Lycopus lucidus Turcz exerts neuroprotective effect against H$_2$O$_2$-induced neuroinflammation by inhibiting NLRP3 inflammasome activation in cortical neurons

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Research

Abstract

Background

Central nervous system (CNS) injuries are a leading cause of permanent functional impairment in humans. Nerve damage can be aggravated by neuroinflammation mediated by protein complexes known as inflammasomes, such as the NLRP3 inflammasome which is a key mediator of caspase-1 and interleukin-1β (IL-1β) /interleukin-18 (IL-18) activation. *Lycopus lucidus Turcz* (LLT) is a traditional medicinal herb that exerts therapeutic effects against oxidative stress, inflammation, and angiogenesis; however, it remains unclear whether LLT can directly protect neurons against damage, and the underlying molecular mechanisms are poorly understood.

Methods

We investigated the neuroprotective effect of LLT against hydrogen peroxide (H_2O_2)-induced neuronal damage in cultured primary rat cortical neurons, as well as the potential underlying mechanisms. Neuronal viability and cell death assays were used to determine the effects of LLT on neuroprotection, while the mode of cell death was confirmed using flow cytometry. Changes in the expression of inflammatory factors involved in activation of the NLRP3 inflammasome were measured using immunocytochemistry (ICC) and confirmed by real-time PCR. And, we analyzed that the effect of LLT on neurotrophic factors secretion and synaptic connectivity using ICC in H_2O_2-induced neuron at 7 days *in vitro*.

Results

LLT effectively protected cultured rat cortical neurons from H_2O_2-induced injury by significantly inhibiting NLRP3 inflammasome activation. In addition, LLT significantly reduced caspase1 activation, which is known to be induced by inflammasome formation, and consequently regulated the secretion of IL-1β/IL-18. We demonstrated that LLT enhances axonal elongation and synaptic connectivity against H_2O_2-induced injury of rat primary cortical neuron.

Conclusions

Together, these results demonstrate that LLT can directly protect cultured cortical neurons from H_2O_2-induced neuronal damage by inhibiting NLRP3 inflammasome activation and the secretion of caspase-1 and IL-1β/IL-18. Thus, our study provides new insights into the therapeutic mechanisms of LLT and suggests that the NLRP3 inflammasome could be a promising target for treating neurological diseases.

Background

Central nervous system (CNS) inflammation alters neurotransmission, affects neuronal death, regeneration, and neuroplasticity, and is involved in the pathophysiology of neurological diseases [1, 2]. When activated by harmful stimuli, such as trauma, infection, and oxidative factors, inflammatory cells secrete abnormal levels of pro-inflammatory cytokines and amplify the response of other immune cells, leading to clinical symptoms of inflammation [3, 4].

Recent studies have reported that neuroinflammation is related to inflammasome activation and IL-1β, IL-18, and IL-33 production [5, 6]. Inflammasomes are complexes formed from homogeneous proteins expressed in inflammatory cells that are activated by specific stimuli. Activated inflammasomes induce the cleavage of procaspase-1 into active caspase-1, which cleaves IL-1β precursors generated by pattern recognition receptor (PRR)-mediated signaling into IL-1β, the active form [2, 7, 8]. Thus, active cytokines produced by inflammasome activation are important drivers of inflammation that interact with other cytokine pathways to activate immune responses against infection and injury [9].

Inflammasomes have recently been implicated in the etiology, onset, and progression of several important diseases as well as a novel biological defense mechanism against pathogens [10]. To date, multiple types of inflammasomes have been identified and studied, among which NLRP3 inflammasomes have been closely associated with the progression and pathophysiological mechanisms of CNS disorders, such as Parkinson's disease, Alzheimer's disease, and stroke [11–15]. Previous studies have shown that inhibiting inflammasome activation after traumatic brain injury (TBI) has been confirmed to regulate neuroinflammatory responses by significantly reducing neurodegeneration and cortical damage induced by injury [16]. Moreover, blocking the NLRP3 inflammasome and its adaptor protein ASC has been shown to induce against injury-induced neuroinflammatory responses by decreasing caspase1, iNOS, and IL-1β activation [17, 18]. Therefore, it is important to understand these mechanisms in order to develop treatments for neurological diseases related to neuroinflammation.

Based on these mechanisms, current studies are developing treatments for neuropathy based on natural substances with few side effects, whose safety and effectiveness have already been empirically verified. In this study, we examined the neuroprotective and regenerative effects of LLT, a traditional medicinal herb that has been reported to exert therapeutic effects against oxidative stress, inflammation, and angiogenesis with few side effects. Importantly, we confirmed that LLT exerts neuroprotective effects against neuronal damage induced by hydrogen peroxide (H_2O_2) and elucidated the underlying mechanisms. Therefore, the findings of this study will be allow the utilization of this natural substance to treat various neurological diseases in the future.

Methods

In vitro culture of cortical neurons

All animals used in this study were maintained in accordance with the Jaseng Animal Care and Use Committee (JSR-2020-03-004). Primary cortical neurons were prepared from Sprague-Dawley rat embryos

(embryonic day 17, Daehan Bio Link, Chungbuk, Korea). Briefly, the isolated cortices were placed in Hank's balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY, USA), and the meninges were manually removed from the cerebral hemispheres. The tissues were rinsed twice in HBSS, digested with 2 mL of 2.5 mg/mL papain solution (Sigma-Aldrich, St. Louis, MO, USA) in HBSS for 15 min at 37 °C, and the supernatant discarded. The tissues were then rinsed twice in 2 mL HBSS and centrifuged at 1500 rpm for 3 min to obtain the cell pellet. Cells were triturated in 1 mL cortical neuron culture medium containing neurobasal medium (Gibco BRL) supplemented with 2 % B27 (Gibco BRL), 1 % Gluta-MAX (Gibco BRL), and 1 % penicillin/ streptomycin (Gibco BRL). Single cells were then seeded onto 12 mm circular cover slips for immunocytochemical (ICC) analysis, a 6-well plate for FACS analysis, and 96-well plates for the cell viability assay, which were then coated with 20 mg/mL poly-D-lysine (Gibco BRL) overnight followed by 10 mg/mL laminin (Sigma-Aldrich) for 2 h at 4 °C.

Preparation of LLT

First, LLT was heated to 105 ºC with water by refluxing for 3 h, cooled on ice, and filtered once with filter paper (Hyundai micro, HA-030, Korea). The filtrate was lyophilized using a freeze dryer (Ilshin BioBase, Korea) to obtain dry LLT extract; this extract was weighed, the extract yield calculated, and re-dissolved in phosphate-buffered saline (PBS) to the high dose concentration (10 mg/ml). The extract was moved to a conical flask and maintained at – 70 ºC.

Hydrogen peroxide (H_2O_2)-induced neuronal injury and LLT treatment

Cortical neurons were plated at different densities for various analysis (4×10^5 cells/450 μL in 24 well plate for immunocytochemistry; 2×10^6 cells/1.8 mL in 6 well plate for flow cytometry; 4×10^6 cells/2.7 mL in 60 mm^2 dish for real-time PCR) for 2 h. Next, H_2O_2 (Sigma-Aldrich, 5 mM) was added at 10 % of the total volume to a final concentration of 500 μM. After 1 h, the H_2O_2-containing medium was replaced with LLT extract (10, 25, or 50 μg/mL) in cortical neuron medium and incubated in 5 % CO_2 at 37 °C for 24 h.

Neuronal viability assay

Neuronal viability was analyzed using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) at 24 h after treatment with LLT extract (1, 10, 25, 50, or 100 μg/mL) followed by stimulation with or without H_2O_2. Briefly, CCK-8 solution (10 μL) was added to each well, incubated for 4 h at 37 °C, and then absorbance measured using a microplate reader (Epoch, BioteK, Winooski, VT, USA) at 450 nm. Neuronal viability was expressed as a percentage of the blank group, which was defined as 100 % viability.

Neuronal viability was also determined using a live/dead cell imaging kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The staining solution consisted of two probes measuring recognized cytotoxicity and cell viability parameters: calcein AM indicating live cells and BOBO-3 Iodide (EthD-1) indicating dead cells (red). The culture medium was

discarded, and each sample was incubated in 100 μL of staining solution for 15 min at 20–25 °C. To quantify neuronal viability, ten random images per group were captured at 10× magnification using a confocal microscope (Eclipse C2 Plus, Minato, Tokyo, Nikon, Japan). Live and dead cells were manually counted using Image J software (National Institutes of Health).

Immunocytochemistry (ICC)

Changes in the expression of inflammatory factors related to the NLRP3 inflammasome in H_2O_2-treated cortical neurons were observed using ICC. After 24 h, the samples were fixed with 4 % paraformaldehyde for 30 min, rinsed three times with PBS for 5 min each, and then incubated with 0.2 % Triton X-100 in PBS for 5 min. After two rinses with PBS for 5 min and blocking with 2 % normal goat serum (NGS) in PBS for 1 h, the cells were incubated overnight at 4 °C. The primary antibodies used were as follows: NLRP3 (1:100; abcam, Cambridge, MA, USA), ASC (1:200; Santa Cruz Biotechnology, CA, USA), Caspase1 (1:200; abcam), IL-1β (1:200; Novus Biologicals, CO, USA), BDNF (1:200; abcam), NGF (1:100; abcam), Synaptophysin (1:500; Sigma-Aldrich), Tuj1 (1:2000; R&D systems, McKinley Place NE, USA) and it had been incubated for 2 h with fluorescent secondary antibodies (FITC-conjugated goat anti-rabbit IgG, 1:300; FITC-conjugated goat anti-mouse IgG, Jackson Immuno-Research Labs, West Grove, PA, USA). The samples were treated with 4 – 6-diamidino-2-phenylindole (DAPI, Tokyo Chemical Industry Co. (TCI), Tokyo, Japan) containing PBS for 10 min at room temperature. Next, the cells were washed three times with PBS for 5 min, mounted with fluorescence mounting medium (Dako Cytomation, Carpinteria, CA, USA), and imaged using a confocal microscope (Eclipse C2 Plus, Nikon). To quantify fluorescence intensity, ten representative images were captured at 400 × magnification using confocal microscopy with fixed acquisition settings. Average intensity was measured using Image J software (1.37 v, National Institutes of Health, Bethesda, MD, USA).

Real-time PCR

We analyzed the changes in the expression level of genes related to the neuroinflammation and neuronal growth in each group using Real-time PCR technique. Total RNA was isolated from cells using Trizol reagent (Thermo Fisher Scientific) and cDNA synthesized using random hexamer primers and Accupower RT premix (Bioneer, Korea). All primers pairs were designed using UCSC Genome Bioinformatics and the NCBI database (Table 1). Real-time PCR was performed using iQSYBR green supermix (Bio-Rad, Hercules, CA, USA) on an CFX Connect Real-Time PCR Detection System (Bio-Rad). Each assay was performed at least three times. Target gene expression was normalized to GAPDH and expressed as a fold change relative to the control.

Table 1
Primer sequences used for real-time PCR analysis.

Gene	5'-3'	Primer sequence
Akt1	Forward	ACCTCTGAGACCGACACCAG
	Reverse	AGGAGAACTGGGGAAAGTGC
BDNF	Forward	CTTGGAGAAGGAAACCGCCT
	Reverse	GTCCACACAAAGCTCTCGGA
Caspase-1	Forward	ACTCGTACACGTCTTGCCCTCA
	Reverse	CTGGGCAGGCAGCAAATTC
iNOS	Forward	ATGGCTTGCCCCTGGAAGTT
	Reverse	TGTTGGGCTGGGAATAGCAC
IL-1β	Forward	TTGCTTCCAAGCCCTTGACT
	Reverse	GGTCGTCATCATCCCACGAG
IL-18	Forward	GGACTGGCTGTGACCCTATC
	Reverse	TGTCCTGGCACACGTTTCTG
NT3	Forward	CCGACAAGTCCTCAGCCATT
	Reverse	CAGTGCTCGGACGTAGGTTT
NGF	Forward	CCAAGGACGCAGCTTTCTATC
	Reverse	CTGTGTCAAGGGAATGCTGAAG
NLRP3	Forward	GCTCCAACCATTCTCTGACC
	Reverse	AAGTAAGGCCGGAATTCACC
mTOR	Forward	GCAATGGGCACGAGTTTGTT
	Reverse	AGTGTGTTCACCAGGCCAAA
GAPDH	Forward	CCCCCAATGTATCCGTTGTG
	Reverse	TAGCCCAGGATGCCCTTTAGT

Flow cytometry

The mode of cell death was determined using flow cytometry. Apoptotic cell death was detected using an Annexin V- Flourescein isothiocyanate (FITC)/ Propidium iodide (PI)-PE (phycoerythin) apoptosis detection kit (abcam) as described previously. Briefly, cells were collected, incubated with 1% Annexin V and 1% PI in binding buffer, and then analyzed directly using fluorescence-activated cell sorting (FACS, Accuri C6 plus flow cytometer, BD Bioscience, Franklin Lakes, NJ, USA).

Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). Comparisons among each group were analyzed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Differences were considered statistically significant if the P value was < 0.05.

Results

LLT protects cortical neurons against H_2O_2-induced neuronal death

First, we investigated the induction of neuronal death in cortical neurons by H_2O_2 exposure, confirmed whether LLT extract was non-toxic in cortical neurons, and evaluated the optimal dose of LLT by screening cell viability (Fig. 1a). LLT extract alone was not toxic to cortical neurons at concentrations ranging from 1 to 100 μg/mL and actually increased cell viability compared to the control group at 25 to 100 μg/mL The optimal concentration for this neuroprotective effect was evaluated 24 h after H_2O_2-induced neurons had been treated with LLT. We found that neuronal viability decreased to about one-thirds when cortical neurons were treated with H_2O_2 (500 μM; Fig. 1b); however, treatment with LLT (1 to 100 μg/mL) for 24 h significantly and dose-dependently increased neuronal viability. These findings demonstrate that LLT extract has neuroprotective effects in H_2O_2−induced neuronal injury.

Live/dead cell assays were also performed to quantify the dead (red) and live cells under the same culture conditions. The neurons treated with LLT were mostly positive for calcein-AM (green fluorescence; Fig. 1c), indicating that LLT significantly and dose-dependently increased the number of live cells compared to the H_2O_2 group (Fig. 1d). We further confirmed the mode of neuronal death using flow cytometry with the calcium-dependent phospholipid adhesion protein, Annexin V, used to confirm apoptosis and propidium iodide (PI) used to detect late apoptosis/necrosis (Fig. 1e, g). When treated with H_2O_2, more of the primary cultured cortical neurons underwent apoptosis (annexin V+/PI-); however, LLT gradually decreased the population of apoptotic cells induced by H_2O_2 in a dose-dependent manner. Therefore, we examined the effects of LLT extract inhibition on H_2O_2-induced neuronal death in cortical neurons.

Effect of LLT on H_2O_2-induced neuroinflammation in cortical neurons

To assess the effect of LLT extract on inducible nitric oxide synthase (iNOS) expression, a major mediator of inflammation, we examined iNOS expression in H_2O_2-induced neurons using ICC (Fig. 2a). iNOS expression was substantially increased by H_2O_2 treatment but significantly decreased by LLT (Fig. 2b). Consistently, iNOS mRNA expression was also markedly higher in the H_2O_2 group and significantly decreased by LLT in a dose-dependent manner (Fig. 2c). We then assessed whether the LLT treatment

regulates IL-6 and IL-10 cytokine release in the culture medium using cytokine-specific ELISAs. IL-6 is popularly well-known as the another proinflammatory cytokine present in neuron and play a key role in neuronal response to an injury [19]. The level of IL-6 was upregulated in the H_2O_2 group, while much lower level of IL-6 is detected in the LLT groups. In particular, IL-6 level in medium did have significant LLT-dose dependent effect (Fig. 2d). Moreover, LLT enhanced expression of anti-inflammatory cytokine IL-10 and leads to a dose-dependent induction in cortical neurons (Fig. 2e). Based on these results, application of LLT in cortical neuron inhibited inflammatory response with the decrease in the expression of pro-inflammatory cytokines (iNOS and IL-6) and increase in the expression of anti-inflammatory cytokine (IL-10).

Effect of LLT on NLRP3 inflammasome activation in cortical neurons

To determine whether LLT extract inhibited NLRP3 inflammasome activation in neurons, we used ICC to assess the expression of NLRP3 inflammasomes (Fig. 3a), which were identified morphologically as dots inside the cell body. H_2O_2 treatment strongly enhanced NLRP3 inflammasome expression in the neuronal cell body and increased the percentage of NLRP3$^+$ neurons by around 20 % increase compared to the groups treated with LLT, which significantly and dose-dependently inhibited NLRP3 expression (Fig. 3c). We also examined NLRP3 mRNA expression using real time-PCR following H_2O_2 treatment and 24 h after LLT treatment in H_2O_2-treated cortical neurons (Fig. 3d). NLRP3 mRNA expression was significantly higher in the H_2O_2 group than in the blank group; however, LLT dose-dependently decreased NLRP3 mRNA expression in H_2O_2-treated cortical neurons.

Inflammasomes form protein domain bonds with caspase-1 either directly or via the adapter protein ASC; therefore, inflammatory activity causes a multi-step association with Nod-like receptors (NLRs) and the IL-1β cleavage enzyme caspase-1 through ASC [20]. Consequently, we analyzed ASC expression using ICC, observing a correlation with the ICC staining result for NLRP3 (Fig. 3b). Moreover, the number of cells expressing ASC was significantly higher in the H_2O_2 group than in the blank group and was lower following LLT treatment (25 and 50 μg/mL; Fig. 3e). Consistently, ASC mRNA expression was significantly higher in the H_2O_2 group compared to the control group and decreased following LLT treatment in a dose-dependent manner (Fig. 3f). Therefore, LLT extract appears to effectively inhibit ASC-dependent NLRP3 inflammasome activation in cortical neurons.

Effect of LLT on caspase-1 and IL-1β secretion via the NLRP3 inflammasome pathway

The inflammasome is a multi-protein complex that mediates caspase-1 activation, which subsequently promotes the secretion of the proinflammatory cytokines IL-1β and IL-18 [21]. Therefore, we examined caspase-1 activation in cortical neurons using fluorescence-based ICC with specific antibodies (Fig. 4a). Caspase-1 fluorescence intensity was significantly increased by H_2O_2 treatment; however, this effect was

completely inhibited by LLT treatment (Fig. 4b). Subsequent analysis of caspase-1 mRNA expression after LLT treatment in H_2O_2-treated cortical neurons confirmed that caspase-1 expression was significantly higher in the H_2O_2 group than in the blank group and was downregulated significantly and dose-dependently by LLT treatment (Fig. 4c).

We also examined IL-1β levels using ICC in the H_2O_2 and LLT-treated groups (Fig. 4d), finding that the relative intensity of IL-1β was elevated after H_2O_2 treatment but reduced in a dose-dependent manner in the LLT group (Fig. 4e). Real time PCR analysis of IL-1β and IL-18 gene expression confirmed that H_2O_2 treatment significantly increased the relative mRNA levels of IL-1β and IL-18, whereas all LLT doses significantly downregulated their mRNA expression in a dose-dependent manner (Fig. 4f, g). Therefore, these findings confirm that LLT can inhibit caspase-1 expression mediated by inflammasome action and thereby block IL-1β and IL-18 secretion in H_2O_2-induced neuronal damage.

Effect of LLT on neurotrophic factor induction in H_2O_2-induced cortical neurons

Neurotrophic factors stimulate neuroprotection and thus have been classically considered as a good source of treatments for neurodegenerative diseases. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are powerful factors associated with neuroprotection and neuroplasticity [22]. We found that neurons treated with H_2O_2 displayed significantly lower BDNF and NGF expression, whereas LLT treatment dose-dependently increased BDNF and NGF expression (Fig. 5a, b). Similarly, image-based quantification revealed that LLT treatment significantly increased BDNF and NGF expression intensity (Fig. 5c, d), and the mRNA level of BDNF and NGF genes were also significantly increased after LLT treatment under H_2O_2 -induced neuronal damage (Fig. 5e, f). Real-time PCR yielded results consistent with those of ICC experiments, confirming that LLT exerts protective effects against H_2O_2-induced neuronal damage, promoting nerve function restoration by increasing the expression of neurotrophic factors such as BDNF and NGF. Also we confirmed that LLT only treatment without H_2O_2, significantly increase BDNF[+] or NGF^{+} cortical neurons by FACS (Supplementary Figure S1). Together, these results suggest that the therapeutic effects of increased BDNF and NGF secretion could effectively stimulate the regrowth of injured axons.

Neuroprotective effect of LLT on synapse formation in H_2O_2-induced cortical neurons

Synaptogenesis describes the critical process of synapse formation between neurons that allows the assembly of neuronal circuits. Synaptophysin (Syn) is a major synaptic protein that is used as a biomarker of synaptogenesis in cultured neurons [23]. We confirmed Syn expression in H_2O_2-induced neurons after LLT treatment (Fig. 6a) and found that its expression was promoted by LLT but abolished by H_2O_2. The Synaptic densities per field of view was greater in the LLT groups than in the H_2O_2 group (Fig. 6b). In addition, we compared the longest neurite length in cortical neurons after treatment with

various concentrations of LLT under H_2O_2 condition by determining Tuj1 immunoreactivity at 7 days *in vitro* (Fig. 6c). Notably, LLT enhanced axonal elongation compared to H_2O_2 and significantly increased the length of the longest neurite in a dose-dependently. Also we confirmed that LLT only treatment without H_2O_2, significantly increase Syn^+ cortical neurons by FACS (Supplementary Figure S2). Neurotrophin3 (NT3) is a neurotrophic factor belonging to the NGF family that has been shown to display neuroprotective effects [24]. We found that NT3 mRNA expression was significantly higher in the LLT groups than in the H_2O_2 group and increased dramatically as the LLT dose increased (Fig. 6d). The Akt/mTOR signaling pathway is a well-known biomarker of neuroprotection and regeneration, and previous studies have reported that neuronal injury can be prevented due to neuroprotection by regulating AKT/mTOR expression. We also performed the mRNA expression analysis of the AKT and mTOR genes (Fig. 6e, f). These findings revealed that that LLT treatment after H_2O_2 induction improved AKT and mTOR expression dose dependently, whereas AKT and mTOR expression were decreased in the H_2O_2 group. Based on these results, increasing doses of LLT significantly enhanced the axonal regrowth and synaptic connectivity in H_2O_2-induced injury of rat primary cortical neuron.

Discussion

In our previous study, we screened 131 medicinal herbs, which possessed bioactive compounds that regulate nitric oxide (NO) production and cell proliferation in LPS-treated RAW 264.7 macrophages. The top ten medicinal herbs that were associated with the inhibition of NO and increase of cell viability were further screened in H_2O_2-treated cortical neurons. We hypothesized that LLT might modulate neuronal damage through inhibition of inflammation in H_2O_2-treated cortical neurons. In addition, we also elucidated a specific mechanism that regulates the NLRP3 inflammasome based on recently described mechanisms underlying the inflammatory response. Inflammasomes are multiprotein intracellular complexes, which activate inflammatory responses. Among the various subtypes of inflammasomes, the most well studied inflammasome is currently the NLRP3 inflammasome, which consists of NLRP3, the adapter protein ASC, and caspase-1. Although the majority of studies on neurodegenerative diseases show that NLRP3 inhibition is beneficial for the disease progression, the management of NLRP3 inflammasome in the cortical neuron is not very well understood. Recently, published evidence have reported that NLRP3 inflammasome play important roles in regulating neuroinflammatory responses, which can ultimately induce neuronal cell death and associated with various neurological diseases [25]. In particular, NLRP3 inflammasome can cause the cerebral cortex injury [26]. Therefore, it is thought that inhibiting NLRP3 inflammasome activation could be critical for the prevention and treatment of nerve injury. This study identified a new insight of NLRP3 inflammasome and reveals it role in LLT. We therefore aimed to determine whether LLT could positively control the progression of neuronal death by inhibiting excessive neuroinflammatory responses caused by NLRP3 inflammasome activation. We elucidated the efficacy and mechanism of action of LLT, which has been historically used to treat postpartum abdominal pain, edema, menstrual disorders, wounds, bruises, and wheal due to its excellent anti-inflammatory and antioxidant effects. We demonstrated a decreased expression of ASC, caspase-1 after

LLT treatment by inhibiting NLRP3 inflammasome activation, which was increased by H_2O_2. And, we observed that LLT inhibits the expression of inflammatory mediators, such as IL-1β and IL-18 production. A recent report showed that secretion of IL-1β and other cytokines, such as IL-18, IL-1α and TNFα. IL-1β and IL-18 is NLRP3 and ASC-dependent. We confirmed that these markers were downregulated by LLT in H_2O_2-induced neural injury. In addition, LLT not only exhibited anti-inflammatory effects through inhibition of NLRP3 inflammasome, but also directly increased BDNF$^+$ and NGF$^+$ cortical cells. Consequently, LLT exerts excellent neuroprotective effects that induce neuron growth and regeneration by increasing neurotrophic factors secretion and synaptic connection. LLT may be a promising therapeutic approach for various neurological diseases. And, targeting inhibition of NLRP3 activation may also plays an important role in the process of neural regeneration. Therefore, future studies should verify the efficacy and precise mechanism of action of NLRP3 function to better understand the importance of inflammasomes in neurons and neuronal disorders using animal models such as Alzheimer's disease, Parkinson's disease, and cerebral infarction.

Conclusions

In summary, our findings demonstrate that LLT can effectively suppress the inflammatory response by inhibiting NLRP3 inflammasome activation and the secretion of caspase-1 and pro-inflammatory cytokines (IL-1β and IL-18), thereby effectively alleviating or suppressing the mRNA expression of related factors. Therefore, LLT can promote neuronal survival and growth by exerting these neuroprotective effects in H_2O_2-induced neuronal injury.

Abbreviations

ANOVA, Analysis of variance; CNS, Central nervous system; FACS, Fluorescence-activated cell sorting; HBSS, Hank's balanced salt solution; LLT, *Lycopus lucidus Turcz*; H_2O_2, Hydrogen peroxide; NGS, Normal goat serum; NLR, Nod-like receptors; PBS, Phosphate buffered saline; PI, Propidium iodide; RT, Room temperature; SEM, Standard error of the mean

Declarations

Ethics approval and consent to participate: JSR-2020-03-004

Consent for publication: The authors declare no conflict of interest.

Availability of data and materials: Not applicable.

Competing interests: Not applicable.

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References

1. Kyritsis N, Kizil C, Brand M: **Neuroinflammation and central nervous system regeneration in vertebrates.** *Trends Cell Biol* 2014, **24**:128-135.
2. Erickson MA, Dohi K, Banks WA: **Neuroinflammation: a common pathway in CNS diseases as mediated at the blood-brain barrier.** *Neuroimmunomodulation* 2012, **19**:121-130.
3. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L: **Inflammatory responses and inflammation-associated diseases in organs.** *Oncotarget* 2018, **9**:7204-7218.

4. Medzhitov R: **Inflammation 2010: new adventures of an old flame.***Cell* 2010, **140:**771-776.

5. Saresella M, La Rosa F, Piancone F, Zoppis M, Marventano I, Calabrese E, Rainone V, Nemni R, Mancuso R, Clerici M: **The NLRP3 and NLRP1 inflammasomes are activated in Alzheimer's disease.***Mol Neurodegener* 2016, **11:**23.

6. Mantovani A, Dinarello CA, Molgora M, Garlanda C: **Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity.***Immunity* 2019, **50:**778-795.

7. Kelley N, Jeltema D, Duan Y, He Y: **The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation.***Int J Mol Sci* 2019, **20**.

8. Broz P, Dixit VM: **Inflammasomes: mechanism of assembly, regulation and signalling.***Nat Rev Immunol* 2016, **16:**407-420.

9. Davis BK, Wen H, Ting JP: **The inflammasome NLRs in immunity, inflammation, and associated diseases.***Annu Rev Immunol* 2011, **29:**707-735.

10. Yang Y, Wang H, Kouadir M, Song H, Shi F: **Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors.***Cell Death Dis* 2019, **10:**128.

11. Haque ME, Akther M, Jakaria M, Kim IS, Azam S, Choi DK: **Targeting the microglial NLRP3 inflammasome and its role in Parkinson's disease.***Mov Disord* 2020, **35:**20-33.

12. Hong P, Gu RN, Li FX, Xiong XX, Liang WB, You ZJ, Zhang HF: **NLRP3 inflammasome as a potential treatment in ischemic stroke concomitant with diabetes.***J Neuroinflammation* 2019, **16:**121.

13. Lee E, Hwang I, Park S, Hong S, Hwang B, Cho Y, Son J, Yu JW: **MPTP-driven NLRP3 inflammasome activation in microglia plays a central role in dopaminergic neurodegeneration.***Cell Death Differ* 2019, **26:**213-228.

14. Li SJ, Zhang YF, Ma SH, Yi Y, Yu HY, Pei L, Feng D: **The role of NLRP3 inflammasome in stroke and central poststroke pain.***Medicine (Baltimore)* 2018, **97:**e11861.

15. Tan MS, Yu JT, Jiang T, Zhu XC, Tan L: **The NLRP3 inflammasome in Alzheimer's disease.***Mol Neurobiol* 2013, **48:**875-882.

16. Kuwar R, Rolfe A, Di L, Xu H, He L, Jiang Y, Zhang S, Sun D: **A novel small molecular NLRP3 inflammasome inhibitor alleviates neuroinflammatory response following traumatic brain injury.***J Neuroinflammation* 2019, **16:**81.

17. Ismael S, Zhao L, Nasoohi S, Ishrat T: **Inhibition of the NLRP3-inflammasome as a potential approach for neuroprotection after stroke.***Sci Rep* 2018, **8:**5971.

18. Ismael S, Ahmed HA, Adris T, Parveen K, Thakor P, Ishrat T: **The NLRP3 inflammasome: a potential therapeutic target for traumatic brain injury.***Neural Regen Res* 2021, **16:**49-57.

19. Erta M, Quintana A, Hidalgo J: **Interleukin-6, a major cytokine in the central nervous system.***Int J Biol Sci* 2012, **8:**1254-1266.

20. He Y, Hara H, Nunez G: **Mechanism and Regulation of NLRP3 Inflammasome Activation.***Trends Biochem Sci* 2016, **41:**1012-1021.

21. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G: **The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis.**_Nat Immunol_ 2009, **10**:241-247.

22. Weissmiller AM, Wu C: **Current advances in using neurotrophic factors to treat neurodegenerative disorders.**_Transl Neurodegener_ 2012, **1**:14.

23. McAllister AK: **Dynamic aspects of CNS synapse formation.**_Annu Rev Neurosci_ 2007, **30**:425-450.

24. Keefe KM, Sheikh IS, Smith GM: **Targeting Neurotrophins to Specific Populations of Neurons: NGF, BDNF, and NT-3 and Their Relevance for Treatment of Spinal Cord Injury.**_Int J Mol Sci_ 2017, **18**.

25. Voet S, Srinivasan S, Lamkanfi M, van Loo G: **Inflammasomes in neuroinflammatory and neurodegenerative diseases.**_EMBO Mol Med_ 2019, **11**.

26. Liu HD, Li W, Chen ZR, Hu YC, Zhang DD, Shen W, Zhou ML, Zhu L, Hang CH: **Expression of the NLRP3 inflammasome in cerebral cortex after traumatic brain injury in a rat model.**_Neurochem Res_ 2013, **38**:2072-2083.
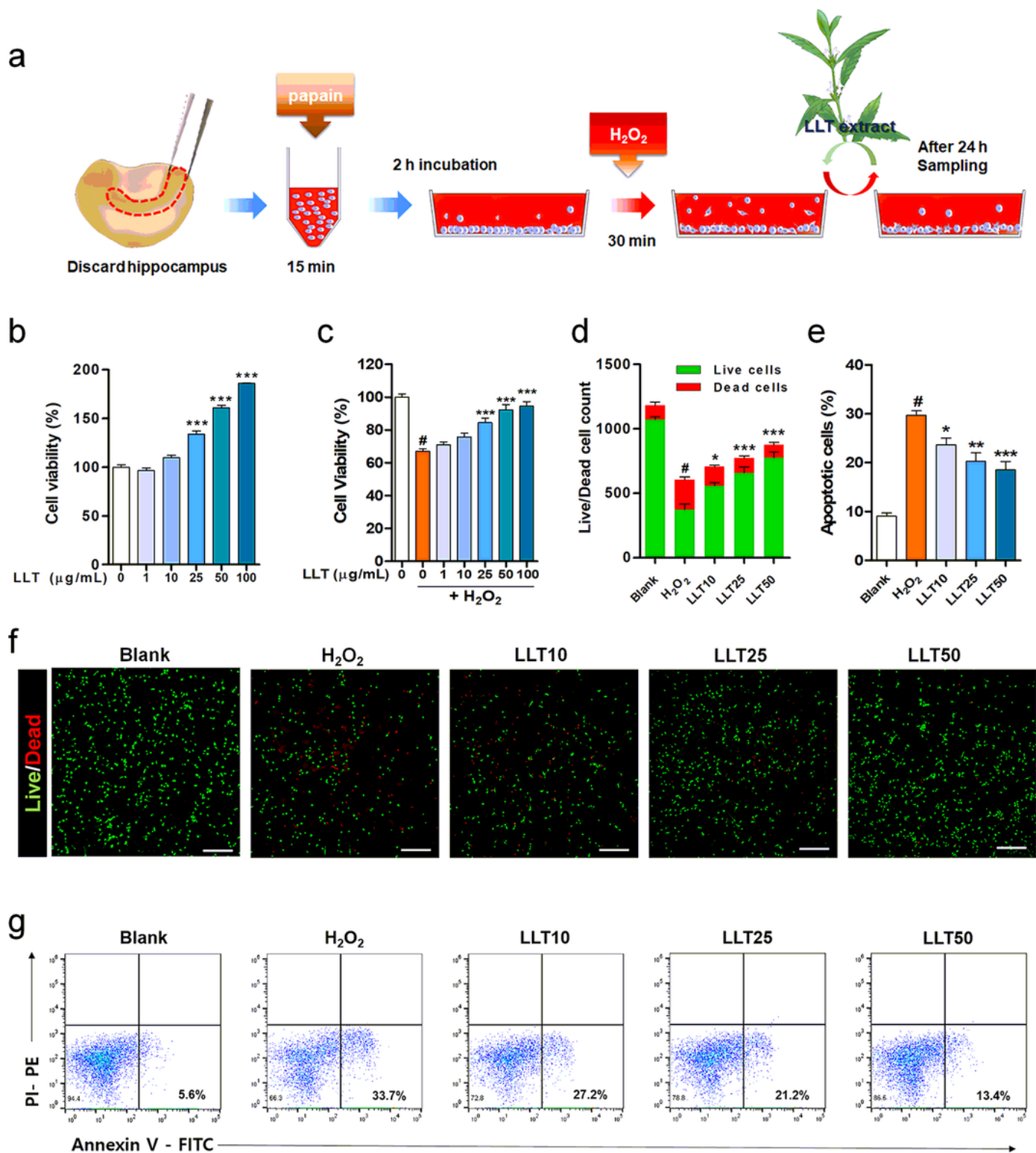
Figures

Figure 1

LLT protects cortical neurons against H2O2-induced neuron damage. (a) Schematic depiction of experimental design in primary cortical neuron (b) Cell viability assay results of cortical neurons treated with various concentration of LLT without H2O2 induction at 24 h, n=5; (c) CCK-8 results of H2O2-induced cortical neuron treated with various concentration of LLT at 24 h, n=6; (d) The total number of live and dead cells calculated from cellular fluorescence images on each group. n=10; (e) The percentage of

apoptotic cells analyzed by flow cytometry of Annexin-V-FITC/PI-PE double staining. n=5; (f) Fluorescence images of live and dead (red) cells for analysis of neuroprotective effect of LLT with different concentration on H2O2-induced cortical neuron. Scale bar = 200 μm; (g) Representative flow cytometry plot using Annexin-V/PI to confirm the mode of cell death. Blank = non-treatment, H2O2 = H2O2 only, LLT = H2O2+LLT extract. Significant differences indicated as #p < 0.001 vs. the Blank group; * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. the H2O2 group were analyzed via one-way ANOVA with Tukey's post-hoc test.
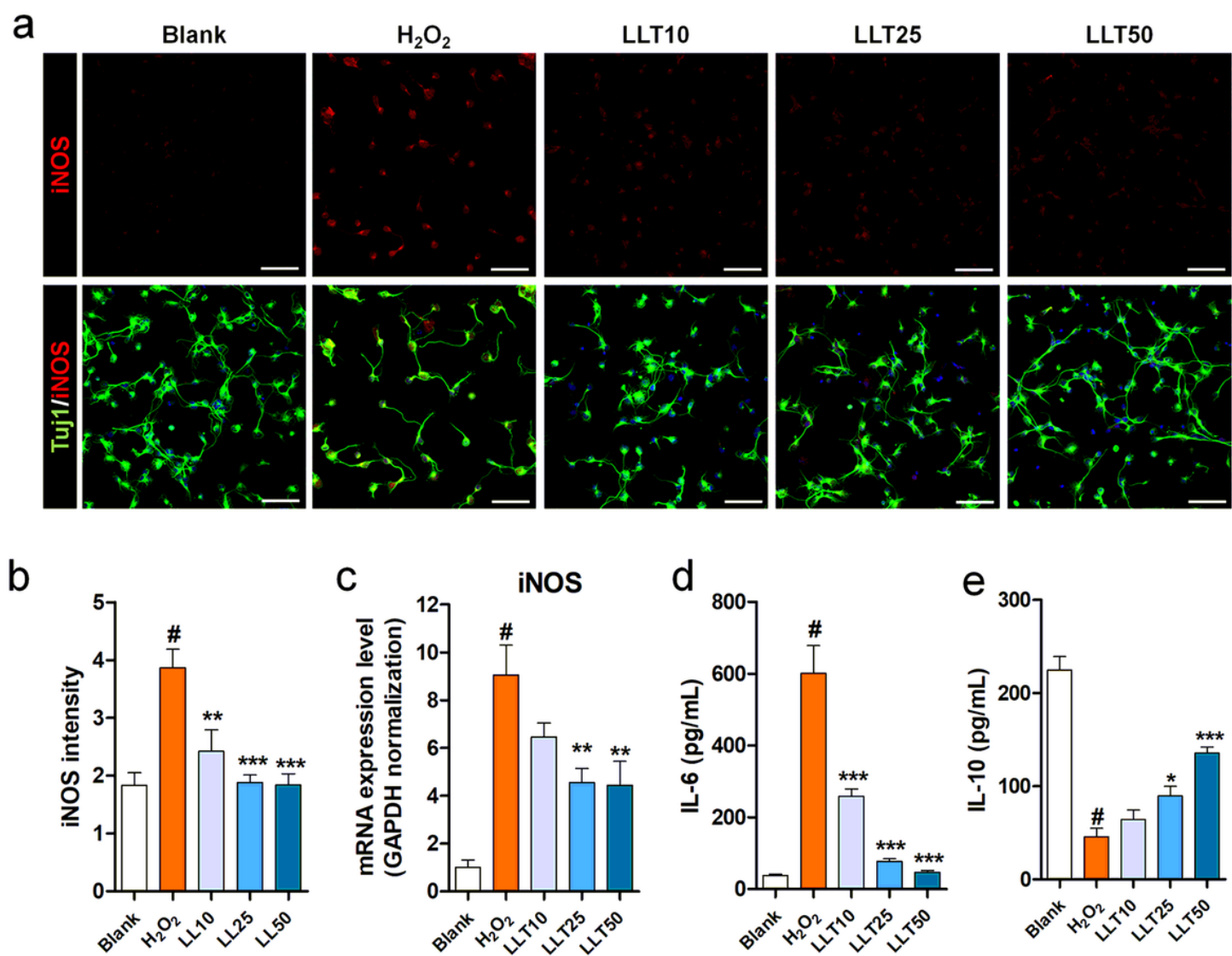
Figure 2

LLT suppress inflammatory responses in H2O2-treated cortical neuron. (a) Representative images of iNOS (red) for analysis of inflammatory response in H2O2-induced cortical neuron after LLT treatment, stained with Tuj-1 for neurites and DAPI (blue) for nuclei, Scale bar = 50 μm; (b) The relative intensity of iNOS per field of view was quantified from iNOS fluorescence images on each group. n=6; (c) Quantitative data from real-time PCR for iNOS after LLT treatment under H2O2 condition. (d, e) The levels of IL-6 (d)

and IL-10 (e) in culture medium by ELISA. n = 6; Blank = non-treatment, H2O2 = H2O2 only, LLT = H2O2+LLT extract. Significant differences indicated as #p < 0.001 vs. the Blank group; * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. the H2O2 group were analyzed via one-way ANOVA with Tukey's post-hoc test.
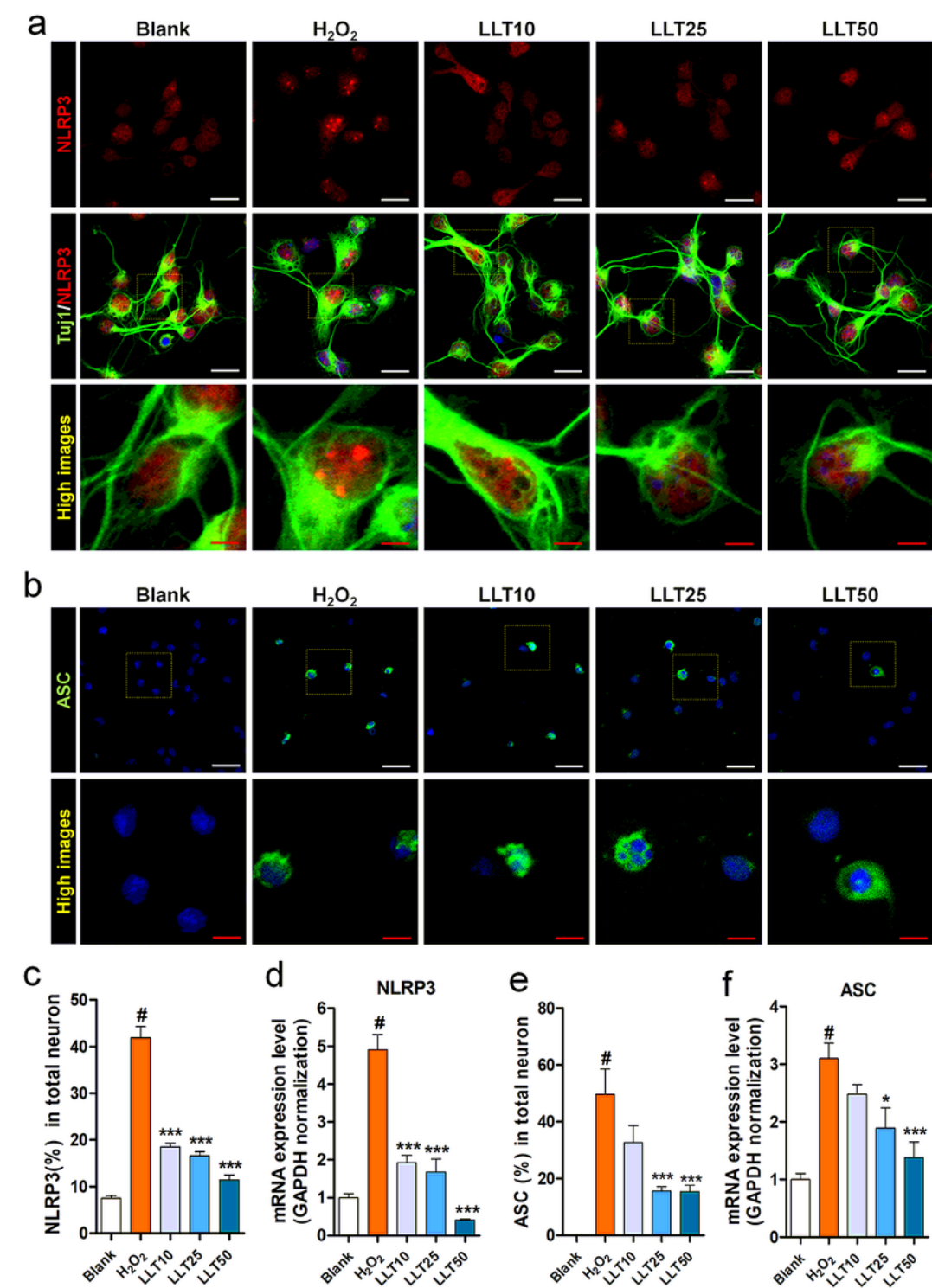

Figure 3

LLT inhibits the NLRP3-ASC activation by H2O2 in cortical neuron. (a) Representative immunocytochemical images of NLRP3 inflammasome (red) in H2O2-induced cortical neuron after LLT

treatment, stained with Tuj-1 for neurites and DAPI (blue) for nuclei. And (b) ASC following H2O2 treatment and the application of various concentration of LLT, stained with DAPI (blue) for nuclei. White scale bar = 50 μm, red scale bar = 20 μm; (c) The relative percentage of NLRP3 positive neurons for the primary cortical neuron of blank, H2O2, and LLT groups (10, 25, 50 μg/ml) at 24 h. (d) Quantitative data from mRNA analysis of NLRP3 levels in LLT groups compared with the H2O2 group. (e) The relative percentage of ASC positive neurons for the primary cortical neuron of blank, H2O2, and LLT groups at 24 h. (f) Quantitative data from mRNA analysis of ASC levels in LLT groups compared with the H2O2 group. n=6; Blank = non-treatment, H2O2 = H2O2 only, LLT = H2O2+LLT extract. Significant differences indicated as #$p < 0.001$ vs. the Blank group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the H2O2 group were analyzed via one-way ANOVA with Tukey's post-hoc test.
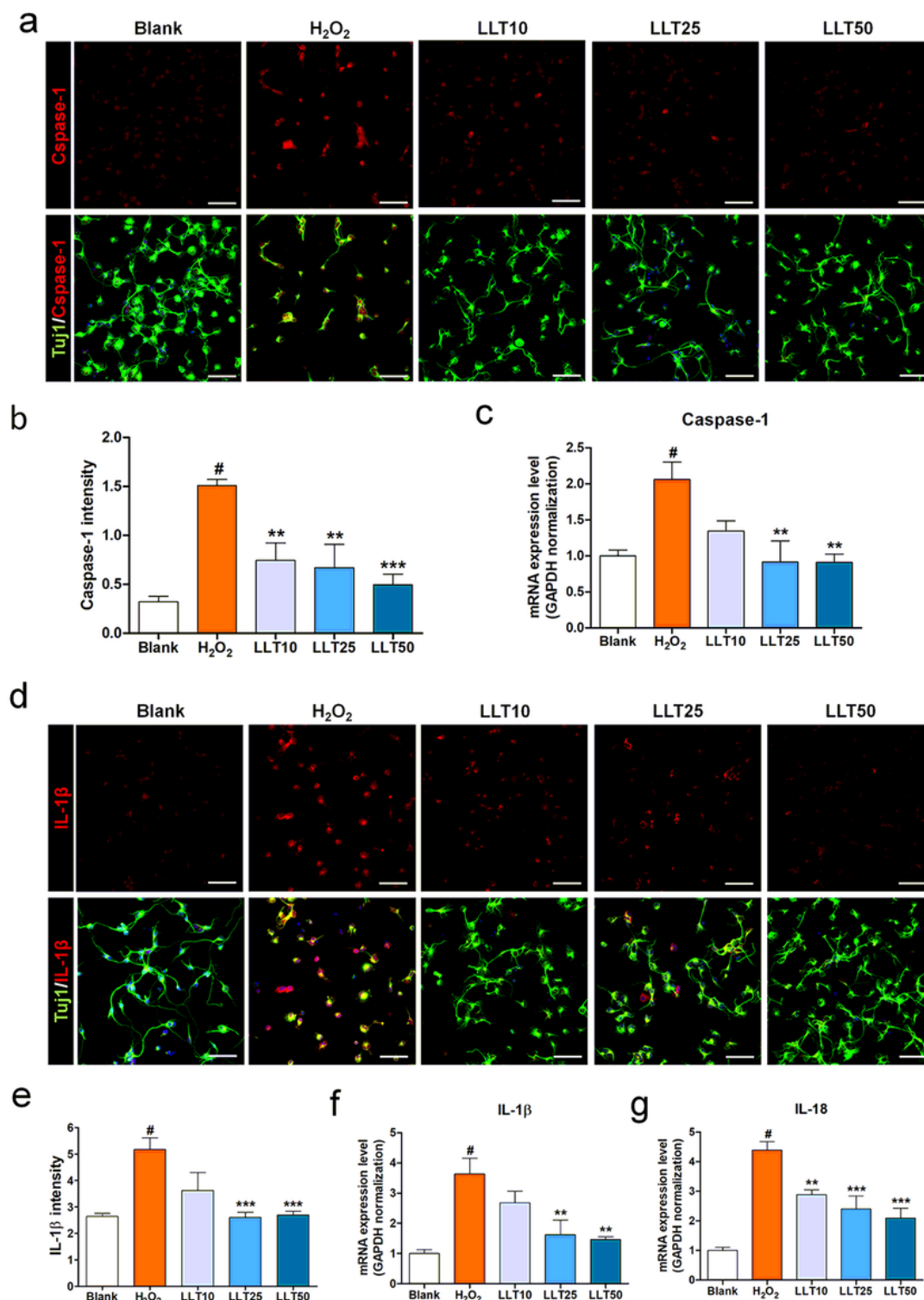
Figure 4

LLT inhibits the IL-1β and caspase-1 activation triggered by the NLRP3 inflammasome. (a) Representative fluorescence images of primary cortical neurons stained with caspase-1 (red), Tuj-1 for neurites and DAPI (blue) for nuclei in each group. Scale bar = 50 μm; (b) The quantitative representation of change in caspase-1 intensity after LLT treatment of various concentration. n = 6; (c) Quantitative data from real time PCR for caspase-1 in each group n = 6; (d) Representative fluorescence images of IL-1β (red) in

H2O2-induced cortical neuron after LLT treatment, stained with Tuj-1 for neurites and DAPI (blue) for nuclei. Scale bar = 50 μm; (e) The quantitative representation of change in IL-1β intensity after LLT treatment of various concentration. n = 6; (f-g) Quantitative data from real time PCR for IL-1β (f) and IL-18 (g) in each group n = 6; Blank = non-treatment, H2O2 = H2O2 only, LLT = H2O2+LLT extract. Significant differences indicated as #p < 0.001 vs. the Blank group; * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. the H2O2 group were analyzed via one-way ANOVA with Tukey's post-hoc test.
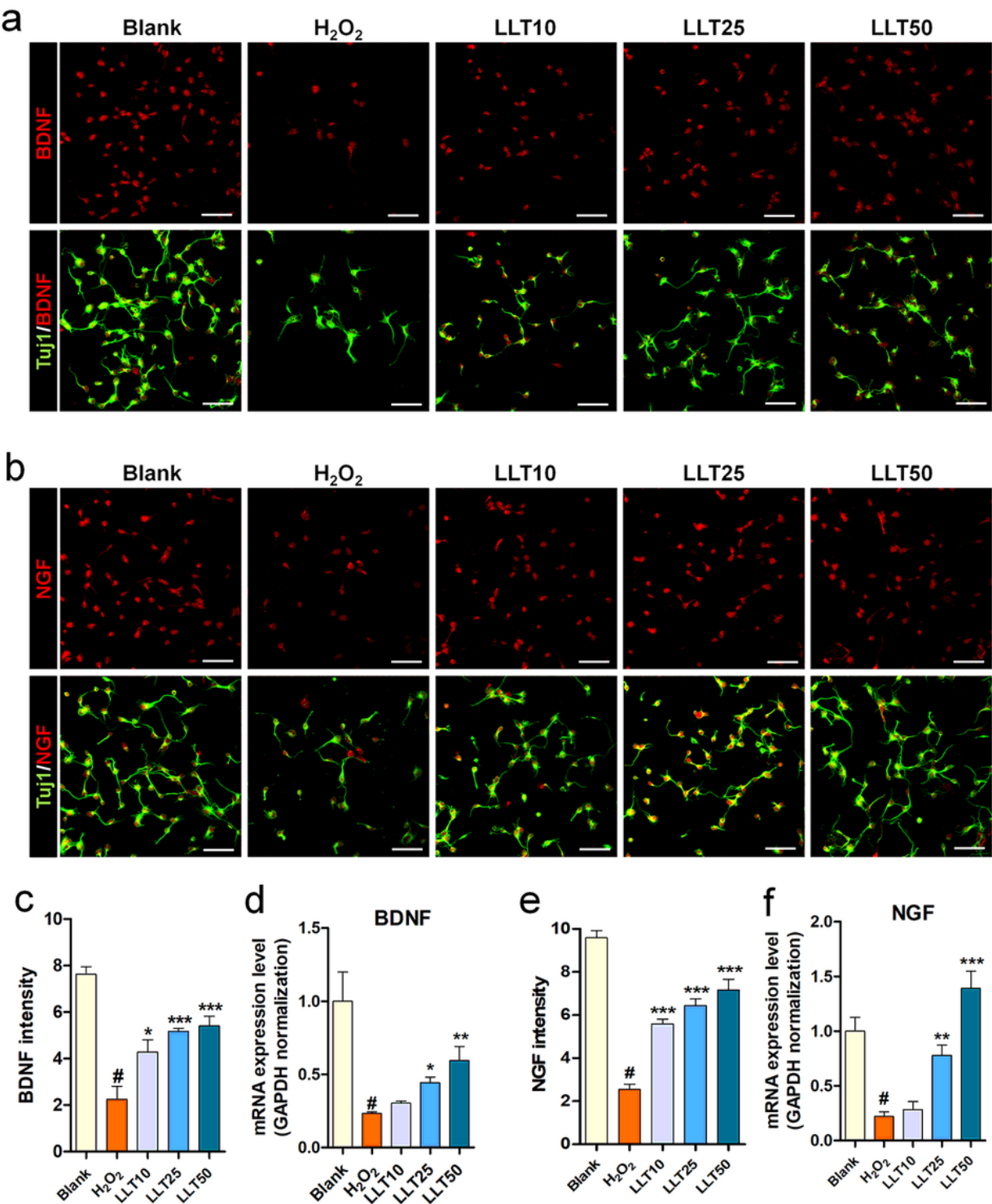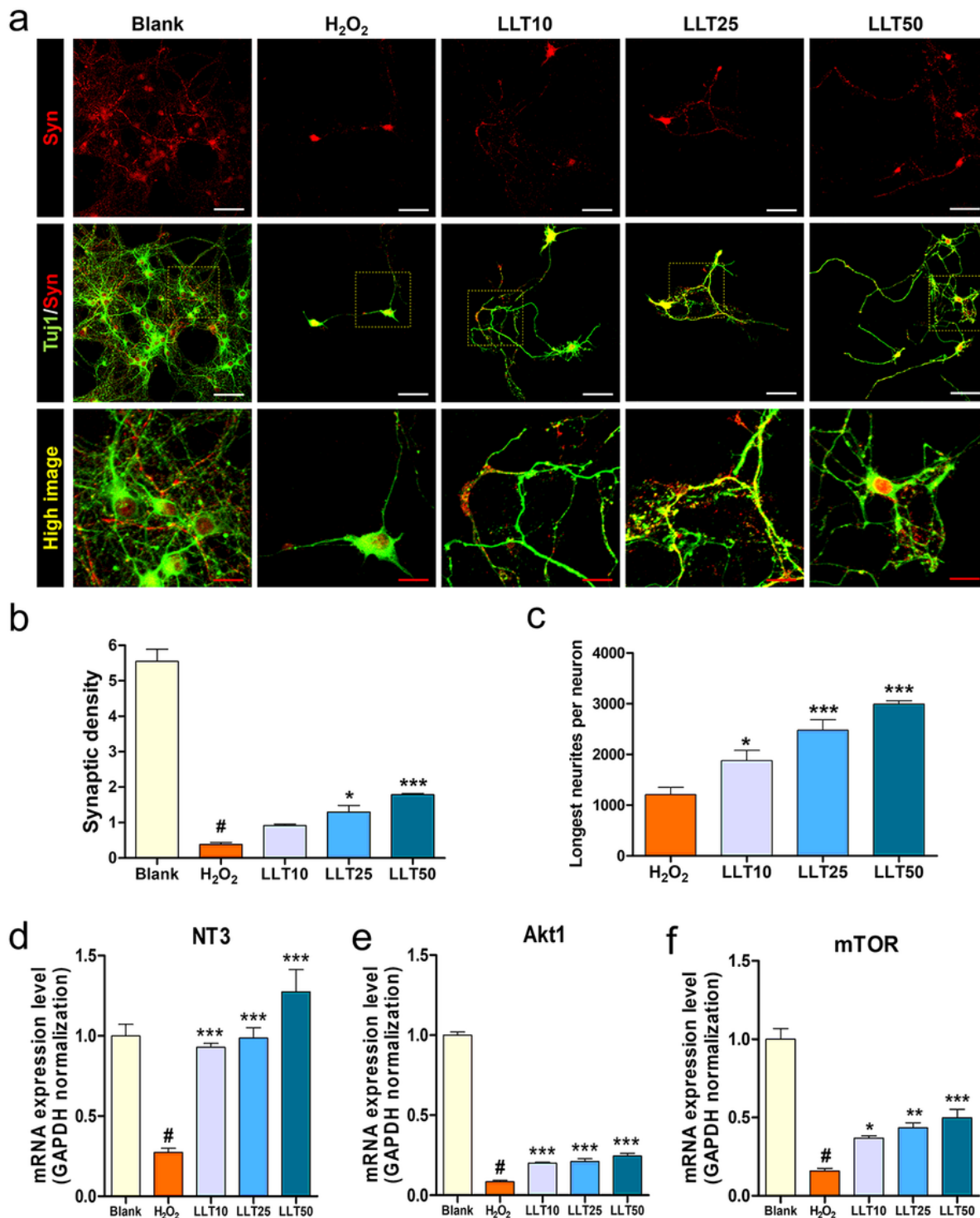

Figure 5

LLT enhances the neurotrophic factors expression in H2O2-treated cortical neuron. (a) Representative fluorescence images of primary cortical neurons stained with BDNF (red) and Tuj-1 in each group. (b) Representative fluorescence images of primary cortical neurons stained with NGF (red) and Tuj-1 in each group. (c-d) Quantification of (c) BDNF and (d) NGF intensities based on immunofluorescence images using image J. n = 6; (e-f) Relative quantification of (e) BDNF and (f) NGF genes by real-time PCR in each group. n = 6; Blank = non-treatment, H2O2 = H2O2 only, LLT = H2O2+LLT extract. Significant differences indicated as #$p < 0.001$ vs. the Blank group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the H2O2 group were analyzed via one-way ANOVA with Tukey's post-hoc test.

Figure 6

LLT enhances the axonal regrowth and synaptic connection in H2O2-treated cortical neuron. (a) Representative fluorescent images of synaptophysin (red) expression in Tuj1-positive neurons treated with LLT under H2O2 condition at 7 days. White scale bar = 50 µm, red scale bar = 20 µm; (b) Quantitative analysis of synaptic density per field of view in cortical neuron treated with LLT in the presence of H2O2. (c) Quantitative analysis of the longest neurite length per field of view in cortical neuron treated with LLT

under H2O2 condition. n=6; (d-f) Relative quantification from real time PCR for neuronal growth related genes, (d) NT3, (e) AKT and (f) mTOR at 24 h in H2O2-induced cortical neuron treated with LLT at various concentration (5, 10, 20 μg/ml) at 24 h. n=6; Blank = non-treatment, H2O2 = H2O2 only, LLT = H2O2+LLT extract. Significant differences indicated as #p < 0.001 vs. the Blank group; * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. the H2O2 group were analyzed via one-way ANOVA with Tukey's post-hoc test.
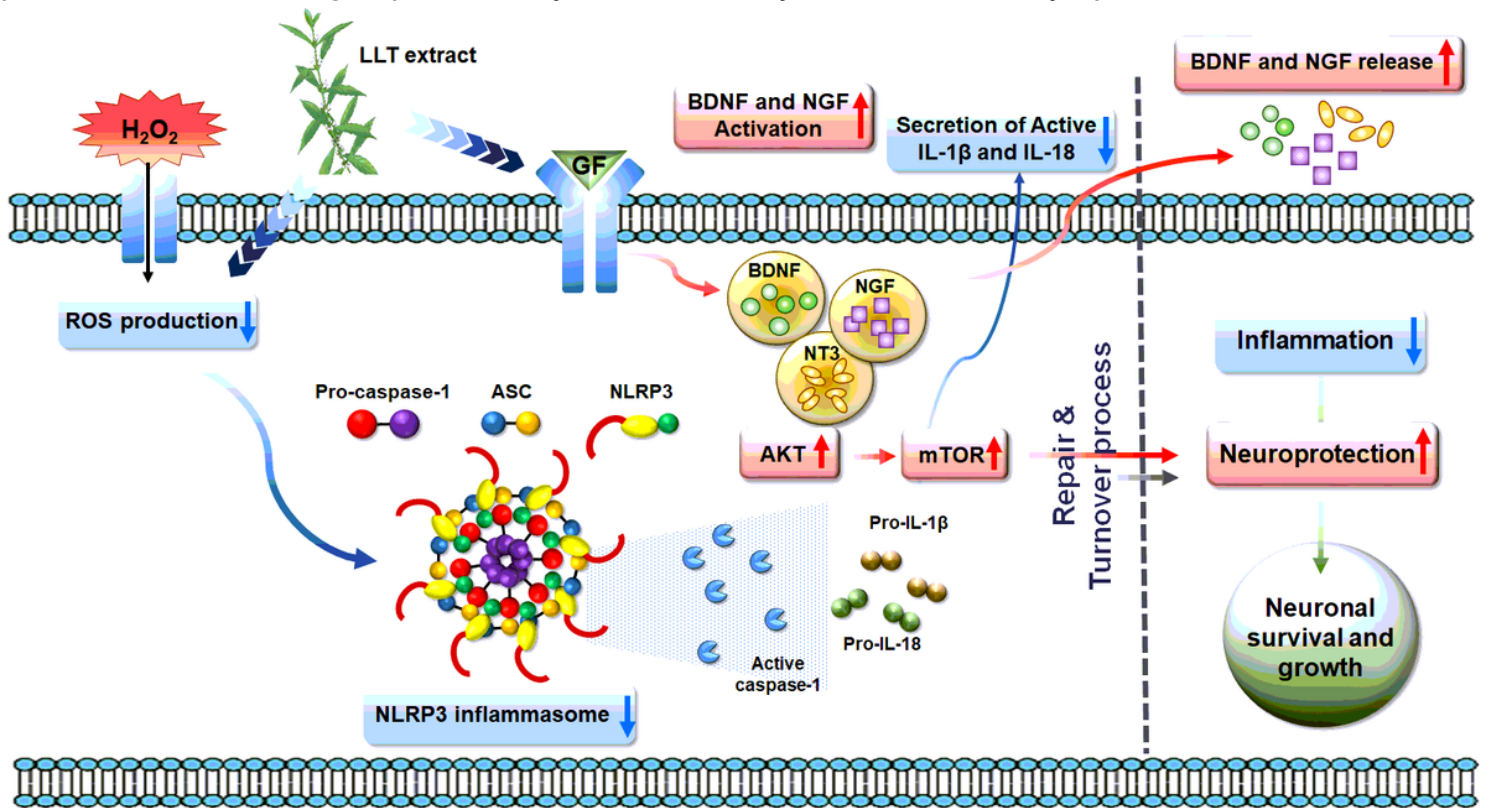

Figure 7

Schematic illustration showing the series of events associated with NLRP3 inflammasome activation in anti-inflammatory and neuroprotective effects of LLT extract through ROS scavenging, suppressing inflammation and apoptosis, enhancing neuronal cell growth and axonal regeneration, and promoting growth factors secretion.

Supplementary Files

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