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Synergism between Luteolin and Sulforaphane in Anti-inflammation

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Abbreviations: COX-2, cyclooxygenase-2; HO-1, heme oxygenase-1; IL-1β, interleukin-1β; IL-, interleukin-6; IκB, inhibitory κB; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LUT, luteolin; NO, nitric oxide; Nrf2, nuclear transcription factor erythroid 2p45 - related factor2; p-IκB, phosphorylated IκB; p-STAT3, phosphorylated STAT3; ROS, reactive oxygen species; SFN, sulforaphane; STAT3, signal transducer and activator of transcription3.

Abstract

Luteolin and sulforaphane are well-known food bioactives with anti-inflammatory properties. Herein, we determined their combinational effects in inhibiting inflammation in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Both luteolin and sulforaphane showed dose-dependent inhibition on LPS-induced production of nitric oxide (NO) in the macrophages. The combined treatments led to a stronger inhibition on NO production compared to the singular treatments. Isobologram analysis confirmed that the combined treatments produced a synergy. Western blotting and ELISA showed that the combined treatment reduced the expression levels of pro-inflammatory proteins involving NF-κB pathway, and STAT3 activation, which regulated expression of other inflammatory proteins such as iNOS, COX-2, IL-6, and IL-1β. Moreover, the combination treatments reduced reactive oxygen species in cells and increased the expression of Nrf2 and HO-1, which are cellular antioxidant proteins. In conclusion, our findings support the notion that certain bioactive food components may act synergistically to produce enhanced health effects such as anti-inflammation.

Keywords: Luteolin, Sulforaphane, Combination, Synergy, Anti-inflammation, Antioxidant

1. Introduction

Human diet consists of many different food components that are likely to interact with each other to affect physical, chemical, and biological properties of these components in the human body. Accumulating evidence suggested that different bioactive food components might interact with each other to produce synergism that results in more potent health benefits than those individual food components. Studies have suggested that increased health effects by the combination of different food bioactives might decrease the dose of these bioactives needed in the diet to manifest the health effects [1-5]. Therefore, to have a meaningful understanding on the role of certain food bioactives as a part of the diet in promoting health, it is important to understand their potential interactions with other food bioactives in biological functions. However, currently there is only very limited information available on the potential interactions between different food bioactives, which greatly limited the development of effective diet-based strategies for health promotion.

Dysregulation of the inflammatory response can induce chronic inflammation leading to many disorders and diseases, such as neurodegenerative diseases, cardiovascular diseases and cancers [6]. During inflammation, there is an accumulation of the reactive oxygen species (ROS) due to an increased oxygen uptake. ROS activate cellular survival signaling pathways including nuclear factor-κB (NF-κB) and the upstream kinase cascades which are known to have promotional roles in inflammation and other related diseases conditions [7]. Inhibition of cellular inflammation and ROS accumulation has been considered as a promising approach to lower the risk of inflammation-driven diseases.

Sulforaphane (SFN; figure 1) is a well-known bioactive isothiocyanate compound that is produced during the myrosinase-catalyzed conversion of glucosinolates in cruciferous vegetables.

SFN have demonstrated potent antioxidative and anti-inflammatory properties in different experimental models [8-10]. Luteolin (LUT) is an abundant flavone found in ranges of vegetables such as thyme, parsley, artichokes, celery, spinach, as well as in some cruciferous plants (*Brassica oleracea*). Broccoli, cauliflower, and cabbage contain both SFN and LUT[11-13]. *In vitro* and *in vivo* experiments have revealed anti-inflammatory activity of LUT. This bioactive is also known as a good ROS scavenger due to the containment of two hydroxyl groups in its ring structure [8]. To understand mode of interaction between SFN and LUT at the cellular level representing consumption of specified vegetables, we studied the combination effects of these two dietary components on the inhibition of inflammation and ROS accumulation in LPS-stimulated RAW 264.7 macrophages.

2. Materials and Methods

2.1. Cell Culture and Treatments

Raw 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Mediatech, Manassas, VA, USA) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Cells were treated with LUT (98%, Quality Phytochemicals, Edison, NJ, USA), SFN (> 98%, Quality Phytochemicals), or their combinations concomitant with lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) stimulation for 24 hours prior to the analysis in different assays. The test compounds were dissolved in dimethyl sulfoxide (DMSO). All treatments contained a final concentration of 0.1 % v/v DMSO in the culture media.

2.2. Cell Viability and Nitric Oxide Assays

Twenty four hours after the seeding of RAW 264.7 cells in 96-well plates (1.0×10^5 cells/well), cells were treated with LPS (1 μg/mL) with and without the test compounds followed by the measurements of cell viability and NO production after 24 hours of incubation [3]. To perform NO assay, 150 μL of the culture medium was mixed with 100 μL of Griess reagent (2% sulfanilamide and 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride in phosphoric acid), and the mixture was incubated at room temperature for 30 minutes before the absorbance at 540 nm was measured using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich). Cells in each well were incubated with 100 μL of culture medium containing 0.1 mg/mL MTT at 37 ^{o}C for 2 hours. MTT containing media were removed prior to the solvation of reduced formazan dye using 100 μL/well of DMSO, and the absorbance was measured at 570 nm using a microplate reader.

2.3. Analysis of Synergy

The mode of interaction (synergism, additivity or antagonism) between LUT and SFN at different concentrations was determined based on Chou and Talalay's method [1] with modifications as we previously described [2, 3], using an R program. This model is used for combination of two compounds at a constant ratio. When the combination dose of compund1 and compound2 at d_1 and d_2, respectively, provides the same effect x as Compound1 alone at dose $D_{x,1}$ and Compound2 alone at dose $D_{x,2}$, the combination index (see equation below) indicates

synergism, additivity, or antagonism of the combinatorial effect when the index <1, =1, or >1, respectively.

$$\text{Combination index} = d_1/D_{x,1} + d_2/D_{x,2} \quad (1)$$

The median-effect plot demonstrated by the equation below was used to determine D value that is the dose of a test compound that demonstrates the E effect. E was the fraction of NO production in this study while α is a slope parameter, and D_m presents the median effective dose of the compound.

$$\log [E/(1-E)] = \alpha(\log D + \log D_m) \quad (2)$$

2.4. Enzyme-Linked Immunosorbent (ELISA) Assay

Twenty-four hours after the seeding of RAW 264.7 cells in 6-well plates (3.75×10^6 cells/well), cells were treated with LPS (1 μg/mL) with and without the test compounds. The levels of inflammatory cytokines such as IL-1β, and IL-6 were determined in the cytoplasmic fraction of the cells or in the culture media by ELISA kits according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA; eBioscience, San Diego, CA, USA).

2.5. Cellular Sample Preparation and Western Blotting

Twenty-four hours after the seeding of RAW 264.7 cells in 100 mm petri dishes, cells were treated with LPS (1 μg/mL) with and without the test compounds. To determine the expression levels of p-p65, p65, and p-IκB, cells were collected at 1 hour after different treatments. Expression of other proteins was monitored after 24 hours of different treatments. Nuclear and cytoplasmic fractions were extracted using NE-PER extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). Whole cell lysate was collected in radioimmunoprecipitation assay buffer (RIPA buffer) containing protease and phosphatase inhibitors (Boston BioProducts, Ashland, MA, USA). Cell suspensions were sonicated and lysed on ice for 20 minutes. Supernatants were collected after centrifugation at 20,817 g for 10 minutes. Based on bicinchoninic acid (BCA) protein assay, equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GVS Filter Technology, Indianapolis, IN, USA). After blocking, specific antibodies were used to detect target proteins at the manufacturer's recommended concentrations. Protein bands were visualized using Odyssey system (LI-COR, Lincoln, NE, USA) after incubation with appropriate secondary antibodies. Antibodies for p65, p-p65, p-IκB, STAT3, and p-STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for iNOS, COX-2, Nrf2, and HO-1 were from Santa Cruz Biotechnology (Dallas, TX, USA). β-Actin antibody (Sigma-Aldrich) was used to show whole cell lysate loading control. PARP antibody (Cell Signaling) was used to show loading controls in cytoplasmic, and nuclear fractions.

2.6. Dichlorofluorescein-Diacetate Assay

Twenty-four hours after the seeding of RAW 264.7 cells in black 96-well plates (1.0×10^5 cells/well), cells were treated with LPS (1 μg/mL) with and without the test compounds followed by the measurements of intracellular oxidative stress using dichlorofluorescein-diacetate (DCFH-DA) assay as previously reported [14]. After removal of cell culture media, cells were incubated with 5 μM DCFH-DA in serum-free RPMI media for 20 minutes in the dark at room temperature. Excess DCFH-DA was washed off with serum-free media. Intracellular esterase cleaved the ester bond in DCFH-DA yielding DCFH, which was oxidized by reactive oxygen species and become a fluorescent. Fluorescence at the excitation and emission wavelengths of 485/528 nm was measured using a microplate reader.

2.7. Statistical Analysis

All data were presented as mean + SD. The values were compared to the control using one-way analysis of variance (ANOVA) and Student's t-test. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Determination of non-cytotoxic concentrations of LUT, SFN, and their combinations in LPS-treated RAW 264.7 macrophages

Cell viability (MTT) assay was performed to determine the potential cytotoxic effect of the LUT (5-25 μM), SFN (0.25-1.25 μM) or their combinations at the half doses. As shown in Figure 2, none of the treatments caused any noticeable cytotoxicity on LPS-treated macrophages with cell viability greater than 95% in comparison to that of the control cells. Subsequently,

these non-toxic concentrations were used to determine their anti-inflammatory properties in LPS-treated macrophages.

3.2. Synergistic effect of LUT/SFN combination in inhibiting LPS-induced overproduction of NO in macrophages.

To determine the anti-inflammatory effects, RAW 264.7 macrophages were treated with LUT, SFN or their combinations at various concentrations for 24 hours in the presence of LPS. As shown in Figure 3A, a dose-dependent inhibition of LPS-induced NO production by the individual or combination treatments of LUT and SFN was observed. LUT at the concentrations of 5, 10, 15, 20, and 25 μM caused 7, 17, 28, 34 and 46 % inhibition on NO production, respectively. SFN at the concentrations of 0.25, 0.50, 0.75, 1.00, and 1.25 μM demonstrated 12, 21, 37, 45 and 51 % inhibition, respectively. Most importantly, the combined treatments with half does of LUT + SFN (at a ratio of 20: 1) produced 12, 24, 35, 46, and 56 % of inhibition on NO production, which were generally similar or stronger than the inhibition caused by individual treatment of LUT or SFN at their full doses. For example, LUT at 25 μM and SFN at 1.25 μM led to 46% and 51% inhibition, respectively, while LUT at 12.5 μM plus SFN at 6.25 μM caused 56% inhibition.

Based on Chou and Talalay's method [1], we further determined the mode of interaction between LUT and SFN in inhibiting NO production using an isobologram-based analysis (Figure 3B). All concentration pairs of LUT/SFN combination treatments produced combination indexes (CI) between 0.82 to 0.87, indicating a synergistic interaction between LUT and SFN in inhibiting NO production in LPS-treated macrophages. IC_{50} values of the combination treatments

(11.25 ± 1.43 μM of LUT + 0.56 ± 0.07 μM of SFN) obtained from the median-effect plot (Figure 3C) were lower in comparison to the IC_{50} values of LUT (29.65 ± 4.92 μM) or SFN (1.22 ± 0.09 μM) alone. The median-effect plot showed that the mathematic model used herein fitted well the data obtained from the experiment.

3.3. Synergistic effect of LUT/SFN combination in inhibiting LPS-induced overproduction of pro-inflammatory cytokines in macrophages.

IL-6, and IL-1β, are cytokines known to play key roles in inflammation [15, 16]. By ELISA, the levels of these cytokines were assessed to determine the effects of LUT, SFN and their combination on the production of these cytokines in the macrophages. As shown in Figure 4A, LPS significantly induced the extracellular release of IL-6 by macrophages. LUT at 5 μM did not produce any significant effect, but at the higher concentration of 20 μM decreased IL-6 production by 26 %. SFN at 0.25 and 1.0 μM showed a dose-dependent inhibition of IL-6 production by 6 and 20 %, respectively. The combination of LUT and SFN (5 μM LUT + 0.25 μM SFN and 10 μM LUT + 0.50 μM SFN) decreased IL-6 production by 13 and 27 %, respectively. Isobologram analysis showed that the two combination treatments produced the combination index of 0.78 and 0.82, which suggested that the combination treatments with LUT and SFN synergistically inhibited the IL-6 production by LPS-treated macrophages.

Due to a low level of extracellular IL-1β found in the culture media, the treatment effects were determined on the intracellular level of IL-1β in the cytoplasmic fraction of the LPS-treated macrophages [17]. LPS significantly increased IL-1β level in macrophages (Figure 4B). SFN at 0.25 μM did not significantly reduce the level of IL-1β but its higher concentration at 1 μM

decreased IL-1β by 43 %. LUT at 5 and 20 μM inhibited IL-1β level by 42 and 83 %, respectively. The combination of 0.25 μM SFN + 5 μM LUT, 0.5 μM SFN + 10 μM LUT, and 1 μM SFN + 20 μM LUT dose-dependently inhibited the intracellular level of IL-1β by 42, 73, and 97 %, respectively. Based on the CI obtained for each combination treatment, combination of 1 μM SFN + 20 μM LUT produced CI of 0.26, indicating a strong synergistic effect between LUT and SFN in inhibiting LPS-induced overproduction of IL-1β in macrophages, while LUT/SFN combinations at the lower doses produced additive or moderately synergistic effects.

3.4. Combination of LUT and SFN decreased expression levels of pro-inflammatory proteins

Since the LUT/SFN combination exerted synergistic inhibitory effects on multiple pro-inflammatory biomarkers as mentioned above, to better understand their bioactivities, next we investigated the underlying molecular mechanisms. NF-κB pathway is essential for inflammatory control. The expression levels of a subunit of NF-κB, p65, its phosphorylated form (p-p65), p-IκB as well as downstream proteins under the regulation of NF-κB, including iNOS and COX-2, were determined by Western blotting. The results in Figure 5A demonstrated that both single and combination treatment of SFN and LUT decreased the expression level of LPS-stimulated p-p65 and p65 in the nucleus. However, the combination treatment of SFN and LUT showed more potent inhibition than their single treatments, especially at the lower doses. For example, half dose combination treatment with 0.25 μM SFN + 5 μM LUT decreased p-p65 and p65 expression level by 42 and 39 %, respectively, while full dose single treatment with 0.5 μM SFN or 10 μM LUT only decreased these levels about 0 to 23%. Both single and combination

treatments with SFN and LUT caused dose-dependent inhibition on the expression level of cytosolic p-IκB. SFN alone at 0.5 and 1 μM decreased p-IκB expression by 19 and 33%, respectively. LUT alone at 10 and 20 μM decrease p-IκB expression by 46 and 57 %, respectively. The combination of 0.25 μM SFN + 5 μM LUT, 0.5 μM SFN + 10 μM LUT, and 1 μM SFN + 20 μM LUT decreased the expression of cytosolic p-IκB by 35, 37 and 70 %, respectively.

Compared to the effects on NF-κB and p-IκB expression, the inhibitory effects of LUT/SFN combinations on LPS-induced iNOS expression showed stronger trend of synergism between SFN and LUT (Figure 5B). The expression of iNOS was decreased by a single treatment of SFN (0.5 and 1 μM) and LUT (10 and 20μM) by 52, 69, 57, and 76%, respectively. The combination of 0.25 μM SFN + 5 μM LUT, 0.5 μM SFN + 10 μM LUT, and 1 μM SFN + 20 μM LUT decreased the expression of iNOS by 57, 79, and 94 %, respectively. In contrast, both single and combination treatments with SFN and LUT showed moderate inhibition (<28%) on the expression level of COX-2. No significant enhancement by the combination was observed.

Another major transcription factor that regulates inflammatory process is STAT3. As shown in Figure 5C, LPS activated STAT3 signaling by increasing the expression of phosphorylated form, p-STAT3, by almost 5-fold. LUT or SFN alone as well as their lower doses of combined treatment did not significantly alter the expression of STAT3 in LPS-treated macrophages. Only the combination of 1 μM SFN + 20 μM LUT significantly decreased STAT3 expression. LUT at the low concentration did not alter the expression level of p-STAT3. SFN (1 μM) and LUT (20 μM) alone at high concentration decreased p-STAT3 expression by 14, and 55 %, respectively, while combination of SFN and LUT at three different concentrations produced a dose-dependent decrease of p-STAT3 expression by 29, 49, and 73%.

3.5. Combination of LUT and SFN activated Nrf2 signaling and increased expression of HO-1, a phase-2 antioxidant and detoxifying enzyme.

According to considerable evidence suggesting links between oxidative stress and inflammation [7, 18], the expression of HO-1, which is an antioxidant protein known to possess anti-inflammatory function [19], as well as its transcriptional regulator Nrf2 were monitored through Western blotting. We found that both single and combined treatment of SFN and LUT increased the levels of nuclear Nrf2 (Figure 6A), but the combined treatment did not lead to stronger effects than the single treatments. SFN or LUT alone increased nuclear Nrf2 by as much as 42 and 79%, respectively, while their combination increased the nuclear Nrf2 level by up to 54%. In figure 6B, the expression of HO-1 was increased by both single and combined treatments, especially the combination of 1 μM SFN with 20 μM LUT increased HO-1 expression by 123% compared to the control, and this effect was stronger than that produced by single treatments.

3.6. Combination of LUT and SFN attenuated cellular oxidative stress

DCFH-DA, a fluorescent indicator of ROS was used to quantify the level of cellular oxidant in LPS-stimulated RAW 264.7 cells. Our results demonstrated that single and combination treatments of LUT and SFN suppressed the cellular ROS in a dose-dependent manner (Figure 7). LUT alone (5, 10, and 20 μM) decreased ROS by 35, 50, and 59%, respectively. SFN alone (0.25, 0.5, and 1.0 μM) decreased ROS by 17, 42, and 56%, respectively. Three LUT/SFN combined treatments with low to high concentrations produced a significant and

synergistic effect by decreasing the cellular ROS level by 45, 61, and 81%, respectively, with combination index of 0.75 for the combination of 1 μM SFN with 20 μM LUT.

4. Discussion

Utilizing bioactive in combination to inhibit diseases has received an increasing amount of attention due to the possibility of producing enhanced efficacy while reducing the dosage in order to avoid the risk of potential side effects and to reduce the development of treatment resistance [1, 20]. In this study, we aimed to evaluate the potential synergistic anti-inflammatory activities from the combination of LUT and SFN and elucidate the molecular mechanisms by which LUT/SFN co-treatment suppressed inflammatory response in RAW 264.7 macrophages.

LPS-stimulated RAW 264.7 macrophages were used as a model in the inflammation study. Cell viability under single and combinatorial treatments was firstly obtained to ensure non-cytotoxic effects from the treatments. In terms of single treatments, SFN demonstrated higher efficacy than LUT to reduce NO production. When SFN and LUT were combined at their half-dose with the ratio of LUT: SFN being 20: 1 based on their IC_{50} values, the combination treatment produced a synergy in the inhibition of NO production induced by LPS. The IC_{50} values of the combined treatment (11.25 μM LUT + 0.56 μM SFN) were lower by 2-3-fold in comparison to the IC_{50} values of LUT or SFN alone. The combinations produced a higher anti-inflammatory effect than the full-dose treatment with LUT or SFN alone, which suggested that lower doses of each bioactive compounds could be utilized in combination to produce satisfactory efficacy. This is meaningful because it could avoid potential adverse effects associated with high doses of these compounds [21]. Based on isobologram analysis, LUT/SFN

combination displayed a moderate synergism with the combination index ranging from 0.82 – 0.87 over concentrations tested in this study. Similar to our findings, other studies demonstrated synergistic anti-inflammation in RAW 264.7 cells when SFN was combined with other bioactives including nobiletin, curcumin, or phenyl isothiocyanate [3, 22]. LUT was also demonstrated to produce a synergism in anti-inflammation when combined with chicoric acid or tangeretin [2, 5]. This information supports the hypothesis that different dietary components could interact synergistically to produce enhanced health benefits.

Besides NO, other inflammatory cytokines that are biological markers related to inflammation and other chronic diseases including cancers, cardiovascular disease, and diabetes were also quantified to further substantiate the anti-inflammatory effects of the LUT/SFN combination treatments [23-25]. It was found that LUT/SFN in combination synergistically suppressed the extracellular secretion of IL-6 and reduced the intracellular level of IL-1β in macrophages, except at the low combinatorial concentrations, at which the combination produced additive effects on the inhibition of IL-1β production. In comparison to the result of NO inhibition, whose combination indexes were relatively constant over a range of the treatments, combination indexes for the inhibition of IL-6 were similar with the values ranging from 0.78-0.82 while the combination indexes for the inhibition of IL-1β decreased when concentrations of the combinatorial treatment increased. These findings suggested that LUT/SFN combinations had different inhibitory effects on these pro-inflammatory cytokines.

To further clarify the molecular mechanisms of the inhibitory effects of the LUT/SFN combination on inflammatory mediators, we investigated the effects of the combination on the activation of two transcription factors, NF-κB, and STAT3, and their related proteins in LPS-stimulated macrophages. The Western blotting results showed that LUT, SFN, and their

combinations dose-dependently inhibited LPS-induced expression of p65 subunit of NF-κB, p-p65 and p-IκB which is a feedback control of NF-κB activation. This observation corresponded with previous studies reporting that LUT or SFN alone inhibits NO production through the NF-κB pathway [3, 5]. Similar trends were also observed in the expression of iNOS and COX-2, which are known to be regulated through this pathway [26]. However, LUT, SFN, and their co-treatment had higher efficacy to decrease protein expression of iNOS than COX-2. The result was obvious when 1 μM SFN was combined with 20 μM LUT. They decreased iNOS expression by as much as 94% while they only decreased COX-2 expression by 20%. These compounds, especially their combination targeting iNOS relatively stronger than COX-2, introducing an idea of using a COX-2 inhibitor to combine with LUT or SFN or both in order to improve anti-inflammation efficacy, especially against COX-2 driven inflammation.

The other major transcription factor induced by inflammatory mediators is STAT3. LPS has been shown to induce STAT3 phosphorylation [27], through the presence of IL-6 and IL-1β [28, 29]. Correspondingly, our data showed that LPS increased expression of STAT3 and p-STAT3. The LUT/SFN combined treatment does-dependently decreased the expression of these proteins, especially p-STAT3 that is an activated form of STAT3. The trend of p-STAT3 expression under combination treatments corresponded with the trend of IL-6 and IL-1β levels, suggesting a potential correlation between these inflammatory cytokines with STAT3 activation in this system.

Studies have shown a sustained relationship between oxidative stress and inflammation. ROS can activate the NF-κB pathway responding to many cellular activities including inflammation [7]. Therefore, Nrf2 and HO-1, which are important phase-2 antioxidant and detoxifying proteins and the level of cellular reactive oxygen species, were examined. The LUT/SFN combination dose-dependently increased Nrf2 and HO-1 expression. However, a

synergy was observed only in increasing the expression of HO-1, suggesting a potential presence of other transcription factor besides Nrf2 that regulated phase 2 antioxidant and detoxification proteins under LUT/SFN treatment. Actually, there has been evidence in various cell lines demonstrating complexed regulatory control of HO- 1 through transcription factors including NF-κB, and STATs[30, 31]. Our data were consistent with a previous study demonstrating that single treatment of SFN dose-dependently upregulated HO-1 at the transcriptional level [10]. Corresponding with an increase of HO-1 expression, LUT, SFN and their combinations significantly and dose-dependently decreased reactive oxygen species in LPS-treated RAW 264.7 cells. According to its chemical structure, LUT possesses antioxidant activity by scavenging free radicals [32], chelating metal ions [33], and also upregulating antioxidant enzymes [34, 35]. Unlike LUT, SFN is not a direct free radical scavenger . Its antioxidant activity is associated with the induction of phase-2 detoxifying enzymes including HO-1 [36]. These different antioxidant mechanisms between LUT and SFN may explain a synergistic attenuation of cellular oxidative stress, which could be related to the regulation of inflammatory mediators in the anti-inflammatory system[7].

5. Conclusion

In summary, the present study showed for the first time that LUT and SFN in combination synergistically suppressed inflammation in LPS-treated RAW 264.7 macrophages. Our results also demonstrated that the combined anti-inflammatory effects of LUT/SFN involved mechanisms including inactivation of NF-κB and STAT3 pathway, which subsequently regulated expression of other pro-inflammatory proteins such as iNOS, COX-2, IL-6, and IL-1β.

In addition, the combined treatment also increased the expression of cellular antioxidant proteins Nrf2 and HO-1 to counteract ROS generated during inflammation. These findings suggest an alternative notion of bioactive compounds in combination including the combination of LUT and SFN that may be used to produce enhanced inhibitory effects against inflammation-driven diseases.

Conflict of interest

The authors declare that there is no conflict of interest.

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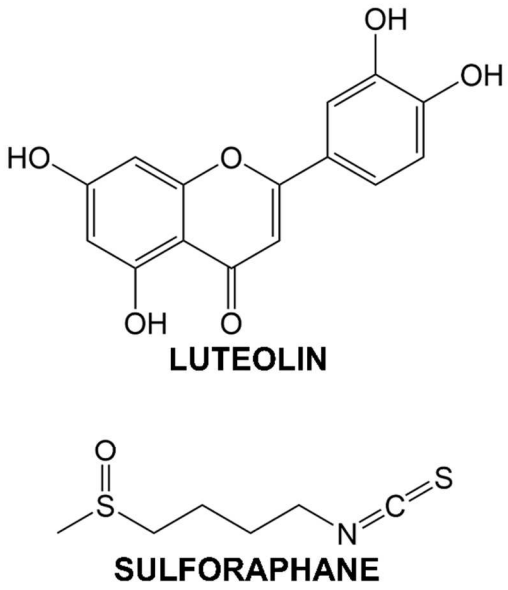


Figure 1. Chemical structures of luteolin (LUT) and sulforaphane (SFN).

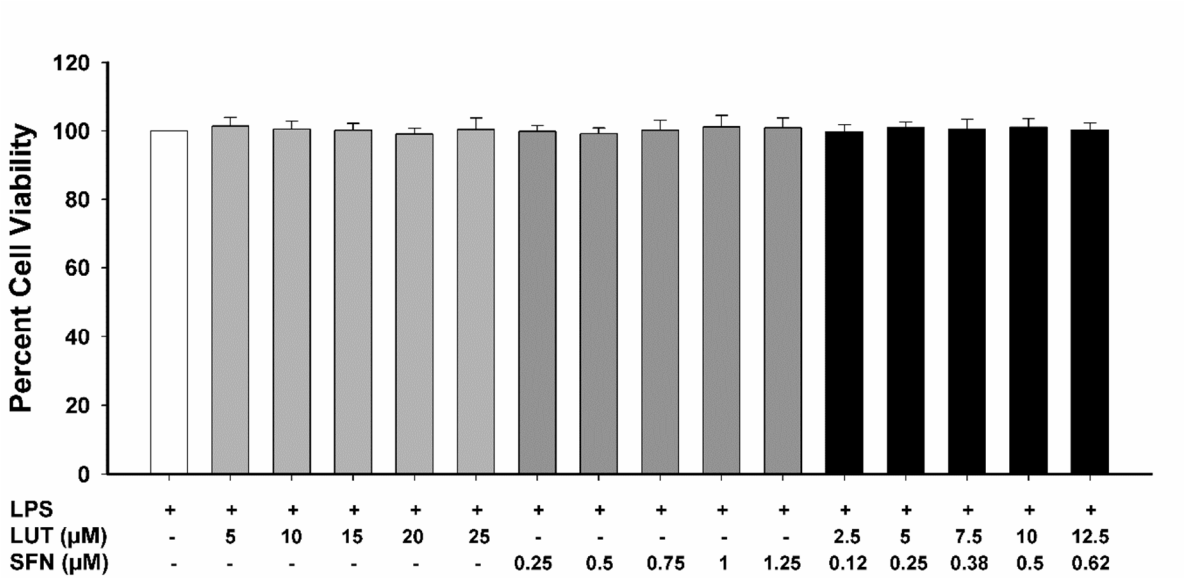
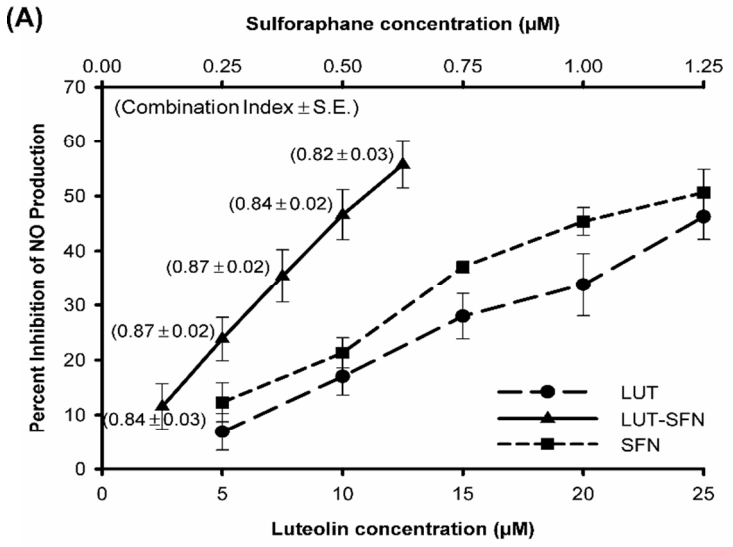
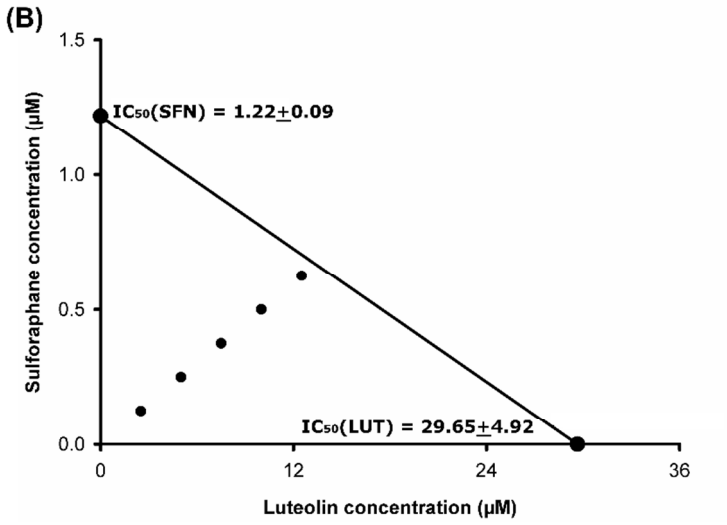


Figure 2. Cytotoxicity profile of LPS-induced RAW 264.7 macrophages with and without 24-hour treatments of LUT, SFN, and their combinations. Cell viability was determined using MTT assay. Results are expressed as mean ± SD from six replicates.


(A)
Sulforaphane concentration (μM)
0.00
0.25
0.50
0.75
1.00
1.25
(Combination Index ± S.E.)
(0.82 ± 0.03)
(0.84 ± 0.02)
(0.87 ± 0.02)
(0.87 ± 0.02)
(0.84 ± 0.03)
Percent Inhibition of NO Production
LUT
LUT-SFN
SFN
Luteolin concentration (μM)


(B)
IC50(SFN) = 1.22±0.09
IC50(LUT) = 29.65±4.92
Sulforaphane concentration (μM)
Luteolin concentration (μM)

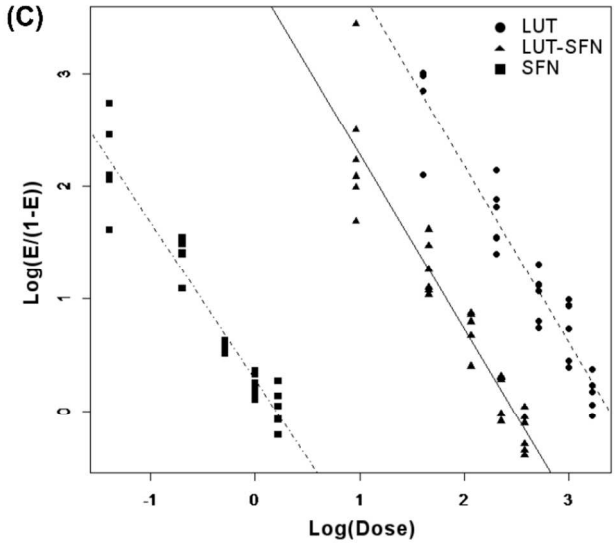

(C)
LUT
LUT-SFN
SFN
Log(E/(1-E))
Log(Dose)

Figure 3. (A) Percent inhibition of NO production by LUT, SFN, and their combinations in LPS-induced RAW 264.7 macrophages with their combination indexes shown in parentheses. After 24-hour treatments, NO assay was performed on the medium. Results are presented as mean ± SD from six replicates. (B) Isobologram analyses of synergy between the combination of LUT and SFN at different concentrations (2.5 μM LUT + 0.125 μM SFN, 5 μM LUT + 0.25 μM SFN, 7.5 μM LUT + 0.375 μM SFN, 10 μM LUT + 0.5 μM SFN, and 12.5 μM LUT + 0.625 μM SFN) with in the ratio of 20:1. (C) Median-effect plot constructed from percent NO in the medium of LPS-induced RAW 264.7 cells after being treated for 24 hours.

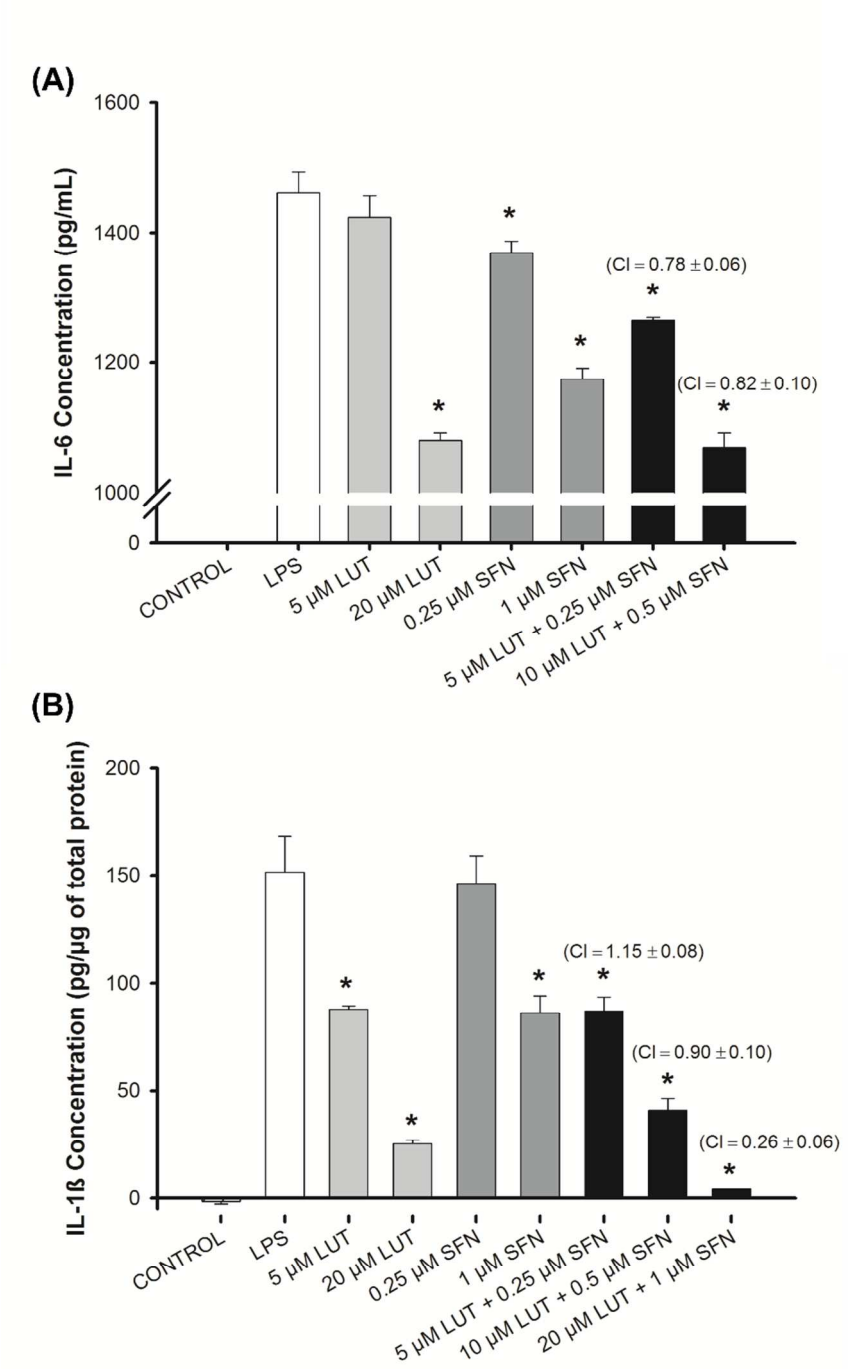


Figure 4. Combination effects of LUT and SFN on IL-6 (A) and IL-1β (B) levels in cell growth medium and cytoplasmic fraction, respectively after 24-hour treatment in LPS-induced RAW 264.7 macrophages. Protein levels of IL-6 and IL-1β were analyzed using ELISA. Combination index (CI) ± SE are present in parentheses. Results are presented as mean ± SD from triplicates (*$p < 0.05$).

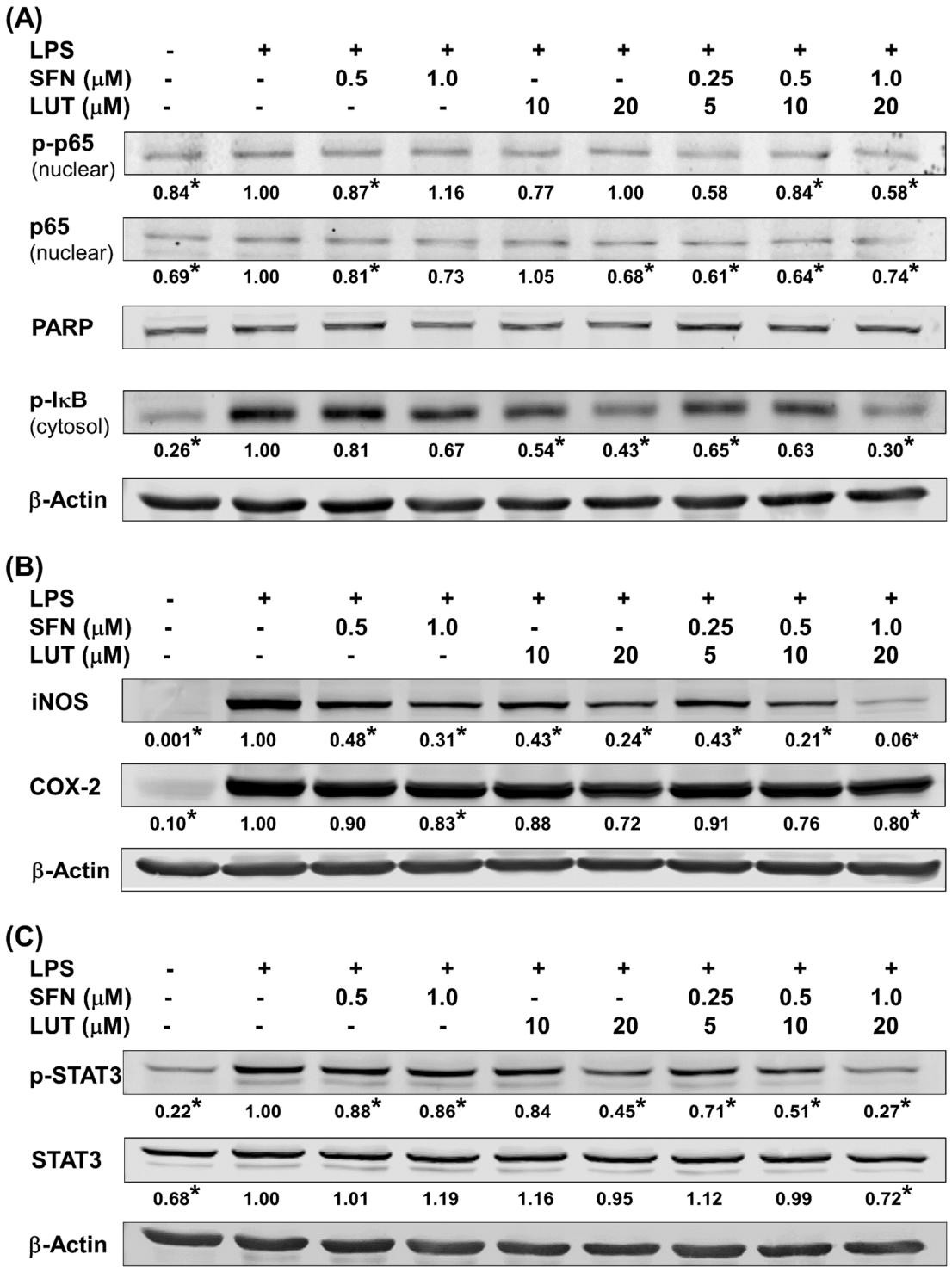


Figure 5. Representative Western blots demonstrating combination effects of LUT and SFN in LPS-induced RAW 264.7 on the expression of p65 subunit of NF-κB in both phosphorylated and non-phosphorylated forms in the nucleus and the expression of p-IκB in cytoplasm after an hour of treatment (A). Protein expression of iNOS, COX-2 (B), p-STAT3 and STAT3 (C) from whole cell lysates were monitored after 24-hour treatment. The protein band intensities underneath the

blots were quantified using Image Studio software. Standard deviations (within ± 20% of the mean) were not shown. * indicates statistical significance ($p < 0.05$; $n = 3$) in comparison to the LPS-treated group. PARP and β-Actin served as internal controls for nuclear fraction and cytosolic fraction or whole cell lysate, respectively.

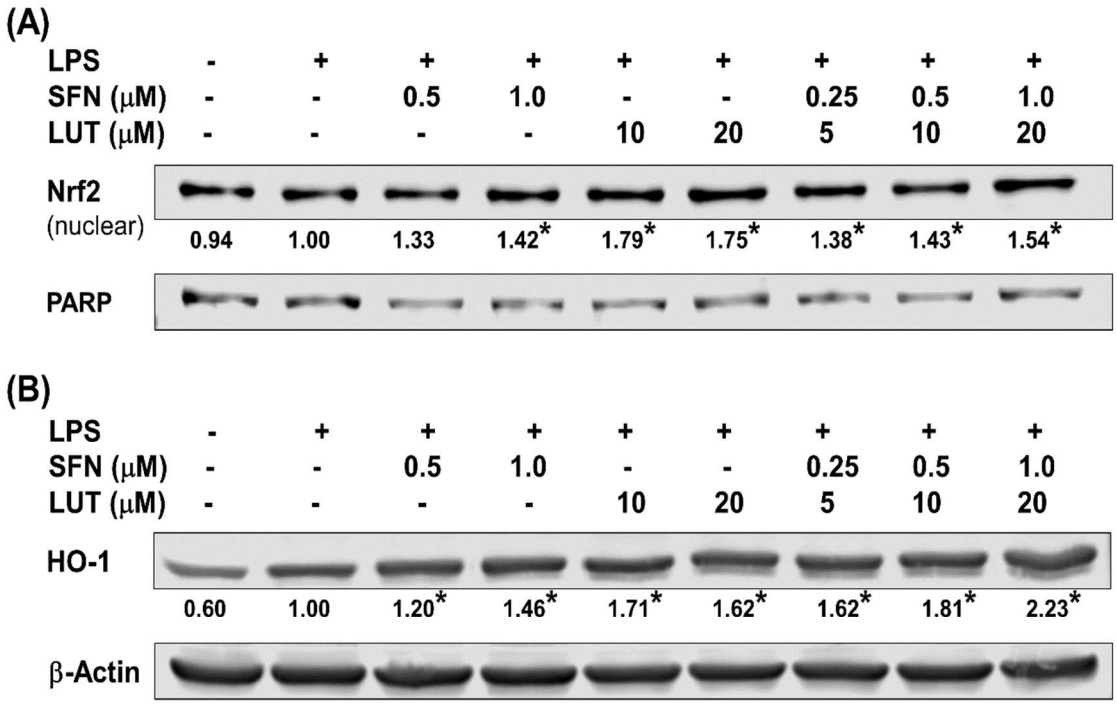


Figure 6. Representative Western blots demonstrating protein expression of Nrf2 (A) and HO-1 (B) after 24-hour treatment from cell nucleus and whole cell lysate, respectively. The protein band intensities underneath the blots were quantified using Image Studio software. Standard deviations (within ± 20% of the mean) were not shown. * indicates statistical significance ($p < 0.05$; $n = 3$) in comparison to the LPS-treated group. PARP was used as an internal control for nuclear fraction and β-Actin was an internal control for the whole cell lysate.

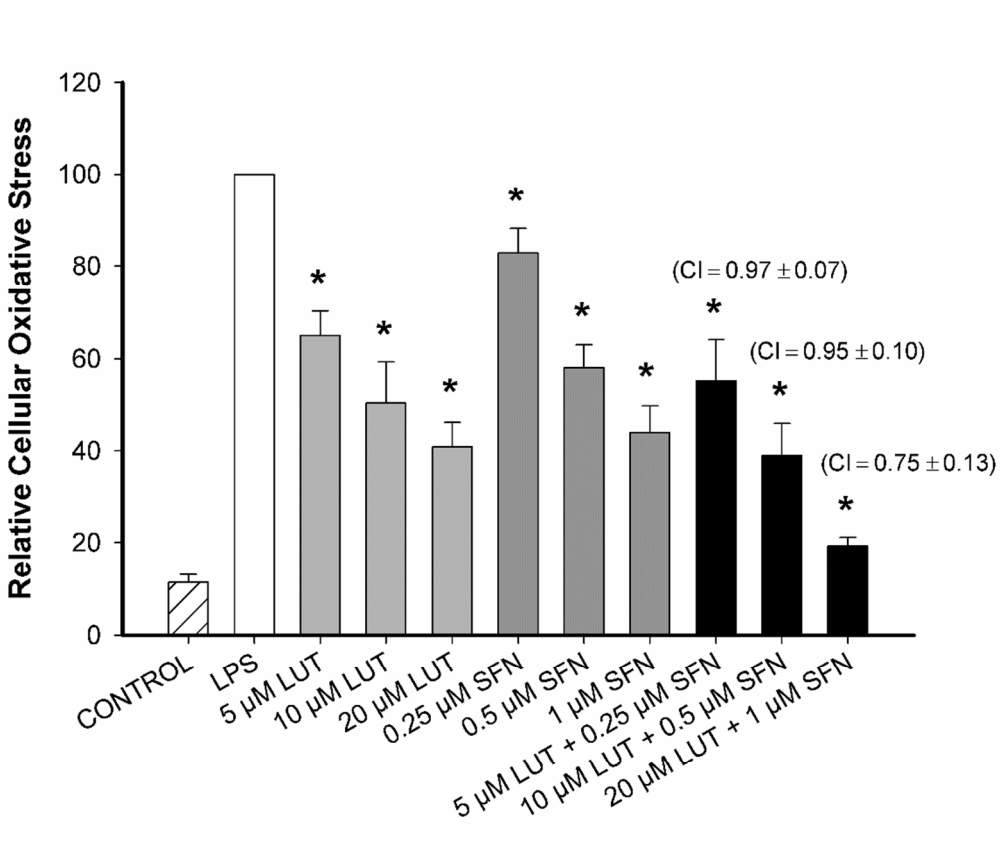


Figure 7. Combination effects of LUT and SFN on the level of cellular ROS after 24-hour treatment in LPS-induced RAW 264.7 macrophages. Intracellular ROS was relatively determined by DCFH-DA assay. Results are presented as mean + SD from five replicates (*$p < 0.05$). Combination index (CI) ± SE are present in parentheses.

Graphical Abstract