Received: 2016.11.22
Accepted: 2016.12.27
Published: 2017.07.12

Epigallocatechin Gallate Attenuates Hip Fracture-Induced Acute Lung Injury by Limiting Mitochondrial DNA (mtDNA) Release

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF **Xiao-dan Zhao**
AEFG **Hao Liu**
CDE **Tao Li**
CDEF **Quan Gong**
BCD **Wen-li Zhang**

Department of Orthopedics, West China Hospital of Sichuan University, Chengdu, Sichuan, P.R. China

Corresponding Author: Hao Liu, e-mail: 229554253@qq.com
Source of support: Departmental sources

Background: The aim of this study was to assess the protective effects and explore the mechanism of epigallocatechin gallate (EGCG) in hip fracture-induced acute lung injury.

Material/Methods: Thirty male Sprague-Dawley (SD) rats were randomly divided into the control group, hip fracture group, and hip fracture + EGCG (10 mg/Kg) group. After 24 h, blood samples, bronchoalveolar lavage fluid (BALF), and lung tissue were collected. Serum mitochondrial DNA (mtDNA) was measured by RT-PCR and BALF was used to perform cytological analysis and enzyme-linked immunosorbent assay (ELISA) assay. Lung tissue was used to evaluate the injury level.

Results: EGCG significantly reduced the hip fracture-induced high level of serum mtDNA ($p < 0.05$). HE staining showed protective effects of EGCG. Lower lung injury score and wet/dry ratio were identified in the hip fracture + EGCG group than in the hip fracture group ($p < 0.05$). We found significantly lower levels of infiltration of inflammatory cells and production of inflammatory cytokines in the BALF of the hip fracture + EGCG group than in the hip fracture group ($p < 0.05$).

Conclusions: Our study found that EGCG had protective effects on hip fracture-induced acute lung injury and suggests that EGCG exerts its protective effects through limiting the release of mtDNA. Our results provide a novel pharmacological agent to attenuate hip fracture-induced acute lung injury, as well as a potential theory to better explain the anti-inflammatory property of EGCG.

MeSH Keywords: **Acute Lung Injury • Genes, Mitochondrial • Hip Fractures**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/902477>

1744 — 4 25

Background

Acute lung injury is a severe complication of hip fracture patients, especially in elderly ones [1]. The overall in-hospital mortality of elderly patients is almost 5.0% [2]. However, the detailed intracellular biochemical and molecular signals remain only partially understood. Long bed rest used to be regarded as the major risk of acute lung injury after fracture [3]. Nevertheless, recent studies showed severe inflammatory responses may be responsible for the hip fracture-induced acute lung injury [4,5].

There has been increasing interest in studying the role of endogenous danger signals in inflammatory damages. Recent studies showed that the mitochondria, consisting of formyl peptides and mitochondrial DNA (mtDNA), can activate neutrophils and induce inflammation [6,7]. In addition, it was reported that the elevated mtDNA levels were related to the severity of systemic inflammatory response syndrome and secondary organ injuries in injured patients or animals [5–8]. Epigallocatechin gallate (EGCG) is a flavonoid belonging to the chemical class of falvan-3-ols esterified gallic acid present in green tea. Studies showed that EGCG possesses beneficial effects of this compound for organ protection due to its anti-inflammatory and anti-oxidant properties through enhancing mitochondrial function [9–12]. More importantly, Kasper et al. reported that EGCG has protective effects on inflammatory lung injury [13].

Given the above evidence, in the present study we studied the protective effects of EGCG on hip fracture-induced acute lung injury and explored a possible mechanism by which EGCG exerts its effects through limiting mtDNA release.

Material and Methods

Establishment of a hip fracture-induced lung injury model

Thirty male Sprague-Dawley (SD) rats (250–300g) were obtained from the Animal Center of the West China Medical Center (Chengdu, China). All rats were randomly divided into 3 groups: control group (N=10), hip fracture group (N=10), and hip fracture + EGCG group (N=10). Rats in the hip fracture and hip fracture + EGCG groups were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and fentanyl (0.05 mg/kg), and then placed in a prone position on the base of a blunt guillotine ramming apparatus. Aimed at the proximal femur, the 500 g blunt guillotine was lifted to a 15 cm height followed by free-fall along the axis, causing a unilateral closed hip fracture [5]. Rats in the hip fracture + EGCG group were peritoneally injected with EGCG (10 mg/Kg) at 10 min before the fracture was induced [13]. Rats in the control

group were received the same anesthesia only. After 24 h, all animals were sacrificed by cervical dislocation, followed by collection of blood and lung tissue samples. The study was performed in accordance with the guiding principles of the Bioethics Committee of West China Medical Center for the care and use of laboratory animals.

BALF collection and cytological study

For bronchoalveolar lavage fluid (BALF) collection, the left lung was lavaged 3 times with 2 ml of lavage buffer (PBS with 0.6 mm EDTA). The lavage was centrifuged at 1500 rpm for 10 min at 4°C, then the cell pellet at the bottom was re-suspended in 1 ml PBS. The total number of BALF cells was counted by a hemocytometer. Next, approximately 5×10^4 cells were placed onto glass slides. Afterwards, the slides were air-dried and stained with Giemsa, and at least 300 cells were counted under a light microscope for differential cell analysis. The supernatants were frozen immediately and stored at –80°C for enzyme-linked immunosorbent assay (ELISA) assay.

Serum mtDNA measurement by real time-PCR (RT-PCR)

The blood was centrifuged at 3000 rpm for 15 min at 4°C to collect serum. The whole DNA was isolated from serum by using the DNeasy Blood and Tissue Kit (#69504, Qiagen, Hilden, Germany). We used 100-μl serum samples to isolate the whole DNA. The procedures were performed carefully according to the manufacture's protocol. At the last step, 100 μl elution buffer were added to resolve DNA.

mtDNA levels were measured by SYBR-green dye-based RT-PCR assay using a PRISM 7300 sequence detection system. The primer sequences were human NADH dehydrogenase 1 gene (mtDNA): forward CGAGCAGTAGCCCAACAAT, reverse TGTGATAAGGGTGGAGAGGTT; rat NADH dehydrogenase 1 gene (mtDNA): forward CGCCTGACCAATAGCCATAA, reverse ATTCGACGTTAAAGCCTGAGA. Concentration of serum mtDNA were converted to copy number via a DNA copy number calculator (<http://cels.uri.edu/gsc/cndna.html>; University of Rhode Island Genomics and Sequencing Center). All samples were measured with standards at the same time. Serum mtDNA levels are shown in copies per microliter of serum according to the following formula:

$$c = Q \times V_{\text{DNA}} / V_{\text{PCR}} \times 1 / V_{\text{ext}}$$

Where c is the concentration of serum mtDNA (copies/μl); Q means quantity of DNA measured by RT-PCR; V_{DNA} means the total volume of serum DNA solution obtained from extraction, 100 μl in this study; V_{PCR} means the volume of serum DNA solution for RT-PCR, 1 μl in this study; V_{ext} means the volume of serum used for extraction, 100 μl in this study [14].

Pathological analysis of the lung tissue

Lung tissue was collected in all groups and stored in formalin for 24 h. Part of the lung tissue was embedded in paraffin. Tissue sections (5 μ m) were obtained and stained with hematoxylin and eosin (HE). Five slices were randomly selected from each rat and 5 fields of each slices were examined. Using the lung injury scoring system, the average score was calculated [15].

Lung wet/dry ratio was used as an index of pulmonary edema. The same portion of lung tissue (right middle lobe) was weighed to measure the wet weight and then placed in an 80°C dry oven for 2 days to get the dry weight.

Measurement of inflammatory cytokines

BALF inflammatory cytokines (TNF- α and IL-6) were measured by ELISA system (Elabscience, China).

Statistical analysis

All descriptive data are presented as mean $\pm$ SD. Statistical significance was evaluated by the *t* test and one-way ANOVA

Figure 1. EGCG attenuates hip fracture-induced elevated serum mtDNA level. Significantly increased mtDNA level in the hip fracture group ($p < 0.05$). EGCG treatment significantly decreased mtDNA ($p < 0.05$). * $p < 0.05$ vs. control group, # $p < 0.05$ vs. the hip fracture group, $N = 10$.

Figure 2. EGCG attenuates hip fracture-induced acute lung injury. (A–C) HE staining images of 3 groups are shown. (D) The lung injury scores in the hip fracture group were much higher than in the control and hip fracture + EGCG groups. (E) The higher ratio in the hip fracture group than in the control and hip fracture + EGCG groups. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. the hip fracture group, $N = 10$.

Figure 3. EGCG attenuates hip fracture-induced inflammatory cells infiltration in the BALF. Rats with EGCG pre-treatment all showed decreased numbers of total cells, monocyte/macrophage number, and neutrophile number compared with the hip fracture group. * $p < 0.05$ vs. control group, # $p < 0.05$ vs. hip fracture group, $N = 10$.

followed by the Bonferroni post hoc test. Differences were considered significant at the level of $p < 0.05$.

Results

EGCG attenuates hip fracture-induced elevated serum mtDNA level

Figure 1 shows the significant increase of mtDNA level in the hip fracture group ($p < 0.05$). More importantly, treatment with EGCG resulted in a significant decrease in mtDNA ($p < 0.05$). The data demonstrate that EGCG can effectively limit the release of mtDNA.

EGCG attenuates hip fracture-induced acute lung injury

HE staining images of lung tissue are shown in Figure 2A–C. Normal pulmonary histology was found in the control group, but the hip fracture group had excessive infiltration of inflammatory

cells and thickening of the alveolar wall. With the administration of EGCG, HE staining showed better lung condition compared to the hip fracture group. The lung injury scores in the hip fracture group were much higher than in the control and hip fracture + EGCG groups (Figure 2D, $p < 0.05$). Lung wet/dry ratio (Figure 2E) also had a higher ratio in the hip fracture group than in the control and hip fracture + EGCG groups ($p < 0.05$).

EGCG attenuates hip fracture-induced inflammatory cells infiltration in the BALF

We further detected inflammatory cells infiltration in the lung through cytological study of BALF. As shown in Figure 3, hip fracture resulted in significant increases in total cell count, monocyte/macrophage numbers, and neutrophile numbers ($p < 0.05$). As expected, rats with EGCG pre-treatment all showed decreased number of total cells, monocyte/macrophage numbers, and neutrophile numbers ($p < 0.05$).

Figure 4. EGCG attenuates hip fracture-induced inflammatory cytokines production in the BALF. Significant increases of inflammatory cytokines (TNF- α and IL-6) are shown in the hip fracture group, and pre-treatment with EGCG significantly inhibited the production of inflammatory cytokines ($p < 0.05$). * $p < 0.05$ vs. control group, # $p < 0.05$ vs. the hip fracture group, N=10.

EGCG attenuates hip fracture-induced inflammatory cytokines production in the BALF

Significant increases of inflammatory cytokines (TNF- α and IL-6) were shown in the hip fracture group ($p < 0.05$, Figure 4). Pre-treatment with EGCG showed significantly inhibited effects on the production of inflammatory cytokines ($p < 0.05$).

Discussion

In the present study, by studying the HE staining of lung tissue, cytological study, and BALF inflammatory cytokines analysis, we found protective effects of EGCG on hip fracture-induced acute lung injury. We also found that EGCG limited the release of mtDNA. Given the pro-inflammatory property of mtDNA, our results suggest that EGCG can attenuate hip fracture-induced acute lung injury through limiting the release of mtDNA.

It is well known that inflammatory responses are responsible for the development of acute lung injury after trauma [16,17]. In our study, we established the hip fracture-induced acute lung injury rat model without bacteria or endotoxin challenges. In the hip fracture group, significant lung injury and inflammatory responses were identified. Consistent with other studies, our results suggest that hip fracture can result in non-infectious inflammatory responses leading to acute lung injury [18]. Additionally, some studies reported mitochondrial oxidative damage in hip fracture-induced systemic inflammatory responses [19]. It is reported that mtDNA can be released after cells or mitochondria are insulted [20]. Further studies demonstrated that mtDNA-mediated activation of neutrophils and formation of neutrophil extracellular traps, as well as activation of the inflammatory signaling pathway,

may aggravate the inflammatory responses in several pathological conditions [5,21]. In the present study, we also confirmed elevated mtDNA levels after hip fracture. In agreement with other studies, we showed that elevated mtDNA levels play a key role in the development of hip fracture-induced acute lung injury through activating the Toll-like receptor-9 signaling pathway [5,6,22,23].

Interestingly, the administration of EGCG, the main constituent of green tea, remarkably attenuated lung injury and decreased inflammatory responses in lung tissue of subjects with hip fracture. Surprisingly, as an initiator of inflammatory responses, mtDNA level was also reduced by EGCG treatment. It is well known that EGCG has anti-inflammatory effects [24], but the detailed mechanism is still unclear. Recently, increasing numbers of studies have shown the anti-oxidative effects of EGCG, which inhibited endothelial dysfunction and formation of reactive oxygen species [9,15,25]. Therefore, our data suggest that the anti-oxidative effects of EGCG may be a mechanism by which it exerts anti-inflammatory effects.

Although we had some interesting results, some limitations need to be noted. First, in the present study, lack of a specific mtDNA antagonist made the interpretation of our data less clear. However, as an initiator of inflammatory responses, the reduction of mtDNA level had its own effects on the decreased inflammatory responses. Second, we did not further explore how EGCG inhibits the release of mtDNA. Finally, further studies are needed to prove the clinical preventive effects of EGCG on hip-fracture-induced lung injury, as well as to study the detailed mechanism of the protective effects of EGCG on hip fracture-induced acute lung injury.

Conclusions

We found that EGCG had protective effects on hip fracture-induced acute lung injury and that EGCG may have exerted its protective effects through limiting the release of mtDNA. Our results provide a novel pharmacological agent to attenuate hip fracture-induced acute lung injury and suggest a theory

that might help to better understand the anti-inflammatory property of EGCG, providing deeper insights into the possible pharmacological utilization of EGCG.

Conflict interest

None.

References:

1. Roche JJ, Wenn RT, Sahota O, Moran CG: Effect of comorbidities and post-operative complications on mortality after hip fracture in elderly people: Prospective observational cohort study. *BMJ*, 2005; 331: 1374
2. Alzahrani K, Gandhi R, Davis A, Mahomed N: In-hospital mortality following hip fracture care in southern Ontario. *Can J Surg*, 2010; 53: 294–98
3. Haleem S, Lutchman L, Mayahi R et al: Mortality following hip fracture: Trends and geographical variations over the last 40 years. *Injury*, 2008; 39: 1157–63
4. Sun T, Wang X, Liu Z et al: Plasma concentrations of pro- and anti-inflammatory cytokines and outcome prediction in elderly hip fracture patients. *Injury*, 2011; 42: 707–13
5. Gan L, Chen X, Sun T et al: The Significance of serum mtDNA concentration in lung injury induced by hip fracture. *Shock*, 2015; 44(1): 52–57
6. Zhang Q, Raoof M, Chen Y et al: Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature*, 2010; 464: 104–7
7. Sun S, Sursal T, Adibnia Y et al: Mitochondrial DAMPs increase endothelial permeability through neutrophil dependent and independent pathways. *PLoS One*, 2013; 8: e59989
8. Zhang JZ, Liu Z, Liu J et al: Mitochondrial DNA induces inflammation and increases TLR9/NF-kappaB expression in lung tissue. *Int J Mol Med*, 2014; 33: 817–24
9. Kim HS, Quon MJ, Kim JA: New insights into the mechanisms of polyphenols beyond antioxidant properties; Lessons from the green tea polyphenol, epigallocatechin 3-gallate. *Redox Biol*, 2014; 2: 187–95
10. Chowdhury A, Sarkar J, Chakraborti T et al: Protective role of epigallocatechin-3-gallate in health and disease: A perspective. *Biomed Pharmacother*, 2016; 78: 50–59
11. Oliveira MR, Nabavi SF, Daglia M et al: Epigallocatechin gallate and mitochondria-A story of life and death. *Pharmacol Res*, 2016; 104: 70–85
12. Pan H, Chen J, Shen K et al: Mitochondrial modulation by Epigallocatechin 3-Gallate ameliorates cisplatin induced renal injury through decreasing oxidative/nitritative stress, inflammation and NF-κB in mice. *PLoS One*, 2015; 10: e0124775
13. Kasper B, Salameh A, Krausch M et al: Epigallocatechin gallate attenuates cardiopulmonary bypass-associated lung injury. *J Surg Res*, 2016; 201: 313–25
14. Qin C, Gu J, Qian H et al: Potential mechanism of post-acute aortic dissection inflammatory responses: The role of mtDNA from activated platelets. *Cardiology*, 2016; 135: 228–35
15. Elbling L, Weiss RM, Teufelhofer O et al: Green tea extract and (–)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *FASEB J*, 2005; 19: 807–9
16. Hirsiger S, Simmen HP, Werner CM et al: Danger signals activating the immune response after trauma. *Mediators Inflamm*, 2012; 2012: 315941
17. Tiansheng S, Xiaobin C, Zhi L et al: Is damage control orthopedics essential for the management of bilateral femoral fractures associated or complicated with shock? An animal study. *J Trauma*, 2009; 67: 1402–11
18. Zhang H, Sun T, Liu Z et al: Systemic inflammatory responses and lung injury following hip fracture surgery increases susceptibility to infection in aged rats. *Mediators Inflamm*, 2013; 2013: 536435
19. Hauser CJ, Sursal T, Rodriguez EK et al: Mitochondrial damage associated molecular patterns from femoral reamings activate neutrophils through formyl peptide receptors and P44/42 MAP kinase. *J Orthopaed Trauma*, 2010; 24: 534–38
20. Mittra I, Nair NK, Mishra PK: Nucleic acids in circulation: Are they harmful to the host? *J Biosci*, 2012; 37: 301–12
21. Itagaki K, Kaczmarek E, Lee YT et al: Mitochondrial DNA released by trauma induces neutrophil extracellular traps. *PLoS One*, 2015; 10: e0120549
22. Gan L, Zhong J, Zhang R et al: The immediate intramedullary nailing surgery increased the mitochondrial DNA release that aggravated systemic inflammatory response and lung injury induced by elderly hip fracture. *Mediators Inflamm*, 2015; 2015: 587378
23. Huang H, Evankovich J, Yan W et al: Endogenous histones function as alarm-ins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology*, 2011; 54: 999–1008
24. Jhang JJ, Lu CC, Yen GC: Epigallocatechin gallate inhibits urate crystals-induced peritoneal inflammation in C57BL/6 mice. *Mol Nutr Food Res*, 2016; 60(10): 2297–303
25. Kim JA, Formoso G, Li Y et al: Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn. *J Biol Chem*, 2007; 282: 13736–45