Original Research *Highlight article*

Natural antioxidants attenuate mycolactone toxicity to RAW 264.7 macrophages

Yvonne A Kwaffo[1,2], Mabel Sarpong-Duah[2], Kwabena Owusu-Boateng[1,2], Winfred SK Gbewonyo[2], Jonathan P Adjimani[2] and Lydia Mosi[1,2]

[1]West African Centre for Cell Biology of Infectious Disease (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Accra 0000, Ghana; [2]Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Accra 0000, Ghana

Corresponding author: Lydia Mosi. Email: lmosi@ug.edu.gh

Impact statement

Buruli ulcer predominantly affects the skin, but is unlimited to the bone in complicated cases, with about 50% of the affected individuals being children under 15 years, who typically live in rural areas with limited accessibility to health facilities. Late presentation to hospital for treatment therefore becomes inevitable, leading to extensive lesions that result in permanent deformities and disabilities. Mycolactone (ML) is reported to stimulate ROS production which could delay wound healing. To counteract the ML-mediated ROS, the search for natural antioxidants which are presumed to be potent, safe, and possessing high therapeutic value is necessary. Ascorbic acid, gallic acid, and green tea kombucha have been shown to exhibit potent antioxidant activities, and we provide here evidence of the role they may play in attenuating the toxicity of ML. Ascorbic acid and green tea kombucha could serve as prophylactic agents to protect macrophages during treatment of Buruli ulcer.

Abstract

Mycobacterium ulcerans produces a macrolide exotoxin, mycolactone which suppresses immune cells activity, is toxic to most cells and the key virulence factor in the pathogenesis of Buruli ulcer disease. Mycolactone is reported to mediate the production of reactive oxygen species in keratinocytes; cells that play critical role in wound healing. Increased levels of reactive oxygen species have been shown to disrupt the well-ordered process of wound repair; hence, the function of wound-healing cells such as macrophages, keratinocytes, and fibroblast could be impaired in the presence of the reactive oxygen species mediator, mycolactone. To ensure regeneration of tissues in chronic ulcers, with proper and timely healing of the wounds, natural antioxidants that can combat the effects of induced reactive oxygen species in wound-healing cells ought to be investigated. Reactive oxygen species activity was determined in mycolactone-treated RAW 264.7 macrophages and the scavenging ability of the antioxidants (ascorbic acid, gallic acid, and green tea kombucha) against mycolactone-induced reactive oxygen species (superoxide anions) was assessed using fluorescein probe (DCF-DA) and nitroblue tetrazolium dye. Cytotoxicity of the antioxidants, mycolactone, and the protective effect of the antioxidants on the cells upon treatment with mycolactone were determined using the Alamar blue assay. The expression levels of endogenous antioxidant enzyme genes (superoxide dismutase, catalase, and glutathione peroxidase) in response to mycolactone-mediated reactive oxygen species were determined using RT-qPCR. Mycolactone induced the production of reactive oxygen species in RAW 264.7 macrophages, and the resulting superoxide anions were scavenged by some of the antioxidants. The selected endogenous antioxidant enzyme genes in the macrophages were upregulated in the presence of the antioxidants and mycolactone. The exogenously supplied ascorbic acid and green tea kombucha offered moderate protection to the macrophages against the toxicity of mycolactone. We conclude that the results provide insights into alternate and adjunct therapeutic approaches in Buruli ulcer treatment, which could significantly attenuate the toxicity of the pathogenic factor; mycolactone.

Keywords: Buruli ulcer, mycolactone, antioxidants, cytotoxicity, kombucha, macrophage

***Experimental Biology and Medicine* 2021; 246: 1884–1894. DOI: 10.1177/15353702211015628**

Introduction

Buruli ulcer (BU) is a skin disease characterized by apoptosis and necrosis of affected cells and is caused by the bacterium *Mycobacterium ulcerans* (MU). The disease starts as nodule or plaque and progresses to an ulcer when there is delayed diagnosis and treatment.[1] It characteristically affects the extremities more than the trunk, partly due to the low and restrictive growth temperature requirements of the bacterium. Although its transmission mode remains unknown, BU is considered a non-contagious disease restricted to tropical and sub-tropical climates with close association to slow moving water.[2]

MU secretes a macrolide toxin called mycolactone (ML) which is cytotoxic, and the main virulence factor in the pathogenesis of BU due to its ability to prime cells for apoptosis and necrosis leading to ulcers. Concentrations of ML between (15 ng/mL to 150 ng/mL) or above 15 μg/mL have been reported to induce apoptosis and necrosis respectively in L929 fibroblast after 4–24 h of exposure.[3] While a few numbers of cells exhibit resistance to ML,[4] several cell types have remained susceptible to the cytopathic effects of ML some of which are adipocytes,[5] monocytes,[6] epithelial cells.[4,7] Fibroblasts[8] and keratinocytes[7] have also been demonstrated to be killed at lower concentrations of the ML. Apart from the cytotoxic property of ML on most cell types, ML also modulates the activity of immune cells.[6] Studies by Simmonds *et al*.[9] and Hall *et al*.[10] have also reported of macrophage sensitivity to ML *in vitro,* occurring at high concentrations and prolonged exposure. This is consistent with other studies where J774 mouse macrophage cells were induced to death via apoptosis after three to five days of ML exposure.[6] ML has been proven to be non-localized, with the capacity of migrating beyond the region of MU infection, and it has been detected in the sera of BU patients via mass spectrometry.[11] Furthermore, the subcutaneous injection of mice with 300 μg of C_{14}-labeled ML could be traced in peripheral blood mononuclear cells and all tissues of the mice, with the exception of the brain.[12] A study by Sarfo *et al*.[13] were able to demonstrate for the first time the presence of ML in the lesions of humans.

The most widely accepted mechanism of action for ML is linked to the blockade of a cellular target, Sec 61 translocon, thus affecting its function of translocating about 30–50% of nascent cytoplasmic proteins required for modification in the endoplasmic reticulum.[10] A recent study by Ogbechi *et al*.[14] reported of the sensitivity of endothelial cells' thrombomodulin to ML in both *in vitro* and *in vivo* studies. This was suggested to lead to loss of coagulation control and eventual fibrin deposition in untreated BU lesions. Another school of thought for the toxicity of ML is its reactive oxygen species (ROS) production, which has been evidenced in keratinocytes. The direct effect of ML toxicity in keratinocytes was mediated by antioxidants, thus offering a level of cytoprotection.[7]

High levels of ROS have been reported to delay wound healing, and[15] therefore ML-mediated ROS generation may be implicated in the delayed wound healing associated with BU disease by disrupting the well-ordered processes of tissue repair. It is therefore necessary to investigate alternative agents such as natural antioxidants that can sequester the ROS and prevent their deleterious effects[16] on cells that play critical roles in wound healing such as macrophages, fibroblasts, and keratinocytes.

Two phenotypes of macrophages have been reported; the pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2), the latter being pro-wound-healing and important in tissue repair and regeneration.[17] Macrophages therefore play significant roles in the repair processes of wound healing; through the production of cytokines and growth factors, stimulation of fibroblasts and keratinocytes' proliferation and activation of fibroblasts to produce collagen for tissue remodeling or re-epithelization. All of these processes are important in the four phases of the repair processes during wound healing—hemostasis, inflammation, proliferation, and re-epithelialization or remodeling.[18]

Despite the key role macrophages play in modulating fibroblast and keratinocyte numbers during wound healing, no studies have been reported on ML-induced ROS activity in macrophages and the effect natural antioxidants could have on them. Furthermore, the direct effects of ML on the level of expression of antioxidant enzyme genes have also not been investigated. In this study, ROS activity was determined in RAW 264.7 macrophages after induction with ML. Locally available and affordable ascorbic acid (ASC), gallic acid (GA), and green tea kombucha (GTK) were used as the natural antioxidants to ascertain their ROS scavenging abilities and protective effects on macrophages *in vitro*, against the damaging effect of ML-mediated ROS. The levels of expression of selected endogenous antioxidant enzyme genes in response to ML were also determined.

Materials and methods

Materials

Synthetic ML A/B (0.1 mg/mL ampule) was provided by the Chemistry Department, University of Ghana (obtained as a kind gift from Professor Yoshito Kishi, Harvard University, USA). The antioxidants; gallic acid (GA) and ascorbic acid (ASC) were purchased from Sigma Aldrich. The culture medium, Dulbecco's Modified Eagle Medium (DMEM) was also purchased from Gibco®. The cell line RAW 264.7 macrophage (RIKEN BioResource Centre Cell Bank, Japan) was a kind gift from Prof Regina Appiah-Opong, while the kombucha starter culture was provided by Dr W.S.K Gbewonyo of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon.

Preparation of green tea kombucha

Green tea kombucha (GTK) was prepared as described by Abass *et al*.[19] A 1-L solution of sucrose (approx. 9.63 g/L) was brought to a boil. One Ceylon green tea bag was infused into the sugared boiled water for 2 min. The sugared tea was transferred into a separate container and allowed to cool to room temperature, and then seeded with 100 mL of a previous ferment containing a "baby mat" (Symbiotic Colony of Bacteria and Yeast [SCOBY]).

The container was then covered with a linen cloth and allowed to ferment for 14 days at room temperature. The fermented tea was filter-sterilized, filtrate lyophilized by freeze-drying, and kept at −20°C until further use.

Determination of antioxidant activity of green tea kombucha

The antioxidant activity of the GTK was measured using DPPH radical quenching activity, as described previously by Jayabalan *et al*.[20] Different concentrations of GTK ranging from 0.625 to 5 mg/mL at volumes of 200 µL were aliquoted into a 96-well plate. A 100-µL methanolic DPPH solution of 0.5 mM was added to the extracts in the wells and incubated for 20 min. Absorbance was measured at 517 nm using a Varioskan Lux spectrophotometer. Freshly prepared butylated hydroxytoluene (BHT) (0.0625–1 mg/mL) was used as a reference standard with methanolic DPPH solution as blank. Three independent experiments were done, each in triplicates. Percentage antioxidant activity was determined as follows

$$\%\ Antioxidant\ Activity = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of blank}}$$

Cell culture

RAW 264.7 macrophages were cultured in T-25 flasks (Eppendorf) containing DMEM (supplemented with 10% FBS, L-glutamine and 100× streptomycin/penicillin) at 37°C in the presence of 5% CO_2. Detachment of cells was done by treating with 0.25% trypsin-EDTA (Gibco, USA), followed by treatment with complete medium to stop the trypsin action.

Cytotoxicity of antioxidants and mycolactone against RAW 264.7 macrophages

To evaluate the cytotoxic concentration of ML and the antioxidants (ASC, GA GTK), varying concentrations of each were prepared and treated against RAW 264.7 macrophages. Briefly, the cells were seeded overnight into 96-well plates (Eppendorf) at $\sim 1 \times 10^5$ cells/mL in DMEM supplemented with 10% FBS, 100× penicillin/streptomycin, and 2 g/L $NaHCO_3$. Cells were incubated with serial dilutions of ML (0.00001 µg/mL to 1.0 µg/mL) for 24–72 h at 37°C in 5% CO_2. Treatment of cells with serial concentrations of antioxidant compounds (ASC (31.25 to 1000 µM); GA (3.75 to 60 µM), and GTK (0.625 to 20 mg/mL)) was also done for 24 h at 37°C in 5% CO_2. Cell viability for both ML and antioxidant treatments was then estimated using Alamar blue assay. Cell viability for the chosen concentrations of the antioxidants; ASC (500 µM), GTK (2.5 mg/mL), and GA (30 µM) was also done. Three independent experiments were done for all treatments, each in triplicate. The inhibition concentration [IC_{50}] value (1.037 µg/mL) for the ML-treated cells was estimated using GraphPad prism 7 software.

Measurement of reactive oxygen species

RAW 264.7 macrophages at a density of 1×10^5 cells/mL were seeded into a 96-well plate (black) and left overnight. Cells were then labeled with 100 µL of 20 µM of fluorescent probe 2′-7′- dichlorodihydrofluorescein diacetate (DCF-DA) (ab113851, Abcam, Cambridge, UK) for 45 min at 37°C in the dark, to detect cellular oxidation (following the manufacturer's instructions). The medium was decanted, washed with 1× PBS, and replaced with 100 µL of 0.1 and 1.0 µg/mL ML-containing medium (90 µL medium: 10 µL ML) for 24 h. As a positive control, cells were treated with 100 µL of 100 µM tert-butyl hydroperoxide (TBHP)-containing media (90 µL medium: 10 µL TBHP) and incubated for 24 h. Untreated cells served as negative control and fluorescence (485 nm excitation/535 nm emission) was measured using a Varioskan Lux plate reader. Three independent experiments were done, each in triplicate.

The percent of control for ROS was calculated as follows

$$ROS\ (\%\ of\ control) = \frac{\left\{\begin{pmatrix}\text{Fluorescence of treated cells}\\ -\text{Fluorescence of blank}\end{pmatrix} \times 100\right\}}{\left\{\begin{matrix}\text{Fluorescence of TBHP treated cells}\\ -\text{Fluorescence of blank}\end{matrix}\right\}}$$

Measurement of superoxide anions

RAW 264.7 macrophages were seeded at a density of 1×10^5 cells/mL in a 96-well plate (black) overnight. Media was decanted and replaced with 90 µL each of 0.1 and 1.0 µg/mL ML-containing media and 10 µL of 2.7 mg/mL nitroblue tetrazolium (NBT) for a 24-h incubation period. Cells were then washed three times with PBS to remove any traces of extracellular NBT solution and ML. Cells were lysed with 60 µL of 2 M KOH to release intracellular formazan, while 70 µL of DMSO was added to solubilize the released formazan and gently shaken for 10 min at room temperature.[21] Absorbance was read at 620 nm using a Varioskan Lux plate reader. TBHP-treated cells were used as positive control, while untreated cells served as negative control. Three independent experiments were done, each in triplicate. The level of superoxide anion (% control) was calculated as follows

$$\text{Superoxide anions (\% of control)} = \frac{\left\{\begin{pmatrix}\text{Absorbance of treated cells}\\ -\text{Absorbance of blank}\end{pmatrix} \times 100\right\}}{\left\{\begin{matrix}\text{Absorbance of TBHP treated cells}\\ -\text{Absorbance of blank}\end{matrix}\right\}}$$

Determination of antioxidant effect on induced intracellular superoxide anions

Superoxide anions were detected with nitroblue tetrazolium (NBT) assay in three-treatment models (pre-treatment, co-treatment and post-treatment). Pre-treatment served as a preventive model (before contact with ML), co-treatment represented a concurrent model, and post-treatment represented curative model (after contact with ML). Final non-toxic concentrations of the antioxidants that were used for the treatments were ASC (500 μM), GTK (2.5 mg/mL), and GA (30 μM) and for ML, 1.037 μg/mL. Cells were seeded in a 96-well-plate overnight in 100 μL growth medium. For pre-treatment of cells, the medium was decanted and replaced with 90 μL of fresh medium and 10 μL of antioxidants. Incubation was done for 24 h at 37°C in the presence of 5% CO_2 after which the antioxidant-containing medium was decanted. Cells were washed with PBS twice to remove any traces of antioxidants and finally replaced with ML−NBT-containing medium (80 μL fresh medium, 10 μL of ML and 10 μL of 2.7 mg/mL NBT) and further incubated for 24 h. Cells were washed with 1× PBS twice and subjected to superoxide anions detection.

In the co-treatment model, the cells were cultured with 70 μL of medium, 10 μL of the antioxidants, 10 μL of ML, and 10 μL of 2.7 mg/mL NBT concurrently and incubated at 37°C in the presence of 5% CO_2 for 24 h. Cells were then washed with 1× PBS twice to remove traces of the antioxidants, ML, and NBT. Cells were then subjected to superoxide anion detection.

Post-treatment model involved prior treatment of the cells with ML-NBT-containing medium (80 μL medium, 10 μL ML and 10 μL of 2.7 mg/mL NBT) for 24 h, washing of cells with 1× PBS to remove traces of the ML and NBT and subsequently, the addition of the antioxidants for another 24 h. Cells were again washed with 1× PBS twice to remove traces of the antioxidants prior to superoxide anion detection. Three independent experiments were done, each in triplicate for the different models of treatments.

Cell viability of the pre-, co-, and post-treatment models using Alamar blue assay

Cell viability for each of the models of treatments (pre-, co- and post-treatments) was estimated using the Alamar blue assay. After the treatment of each model, a volume of 10 μL of 500 μM resazurin solution (114.55 g of resazurin sodium salt (Sigma, USA) dissolved in 1 mL phosphate buffered saline) was added to a 90 μL of fresh medium to obtain a final concentration of 50 μM. Incubation was done for 24 h and fluorescence was measured at excitation and emission wavelengths of 530/590 nm, respectively, using the Varioskan Lux plate reader. Final concentrations of antioxidants used for the treatments were ASC (500 μM), GTK (2.5 mg/mL), and GA (30 μM) and for ML, 1.037 μg/mL. Cells were seeded in a 96-well-plate overnight in 100 μL growth medium.

During the pre-treatment of cells, the medium was decanted and replaced with 90 μL of fresh medium and 10 μL of antioxidants. Incubation was done for 24 h at 37°C in the presence of 5% CO_2 after which the antioxidant-containing medium was decanted. Cells were washed with PBS twice to remove any traces of antioxidants and finally replaced with ML-containing media (90 μL fresh medium and 10 μL of ML) and further incubated for 24 h. Cells were washed with 1× PBS twice and cell viability was determined using Alamar blue assay.

In the co-treatment model, the cells were cultured concurrently with 80 μL of medium, 10 μL of the antioxidants as well as 10 μL of ML and incubated at 37°C in the presence of 5% CO_2 for 24 h. Cells were then washed with 1× PBS twice to remove traces of the antioxidants and ML. Cells were then subjected to the Alamar blue assay to determine cell viability.

Post-treatment model involved prior treatment of the cells with ML-containing media (90 μL media and 10 μL ML) for 24 h, washing of cells with 1× PBS twice to remove traces of the ML and subsequently, the addition of the antioxidants for another 24 h. Cells were again washed with 1× PBS twice to remove traces of the antioxidants and cell viability was determined. Tests for cell viability, in each of the treatment models are representative of three independent experiments done in triplicate.

The percent of control of cell viability was calculated as follows

$$\text{Cell viability (\% of control)} = \frac{\left\{\left(\begin{array}{l}\text{Fluorescence of treated cells}\\ -\text{Fluorescence of blank}\end{array}\right)\times 100\right\}}{\left\{\begin{array}{l}\text{Fluorescence of untreated cells}\\ -\text{Fluorescence of blank}\end{array}\right\}}$$

Gene expression analysis

Total RNA extraction

Total RNA of both treated and untreated RAW 264.7 macrophages at density of $\sim 1 \times 10^5$ cells/mL was isolated from each of the three models of treatment (pre-, co-, and post-treatments), using ZymoQuick-RNA MiniPrep Plus Mini kit (Invitrogen). Briefly, and following the manufacturer's instructions, cells were lysed and homogenized using 300 μL of RNA lysis buffer, followed by centrifugation at 10,000*g* for 30 s to clear the homogenate and remove a majority of gDNA. The resulting supernatant was then used for RNA purification by mixing with equal volume of ethanol (1:1). RNA was sieved through a column by centrifuging at 10,000*g* for 30 s and treated with 80 μL of DNAse 1 reaction mix for 15 min at room temperature (20–30°C), to ensure complete removal of any DNA contaminants. Sieved RNA was washed three times with RNA Prep buffer to ensure complete clearance of DNAse 1 reaction mix. RNA was eluted into an RNAse free tube using 100 μL of DNAse- and RNAse-free water and concentrations were determined using Nanodrop.

Quantitative reverse transcription PCR assay

Quantitative reverse transcription PCR was performed using Luna Universal One-Step RT-qPCR kit (New England Biolabs), following the manufacturer's instructions. Briefly, an assay mix of all the components was prepared and well mixed by vortexing and aliquoted into qPCR tubes followed by addition of RNA templates of the various cell treatments. Samples were assayed in triplicate in each run (40 cycles), which was composed of four stages, 55°C for 10 min (reverse transcription), 95°C for 1 min for each cycle (initial denaturation), 95°C for 10 s (denaturation), and finally the extension step at 60°C for 30 s. Gene expression was calculated relative to the endogenous control sample (β-actin) to determine the relative expression values using the $2^{-\Delta\Delta Ct}$ method (where Ct is the threshold cycle). A PikoReal 24 Real-Time PCR System (Thermo Scientific) was programmed with the indicated thermocycling protocol and the generated data were analyzed. Three independent experiments were done, each in triplicate for the different models of treatments.

Primer sets for PCR

PCR primers used were designed to amplify superoxide dismutase 2 (*SOD2*), catalase (*CAT*), and glutathione peroxidase 1 (*GPX1*) as well as *β-actin* which served as the internal reference primer (control) as shown in Table 1.

Statistical analysis

Data were analyzed using GraphPad Prism version 8.2 software (GraphPad Software, CA, USA). Differences between treatments and treatments for combined data sets were tested for statistical significance using Dunnett's multiple comparison test in one-way and two-way ANOVAs, and $P < 0.05$ were considered significant.

Results

Antioxidant activity of green tea kombucha and butylated hydroxytuolene

The antioxidant activity of prepared GTK was assessed by determining its scavenging ability against DPPH. Butylated hydroxytuolene (BHT) was used as reference standard. It was observed that the antioxidant activities of both GTK and BHT increased with increasing concentrations. A plot of antioxidant activity (%) against concentration was used to extrapolate the effective concentration (EC_{50}) of the tea as 2.165 mg/mL; however, the EC_{50} was about 5× less potent as an antioxidant compared to BHT (Figure 1).

Cytotoxicity of antioxidants and mycolactone on RAW 264.7 macrophages

Varying concentrations of antioxidants ranging from 31.25 μM to 1000 μM ASC, 3.75 μM to 60 μM GA, and 0.625 mg/mL to 20 mg/mL GTK were prepared and used in treating RAW 264.7 macrophages to eliminate antioxidant-induced cell death. Concentrations of ASC, GTK, and GA above 500 μM, 2.5 mg/mL, and 30 μM, respectively, decreased cell viability in the 24-h treatment (Figure 2(a) to (c)). Concentrations of the antioxidants that were non-toxic to RAW 264.7 cells were therefore used for the pre-/co- and post-treatment experiments. Based on this, the following concentrations of ASC (500 μM), GTK (2.5 mg/mL), and GA (30 μM) were chosen as non-toxic levels for subsequent investigations (Figure 2(a) to (c) and (e)). The cytotoxic effect of ML on RAW 264.7 macrophages was also assessed after 24, 48, and 72 h of incubation at varying concentrations of the ML (0.00001 μg/mL to 1.0 μg/mL). Higher concentrations (0.1 and 1.0 μg/mL) with prolonged exposures (48 and 72 h) resulted in more than 50% reduction in cell viability, while lower

Table 1. List of primers used for PCR amplifications.

Gene	Forward primer sequences	Reverse primer sequences
SOD2	5′-ATGTTGTGTCGGGCGGCG-3′	5′-AGGTAGTAAGCGTGCTCCCACACG-3′
CAT	5′-GCAGATACCTGTGAACTGTC-3′	5′-GTAGAATGTCCGCACCTGAG-3′
GPX1	5′-AAGGAGGTGCAGGCGGCTGTGAGG-3′	5′-GCGCGGAGAAGGCATACACGGTGG-3′
β-actin	5′-TGGAATCCTGTGGCATCCATGAAAC-3′	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

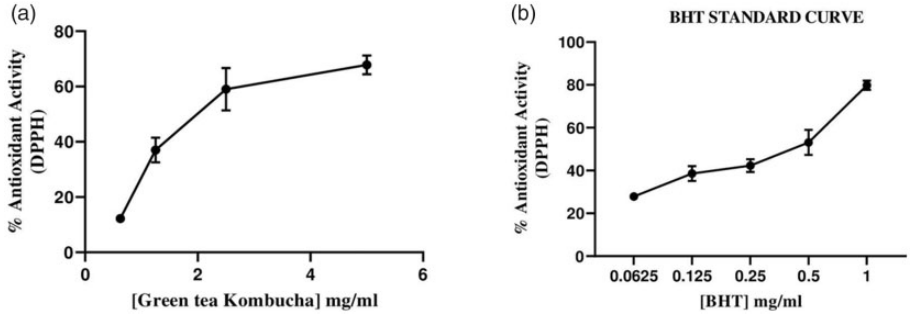

Figure 1. Antioxidant activity of green tea kombucha (GTK) and BHT. Methanolic DPPH solution of 0.5 mM was added to (a) prepared GTK (0.625–5 mg/mL) and (b) BHT solutions (0.0625–1 mg/mL), incubated for 20 min and absorbance read at 517 nm. Results are representative of three independent experiments with three replicates each (means ± SEM). BHT: butylated hydroxytoluene.

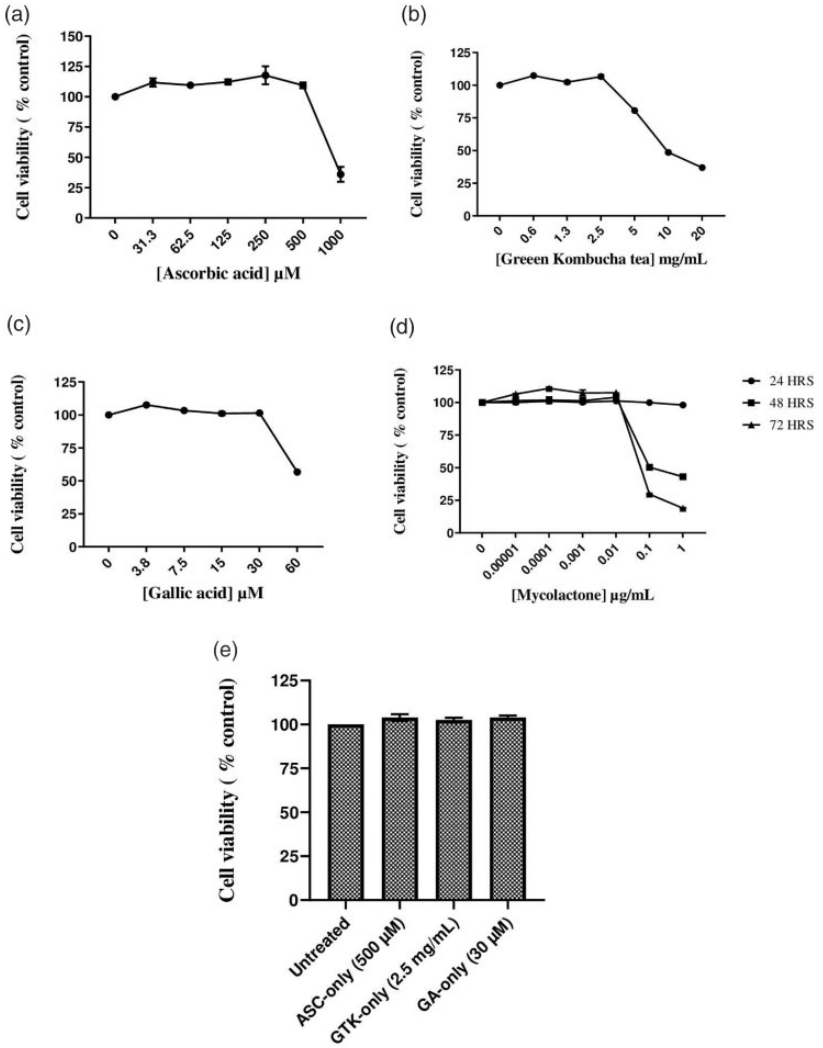

Figure 2. Cytotoxicity of antioxidants and mycolactone against RAW 264.7 macrophages. Cells were seeded overnight and separately treated with varying concentrations of (a) ascorbic acid; (b) green tea kombucha, and (c) gallic acid for 24 h. (d) Mycolactone treatment of cells was also done at varying concentrations for 24, 48, and 72 h. (e) Cell viability of chosen non-toxic concentrations of the antioxidants (ASC = 500 µM; GTK = 2.5 mg/mL; GA = 30 µM) after 24 h. Cell viability was determined using the Alamar blue assay. Results are representative of three independent experiments with three replicates each (means ± SEM).

concentrations (0.00001–0.01 µg/mL) had no toxic effects, irrespective of the duration of exposure (Figure 2(d)).

Mycolactone induces ROS production

Reactive oxygen species (ROS) and superoxide anions production in RAW 264.7 macrophages were determined using DCFDA (fluorescent probe) and nitroblue tetrazolium (NBT) assay, respectively, after 24 h of treatment with ML and tert-butyl hydroperoxide (TBHP). The TBHP (100 µM), which served as a positive control induced a substantial increase in ROS production compared to the untreated control (6%). The cells treated with ML at 0.1 µg/mL and 1.0 µg/mL had a significant increase in the levels of ROS (40% and 59%, respectively), relative to untreated control (Figure 3(a)).

In determining the specific ROS, superoxide anions which are the leading actors of ROS were probed. Levels of generated superoxide anions were no different when the macrophages were treated with ML at concentrations of 0.1 µg/mL and 1.0 µg/mL for 24 h, generating 79% and 76%, respectively but were significantly higher than the untreated control (Figure 3(b)).

Effect of antioxidants on induced-superoxide anions and the level of antioxidant protection of cells against ML toxicity in pre-, co-, and post-treatment experiments

To assess the ML-induced superoxide anions scavenging ability of the antioxidants, ASC (500 µM), GTK (2.5 mg/mL), and GA (30 µM) as well as ML (1.037 µg/mL) were used in the three antioxidant-treatment models (pre-, co-, and post-treatments). Based on the superoxide scavenging effects of the antioxidants, it became necessary to investigate whether the antioxidants could protect cells from death. Cell viability assays were performed for the pre-, co- and post-treated experiments (Supplementary Figure 1). ASC potently reduced the levels of superoxide anions in all the treatment models and showed significant

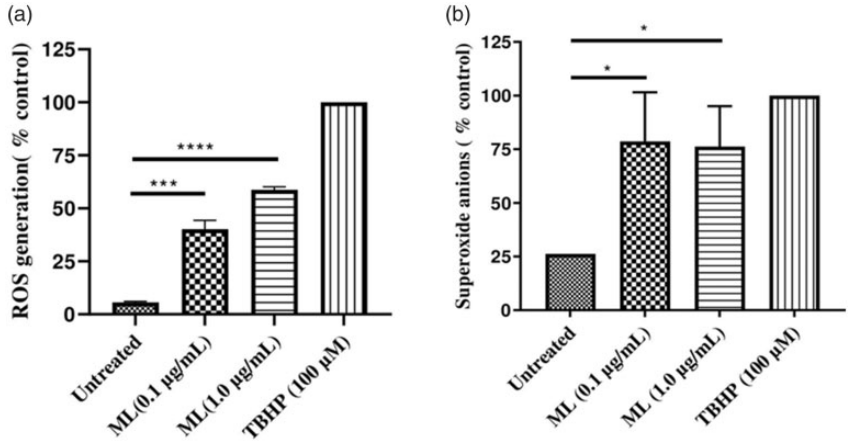

Figure 3. Levels of ROS and superoxide anions in mycolactone-treated RAW 264.7 macrophages. (a) Cells were labeled with DCFDA (20 μM) followed by ML treatments (0.1 and 1.0 μg/mL) for 24 h to detect ROS. (b) Cells were treated with ML (0.1 and 1.0 μg/mL) and NBT solution (0.27 mg/mL) concurrently for 24 h to determine levels of superoxide anions. Results are representative of three independent experiments with three replicates each (means ± SEM). Values ($P < 0.05$) were considered statistically significant compared to negative control. (****$P < 0.0001$; ***$P < 0.001$; *$P < 0.05$). Data were analyzed using Dunnett's multiple comparison test. TBHP: tert-butyl hydroperoxide, which served as positive control. ML: mycolactone.

protection of the macrophages when pre-treated with the antioxidants as observed with a survival rate of about 56% compared to the ML-only treated control cells (40%). However, GTK exhibited poor scavenging activity in the pre-treatment experiment and could not significantly sustain viability of the cells relative to the ML-only treated control. Despite GA also showing a significant scavenging activity in this pre-treatment model, the survival rate of the cells was reduced (Figure 4(a)).

In the co-treatment experiment, a significant cell preservation (survival rate) was observed when the cells were co-treated with the GTK and ML compared to ML-only treated control. There was reduced cell viability when co-treated with both ASC and GA despite the effective scavenging of superoxide anions in this treatment model (Figure 4(b)). In the post-treatment experiments, effective scavenging of superoxide anions was observed in all the three antioxidant-treatments, but an interesting observation was made where addition of the antioxidants (ASC, GTK, GA) after the 24 h of ML treatment, rather, enhanced cell death compared to ML-only treated controls (Figure 4(c)).

Antioxidants enhance activity of endogenous antioxidant genes in the pre- and co-treatment models, while overly expressed in the post-treatment model

The levels of expression of antioxidant enzyme genes (manganese superoxide dismutase (*SOD2*), catalase (*CAT*), and glutathione peroxidase (*GPX1*)) were determined to investigate the compensatory response mechanism mounted by the cells, to combat the toxic effects of reactive oxygen species (ROS). Here, total transcript levels of cells treated with ASC, GTK, and GA in the pre-, co-, and post-treatment models were analyzed relative to ML-only treated controls. ML-treated cells with a survival rate of 40% had moderate expression of the antioxidant enzyme genes with 7- and 8-fold increases of *SOD2* and *CAT*, respectively, while *GPX1* was a 3-fold increase.

In the pre-treatment model, ASC-treated cells with a survival rate of 56% had an enhanced expression of *SOD2*; with minimal expressions of *CAT* and *GPX1*, compared to ML-treated cells. Cells treated with GTK (survival rate of 49%) also had increased expressions of *SOD2, CAT,* and *GPX1,* relative to ML-treated cells. The expression of *CAT* gene was relatively lower in each of the antioxidant-treated cells with the highest *CAT* expression being 13-fold in GTK-treated cells. The expression levels of all three antioxidant genes in GA-treated cells (survival rate of 48%) were similar compared to ML-treated cells (Figure 5(a)).

In the co-treatment model, cells treated with GTK had the highest survival rate of 57% with an exceptionally enhanced expression of *CAT* gene (146-fold), while *SOD2* and *GPX1* were expressed up to 27-fold and 19-fold, respectively. Although ASC and GA also showed moderate increases in *CAT* expression with 65- and 21-folds, respectively, the survival rates of their cells remained similar to that of the ML-treated cells (41%). Moderate expressions of the *SOD2* were observed in ASC- and GTK-treated cells with the exception of GA. The expression of *GPX1* was relatively lower when the cells were treated with all the three antioxidants (Figure 5(b)).

The post-treatment model, which involved the treatment of the cells with ML prior to antioxidants treatment (ASC, GTK and GA), generated several 1000-fold increases in the expression levels of the antioxidant enzyme genes; *SOD2*, *CAT,* and *GPX1,* relative to the ML-only treated controls. *SOD2* gene was substantially over-expressed (over 70,000-folds) when the cells were post-treated with ASC. The expression of *GPX1* in ASC treated cells was also highly expressed with over 40,000-fold increase. While the expression of *SOD2*, *CAT*, and *GPX1* had over 20,000-fold increases in GTK post-treated cells, their expression in GA-treated cells were relatively lower with less than 5000-fold increases. Despite the excessive expression of these antioxidant genes in each of the antioxidant treated cells, their survival rates were markedly reduced, far less than even the ML-treated cells (Figure 5(c)).

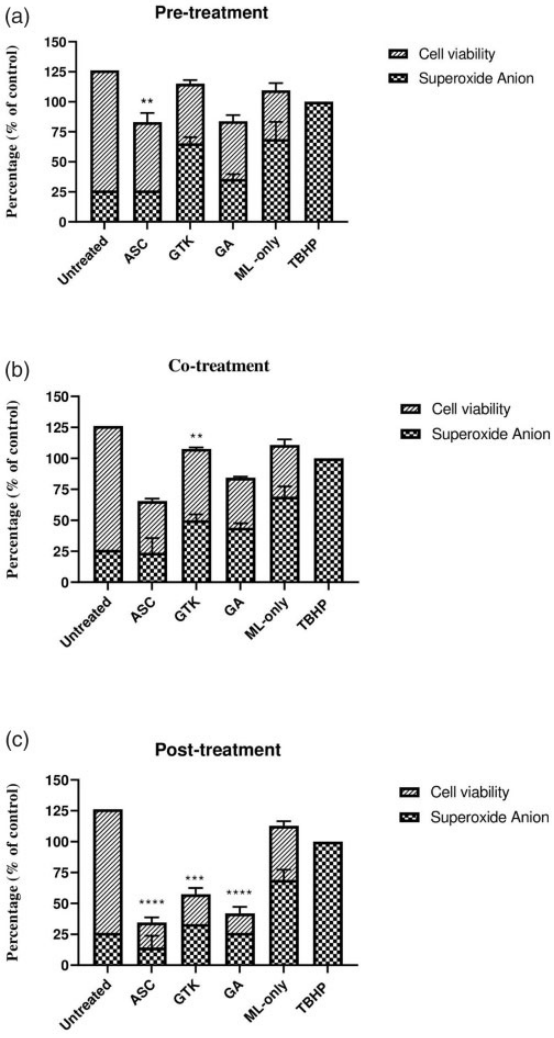

Figure 4. Effect of antioxidants on superoxide anion production and cell viability in RAW 264.7 macrophages. (a) For superoxide anions detection, cells in pre-treatment model were treated with the antioxidants (ASC = 500 μM; GTK = 2.5 mg/mL; GA = 30 μM.) for 24 h, followed by treatments with ML (1.037 μg/mL) and NBT for 24 h. In a separate experiment, cell viability was also determined in cells pre-treated with antioxidants (24 h) and followed by ML exposure (24 h). (b) For superoxide anions detection in co-treatment model, cells were simultaneously exposed with the antioxidants, ML (1.037 μg/mL) and NBT for 24 h. Cell viability assay was also done after co-treatment of antioxidants and ML (24 h) in separate experimental set up. (c) For superoxide anions detection in the post-treatment model, the cells were exposed to ML (1.037 μg/mL) and NBT for 24 h followed by post-treatment with the antioxidants for 24 h. Cell viability assay was done in a separate experiment for cells post-treated with antioxidants (24 h) after initial exposure to ML (24 h).
TBHP-treated cells served as positive control, while untreated cells represented negative control for superoxide anion production. Superoxide anions generated from cells treated with only ML, also served as experimental controls. TBHP: tert-butyl hydroperoxide (100 μM); ASC: 500 μM; GTK: 2.5 mg/mL; GA: 30 μM, and ML: 1.037 μg/mL. Untreated cells served as positive control, while cells treated with only ML served as experimental controls. Results are representative of three independent experiments with three replicates (means ± SEM) in each of the models. For cell viability, values ($P < 0.05$) were considered statistically significant compared to ML-treated control. (****$P < 0.0001$; ***$P < 0.001$; **$P < 0.005$). Data were analyzed using Dunnett's multiple comparison test.

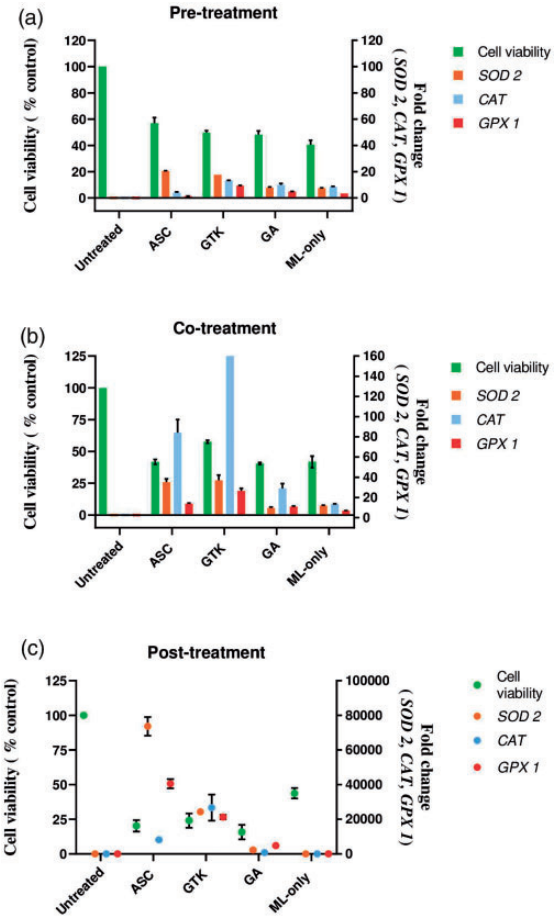

Figure 5. Expression of endogenous antioxidant genes in antioxidant pre-/co-/post-treated cells. (a) The cells were pre-treated (24 h) with antioxidants (ASC = 500 μM; GTK = 2.5 mg/mL; GA = 30 μM.) followed by ML (1.037 μg/mL) for 24 h; (b) Cells were co-treated with antioxidants and ML concurrently (24 h), and (c) Cells were post-treated with the antioxidants (24 h) after initial ML treatment (24 h). Untreated cells served as positive control, while cells treated with only ML served as experimental controls. Total RNA was extracted from both untreated and treated cells in each of the models of treatment (pre-, co-, and post-treatments) and subjected to RT-qPCR to determine the expression levels of *SOD2, CAT*, and *GPX1*. The total RNA expression profiles were normalized with respect to *β-actin*. Fold increase of each gene was calculated using the $2^{-\Delta\Delta CT}$ *method*. Fold increase was based on levels obtained for ML. Results are representative of three independent experiments with three replicates each. Cell viability was determined in each of the models of treatment in a separate experiment using Alamar blue assay. Untreated cells served as positive control, while cells treated with only ML served as experimental controls. Results are representative of three independent experiments with three replicates each (means ± SEM). (A color version of this figure is available in the online journal.)

Discussion

Mycolactone, the main virulence factor in the pathogenesis of Buruli ulcer causes chronic ulcers, which are manifestations of its cytotoxicity. It has been implicated in ROS production and its persistence in tissues has been shown to delay wound healing. The search for therapeutic agents that can combat the effects of ML-mediated ROS and attenuate its toxicity to wound-healing cells (macrophages, keratinocytes, and fibroblast) is necessary. The cytotoxic effect of ML was therefore studied in RAW 264.7 macrophages in the presence of selected antioxidants. The scavenging ability of some of the antioxidants, in neutralizing ML-induced ROS was demonstrated. The antioxidants up-regulated the expression of endogenous antioxidant enzyme genes in the macrophages, as the ASC and GTK slightly attenuated the toxic effects of ML in the preventive and concurrent models,

respectively. Macrophage protection and survival may therefore auger well in the fight against MU infection.

The generation of ML-induced ROS in the macrophages, re-affirmed the study by Gronberg *et al*.,[7] which indicated ML as a ROS-stimulant. Since increasing concentrations of toxic substances have been shown to reduce the expression and activity of antioxidant enzyme genes,[22] we sought to ascertain whether ML could alter the expression of endogenous antioxidant enzyme genes (*SOD2*, *CAT*, and *GPX1*). In this study, ML concentration at 1.037 μg/mL could not trigger adverse down regulation of the endogenous antioxidant enzyme genes within the macrophages, but rather, increased their expressions relative to the untreated macrophages, which could be a possible compensatory mechanism initiated by the cells to quench the ML-induced ROS (Figure 5). Although macrophages have been characterized as ROS-resistant, due to their increased level of ROS reductase and DNA repair proteins during excessive ROS exposure, they are still susceptible to ROS-associated death.[23] This can abrogate their stimulatory function on fibroblasts and keratinocytes' proliferation, which are key players in wound healing.

Macrophages promote cellular proliferation by producing numerous growth factors such as TGF-β1, IGF-1, and VEGF-α,[24] and by stimulating fibroblast differentiation to myofibroblast. This yields extracellular matrix components as well as protein mediators for wound contraction and closure.[25] In the presence of excessive ROS, proteins of the extracellular matrix can be degraded and impair the function of dermal fibroblast and keratinocytes,[26] thus disrupting wound repair processes with associated chronic non-healing wounds.[15] This unique dimension of macrophage function can therefore ensure wound healing if macrophages can be protected from ROS-induced cell death using natural antioxidants, and survive to exploit their functions appropriately during wound healing.

To determine whether the reduction of ROS by the antioxidants was translated into cyto-protection, cell viability assays were done. Treatment of cells with ASC was effective in protecting the cells in the pre-treatment model but was ineffective in the co-treatment model, despite having a significant reduction of superoxide anions and up-regulation of the antioxidant enzyme genes *SOD 2* (pre-treatment model), *CAT*, and *SOD 2* (co-treatment model).

Similarly, the increased expression of the antioxidant genes during the pre- and co-treatment models with GTK could not provide the same level of cellular protection, but rather a decline in the survival rates of cells in the pre-treatment model. The mode of the treatments may therefore be eliciting different mechanisms of action for each antioxidant based on the microenvironment of the reaction medium and the initiation conditions. An exceptionally enhanced expression of the *CAT* gene was observed in the co-treatment model, in the presence of both ASC and GTK; however, this was not translated into cellular protection in the case of the ASC-treatment. Even though GTK has been widely reported as a strong quencher of superoxide anions in cell free systems, as demonstrated in a study by Bhattacharya *et al*.,[27] it could not quench highly significant levels of superoxide anions in the pre- and co-treatment models of this study; hence, its cellular protection in the co-treatment can be attributed to factors, other than direct ROS neutralization (Figure 4).

Gallic acid, as shown to be a good scavenger of superoxide anions,[28] duly mopped-up the superoxide anions to a certain extent, and caused an up-regulation in the expression levels of the antioxidant gene *CAT* (co-treatment model), though minimal when compared to ASC and GTK. This, however, could not exert any protection to the cells.

These observations lead to the speculation that the up-regulation of the antioxidant genes in both models of treatment by ASC, GTK, and GA does not essentially provide protection to the cells. The protection of the cells by ASC and GTK in the pre-treatment and co-treatment models, respectively, may possibly be due to other factors, rather than just superoxide anions scavenging and enhanced antioxidant gene expressions. Further investigation will therefore be required to ascertain the exact molecular mechanism that is being elicited by both ASC and GTK to be providing such protections.

The antioxidant genes may have been enhanced, albeit not certainly being translated to proteins (Sec61 translocon blockade), thus any process of protein translocation and modification in the endoplasmic reticulum for subsequent secretion and localization may be aborted.[10] The up-regulation could therefore be a mere compensatory mechanism by which the cells were enabled to counteract the deleterious effects of the superoxide anions, via dismutation to hydrogen peroxide using SOD2. Up-regulation of the *CAT* gene may be indicating the presence of hydrogen peroxide, an end-product of SOD2 dismutation reaction. Glutathione peroxidase 1 (*GPX1*) which also regulates the levels of hydrogen peroxide, by catalyzing its conversion to water, was moderately expressed. A recent study by Förster *et al*. has however, implicated ML in the depletion of the antioxidant gene glutathione.[29]

Despite the potent reduction of the superoxide anions by the antioxidants in the post-treatment model (curative model), the addition of the antioxidants (ASC, GTK, and GA) rather enhanced cell death. This can be attributed to an already impaired physiological fitness of the cells, induced by ML, which could have been exacerbated by the addition of the non-toxic antioxidants. ML mediated ROS may therefore not be the sole mechanism of cell death. This may explain the observed increased cell death despite the effective reduction of superoxide anions by the antioxidants in the post-treatment model. The tremendous over-expression of the antioxidant enzyme genes in the post-treatment model, yet markedly reduced cell survival, may be due to a possible non-translation of the antioxidant enzyme transcripts to proteins, due to blockade of the Sec61 translocon. Further studies may be required to ascertain the protein levels of these endogenous antioxidant enzymes. The enormous over-expression of the antioxidant genes could have led to a disrupted level of physiological oxidant (hydrogen peroxide) required for signalling, as indicated in a previous study that over-expressed catalase can decrease the activation of NFκB survival pathways, and hence the inability to

counteract an apoptotic pathway.[30] This to some extent can explain the swift cell death. The tremendous cell loss in the post-treatment model (curative model for BU) therefore requires a stringent concentration-dependent investigation to determine the best concentrations of the non-toxic antioxidants that would rather not potentiate cell death. The use of both oral and topical applications of the antioxidants may be recommended to complement each other. Therefore, the likely depletion of the cutaneous applied antioxidants by UV light can be complemented by oral ingestion, which on its own may also be insufficient to replenish the cutaneously applied store.

Conclusions

With the moderate protection of the macrophages in the presence of the ASC antioxidant (preventive model against BU) and GTK antioxidant (concurrent model during BU), the toxicity of ML was attenuated to a certain extent. The survival of macrophages may therefore augment the treatment of *M. ulcerans* infections. Macrophage protection could have resulted also from unknown mechanisms, other than superoxide anions scavenging and the expression of endogenous antioxidant enzyme genes, hence requiring further investigation. Results of this *in vitro* study should be replicated *in vivo* to ascertain the importance of antioxidants ASC and GTK as prophylactic agents (oral and/or topical application) that can offer protection in animal models during *M. ulcerans* infection. The use of epithelial cells such as fibroblasts and keratinocytes in further studies is necessary to confirm the protective effect of the exogenous antioxidants in other wound-healing cells. Specific detection of hydrogen peroxide and hydroxyl radicals after ML treatment will be more informative to confirm their involvement in ML cytotoxicity. Investigations focusing on the effect of the exogenous antioxidants on the phagocytic and chemotactic response of ML-treated macrophages will be worthy of consideration.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, analysis of the data and interpretation of the studies. Manuscript review and editing was done by YAK, LM, WSKG, and JPA. The experiments were conducted by YAK, KOB, and MSD and manuscript writing was done by YAK and LM.

ACKNOWLEDGEMENTS

We render appreciation to Prof Regina Appiah-Opong (Clinical Pathology Department of Noguchi Memorial Institute for Medical Research, University of Ghana) for the provision of the RAW 264.7 macrophages. We are most thankful to Gideon Atinga Akolgo (Chemistry Department, University of Ghana) for the provision of the ML, without which this work could not have come into fruition. Our profound gratitude goes to Isaac Tuffour (Clinical Pathology Department of Noguchi Memorial Institute for Medical Research, University of Ghana) for his technical support to this project.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

YAK and KOB were supported by a Masters' fellowship from a World Bank African Centre of Excellence Grant [ACE02-WACCBIP: Awandare]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ORCID iD

Lydia Mosi https://orcid.org/0000-0002-7672-0181

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Wansbrough-Jones M, Phillips R. Buruli ulcer: emerging from obscurity. *Lancet* 2006;**367**:1849–58
2. Yotsu RR, Suzuki K, Simmonds RE, Bedimo R, Ablordey A, Yeboah-Manu D, Phillips R, Asiedu K. Buruli ulcer: a review of the current knowledge. *Curr Trop Med Rep* 2018;**5**:247–56
3. Adusumilli S, Mve-Obiang A, Sparer T, Meyers W, Hayman J, Small PL. *Mycobacterium ulcerans* toxic macrolide, mycolactone modulates the host immune response and cellular location of *M. ulcerans* in vitro and in vivo. *Cell Microbiol* 2005;**7**:1295–304
4. Bozzo C, Tiberio R, Graziola F, Pertusi G, Valente G, Colombo E, Small PL, Leigheb G. A *Mycobacterium ulcerans* toxin, mycolactone, induces apoptosis in primary human keratinocytes and in HaCaT cells. *Microbes Infect* 2010;**12**:1258–63
5. Dobos KM, Small PL, Deslauriers M, Quinn FD, King CH. *Mycobacterium ulcerans* cytotoxicity in an adipose cell model. *Infect Immun* 2001;**69**:7182–6
6. George KM, Pascopella L, Welty DM, Small PL. A *Mycobacterium ulcerans* toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. *Infect Immun* 2000;**68**:877–83
7. Gronberg A, Zettergren L, Bergh K, Stahle M, Heilborn J, Angeby K, Small PL, Akuffo H, Britton S. Antioxidants protect keratinocytes against *M. ulcerans* mycolactone cytotoxicity. *PLoS One* 2010;**5**:e13839
8. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, Small PL. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. *Science* 1999;**283**:854–7
9. Simmonds RE, Lali FV, Smallie T, Small PL, Foxwell BM. Mycolactone inhibits monocyte cytokine production by a posttranscriptional mechanism. *J Immunol* 2009;**182**:2194–202
10. Hall BS, Hill K, McKenna M, Ogbechi J, High S, Willis AE, Simmonds RE. The pathogenic mechanism of the *Mycobacterium ulcerans* virulence factor, mycolactone, depends on blockade of protein translocation into the ER. *PLoS Pathog* 2014;**10**:e1004061
11. Sarfo FS, Le Chevalier F, Aka N, Phillips RO, Amoako Y, Boneca IG, Lenormand P, Dosso M, Wansbrough-Jones M, Veyron-Churlet R, Guenin-Mace L, Demangel C. Mycolactone diffuses into the peripheral blood of buruli ulcer patients – implications for diagnosis and disease monitoring. *PLoS Negl Trop Dis* 2011;**5**:e1237
12. Hong H, Demangel C, Pidot SJ, Leadlay PF, Stinear T. Mycolactones: immunosuppressive and cytotoxic polyketides produced by aquatic mycobacteria. *Nat Prod Rep* 2008;**25**:447–54
13. Sarfo FS, Phillips R, Asiedu K, Ampadu E, Bobi N, Adentwe E, Lartey A, Tetteh I, Wansbrough-Jones M. Clinical efficacy of combination of

rifampin and streptomycin for treatment of *Mycobacterium ulcerans* disease. *Antimicrob Agents Chemother* 2010;**54**:3678–85
14. Ogbechi J, Ruf MT, Hall BS, Bodman-Smith K, Vogel M, Wu HL, Stainer A, Esmon CT, Ahnstrom J, Pluschke G, Simmonds RE. Mycolactone-dependent depletion of endothelial cell thrombomodulin is strongly associated with fibrin deposition in buruli ulcer lesions. *PLoS Pathog* 2015;**11**:e1005011
15. Schafer M, Werner S. Oxidative stress in normal and impaired wound repair. *Pharmacol Res* 2008;**58**:165–71
16. Bhattacharya S. Reactive oxygen species and cellular defense system. In: Rani V, Yadav, U (ed) *Free radicals in human health and disease*. New Delhi: Springer, 2015, pp.17–29
17. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity* 2016;**44**:450–62
18. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008;**453**:314–21
19. Adiza Abass EG, Appiah-Opong R. *WSK gbewonyo, phyllis addo, lydia mosi. Evaluation of the antimycobacterial and antimycolactone efficacy of kombucha tea*. Illinois, IL: American Society of Tropical Medicine and Hygiene, 2020
20. Jayabalan R, Subathradevi P, Marimuthu S, Sathishkumar M, Swaminathan K. Changes in free-radical scavenging ability of kombucha tea during fermentation. *Food Chem* 2008;**109**:227–34
21. Choi HS, Kim JW, Cha YN, Kim C. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *J Immunoassay Immunochem* 2006;**27**:31–44
22. Gechev T, Gadjev I, Van Breusegem F, Inze D, Dukiandjiev S, Toneva V, Minkov I. Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. *Cell Mol Life Sci* 2002;**59**:708–14
23. Lo HM, Chen CL, Yang CM, Wu PH, Tsou CJ, Chiang KW, Wu WB. The carotenoid lutein enhances matrix metalloproteinase-9 production and phagocytosis through intracellular ROS generation and ERK1/2, p38 MAPK, and RARbeta activation in murine macrophages. *J Leukoc Biol* 2013;**93**:723–35
24. Chujo S, Shirasaki F, Kondo-Miyazaki M, Ikawa Y, Takehara K. Role of connective tissue growth factor and its interaction with basic fibroblast growth factor and macrophage chemoattractant protein-1 in skin fibrosis. *J Cell Physiol* 2009;**220**:189–95
25. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 2011;**11**:723–37
26. Moseley R, Stewart JE, Stephens P, Waddington RJ, Thomas DW. Extracellular matrix metabolites as potential biomarkers of disease activity in wound fluid: lessons learned from other inflammatory diseases? *Br J Dermatol* 2004;**150**:401–13
27. Bhattacharya S, Gachhui R, Sil PC. Hepatoprotective properties of kombucha tea against TBHP-induced oxidative stress via suppression of mitochondria dependent apoptosis. *Pathophysiology* 2011;**18**:221–34
28. Masaki H, Atsumi T, Sakurai H. Protective activity of hamamelitannin on cell damage of murine skin fibroblasts induced by UVB irradiation. *J Dermatol Sci* 1995;**10**:25–34
29. Forster B, Demangel C, Thye T. Mycolactone induces cell death by SETD1B-dependent degradation of glutathione. *PLoS Negl Trop Dis* 2020;**14**:e0008709
30. Lupertz R, Chovolou Y, Kampkotter A, Watjen W, Kahl R. Catalase overexpression impairs TNF-alpha induced NF-kappaB activation and sensitizes MCF-7 cells against TNF-alpha. *J Cell Biochem* 2008;**103**:1497–511

(Received November 11, 2020, Accepted April 17, 2021)