

Upregulation and stabilization of senescence marker protein-30 by epigallocatechin gallate against *tert*-butyl hydroperoxide-induced liver injury *in vitro* and *in vivo*

Hirofumi Inoue,[#] Kohta Arakawa,[#] Miori Tanaka, Rie Katsumata-Tsuboi, Nobuyuki Takahashi, and Mariko Uehara*

Department of Nutritional Science and Food Safety, Faculty of Applied Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

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Senescence marker protein-30 (SMP30), a novel ageing marker, suppresses oxidative stress in the liver. However, studies on phytochemical-mediated regulation of SMP30 expression are lacking. Here, we showed that epigallocatechin gallate (EGCg), a polyphenol abundant in green tea, positively regulates SMP30 expression in the rat hepatoma-derived Fao cells. EGCg maintained SMP30 expression even in the presence of cycloheximide, a protein synthesis inhibitor. Furthermore, treatment of cells with *tert*-butyl hydroperoxide (*tert*-BHP), an oxidative promoter, decreased SMP30 expression and ERK1/2 phosphorylation, while EGCg treatment inhibited these effects. Male mice (7-week-old) were divided into 4 groups—Control (saline), *tert*-BHP (1.5 mmol/kg *tert*-BHP), EGCg + *tert*-BHP (30 mg/kg/day of EGCg and 1.5 mmol/kg *tert*-BHP), and EGCg (30 mg/kg/day). After oral EGCg administration for 6 consecutive days, EGCg + *tert*-BHP group mice were administered *tert*-BHP. The *tert*-BHP-administered mice showed decreased SMP30 expression in the liver and increased aspartate aminotransferase and alanine transaminase (hepatic injury marker enzymes) activities; however, EGCg treatment attenuated these changes. Thus, EGCg-induced SMP30 upregulation may alleviate *tert*-BHP-induced liver injury. The findings of this study offer new perspectives of the anti-ageing properties of EGCg.

Key Words: senescence marker protein-30, epigallocatechin gallate, *tert*-BHP-induced liver injury

Ageing is a physiological phenomenon in all living species characterized by a progressive loss of physiological integrity, leading to impaired body functions.^(1,2) Ageing is accompanied by an increased frequency of age-related disorders including cardiovascular disease, neurodegeneration, cancer, and osteoporosis.^(3–7) The fundamental mechanism of ageing is related to the process of cellular senescence. Senescent cells are considered to be in a state of irreversible growth arrest, although they remain metabolically active.⁽⁸⁾ Senescence is caused by increased oxidative stress, including hydrogen peroxide, genetic toxicity, and oncogene activation. Therefore, we must first understand how to suppress oxidative stress to understand the mechanisms driving ageing.

Senescence marker protein-30 (SMP30), also known as regucalcin, is a 34 kDa protein. The gene encoding SMP30 is located on the X chromosome and was originally discovered in the rat liver.⁽⁹⁾ The SMP30 is highly conserved in various species, including human, mouse, and rat, and is highly expressed in the liver and kidney.⁽¹⁰⁾ SMP30 exhibits gluconolactonase activity, involving it in L-ascorbic acid biosynthesis in the liver, while it also suppresses oxidative stress.^(11–13) In fact, acute oxidative stress

such as carbon tetrachloride treatment suppresses SMP30 expression in the liver.⁽¹⁴⁾ In addition, SMP30-knockout mice show increased reactive oxygen species (ROS) accumulation and a decreased lifespan, and exhibit distinct ageing-associated characteristics.⁽¹⁵⁾ In contrast, dietary restriction reduces oxidative stress, prolongs lifespan, and increases SMP30 expression.⁽¹⁶⁾ Handa *et al.*⁽¹⁷⁾ reported that SMP30 over-expression decreases ROS formation and antioxidant enzyme activities, including superoxide dismutase (SOD) and glutathione (GSH), in hepatic carcinoma HepG2 cells. Thus, SMP30 may play a role in anti-ageing by suppressing oxidative stress. However, to the best of our knowledge, there has been no research on the regulation of SMP30 expression using phytochemicals.

Tea and tea polyphenols have been reported to possess potent radical scavenging and anti-inflammatory effects.^(18,19) Specially, the most abundant polyphenol in green tea, (–)-epigallocatechin-3-gallate (EGCg), has diverse pharmacological activities, and is preventative against several chronic diseases including cancer, inflammation, diabetes, fatty liver disease, and neurodegenerative diseases.^(20–24) It also protects cells from ROS-induced injury.⁽²⁵⁾ EGCg could reduce oxidative stress and cell damage by increasing SMP30 expression. However, there is little knowledge about the effect of EGCg on SMP30 expression.

The aim of this study was to determine whether EGCg positively regulates SMP30 expression and prevents liver injury due to *tert*-butyl hydroperoxide (*tert*-BHP), an oxidative stress inducer in Fao cells (*in vitro*) and in mice (*in vivo*).

Materials and Methods

Reagents. EGCg (>98.0%) was purchased from Nacalai Tesque (Kyoto, Japan) for use in both *in vitro* and *in vivo* examinations. Cycloheximide (CHX) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *tert*-BHP and *N*-acetyl cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture. Fao rat liver cell line was cultured in DMEM/F-12 Ham's (Gibco BRL/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL/Invitrogen) at 37°C in 5% CO₂.

[#]Equally contributed first author.

*To whom correspondence should be addressed.

E-mail: mari@nodai.ac.jp

Cell viability assay. Fao cells (1.0×10^5 cells/well) were cultured in 96-well plates. Cells were treated with various concentrations of EGCg for 24 h. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan). The effect of EGCg on cell viability was expressed as percent cell viability, with that of cells treated with a methanol vehicle set at 100%.

Cytotoxicity evaluation (lactate dehydrogenase assay). Fao cells (1.0×10^5 cells/well) were cultured in 96-well plates. Cells were treated with various EGCg concentrations for 24 h. After 24 h, 50 μ l of culture medium from each well was collected and transferred to another 96-well culture plate. A total of 50 μ l of lactate dehydrogenase (LDH) reagent (DOJINDO, Kumamoto, Japan) was added to each well and the solution was incubated at room temperature for 30 min. After the reaction was complete, absorbance at 490 nm was measured using a microplate reader. The effect of EGCg on decreasing LDH release was expressed as a percent with *tert*-BHP-treated cells set at 100%.

Animal studies. This study was approved by the Tokyo University of Agriculture Animal Use Committee, and mice were maintained in accordance with the guidelines of the university for the care and use of laboratory animals. A total of 28 specific pathogen-free male ICR mice (7 weeks old, 32–34 g) were obtained from a commercial animal breeder (Japan SLC, Inc., Hamamatsu, Japan) and acclimated for one week in our facility prior to the experiment. All animals were housed in a controlled environment at $22 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ relative humidity under a 12/12 h light/dark cycle. All mice were fed the same amount of AIN-93G diet with ad-libitum access to tap water. Mice were randomly divided into four groups ($n = 7$ for each group)—Control (saline injection), *tert*-BHP (1.5 mmol/kg *tert*-BHP injection), EGCg + *tert*-BHP (30 mg/kg/day of EGCg and 1.5 mmol/kg *tert*-BHP injection), and EGCg (30 mg/kg/day of EGCg). EGCg and *tert*-BHP were dissolved in saline. Saline alone or saline with EGCg was administered by oral gavage to mice for six consecutive days before *tert*-BHP injection. At 24 h after the final oral administration of EGCg, severe acute liver injury was induced by intraperitoneally injecting 1.5 mmol/kg *tert*-BHP dissolved in saline. Saline alone was also intraperitoneally administered to the control and EGCg groups. Mice were sacrificed 24 h after intraperitoneal administration and whole blood was collected via an abdominal vein under three types of mixed anesthesia. The liver was removed and immediately weighed before fixing or storing at -80°C until downstream western blot analysis.

Western blot analysis. Liver homogenate was prepared by homogenizing 0.5 g of liver in 1.5 ml of RIPA buffer [10 mM Tris-HCl, pH 7.6, 150 mM sodium chloride, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 1.0% (w/v) NP-40 substitute]. Fao cells were washed twice with PBS and lysed with RIPA buffer. Samples were separated using SDS-PAGE before transfer to a PVDF membrane. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with 0.05% Tween-20 (TBST) before incubating with anti-SMP30 (Santa Cruz Biotechnology, Dallas, TX), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Danvers, MA), and β -actin (Santa Cruz Biotechnology) antibodies in TBST. Next, membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) in TBST before visualization using the EzWestLumi Plus (ATTO, Tokyo, Japan). Band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Aspartate aminotransferase and alanine transaminase measurement. Blood was collected from the inferior vena cava and serum was obtained by centrifuging at $3,000 \times g$ for 10 min at 4°C . Aspartate aminotransferase (AST) and alanine transaminase (ALT) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were used as biochemical markers of hepatotoxicity and were

determined using a commercially available spectrophotometric assay ($\lambda_{\text{max}} = 555$ nm) according to the manufacturer's instructions.

Statistical analysis. Results are presented as mean \pm SE. All data presented represent the mean of three independent experiments. Multiple comparisons were performed using Tukey's test or Tukey-Kramer's test after one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

EGCg regulated SMP30 expression in a dose-dependent manner. Fao cells were treated with EGCg at various concentrations, and cell viability was evaluated using the CCK-8 assay. As shown in Fig. 1A, EGCg did not induce toxicity in Fao cells. The results of this assay indicated that the maximum concentration of EGCg that can be used in our subsequent studies was 100 μM . Next, we examined SMP30 expression after EGCg treatment. Interestingly, EGCg treatment caused an increase in SMP30 expression in a dose-dependent manner until 50 μM , but the upregulation stopped at 100 μM (Fig. 1B). Subsequently, we used 30 μM of EGCg as the maximum concentration for further experiments.

EGCg promotes SMP30 stabilization. To determine whether EGCg treatment results in SMP30 stability, Fao cells were pre-treated with CHX, an inhibitor of protein synthesis. Cells were treated with EGCg or a vehicle and protein lysates were collected after 24 h. EGCg was able to promote SMP30 stabilization even in CHX-co-treated cells (Fig. 2A and B).

EGCg attenuates LDH release in *tert*-BHP-treated cells.

Next, we examined whether EGCg attenuates liver injury in *tert*-BHP-treated cells. LDH release, which is a marker of intracellular damage, was measured in Fao cells. Fao cells were pre-treated with EGCg to assess the protective effect of EGCg against *tert*-BHP induced cell damage. Although LDH release was observed after stimulation with *tert*-BHP treatment, LDH release was lower in the EGCg-treated cells than in the cells treated with *tert*-BHP alone (Fig. 3).

EGCg attenuates the downregulation of SMP30 and ERK1/2 phosphorylation in *tert*-BHP-treated cells. Previously, Jung *et al.*⁽¹⁶⁾ reported that downregulation of *SMP30* is likely involved in the ERK signaling pathway. In addition, *tert*-BHP induces ERK1/2 phosphorylation.⁽²⁶⁾ Thus, we examined whether EGCg treatment increases SMP30 expression and/or suppresses ERK1/2 phosphorylation in Fao cells. Interestingly, *tert*-BHP downregulated SMP30 expression and induced ERK1/2 phosphorylation, while EGCg pre-treatment attenuated these effects (Fig. 4A and B). Furthermore, decreased SMP30 expression is accompanied by increased ROS generation.⁽²⁷⁾ Therefore, we assessed whether NAC, a nitroxide antioxidant reagent, could attenuate the downregulation of SMP30 expression by *tert*-BHP treatment in Fao cells, since nitroxides are known to have high anti-oxidative activities.⁽²⁸⁾ The results indicated that SMP30 expression was downregulated in *tert*-BHP-treated Fao cells with 30 μM EGCg, while treatment with 0.5 mM NAC increased SMP30 expression (Fig. 4C and D).

EGCg attenuates the downregulation of SMP30 and liver injury in *tert*-BHP-administered mice. *tert*-BHP has been used to induce oxidative stress in previous *in vivo* and *in vitro* studies.^(29,30) As shown in Fig. 5A, we first examined the effect of EGCg oral administration (30 mg/kg/day) for 1 week on SMP30 expression and serum AST and ALT levels, which reflect hepatic functions, in *tert*-BHP-administered (1.5 mmol/kg) mice. There were no significant differences in food intake, final body weight, or organ weight between the control and the *tert*-BHP-administered mice with or without EGCg (Table 1). Hepatic SMP30 expression in the *tert*-BHP-administered group was decreased compared to that in the control group, while that in the EGCg oral administration group was unchanged (Fig. 5B). Subsequently, AST and ALT levels were higher in the *tert*-BHP

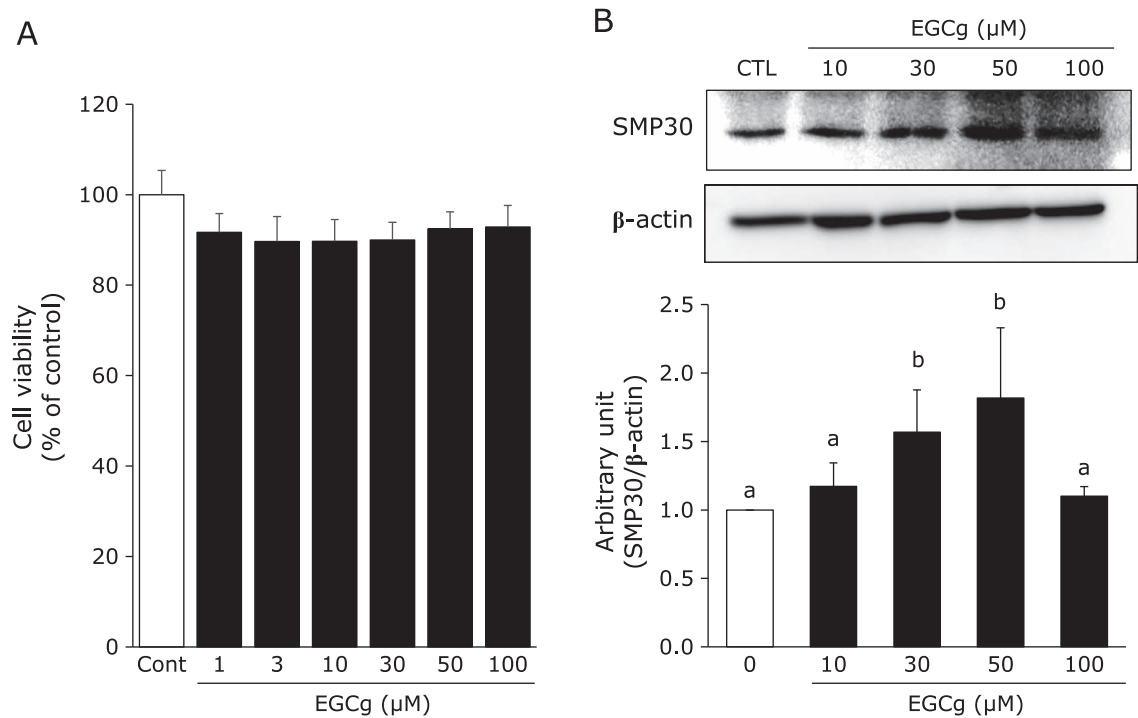


Fig. 1. Effects of EGCg on cell toxicity and SMP30 expression. (A) Cell viability of EGCg: Fao cells (1.0×10^5 cells/well) were cultured in a 96-well plate before treating with various EGCg concentrations for 24 h. Cell viability was assessed using the CCK-8 assay. Cell viability was expressed as a percentage of the values obtained for EGCg-untreated cells. (B) Protein expression of SMP30: Fao cells were treated with EGCg (0–100 μ M) for 24 h, and the cell lysate was immunoblotted with each indicated antibody. Densitometry quantification of SMP30 was performed using ImageJ. SMP30 levels were normalized to β -actin. Data are shown as the means \pm SE from at least three independent experiments. Unmatching letters denote significantly different, $p < 0.05$.

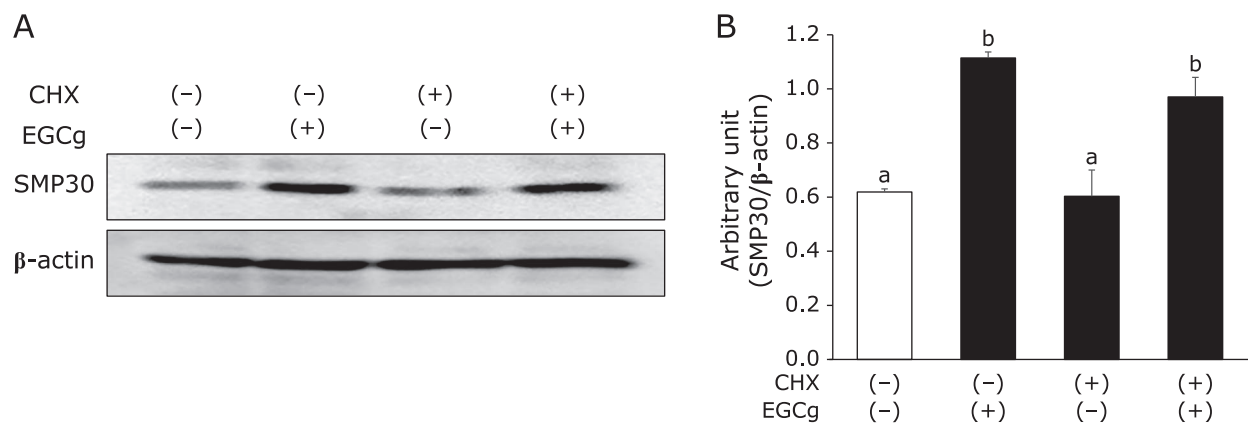


Fig. 2. Effects of EGCg on SMP30 up-regulation and stabilization in Fao cells. (A) Fao cells were pre-treated with or without CHX (10 mg/ml) for 0.5 h before stimulating with or without 30 μ M EGCg for 24 h. (B) Densitometry quantification was performed using ImageJ. SMP30 levels were normalized to β -actin. Data are shown as the means \pm SE from at least three independent experiments. Unmatching letters denote significantly different, $p < 0.05$.

group than in the control group, while EGCg oral administration significantly attenuated this increase (Fig. 5C and D). Importantly, in the EGCg group, liver injury did not occur and liver SMP30 expression was not increased.

Discussion

In the present study, we assessed whether EGCg positively regulates SMP30 expression and prevents liver injury *in vitro* and *in vivo*. Here, we indicated that Fao liver cell lines treated with EGCg have increased SMP30 expression. It has previously been

reported that *SMP30* expression is downregulated by increased ROS.^(14,27) In contrast, EGCg possesses free radical-scavenging activity and inhibits protein aggregation via proteasomal degradation.^(31,32) Here, we determined how EGCg upregulates SMP30 expression. In fact, we found that EGCg can upregulate SMP30 expression even when cells are treated with CHX, an inhibitor of protein synthesis. Therefore, these data propose that the ubiquitin-proteasome pathway is involved in regulating the turnover of SMP30 and that EGCg increases SMP30 stability via post-translational mechanisms, resulting in decreased proteasome-dependent protein degradation. To support this hypothesis, it was

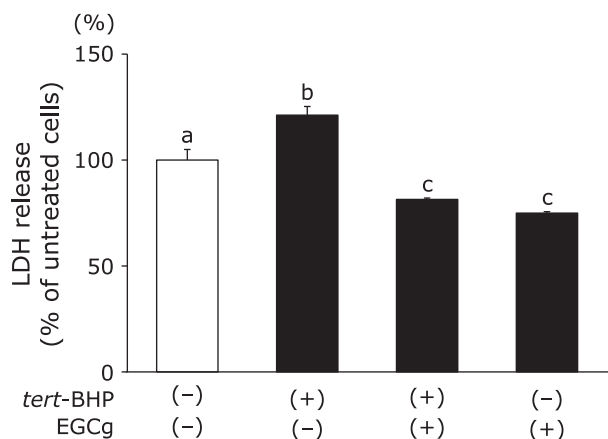


Fig. 3. Effects of EGCg on LDH release with *tert*-BHP-induced oxidative stress in Fao cells. Fao cells were pre-treated with 30 μ M EGCg for 8 h. After incubating with EGCg, cells were treated with 100 μ M *tert*-BHP with or without 30 μ M EGCg for 24 h. Quantitative LDH level analyses were performed in the cell culture supernatant. Data are shown as the means \pm SE from at least three independent experiments. Unmatching letters denote significantly different, $p < 0.05$.

reported that ester bond-containing green tea polyphenols, including EGCg, potentially inhibit cellular proteasome activity.⁽³³⁾ Thus, we suggest a novel mechanism in which EGCg acts to regulate SMP30 stabilization at the intracellular level.

As previously described, *tert*-BHP, an oxidant reagent, can induce acute liver injury.^(29,30) To confirm the positive effect of EGCg under oxidative stress conditions, SMP30 expression was measured in *tert*-BHP-treated Fao cells. We observed that EGCg treatment attenuated the downregulation of SMP30 expression in *tert*-BHP-treated cells.

Jung *et al.*⁽¹⁶⁾ reported that the downregulation of *SMP30* expression is likely involved in the ERK signaling pathway. Thus, we examined whether EGCg attenuated ERK1/2 activation and inhibited the downregulation of SMP30 expression due to *tert*-BHP treatment. In fact, our data suggested that EGCg upregulates SMP30 expression by inactivating ERK1/2 signaling. Interestingly, over-expression of SMP30 in HepG2 cells contributes to a marked decrease in ROS formation accompanied by decreased lipid peroxidation, SOD activity and GSH, whereas SMP30 itself does not exert radical scavenging ability.⁽¹⁷⁾ In contrast, SMP30 protects intestinal epithelial cells from inflammation-induced cell death by enhancing the activity of Nrf2, a key antioxidant molecule.⁽³⁴⁾ In addition, EGCg activates Nrf2 signaling and promotes antioxidant enzymes such as SOD and catalase to work against oxidative stress-induced hepatotoxicity *in vivo* and *in vitro*.⁽³⁵⁾ Therefore, we propose that EGCg may be involved in upregulating and stabilizing SMP30 and could act as an antioxidant signal activator in *tert*-BHP-treated liver cells.

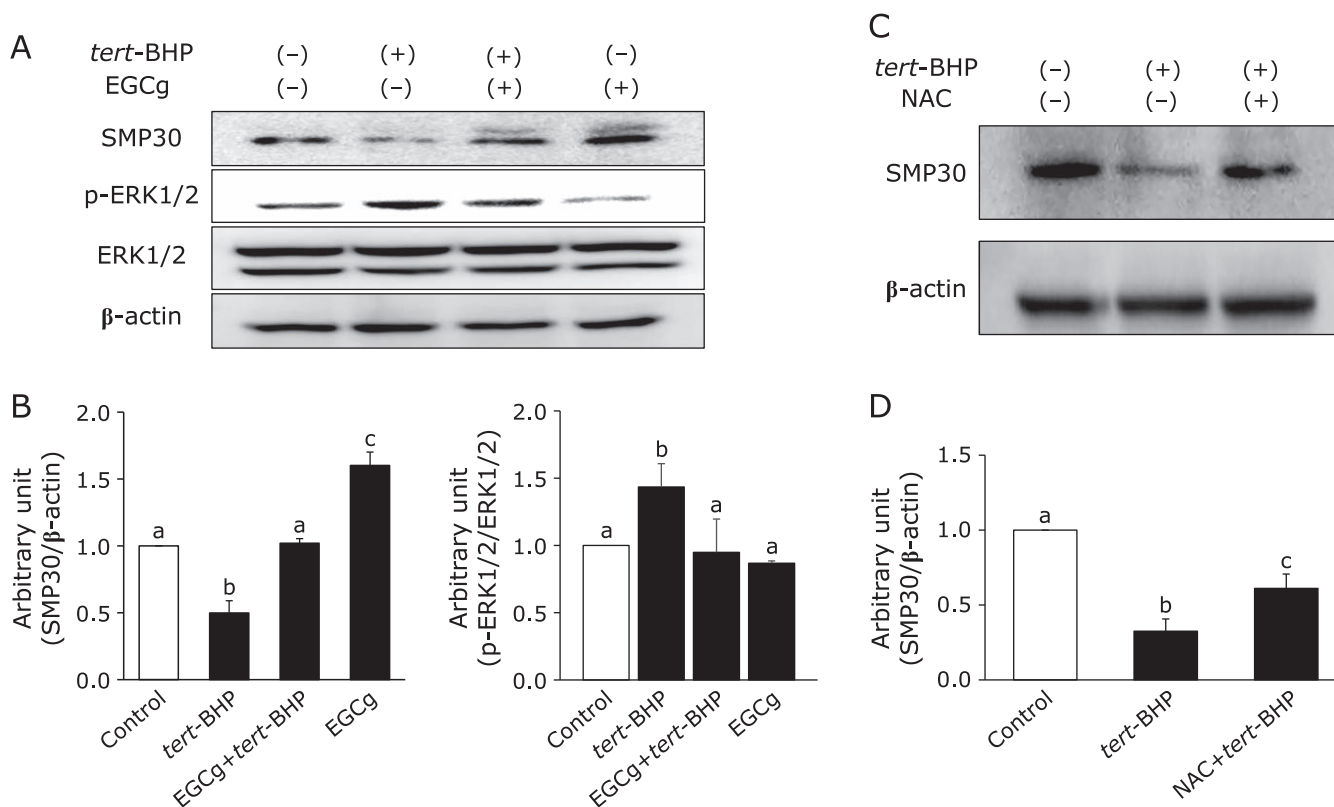


Fig. 4. Effects of EGCg on SMP30 expression and ERK1/2 phosphorylation with *tert*-BHP-treated Fao cells. (A) Cells were pre-treated with 30 μ M EGCg for 8 h. After incubating with EGCg, cells were treated with 100 μ M *tert*-BHP with or without 30 μ M EGCg for 24 h. Cells were homogenized using RIPA buffer, and the supernatants were separated to measure SMP30, phosphorylated ERK1/2 (activation form), and ERK1/2 expression. (B) Densitometry quantification was performed using ImageJ. SMP30 levels were normalized to β -actin. p-ERK1/2 levels (Thr202/Tyr204) were normalized to ERK1/2. (C) Cells were treated with 100 μ M *tert*-BHP with/without 0.5 mM NAC for 24 h. The supernatants were separated for SMP30 expression measurement. (D) Densitometry quantification was performed using ImageJ. SMP30 levels were normalized to β -actin. Data are shown as the means \pm SE from at least three independent experiments. Unmatching letters denote significantly different, $p < 0.05$.

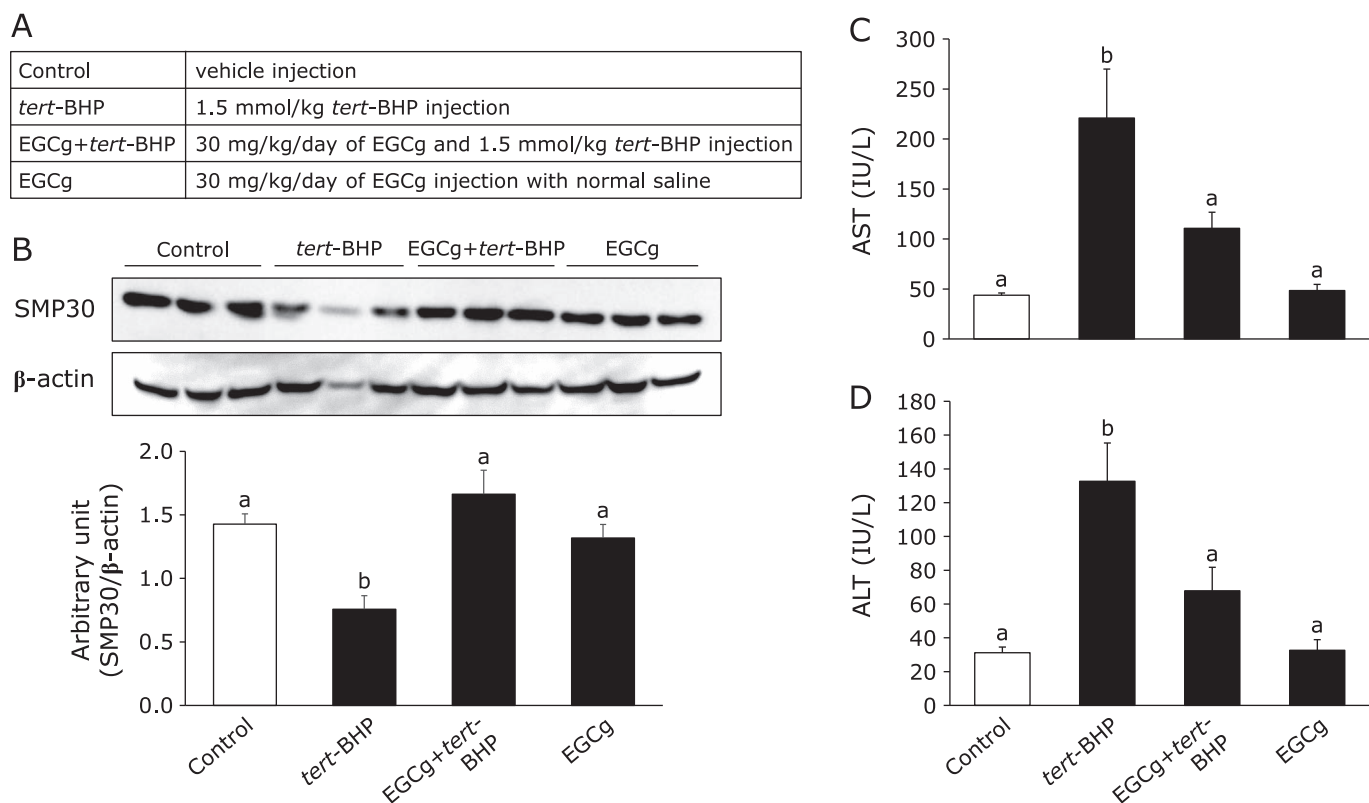


Fig. 5. Effects of EGCg on SMP30 expression and hepatic toxicity in *tert*-BHP-injected mice. (A) All groups were fed an AIN93G diet as a basal diet and given tap water for 7 days. EGCg (30 mg/kg body weight) was orally administered to the EGCg and EGCg + *tert*-BHP groups for 6 days. After 6 days, the *tert*-BHP and EGCg + *tert*-BHP groups were intraperitoneally injected with *tert*-BHP for 24 h. (B) Livers were homogenized using RIPA buffer, and the supernatants were separated for SMP30 expression measurement. Densitometry quantification was performed using ImageJ. SMP30 levels were normalized to β -actin. Data are shown as the means \pm SE. (C) Blood was collected and serum separated to determine serum AST and (D) ALT levels. Unmatching letters denote significantly different, $p < 0.05$.

Table 1. Food intake, body weight, and organ weights

	Control	<i>tert</i> -BHP	EGCg + <i>tert</i> -BHP	EGCg
Total food intake (g)	47.19 \pm 0.90	45.72 \pm 1.78	46.73 \pm 1.82	46.69 \pm 1.14
Final body weight (g)	36.93 \pm 2.30	36.55 \pm 1.62	36.92 \pm 2.73	36.81 \pm 1.39
Liver weight (g/100 g body weight)	1.891 \pm 0.05	1.933 \pm 0.06	1.947 \pm 0.08	1.871 \pm 0.08
Kidney weight (g/100 g body weight)	0.595 \pm 0.03	0.600 \pm 0.04	0.624 \pm 0.02	0.623 \pm 0.02

Data are shown as the means \pm SE ($n = 7$ each).

We confirmed that AST and ALT, liver injury markers, are drastically increased in *tert*-BHP-treated mice compared to that in untreated mice; however, EGCg inhibited the increase in these markers. Of note, we also observed that EGCg administration significantly inhibited the downregulation of SMP30 expression in the liver of *tert*-BHP-administered mice, suggesting that it plays a role in attenuating *tert*-BHP-induced liver injury. In contrast, several studies have reported that high-dose EGCg causes hepatotoxicity.^(36–39) Here, treating mice with EGCg alone did not modulate AST and ALT levels, and EGCg-treated mice did not increase SMP30 expression in the liver compared to that in the control mice. Thus, oral EGCg administration for 6 days may be insufficient. Another study showed that treatment with EGCg (25 mg/kg/day) for long duration extends lifespan by improving age-associated inflammation and oxidative stress in healthy rats.⁽⁴⁰⁾ In any case, we found that EGCg positively regulates SMP30 stabilization at the intracellular level to protect against *tert*-BHP-induced liver injury. Based on *in vitro* data, EGCg could regulate SMP30 turnover and stability via post-translational

mechanisms.

In conclusion, the present study demonstrated that EGCg upregulates and stabilizes SMP30 and reduces *tert*-BHP-induced liver injury *in vitro*. Importantly, our *in vivo* data also shows that EGCg treatment improves the downregulation of SMP30 and liver injury markers in mice with *tert*-BHP-induced liver injury. These findings could offer new perspectives for novel therapeutic approaches against age-related diseases by EGCg-induced upregulation and stabilization of SMP30.

Author Contributions

Study concept and design: HI, KA, and MU; acquisition of data: HI, KA; analysis and interpretation of data: HI, KA, and MU; drafting of the manuscript: HI, KA, and MU; critical revision of the manuscript for important intellectual content: MT and NT; statistical analysis: HI, KA; administrative, technical, or material support: RK-T and NT; study supervision: MU.

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Abbreviations

ALT alanine transaminase
AST aspartate aminotransferase

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CHX cycloheximide
EGCG epigallocatechin gallate
NAC N-acetyl cysteine
ROS reactive oxygen species
SMP30 senescence marker protein-30
tert-BHP tert-butyl hydroperoxide

Conflict of Interest

No potential conflicts of interest were disclosed.

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