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Anti-septic activity of α-cubebenoate isolated from *Schisandra chinensis*

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Sepsis is a life-threatening, infectious, systemic inflammatory disease. In this study, we investigated the therapeutic effect of α-cubebenoate, a novel compound isolated from *Schisandra chinensis* against polymicrobial sepsis in a cecal ligation and puncture (CLP) experimental model. Administration of α-cubebenoate strongly enhanced survival in the CLP model. α-cubebenoate administration also markedly blocked CLP-induced lung inflammation and increased bactericidal activity by enhancing phagocytic activity and hydrogen peroxide generation in mouse bone marrow-derived macrophages and neutrophils. Expression of two important inflammatory cytokines, IL-1β and IL-6, was strongly increased in the CLP model, and this was dramatically blocked by α-cubebenoate. Lymphocyte apoptosis and caspase-3 activation, which are associated with immune paralysis during sepsis, were markedly attenuated by α-cubebenoate. Taken together, our findings indicate that α-cubebenoate, a natural compound isolated from *Schisandra chinensis*, is a powerful potential anti-septic agent. [BMB Reports 2015; 48(6): 336-341]

INTRODUCTION

Sepsis is a life-threatening, infection-induced systematic inflammatory disease (1). Approximately 800,000 cases of sepsis are estimated to occur annually in the USA with a mortality rate of about 27% (2, 3) and estimated cost of over $17 billion per year to treat (4). Despite the severity of this medical problem, there are no effective therapeutics against sepsis. Withdrawal of the only USA-FDA approved anti-septic drug, Xigris® in 2011, has spurred research groups to focus on new therapeutics against sepsis.

With regard to the pathogenesis of sepsis, failure to clear infecting microorganisms can progress to systemic inflammatory response syndrome, leading to multi-organ failure and septic shock (1, 5). Phagocytic cells such as macrophages and neutrophils play key roles in the innate immune response against invading pathogens (6). These cells effectively remove pathogens by engulfing the pathogens and releasing reactive oxygen species such as hydrogen peroxide (6). Infection-induced inflammatory response is mediated by the production of proinflammatory cytokines from inflammatory cells such as macro phages. Excessive production of proinflammatory mediators contributes to lymphocyte apoptosis, which results in immune paralysis (7). Based on the pathological progress of sepsis, effective therapeutics against sepsis should show bactericidal activity, be able to modulate the expression of cytokines, and show anti-apoptotic activity.

Dried fruits of *Schisandra chinensis* (*S. chinensis*) ("five-flavored berry") have been reported to contain diverse pharmacologically active compounds, including C18 dibenzocyclooctadiene lignans (8-10). Some of these lignans have been demonstrated to have anti-oxidative, anti-cancer, and anti-inflammatory effects (8-10). Recently, we identified a novel compound, α-cubebenoate, from *S. chinensis* (11). We found that α-cubebenoate had an anti-inflammatory effect by blocking lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 expression in macrophages (11). In this study, we investigated the *in vivo* therapeutic effect of α-cubebenoate against polymicrobial sepsis in a cecal ligation and puncture (CLP) model. We also determined the mechanisms underlying the anti-septic effects of α-cubebenoate.

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RESULTS

α-Cubebenoate administration has a therapeutic effect against polymicrobial sepsis

To investigate the effects of α-cubebenoate against polymicrobial sepsis, we used a CLP sepsis model. CLP surgery markedly decreased survival rate within 2 days, with a survival rate of approximately 14% (Fig. 1A). α-Cubebenoate administration strongly increased survival rate in a dose-dependent manner, with 57% survival observed at 15 mg/kg (Fig. 1A). Previous studies have reported that mortality in sepsis is closely associated with lung inflammation (12, 13). In this study, we also examined lung inflammation using hematoxylin and eosin staining after CLP. CLP surgery markedly induced lung inflammation, which was almost completely blocked by α-cubebenoate administration (Fig. 1B). Because septic patients receive antibiotics in a clinical setting, we tested the effect of α-cubebenoate against sepsis in the presence of antibiotics (gentamycin + cephalosporin). α-Cubebenoate administration enhanced survival rate even further in the presence of antibiotics than in their absence (Fig. 1C).

α-Cubebenoate administration induces bactericidal activity via stimulation of phagocytosis and reactive oxygen species generation

CLP surgery increases the bacterial burden in peritoneal cavity. Because it is important to control bacterial burden in peritoneal cavity to control polymicrobial sepsis induced by CLP surgery, we investigated the effect of α-cubebenoate on the number of CFUs in peritoneal fluid at 24 h after CLP. As shown in Fig. 2A, α-cubebenoate administration strongly reduced the number of CFUs in peritoneal fluid. Bacterial killing activity is mediated by stimulation of phagocytosis from phagocytic cells such as neutrophils (6, 14). Stimulation of mouse bone marrow-derived macrophages with α-cubebenoate enhanced phagocytic activity (Fig. 2B). Hydrogen peroxide is an important weapon against invading pathogens (6). We therefore tested the effect of α-cubebenoate on hydrogen peroxide production from mouse neutrophils. As shown in Fig. 2C, stimulation of neutrophils with α-cubebenoate significantly enhanced hydrogen peroxide production. These results suggest that α-cubebenoate stimulates bacterial killing activity via phagocytosis and hydrogen peroxide generation from mouse bone marrow-derived macrophages and neutrophils.

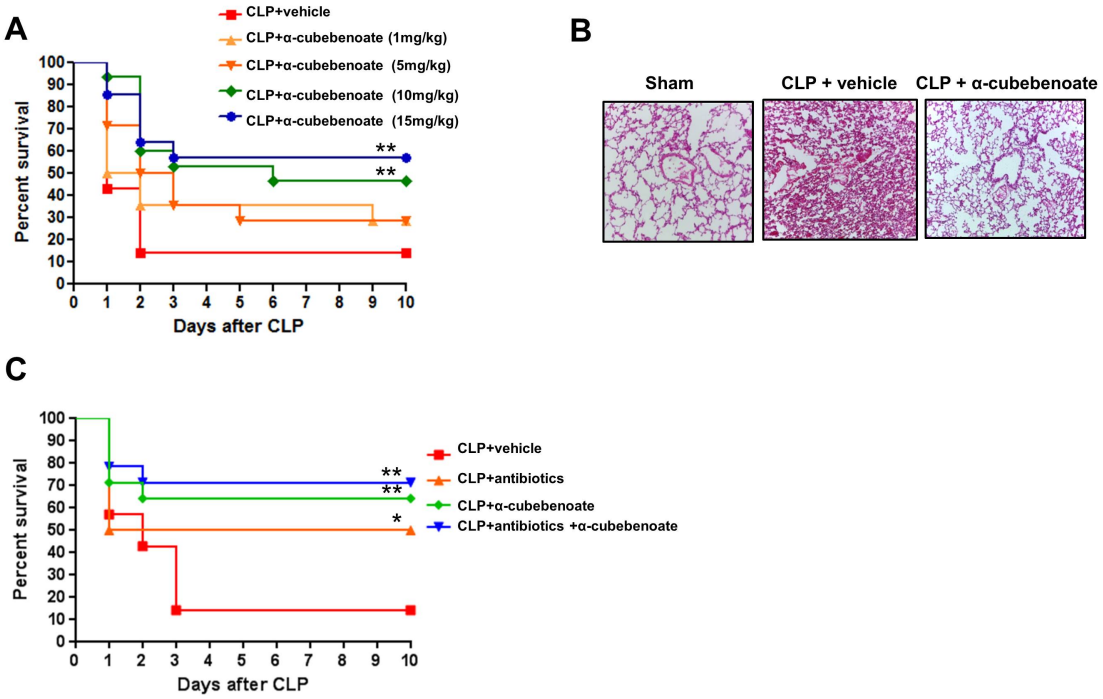

Fig. 1. α-Cubebenoate has therapeutic effects against CLP-induced sepsis. Several doses (0, 1, 5, 10, 15 mg/kg) of α-cubebenoate were injected subcutaneously into CLP mice at 2, 14, 26, and 38 h after CLP surgery (A). Vehicle (0.8% DMSO in PBS) or α-cubebenoate (15 mg/kg) was administered at 2 and 14 h after CLP. Mice were sacrificed 24 h after CLP surgery and lungs were stained with hematoxylin and eosin (magnification, ×100) (B). Vehicle (0.8% DMSO in PBS), α-cubebenoate (15 mg/kg), antibiotics (10 mg/kg gentamycin plus 10 mg/kg cephalosporin), or α-cubebenoate plus antibiotics were injected subcutaneously into CLP mice at 2, 14, 26, and 38 h post-CLP (C). *P < 0.05, **P < 0.01 compared to CLP plus vehicle control (A, C). Sample size: n = 14 mice/group (A) or 10 mice/group (C). Data are representative of eight mice per group (B).

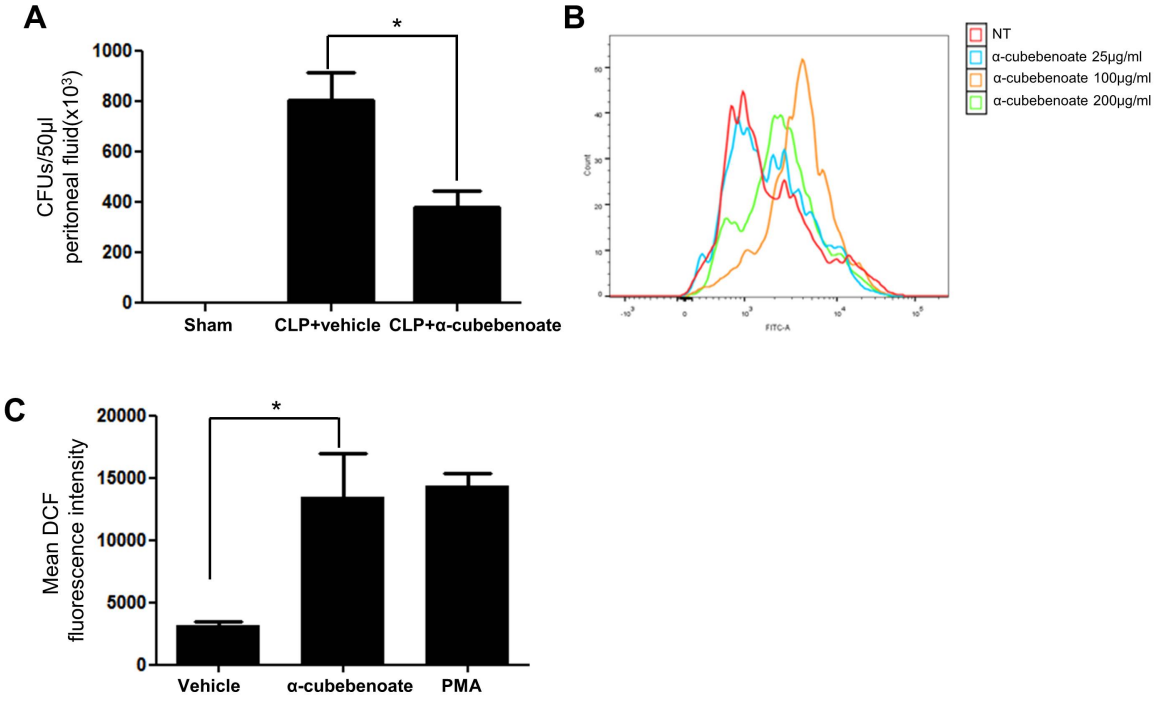

Fig. 2. α-Cubebenoate stimulates bactericidal activity. (A) Vehicle (0.8% DMSO in PBS) or α-cubebenoate (15 mg/kg) was injected into CLP mice 2 and 14 h after CLP surgery. Peritoneal lavage fluid collected 24 h after sham operation, CLP, or CLP plus α-cubebenoate administration was cultured overnight on blood-agar base plates at 37°C, and CFUs were determined. (B) Mouse bone marrow-derived macrophages (2×10^5) were resuspended in 200 μl PBS and preincubated with or without α-cubebenoate for 30 min. Then, cells were incubated with FITC-dextran (1 mg/ml) at 37°C for 30 min. After fixing cells, phagocytic uptake was analyzed on a flow cytometer. The result is representative of three independent experiments. (C) Mouse neutrophils were stimulated with vehicle (0.1% DMSO in PBS) or α-cubebenoate (100 μg/ml) for 15 min. The amount of hydrogen peroxide produced from mouse neutrophils was measured using a flow cytometer. Data are expressed as mean ± SD; n = 5 (A), 3 (C). *$P < 0.05$ (A, C)

α-Cubebenoate administration decreases proinflammatory cytokine production in a CLP sepsis model

Sepsis is an infectious inflammatory disease (1, 15, 16). Infection by pathogenic microorganisms stimulates production of excessive amounts of proinflammatory cytokines, such as IL-1β and IL-6 (1). We found that CLP surgery strongly induced the production of proinflammatory cytokines at 24 h after surgery (Fig. 3A, B). However, α-cubebenoate administration strongly decreased the production of the proinflammatory cytokines IL-1β and IL-6 (Fig. 3A, B). In particular, the level of IL-1β returned to sham levels in response to α-cubebenoate administration (Fig. 3A). These results demonstrate that α-cubebenoate administration has an anti-inflammatory effect in polymicrobial sepsis.

We also tested the effect of α-cubebenoate on LPS-induced cytokine production by mouse splenocytes. Stimulation of mouse splenocytes with LPS induced the production of the pro-inflammatory cytokines such as IL-1β and IL-6 (Fig. 3C, D). Preincubation of mouse splenocytes with α-cubebenoate prior to LPS stimulation blocked the production of these cytokines (Fig. 3C, D). Since LPS stimulates the production of proinflammatory cytokine such as IL-6 via the activation of NF-κB (17, 18), and IκB-α degradation is associated with NF-κB activation (19), we tested the effect of α-cubebenoate on the LPS-stimulated NF-κB activity by monitoring IκB-α degradation. LPS stimulated IκB-α degradation in mouse splenocytes, which was markedly blocked by α-cubebenoate (Fig. 3E). These findings indicate that α-cubebenoate blocks the activation of TLR-induced signaling such as NF-κB, thereby decreasing the expression of inflammatory cytokines.

α-Cubebenoate administration blocks lymphocyte apoptosis in the spleen

Lymphocyte apoptosis is closely associated with the pathological process of sepsis (1, 20). We found that CLP surgery was a strong inducer of lymphocyte apoptosis in the spleen (Fig. 4A). Lymphocyte apoptosis in the spleen was markedly reduced by α-cubebenoate administration (Fig. 4A), suggesting that α-cubebenoate has anti-apoptotic activity against polymicrobial sepsis. The numbers of apoptotic cells were quantified by counting TUNEL-positive cells (Fig. 4B). α-Cubebenoate administration strongly decreased apoptotic cell number

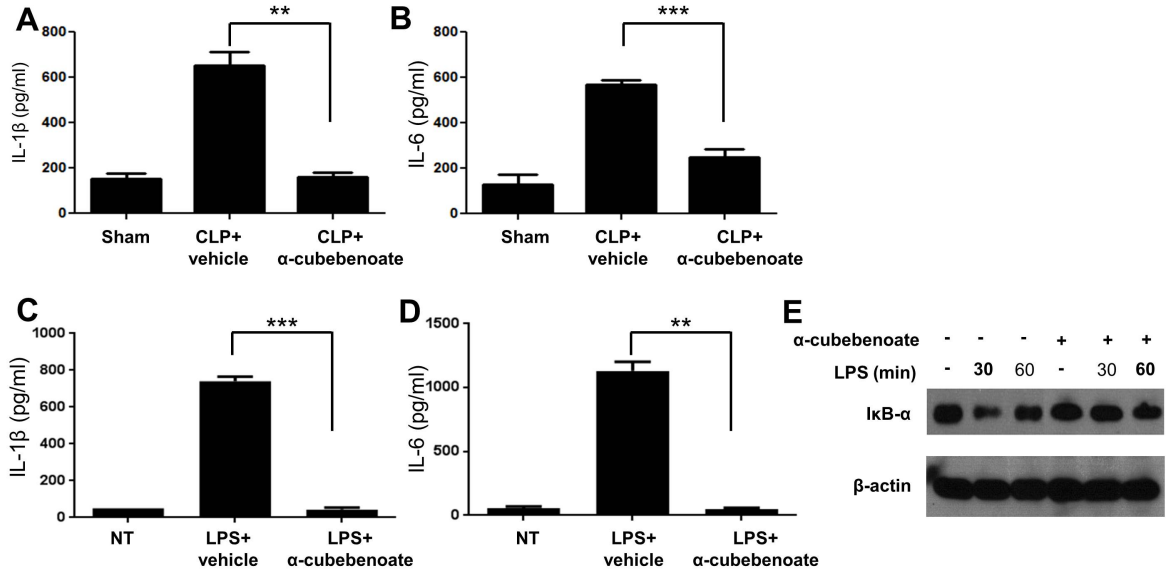

Fig. 3. Effects of α-cubebenoate on CLP- or LPS-induced cytokine production. (A, B) Vehicle (0.8% DMSO in PBS) or α-cubebenoate (15 mg/kg) was injected into CLP mice at 2 and 14 h after CLP surgery. Separate groups of animals were subjected to sham, CLP plus vehicle, or CLP plus α-cubebenoate treatment. Peritoneal fluid was collected at 24 h after CLP. Cytokine levels in peritoneal fluid were determined by ELISA analysis. Panels A, B display results for IL-1β and IL-6 respectively. (C, D) Mouse splenocytes were stimulated with PBS or LPS (100 ng/ml). After 15 min, cells were stimulated with vehicle (0.1% DMSO in PBS) or α-cubebenoate (100 μg/ml) for 4 h. IL-1β (C) and IL-6 (D) levels were measured by ELISA. Data are expressed as mean ± SD (n = 8). **P < 0.01 ***P < 0.001 (A-D). Mouse splenocytes were stimulated with 100 ng/ml LPS in the absence or presence of α-cubebenoate (100 μg/ml) for 0, 30, or 60 min (E). The levels of Iκβ-α were determined by Western blot. Data are presented as representative of three independent experiments (E).

(Fig. 4B).

Lymphocyte apoptosis is mediated by the activation of caspase-3 (20-22). Consistent with this, caspase-3 activity was increased by CLP in our study, and showed a positive correlation with increased lymphocyte apoptosis in the spleen (Fig. 4B). α-Cubebenoate administration strongly decreased CLP surgery-induced caspase-3 activity (Fig. 4C).

DISCUSSION

Despite improvements in health care over the last two decades, the incidence and mortality rates of sepsis are still high (2, 3). The only anti-sepsis agent that was approved by the US FDA to treat sepsis, Xigris®, was withdrawn from the market due to failure to show effective therapeutic effects in clinical settings (23). In addition to determining the mechanisms involved in sepsis, development of therapeutic agents to treat sepsis is paramount. In this study, we demonstrated that a naturally occurring compound isolated from *S. chinensis*, α-cubebenoate, had a strong therapeutic effect against experimentally -induced polymicrobial sepsis. *S. chinensis* has been consumed by humans to enhance immunity against infections for a long period of time; our results provide the scientific rationale for the anti-infective activity of *S. chinensis*.

Sepsis is induced by infection by diverse microorganisms, leading to a systemic inflammatory response (1). Therefore, control of invading microorganism is essential for control of sepsis. Our experimental sepsis model involved CLP surgery to induce the release of microorganisms into the peritoneal cavity, resulting in bacterial dissemination via the blood circu lation. α-Cubebenoate had anti-septic activity, as evidenced by the decrease in bacterial CFUs in the CLP polymicrobial sepsis model after administration of α-cubebenoate (Fig. 2). Because α-cubebenoate strongly stimulated not only phagocytic activity but also hydrogen peroxide production from mouse bone marrow-derived macrophages and neutrophils, we reasoned that the bactericidal activity of α-cubebenoate was due to its enhancement of phagocytic cell activity. Therapeutic effect of α-cubebenoate was further enhanced in mice that received antibiotics versus α-cubebenoate alone or antibiotics alone (Fig. 1C). These results suggest that α-cubebenoate may have a different mode of action from that of antibiotics.

Because sepsis is a systemic inflammatory disease that is characterized by increased levels of proinflammatory cytokines, control of these proinflammatory cytokines is essential to counteract sepsis. In this study, we demonstrated that CLP-induced proinflammatory cytokine production was effectively reduced by α-cubebenoate administration (Fig. 3A). Based on the finding that α-cubebenoate also blocks the LPS-stimulated production of proinflammatory cytokines in

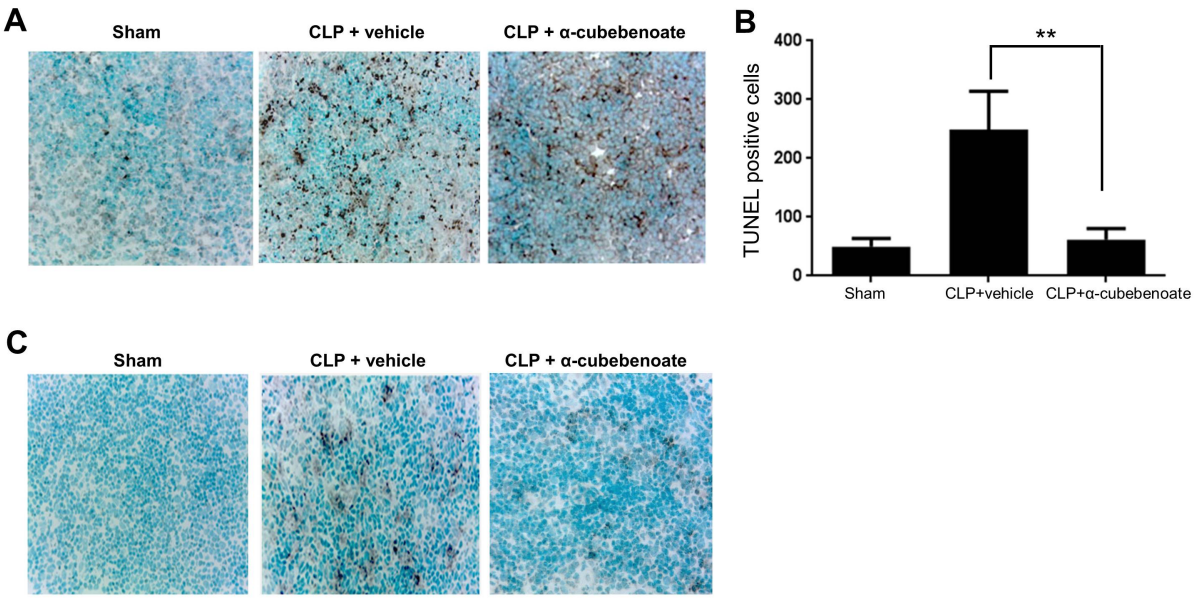

Fig. 4. α-Cubebenoate protects against CLP-induced apoptosis in the spleen. (A) Vehicle (0.8% DMSO in PBS) or α-cubebenoate (15 mg/kg) was injected into CLP mice 2 and 14 h after CLP surgery. Spleens were collected 24 h after sham, CLP plus vehicle, or CLP plus α-cubebenoate administration, and analyzed by TUNEL assay. (B) TUNEL-positive cells from the spleens of mice described in (A) were quantified. Data are expressed as mean ± SD (n = 8). **P < 0.01 (B) Spleens from the mice described in (A) were subjected to immunohistochemistry with cleaved-caspase-3 antibody (magnification, ×100). Data are representative of eight mice per group (A, C).

mouse splenocytes, we hypothesize that α-cubebenoate may lock the signaling pathway induced by LPS in mouse splenocytes. Because LPS-induced proinflammtory cytokine production is mediated by the activation of an important transcription factor, NF-κB (17, 18), we tested the effect of α-cubebenoate on the LPS-stimulated NF-κB activity by monitoring IκB-α degradation. The incubation of mouse splenocytes with α-cubebenoate prior to LPS stimulation markedly blocked LPS-induced IκB-α degradation (Fig. 3E). The result suggests that α-cubebenoate may block LPS-induced TLR4 signaling by inhibiting NF-κB activity. In a previous report, α-cubebenoate decreased LPS-stimulated PGE_2 production, COX-2 expression, and JNK activity in macrophages (11). Taken together, these findings suggest that α-cubebenoate blocks LPS-induced TLR4 signaling, resulting in inhibition of the production of several important inflammatory mediators such as IL-1β, IL-6, and PGE_2. Our results strongly indicate that a natural product isolated from *S. chinensis*, namely α-cubebenoate, may be a useful material to treat sepsis.

MATERIALS AND METHODS

Materials and Methods are described in the online data supplement, available at http://www.bmbreports.org/.

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