**Research**

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**Volume 1 : Issue 1**  
**Article Ref. #: 1000SROJ1101**

**Article History**  
Received: June 17th, 2014  
Accepted: July 9th, 2014  
Published: July 10th, 2014

**Citation**  

**ABSTRACT**

In this study, we investigated the effects of (-)-epigallocatechin-3-gallate (EGCG) on wound healing both in vitro and in vivo with and without infection. EGCG has antimicrobial properties and could be useful as a topical agent to prevent and/or treat wound infections. Normal fibroblasts were isolated from the dermis of C57BL/6 mice and cultured with 0, 0.001 to 0.400 mg/ml of EGCG. In vitro assays demonstrated that migration, proliferation, and apoptosis were inhibited at EGCG concentrations of 0.100-0.400 mg/ml. Expression of α-smooth muscle actin (α-SMA) was also reduced. In vivo experiments measured closure/contraction of full-thickness dorsal wounds that were treated with 0, 0.3, 3.0, and 30.0 mg/ml of EGCG every 24 hours. Macrophages (F4/80), neutrophils (Ly-6G) and myofibroblasts (α-SMA) were assessed at 48 and 168 hours. By 168 hours there was a significant reduction in presence with the 30 mg/ml dose vs. 0.3 and 3.0 mg/ml (p<0.009, p<0.006, respectively). The percentage of wound closure at one week in EGCG treated wounds was 87.87% (0.03 mg), 85.23% (0.3 mg) and 40.06% (30 mg/ml) compared to controls. Reduced quantities of α-SMA myofibroblasts were observed in the 3 mg EGCG treatment group compared to controls at 168 hours. We previously demonstrated that EGCG has antimicrobial properties (MIC50 ~0.3 mg/ml). This data suggests that EGCG could potentially be applied to the wound surface as an antimicrobial without negatively influencing healing. To this end we applied EGCG (10 mg/ml) to a model of an infected traumatic wound. EGCG treatment significantly reduced bacterial load after one and two dose regimens.

**KEYWORDS:** EGCG; (-)-Epigallocatechin-3-Gallate; Wound healing.

**INTRODUCTION**

Epigallocatechin-3-gallate (EGCG) is the predominant polyphenol found in green tea. It is has been widely consumed for three millennia as a beverage and is known to have medicinal properties. Recent work with EGCG, and related catechins, has shown it has powerful anti-oxidant properties\(^1\) and displays anti-tumor;\(^2\) anti-inflammatory;\(^3,4\) antikeloid scar formation;\(^5\) alters apoptosis;\(^6,7\) anti-metastatic;\(^8,9,10\) and anti-atherogenic\(^11\) activity both in vitro and in vivo settings. EGCG also has been demonstrated to inhibit growth of microbes such as Acinetobacter baumannii;\(^12\) Bordetella pertussis;\(^13\) Helicobacter pylori;\(^14,15\) Porphyromonas gingivalis;\(^16,17\) Staphylococcus aureus and Eschericia coli\(^18\) and Salmonella typhimurium.\(^19\) Moreover, EGCG has been shown to synergize with antibiotics in preventing growth of various microbes.\(^13,20,24\) Because EGCG is generally considered safe for use in humans, it may be an attractive compound to augment or treat wounds topically, especially in light of its antimicrobial activity. While recent work with nude mice has demonstrated the effects of EGCG impregnated dressing on sterile wound,\(^25\) it is not known if the concentrations needed to inhibit microbial growth are compatible with wound closure in immune competent mice.
Recent work in our laboratory indicated that the EGCG concentration needed to kill Multi-Drug Resistant (MDR) A. baumanii would be difficult to achieve in the blood. Burns and cutaneous wounds, therefore, represent an ideal situation in which EGCG could be applied directly to the wound. The superficial location of wounds allow for topical applications of high concentrations of the antimicrobial polyphenol. Kim et al. showed in leptin receptor deficient mice used as a model for diabetic wound healing that an implanted collagen sponge infused with EGCG enhanced wound closure. Additionally, EGCG at various concentrations promoted differentiation and proliferation of keratinocytes. These results suggest that EGCG may promote wound healing. However, in this same study the researchers found that high concentrations of EGCG (1000 ppm) appear to inhibit wound contraction and reduce granulation tissue formation. Klass et al. demonstrate that of EGCG (1000 ppm) appear to inhibit wound contraction and reduce granulation tissue formation. Klass et al. demonstrate that EGCG reduces the effects of Transforming Growth Factor beta (TGFβ1) on fibroblast contraction as well as differentiation of myofibroblasts. Furthermore EGCG in a number of systems has been shown to inhibit the production of collagen and fibroblast contraction. In a full-thickness excisional rat model of wounds EGCG increased angiogenesis and Vascular Endothelial Growth Factor (VEGF) expression, decreased arginase activity, and more mature collagen deposition within the wound site.

To determine the effects of EGCG on murine wound healing we investigated its effects following a full-thickness excisional wound made on the dorsal surface of C57BL6 mice. EGCG was applied at various concentrations and wound closure, apoptosis, alpha-smooth muscle actin (α-SMA) expression, migration and proliferation of cultured primary mouse fibroblasts, inflammation and granulation formation were monitored. Finally we wished to determine if the antibacterial properties of EGCG observed in vitro are effective in a surgical wound with infection model.

MATERIALS AND METHODS

General Approach

EGCG has been previously shown to alter many biological functions. In this study we examined the effects of this compound on healing (contraction) of sterile wounds in vitro and in vivo and on viability of grossly contaminated wounds. Some of the potential mechanisms for the results obtained were also examined. Studies performed in vitro included an examination of the effects of EGCG on the migration of skin fibroblasts into the damaged area of a monolayer. These in vitro studies were expanded to include an examination of the effects of EGCG on cell proliferation and cell death by apoptosis. In the next series of experiments two in vivo models were utilized to examine the effects of EGCG: a punch biopsy model to measure the effects on wound closure and an incision/infection model to measure the effects on wound infections. Immunohistochemical methods were applied to the sections from the wound to determine the effects of EGCG on potential scarring and leukocyte infiltration.

Reagents

EGCG was provided by S.H Hyon (Institute for Frontier Medical Sciences, Kyoto University, Japan), with purity in excess of 90%. EGCG was reconstituted at the required concentration in 1X PBS unless otherwise noted. Annexin V-APC was obtained from BD Biosciences (Franklin Lakes, NJ, USA). α-smooth muscle actin rabbit monoclonal (α-SMA, clone E184) was purchased from Abcam (Cambridge, MA, USA). Anti-Gr-1 and anti-F4/80 antibodies were purchased from eBioscience (San Diego, CA, USA). For immunohistochemical staining the Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA, USA).

Cell culture

Primary cell cultures were initiated by removing a 3 by 3 cm section of dorsal skin from 3 female C57BL/6 mice per experiment (Charles River Laboratories Wilmington, MA, USA, 8 week-old, 20-22 g). Before excision, hair was shaved and skin disinfected with povidone-iodine. Dermis was mechanically separated and placed in a 0.1% gelatin coated 6-well plate. After 15 minutes, cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% Fetal Bovine Serum (FBS), penicillin, and streptomycin at 37°C in 5% CO2. Cells at passage 6 were seeded into culture dishes and tested with various concentrations of EGCG.

Cell apoptosis

Apoptotic cells were detected by flow cytometry using Annexin V-APC and propidium iodide. Cells were seeded in 100-mm dishes, and 0, 1.0, 10, 100, 200, and 400 μg/ml of EGCG in culture medium were added for 48 hours. The cells were then trypsinized and 1×106 cells were washed in ice-cold azide free flow buffer (1X PBS, 1% BSA, 25 mM CaCl2). Cells where then stained with Annexin V-APC and 1 μg/ml of propidium iodide in 1X binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM CaCl2), on ice, and in the dark for 15 minutes. Cells were immediately analysed on an LSR II flow cytomter (BD Biosciences) equipped with 488 nm, 633 nm laser excitation and appropriate filters. 10,000 cells were collected per sample.

Flow cytometry

Flow cytometry was performed at the Shriners Hospital for Children in Cincinnati Flow Cytometry Core Facility. All cytometry, with the exception of DNA cell cycle determinations, was performed on an LSRII flow cytomter (BD Biosciences). The 488 and 633 nm lasers on LSR II were utilized and fluorescence was collected with appropriate filters. The data obtained from the LSR II were analyzed using Diva Software v5.0.2 (BD Bioscience). For DNA cell cycle
measures, data were collected on a Coulter Epics XL system (Beckman-Coulter, Fullerton, CA, USA) with 488 nm excitation with System II software (v3) as described previously. Listmode files were analyzed using ModFit v3.1 (Verity Software House, Inc., Topsham, ME, USA) to determine quantities of cells in G0/ G1, S, and G2/M phases of the cell cycle. At least 10,000 cells were measured per sample.

Immunohistochemistry

Immunohistochemical staining was used to detect alpha smooth muscle actin (α-SMA). Cells were grown to 80% confluence with each concentration of EGCG in 24 well culture dishes (six wells/sample), and fixed with 4% paraformaldehyde. Cells were stained with the Vectastain ABC kit according to manufacturer’s directions. Briefly, after washes with PBS, cells were incubated in 0.5% H2O2 in MeOH for 30 minutes and then washed with 1X PBS. Samples were stained with primary antibodies to rabbit monoclonal anti-α-SMA for 1 hour. Anti-mouse biotinylated secondary antibodies were added followed by 1 hour incubation before the strepavidin-HRP was applied for 30 minutes at room temperature. All samples were visualized with 3,3’-diaminobenzidine colorimetric substrate (DAB). Wound tissues, 8 samples per treatment, were fixed with 10% paraformaldehyde in PBS, embedded in paraffin, and cut into three 5-6 μm slices. Sections where then washed with xylene to remove paraffin. The sections were stained with anti-mouse monoclonal Gr-1 anti-mouse monoclonal F4/80 and anti-rabbit smooth muscle actin.

Migration assay

Cells were seeded into 24-well plates at a density of 1×10⁶ cells per well dish, allowed to attach overnight, and then treated with culture medium containing various concentrations of EGCG for 48 hours using 6 samples per concentration. Cell monolayers were then wounded by making a scratch with a sterile 200 μl pipet tip perpendicular to the bottom of the plate. After fixation with 4% paraformaldehyde, cells were stained with hematoxylin & eosin and migration distance measured by light microscopy.

Proliferation assay

For cell-cycle analysis, flow cytometry was performed as previously described by Osterburg et al. Briefly, cells were seeded into duplicate 100-mm dishes at a density of 1×10⁶ cells per dish, allowed to attach overnight, and then treated with 0, 1.0, 10, and 100 μg/ml EGCG in culture medium for 48 hours. Cells were harvested using trypsin and fixed with 90% methanol over night at -20°C. After washing 2X with flow buffer (1X PBS, 1% BSA, 25 mM CaCl2, and 0.1% sodium azide), cells were incubated in propidium iodide staining solution (50 μg PI/mL, 0.1% Triton X-100, 25μg RNase A, 1X PBS) for 30 minutes and analyzed.

Wound healing model

Specific pathogen-free C57BL/6 mice (Charles River, 8-12 week-old, female, 20-22 grams) were purchased from Charles River Laboratories (Wilmington, MA, USA). All animal procedures were approved by the University of Cincinnati’s Institutional Animal Care and Use Committees (IACUC). Eight mice were used for each experiment and the experiments were repeated for consistency. Following hair removal from the dorsal surface, the skin was disinfected and two full-thickness skin wounds were created on each side of the midline using 4-mm biopsy punch. Then 100 μl of EGCG in PBS (0, 0.3, 3.0, and 30 mg/ml) was applied to the wound surface of the right side, and 100 μl of PBS as a control was added to the left side. Each wound was individually covered with a rectangle Theragauze (Soluble Systems, VA, USA) which was sutured into place. Sterile gauze was placed over the Theragauze and both were covered with Opsite (Smith & Nephew Medical Limited, Hull, United Kingdom). Finally, the dressings and wound site were covered with Coban (3M, US). Concentrations of EGCG or PBS were loaded into the Theragauze dressing every 24-hours by injecting through the Opsite using 1 mm syringe. For planimetric and immunohistological wound assessments, tissue biopsies were taken from the wound area and the surrounding normal tissue at 48 hours and 1 week after wounding.

Statistics

Experiments were performed in triplicate, and results are expressed as means ± SE. Data were analyzed using Student’s t-test, ANOVA or Kruskal-Wallis One-way Analysis of Variance. p-values <0.05 were considered to be significant. Kaplan-Meier, with multiple test corrections was utilized in wound with infection model.
RESULTS

Effects of EGCG on scratch assay with cultured fibroblasts

*In vitro* wounding assays (scratch assays) were performed to determine if EGCG altered cellular migration into the scratch. Confluent cultures were perturbed with a pipet tip and migration into the wound measured over a three-day period. The data in figure 1A demonstrate a dose-dependent effect EGCG (200µg/ml and 400µg/ml) with fewer cells appearing in the scratched area with these concentrations versus the lower concentrations of EGCG and the control. Figure 1B quantifies the size of the initial scratch over time. The data indicate that over time the higher doses of EGCG (400, 200, and 100 µg/ml) significantly (P<0.05) reduced the extent of cell migration into the scratch.

Inhibition of migration could be due to increased apoptosis of cells in culture. Therefore we examined the effects of EGCG on apoptosis in treated fibroblasts. Apoptosis was assayed by staining with Annexin V-APC and propidium iodide followed by analysis using flow cytometry. As demonstrated in Figure 2, at the 100 µg/ml dose of EGCG (p<0.003 vs. lower doses) there were increasing quantities of cells in early and late apoptosis/necrosis. The 0, 1 and 10 µg/ml doses of EGCG had near baseline percentages of both early and late apoptosis/necrosis. At the very highest dose of EGCG (400 µg/ml) there was a large increase (75.7% +/- 1.68 SE) in predominantly late apoptosis/necrosis. The reduction in early apoptosis at this quantity of EGCG is due to increased progression to late apoptosis/necrosis in this sample.

Decreased migration into the scratch wound could also be indicative of a decrease in cellular proliferation of the primary fibroblasts. In order to determine the effects of EGCG on cell cycle, cells were treated with various doses of EGCG, stained with propidium iodide, and analyzed by flow cytometry. Figure 3 indicates that cell cycle progression is altered at 100 µg/ml of EGCG. As can be seen in the figure the quantity of cells in G₀/G₁ are significantly increased (P<0.05) vs. the controls, and S and G₂/M phases were significantly decreased (P<0.05) vs. the controls. Lower doses of EGCG did not appear to have any effect on cell cycle kinetics.
Wound healing and Immunochemistry

Previously published studies suggest that EGCG inhibits the α-SMA production. We stained primary fibroblasts cultures with an anti-α-SMA and visualized them by immunohistochemistry. The data in Figure 4 indicate that as the dose of EGCG increases the quantity of α-SMA decreases. At the two highest doses of EGCG (200, and 400 µg/ml) α-SMA appears to be almost entirely absent. There are fewer cells at these doses but this does not account for the reduction in the quantity of α-SMA staining.

Next, a series of animal experiments were performed in which two full thickness excisional wounds were made with 4 mm punch biopsy probes on the backs of C57BL6 mice to determine if EGCG inhibited wound contraction and/or closure. The pictures in Figure 5A depicts the wounds after 7 days. The highest dose of EGCG (30 mg/ml) shows a clearly greater size of wound. The data in Figure 5B quantifies closure rates at day 2 and 7 post-wounding. A significant delay in closure/contraction at the 30 mg/ml is visible at day 7. The high dose of EGCG significantly inhibited closure/contraction 40.06% ± 8.63SE vs. 88.23% ± 5.30SE (p<0.009) for 30 mg/ml vs. 3.0 mg/ml, respectively. Likewise, there was an inhibition of closure/contraction for the 0.3 mg/ml EGCG (87.87% ± 3.46) dose vs. the 30 mg/ml dose (p<0.006).

We sought to determine if the decreased closure/contraction of the 30.0 mg/ml dosed wounds was due to decreased numbers of α-SMA expressing myofibroblasts. Figure 6 depicts the immunohistochemical staining of tissue sections from EGCG treated wounds 7 week post-wounding. There is a clear reduction in the α-SMA positive cells. The lower doses of EGCG do not appear to alter α-SMA staining vs. controls. We then sought to determine if there were changes in F4/80 macrophage and Ly-6G neutrophil populations in EGCG treated wounds. The data in Figure 7 indicate there are no differences in the extent and location of staining at day 2 or 7 for either F4/80 or Ly-6G.
Traumatic Wound with Infection model

Our combined in vitro results (antibacterial and wound healing neutral) culminated with an in vivo application of EGCG to an infected murine wound. The results were far superior to vehicle control, approaching results obtained with Ciprofloxacin. While data in these studies were not put through the same battery of analysis as in wound closure model, we saw no sign that wound closure was inhibited. Tracing data indicated no closure at 7 days whether treated with Ciprofloxacin, EGCG, or PBS.

DISCUSSION

The rise in antibiotic resistant microorganisms has renewed the interest in agents that can supplement current antibiotics. Recent work demonstrates that polyphenol EGCG has significant antimicrobial activity against a number of microorganisms.\textsuperscript{13,32-34} Furthermore, microorganisms with resistance to systemic antibiotics have increased resistance to many topical antimicrobial treatments currently in use.\textsuperscript{35} There is a need to evaluate the efficacy of topical agents that can be used to supplement or enhance current systemic antimicrobial therapies. In the clinical arena, especially following severe trauma, both wound healing and infections are major concerns. Our laboratory has reported that EGCG exhibits broad anti-microbial activity especially against multi-drug resistant organisms such as A. baumannii.\textsuperscript{31} We found that the minimum inhibitory concentration of EGCG against A. baumannii is ~300 µg/ml, and additionally, against clinical isolates of Pseudomonas aeruginosa and MRSA MICs, ~600 µg/ml and ~60 µg/ml, respectively.\textsuperscript{36} To use EGCG as a topical anti-microbial agent on wounds or burns, it should have limited deleterious effects on wound healing. In this study, we examined the effects of EGCG on migration, apoptosis, proliferation and alpha-smooth muscle actin (α-SMA) expression in cultured primary mouse fibroblasts in vitro. Murine wound closure/contraction was also investigated following a full-thickness excisional wound made on the dorsal surface of C57BL6 mice. EGCG was applied in vivo at various concentrations with wound closure, α-SMA, inflammation and granulation tissue formation monitored. It was important to determine not only the effects of EGCG on wound healing but also its effects at concentrations that are both anti-microbial and within the concentrations that may be used clinically.

EGCG significantly inhibited migration and proliferation of fibroblasts using an in vitro scratch assay. This is in agreement with data published by Park et al.\textsuperscript{32} who reported that EGCG inhibited proliferation and migration of keloid and to lesser extent normal fibroblasts in vitro. We also demonstrated that at higher doses of EGCG (100 µg/ml and above) treated fibroblasts demonstrate a statistically significant (P<0.05) increase in the quantity of cells in G₂/M, compared to controls indicating that a smaller fraction of cells were dividing. Absence of cell proliferation is one explanation for the lack of closure of the scratch wound. Additionally, EGCG may have induced programmed cell death in cultured primary fibroblasts. Our studies indicated that apoptosis was significantly increased at high dosages of EGCG. These data indicate that both alterations in cell cycle progression and increased apoptosis are responsible for the reduction in fibroblast proliferation following treatment with EGCG. Increased EGCG-dependent apoptosis of transformed cells is well known. Additionally, EGCG has been reported to increase apoptosis in normal non-transformed cells.\textsuperscript{34,36}

Myofibroblasts produce contractile forces in the wound and promote wound closure. We examined the effects of EGCG on the expression of α-SMA in the wound margins. EGCG could reduce recruitment of fibroblasts/myofibroblasts from circulating bone marrow derived cells. This opens the possibility that EGCG is anti-scarring. However, Barisic-Dujmovic et al. demonstrated that myofibroblasts within the wound are derived from resident fibroblasts.\textsuperscript{35} EGCG has been previously demonstrated to inhibit the differentiation of fibroblasts into myofibroblasts.\textsuperscript{26} In vitro, there was a significant reduction in α-SMA staining at the higher doses of EGCG. In culture, EGCG has been shown to inhibit transforming growth factor beta-receptor signaling as well as inhibiting AP-1 and NF-κB in fibroblasts.\textsuperscript{26} In the animal wound, this would be consistent with our observations of reduced α-SMA staining in sections from the wounds treated with 30 mg/ml EGCG.

The in vivo wound model used in these studies clearly indicated that only at very high concentrations EGCG delayed wound contraction/closure. This is in contrast to several studies that reported that EGCG enhanced wound closure.\textsuperscript{37,32} Kim et al. implanted collagen sponges impregnated with EGCG, and that at low doses (10 ppm), healing was observed in diabetic wounds.\textsuperscript{33} Park et al. examine keloid formation in vivo with and without EGCG.\textsuperscript{32} These are very different wound healing models and while these data suggest an effect of EGCG at low doses, the studies involve the response of a diabetic pathology and not the direct effect of EGCG on normal wound healing. Our group examined the inflammatory infiltrate in EGCG and non-treated tissues and found no difference, indicating the observed differences were not due to alterations in inflammation.

The data presented here demonstrate that concentrations of EGCG used for in vitro and in vivo assays are not directly comparable and thus vary in the amounts necessary to produce biological function. For example, the in vitro data indicated that there are changes in migration, proliferation and apoptosis at EGCG concentrations of 0.1-0.4 mg/ml while the in vivo data indicated that a significantly higher dose of EGCG was needed to alter wound closure (Figure 5A/B). Our laboratory demonstrated that EGCG was bactericidal at concentrations of 0.300 mg/ml. This dose would clearly fall into the concentration that inhibited apoptosis, migration and proliferation in vivo. However, the 0.300 mg/ml concentration had little impact on the degree of wound closure/contraction.
in vivo. Caution should be exercised in evaluating in vitro evidence to interpret the effects of EGCG in vivo. We clearly show that the dose of EGCG that inhibits negatively cell cycle, apoptosis, and scratch wound closure is much lower than the dose required in vivo. The discrepancy between in vitro and in vivo data suggests that cells are directly exposed to EGCG in culture systems in contrast to in vivo exposures in which tissues are more likely exposed to metabolized products of EGCG rather than intact EGCG. Additionally, the wound is a complex environment and the dose of EGCG that cells see may highly depend on the microenvironment of the wound. As a topical agent, EGCG warrants further investigation into its properties in the context of healing and infection control.

In conclusion, the detrimental effects of EGCG on wound closure appear minor, with reduced closure only at high doses. However the antimicrobial effects of EGCG are impressive and the well-known safety of external and internal use of EGCG suggests that further investigations into its use to supplement and treat topical lesions are warranted.

ACKNOWLEDGEMENTS

We would also like to thank Sandy Schwemberger MT and the Shriners Flow Cytometry Core Facility for their assistance in performing the flow cytometry. We would also like to thank Deanna Leslie ASCP HT and the Shriners Hospital for Childrens Microscopy Core for assistance in the preparation of tissue sections. Finally, we wish to thank Karen Domenico and Alexandra Bowles for help with editing and formatting. This study was supported in part by Shriners Hospitals for Children.

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