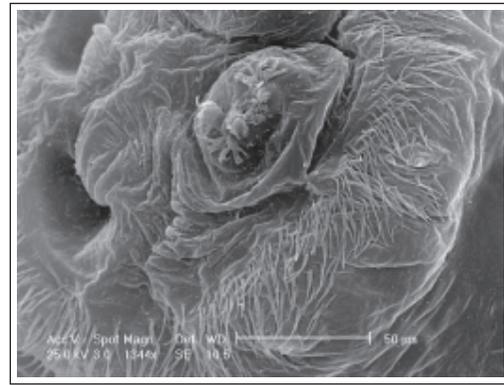


Figure 8. Absence of microbial flora on the anterior head of sterilized *L. cuprina* maggot. M: 1344X

sterilized *L. cuprina* maggot. Figures 9, 11, and 13 indicated bacillus-like bacteria colonizing the hair-like structure on the body of maggot and body segment of non-sterilized *L. cuprina* maggot, however, Figures 10, 12 and 14 showed absence of the bacillus-like bacterial colony on similar body section of *L. cuprina* maggot. In Figures 10, 12 and 14, spots that appeared adhering to the body segment were possibly food debris and lysed materials.

DISCUSSION

The present study showed that scanning electron microscope is also a promising method to confirm the sterility of the sterilized eggs and sterile maggots produced after sterilization before MDT application. Our results indicated that sterility of sterile eggs and maggots was successfully achieved after sterilization.

Sterilization process used in MDT must be a stringent process resulting in total sterility yet yielding high number of viable eggs after sterilization process (Nuesch *et al.*, 2002). This is to ensure large number of sterile maggots can be produced at any one time to maximize the cost-effectiveness of MDT.

The same author (Nuesch, *et al.*, 2002) reported an unusual clustering of bloodstream infection of five of their patients after using contaminated larvae of *Protophormia terranova*. Later on, they improved the

disinfecting procedures used in MDT; utilizing *L. sericata* instead of *P. terranova* and application of MDT was reported safe since then. Baer (1931) also reported that it was necessary to use sterile maggots on patients. In his early experiment, non-sterile maggots were used as MDT debriding agent; however, patients subsequently developed secondary infection such as tetanus (Baer, 1931).

In addition to Baer (1931) and Nuesch *et al.* (2002) also concluded that it was indispensable to use only sterile maggots on patients (Baer 1931; Nuesch, *et al.*, 2002). In our studies, the best sterilization method is to sterilize the eggs as compared to sterilization of maggots and this observation was in line with Baer's finding (Baer, 1931). He reported that the external surface of maggots could be sterilized, but not the contents of the gut and intestines of maggots. Therefore, the maggots continued to be septic as they constantly secreted gut contents into the surrounding environment.

Lucilia cuprina eggs are laid in cluster which must be separated into single egg before sterilization process to ensure disinfectants used can sterilize the entire external surface of the egg (Baer, 1931).

Generally, contamination can be easily detected after sterilization process as sterilized eggs will be deposited onto sterile agar plate and incubated for 24 hours before the maggots hatch from the eggs. Contamination on the agar plates will be

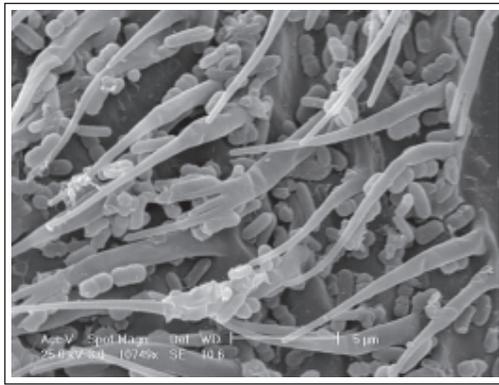


Figure 9. Bacillus-like bacteria colonized the hair-like structure of non-sterilized *L. cuprina* maggot. M: 10749X

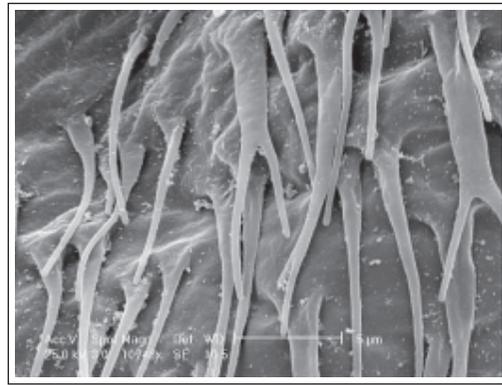


Figure 10. Clean surface observed on the body of sterilized *L. cuprina* maggot. M: 10748X

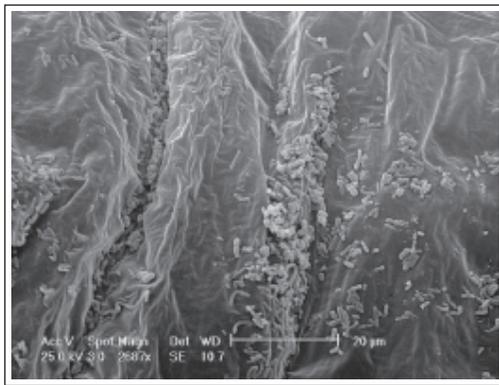


Figure 11. Bacillus-like bacteria colonized the segment of non-sterilized *L. cuprina* maggot. M: 2687X

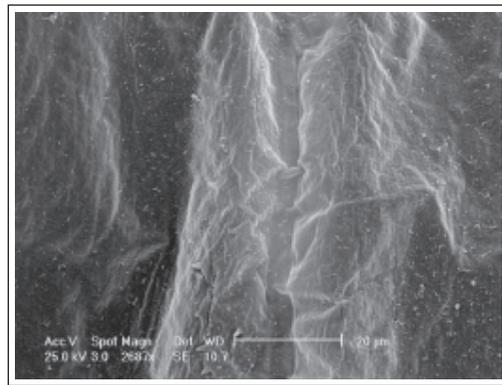


Figure 12. No colonization of bacteria found on the body segments of sterilized *L. cuprina* maggot. M: 2687X



Figure 13. Magnified bacillus-like bacteria colonized on the body of non-sterilized *L. cuprina* maggot. M: 10748X

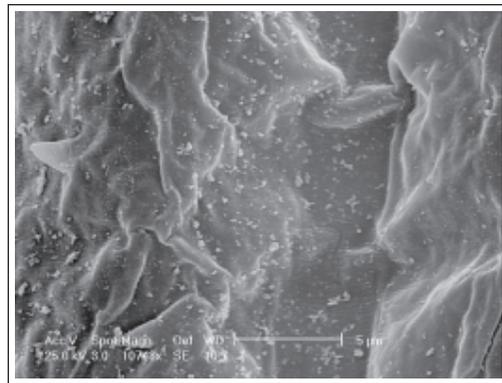


Figure 14. Only debris and possible lysed materials on the body sterilized *L. cuprina* maggot. M: 10748X

indicated by growth of microorganisms which could be easily detected (Sherman & Wyle 1996).

Simmons (1934) suggested that few factors needed to be considered before choosing a disinfectant for sterilization process for MDT (Simmons, 1934). There are agglutination and floating caused by effect of disinfectants and sterilization, time consumed for production of sterile maggots and the age of the eggs used at the time of sterilization process.

Entomologists use sodium hypochlorite to surface sterilize the insect eggs and equipments used in limited or mass rearing of insect (Soo Hoo *et al.*, 1971). Linville & Wells (2002) reported that using 20% bleach to wash or surface sterilize blowfly's maggots appeared to be an effective method to significantly reduce the risk of external contamination of maggots.

Sodium hypochlorite and formaldehyde are antimicrobial agents (Neely, 1963; Estrela & Ribeiro, 2003). Sodium hypochlorite is a potent disinfectant in which the activity occurs when hypochlorous acid is formed with the release of chlorine gas (Penick & Osetek, 1970). Sodium hypochlorite has a broad spectrum of antimicrobial activity and it exhibits robust and rapid activity against both vegetative bacteria and *Mycobacterium tuberculosis* even at relative low concentrations (50 ppm). Sodium hypochlorite inactivated many viruses at 200 ppm of concentrations, such as the Human Immunodeficiency Virus (HIV) which is susceptible to sodium hypochlorite at 50 ppm and Hepatitis B Virus (HBV) which is inactivated at 500 ppm (Sharbaugh, 1998).

Formaldehyde (methanal, CH₂O) is bactericidal, sporicidal, and virucidal. It is an extremely reactive chemical with its well known rapid reaction with protein, DNA and RNA *in vitro*. It has been suggested that formaldehyde acts as a mutagenic agent and as an alkylating agent by reaction with carboxyl, sulfhydryl, hydroxyl groups and

nucleic acid. It also alters Hepatitis B surface antigen (HBsAg) and Hepatitis C core antigen (HBcAg) of HBV (McDonnell & Russell, 1999). Formaldehyde exerts sporostatic and sporicidal effects on *Bacillus subtilis* spores (Trujillo & David, 1972).

We found out that the combination of sodium hypochlorite and formaldehyde used in our sterilization method prevent agglutination and thus reducing egg loss during the sterile maggots production process. This combination too achieves good sterility and high eggs viability (85%-90%) after stringent sterilization process (Yeong, Unpublished data). Our sterilization method is similar to Simmon's sterilization process, however, with some modifications. In his original sterilization method, he used 1% formalin (formaldehyde) with 1% and 5% of sodium hypochlorite. Each combination was exposed to 5 and 10 minutes of sterilization. All combinations used in his study achieved 100% sterility (Simmons, 1934).

Our study is the first description of successful sterilization of blowfly eggs confirmed by SEM. We found that the sterilization method employed in sterilizing eggs of *L. cuprina* was effective and safe to be used therapeutically. In addition, Scanning Electron Microscope (SEM) provided us exceptionally clear and remarkable evidence of the sterility of eggs of *L. cuprina*.

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