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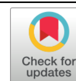
Comparative Anti-Inflammatory and Antioxidant Effects of N-Acetylcysteine, Curcumin, and Coenzyme Q10 in LPS-Activated RAW 264.7 Macrophages



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ABSTRACT

Continuous activation of macrophages is among the significant causes of chronic inflammatory diseases. The long-term sustenance of this process is through redox imbalance, NF-κB activation, extreme secretion of cytokines, and lipid peroxidation. Antioxidants such as N-acetylcysteine (NAC), curcumin, and coenzyme Q10 (CoQ10) have various mechanisms of action, and not much has been done on comparative and synergistic analysis. To compare the anti-inflammatory and antioxidant activity of NAC, curcumin, and CoQ10 individually and in combination in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with NAC, curcumin, or CoQ10, followed by LPS stimulation. Measurement of the levels of nitric oxide (NO) was carried out by the Griess assay, tumor necrosis factor-α (TNF-α) and IL-6 by the enzyme-linked immunosorbent assay (ELISA), and lipid peroxidation by the TBARS assay. The values of the combination index (CI) were calculated with a combination of fixed-ratio binary and ternary combinations employing the Chou-Talalay median-effect method. NAC and curcumin had a significant effect on inhibiting the release of NO and pro-inflammatory cytokines between 70% and 75%, and CoQ10 inhibited the release of malondialdehyde (MDA) between 48% and 60%. Cell viability (> 90%) was maintained by all the treatments. The effects of combination therapy were synergistic (CI < 1) between NAC + curcumin and tertiary NAC + curcumin + CoQ10, which yielded the strongest synergy (CI ≈ 0.65–0.72) and greatest overall inhibition. The redox-sensitive inflammatory pathways of NAC and curcumin have a synergistic effect, and CoQ10 provides additional antioxidant action on the membrane level. They have a synergistic effect that provides a multi-level inhibitory effect on both oxidative and inflammatory signaling, and this could be applied in the translational implications of chronic inflammatory diseases.

1. INTRODUCTION

Excessive or prolonged activation of macrophages is the pathogenesis of chronic inflammatory and metabolic diseases. Pattern-recognition receptors, which are toll-like receptors (TLRs) and NOD-like receptors (NLRs), as well as cytokine and scavenger receptors, are recognized by macrophages as indicators of microbes, damaged cells, and metabolic disorders. They initiate intracellular signaling pathways (NF-κB, IRF/STAT, and MAP kinases) which activate pro-inflammatory genes, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [1, 2].

Macrophages are reprogrammed to an aerobic glycolysis metabolic phenotype (a Warburg-like state), with more pronounced activation of the pentose-phosphate pathway to produce NADPH and biosynthetic precursors and changes in mitochondrial respiration [3]. Such changes facilitate the bioenergetic and biosynthetic requirements of inflammation and also increase intracellular reactive oxygen and nitrogen species (ROS/RNS). These oxidants mediate and amplify inflammation and activate NF-κB signaling, lipid peroxidation (measured as malondialdehyde (MDA)), and tissue damage, and form a feed-forward pathway of oxidative stress and cytokine release, which is self-conserving [4, 5].

There is growing evidence that oxidative stress is not an inevitable outcome but is actually a prime cause of the pathogenesis of chronic diseases. Overproduction of ROS and RNS has been associated with the emergence and progression of metabolic syndrome, neurodegenerative diseases, cardiovascular diseases, and autoimmune diseases [6, 7].

The macrophage-mediated redox dysregulation is becoming a key pathogenic process in a variety of diseases, including inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and atherosclerosis. Strong ROS and RNS that are produced by activated macrophages mediate lipid peroxidation, indicated by the increase in levels of MDA, and oxidative damage to DNA, which is evidenced by the rise of 8-hydroxy-2-deoxyguanosine (8-OHdG). The high levels of MDA and 8-OHdG have been directly related to the severity and tissue damage in IBD, RA, and atherosclerotic lesions [8, 9]. This close connection of oxidative reaction with inflammatory

processes makes macrophages the major target of therapeutic antioxidants and anti-inflammatory agents. N-acetylcysteine (NAC) is a thiol molecule that is small enough to complement the intracellular cysteine levels, thus reinstating glutathione (GSH) synthesis and redox equilibrium in the cell. It is a precursor of antioxidant defenses, and it is also a direct scavenger of ROS and RNS [10].

NAC has many clinical applications that are based on the overdose of acetaminophen and as a mucolytic agent. It significantly decreases the production of ROS/RNS and suppresses the expression of NO, TNF-α, and IL-6 in experimental models of inflammation by inhibiting the activation of redox-sensitive transcription factors like the NF-κB. Recent investigations show NAC as an inhibitor of the NF-κB activation process, and the production of pro-inflammatory mediators in RAW 264.7 macrophages corroborated the findings of their predecessors by showing that NAC blocks the oxidative stress signaling and MAPK pathways [11, 12].

The major bioactive polyphenol in turmeric (*Curcuma longa*) is curcumin, which has a strong antioxidant and anti-inflammatory effect. Curcumin disrupts several signaling pathways, the most significant of which include NF-κB, MAPKs, and Nrf2/Keap1, resulting in the inhibition of iNOS and cytokine expression and stimulation of intrinsic antioxidant systems [13, 14]. Curcumin in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages suppresses IL-6 and TNF-α, and releases NO, enhances the activity of superoxide dismutase (SOD) and catalase (CAT), and promotes Nrf2 translocation [15-17].

Comparative studies of curcumin and its reduced metabolites (tetrahydrocurcumin and octahydrocurcumin) have indicated that all exhibited antioxidative activity by using NF-κB and Nrf2 pathways [18]. Irrespective of its wide pharmacologic activity, low solubility and bioavailability are significant limitations of curcumin, which encourage the utilization of optimized preparations or synergistic combinations with other complementary agents. The ubiquinone, also known as coenzyme Q10 (CoQ10), is a lipophilic antioxidant, as well as an essential cofactor in the mitochondrial electron transport chain. CoQ10 protects cellular and mitochondrial membranes against lipid peroxidation by its reversible redox action between ubiquinone and ubiquinol [19]. The CoQ10 supplement decreases the indicators of oxidative stress (MDA, 4-HNE) and inflammation (TNF-α, IL-6, CRP) in metabolic and cardiovascular diseases [20]. Also, it regulates mitochondrial respiration, prevents the activation of NF-κB, and the expression of pro-inflammatory genes in macrophage models [21, 22]. Moreover, combination supplementation with other antioxidants has shown additive effects on mitochondrial protection and cytokine reduction [23].

Recent studies emphasize multi-target antioxidant strategies as an effective approach to modulate redox-sensitive inflammatory pathways, including NF-κB, MAPKs, and Nrf2 in macrophages [24, 25]. Compounds such as NAC, curcumin, and CoQ10 exhibit complementary mechanisms, including glutathione replenishment, inhibition of inflammatory signaling, and mitochondrial stabilization [26-28]. However, their combined effects remain insufficiently explored, particularly using quantitative synergy models such as the Chou–Talalay combination index in macrophage systems [29].

The current investigation was conducted to compare and contrast the anti-inflammatory and antioxidant properties of NAC, curcumin, and CoQ10 on LPS-stimulated RAW 264.7 macrophages. It also sought to find out whether there was any synergistic effect of these agents when administered as a fixed-ratio combination assay and the Chou–Talalay index analysis. The action of measuring nitric oxide (NO), TNF-α, IL-6, and MDA was conducted to investigate the influence of the treatments on the oxidative and inflammatory stress. These markers were selected to assess how targeting different redox and signaling pathways may confer protection against macrophage-driven oxidative and inflammatory stress.

2. MATERIALS AND METHODS

2.1 Cell culture and experimental design

RAW 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM; high glucose; Gibco), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. Subculture of cells was done every 2-3 days, and the cell lines were verified to be mycoplasma-free. In the case of 96-well assays (MTT, Griess, ELISA), 1.0–1.5 × 10^4 cells/well were placed in 100 μL of complete medium and left to adhere overnight (16-18 hours). In the case of thiobarbituric acid reactive substances (TBARS) and protein quantification, 5.0 × 10^5–1.0 × 10^6 cells/well were grown in 2 mL medium in 6-well plates and incubated under the same conditions.

2.2 Dose selection and preliminary dose–response analysis

Preliminary concentration–response experiments were performed for NAC, curcumin, and CoQ10 to identify working concentrations that produced measurable anti-inflammatory and antioxidant effects without relevant cytotoxicity. A range of concentrations for each agent was screened in LPS-stimulated RAW 264.7 macrophages, and cell viability was assessed by MTT assay together with representative inflammatory/oxidative endpoints. Dose–response curves were generated for each compound across multiple concentrations, and approximate mid-range inhibitory concentrations (~50% effect) were identified from these data to support dose selection. The final concentrations of 3 mM NAC, 10 μM curcumin, and 20 μM CoQ10 were selected because they maintained cell viability above 90% while producing clear but submaximal inhibitory effects, making them suitable for both single-agent comparison and combination index analysis.

2.3 Treatment groups and replicates

The following groups of single-agent treatment groups were used on each plate: Control: unstimulated, no LPS; LPS control: vehicle + LPS (100 ng/mL); NAC + LPS (3 mM); curcumin + LPS (10 μM); CoQ10 + LPS (20 μM). In all wells, vehicle concentration (dimethyl sulfoxide (DMSO) $\leq$ 0.1 % v/v) was maintained. The treatment plan was randomized to reduce the edge effects. Every condition was done three times technically, and the entire experiment was repeated thrice (n = 3 biological replicates). Cells were pre-treated with the compounds in full medium after 1 h, after which the cells were stimulated by LPS (100 ng/mL) for 24 h. Following incubation, the culture supernatants were pooled to perform NO and

cytokine assays. Cell viability and lipid peroxidation were also assessed.

2.4 Preparation of stock solutions

All reagents were prepared as stock solutions in sterile conditions and stored under the conditions of their stability. NAC was dissolved in sterile distilled water to give a 1 M stock solution, which was later filter-sterilized with a 0.22 µm syringe filter and kept at 4 °C. Fresh working solutions were made with a final concentration of 3 mM immediately before each experiment. Curcumin was dissolved in DMSO to a concentration of 10-20 mM, kept away from light, and stored at -20 °C to inhibit degradation. CoQ10 was also prepared in DMSO at different concentrations (10-50 mM) with short-term sonication to facilitate the formation of the solution and stored at -20 °C until the time of use. Lipopolysaccharide was dissolved in sterile distilled water with a final concentration of 1 mg/mL and aliquoted into small portions to prevent a repetitive freeze-thaw cycle and kept at -20 °C to be used in a subsequent cell stimulation analysis.

2.5 MTT viability assay

After 24 h of LPS with or without treatment, the medium was aspirated, and MTT (0.5 mg/mL final; 10 µL of 5 mg/mL stock + 100 µL medium) was added. Plates were incubated for 2-4 h at 37 °C. The medium was removed, and formazan crystals were dissolved in 100 µL DMSO per well. A microplate reader at 570 nm was used as the means of absorbance reading [30].

$$\text{Viability (\%)} = \frac{A_t - A_b}{A_c - A_b} \times 100$$

where, A_t is the absorbance of cells treated with the tested compounds, A_c is the absorbance of untreated/unstimulated control cells, and A_b is the background absorbance of blank wells containing medium and reagents without cells. The results were expressed as mean ± SD (n = 3).

2.6 Nitric oxide estimation by Griess assay

The Griess assay is a method used to estimate the concentration of nitric oxide in the body, which involves measuring the quantity of NO generated under the influence of NO -producing conditions. Supernatants (50–100 µL) were collected and clarified (3,000 × g, 5 min). Griess reagent (1% sulfanilamide, 0.1% NED in 2.5% phosphoric acid) [1:1 v/v] was added and incubated for 10 min in the dark at room temperature. Absorbance was read at 540–550 nm [31]. Nitrite concentration (µM) was interpolated from sodium nitrite standards (0–50 µM).

$$Inhibition\,\%\; vs\; LPS = \frac{(N0_LPS - Nontreated)}{NP_LPS} \times 100$$

Compound–reagent blanks were tested to exclude direct color reactions.

2.7 Evaluation of cytokine levels

ELISA kits of mice were used to estimate the levels of TNF-α and IL-6 following the manufacturer's guidelines [32]. Supernatants were then centrifuged after 24 h to get rid of debris and diluted (1:2-1:5) as needed. The standards and samples were loaded in duplicate, and the absorbance was read at 450 nm (reference 540 nm). Cytokine levels were expressed in pg/mL (TNF-6) or in ng/mL (IL-6). The percentage inhibition vs LPS was determined as above.

2.8 Lipid peroxidation and protein normalization

The cells in 6-well plates were washed with phosphate-buffered saline (PBS), scraped into ice-cold PBS, and pelleted (500 × g, 5 min) after 24 h of treatment. In 200-300 µL of buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 + protease inhibitors), pellets were lysed. The BCA assay was used to determine protein quantity. TBARS reaction was conducted with a commercial kit of MDA (Abcam), according to the instructions of the manufacturer, and the absorbance values were measured at 532 nm. The standard curve was used to obtain the MDA level, which was normalized by protein content (nmol MDA / mg protein).

2.9 Combination testing and combination index analysis

Fixed-ratio drug combinations were employed to evaluate potential synergistic interactions among NAC, curcumin, and CoQ10. The combination index (CI) was calculated based on the Chou–Talalay median-effect principle using CompuSyn software (CompuSyn Inc., USA). Single-agent dose–response curves were first generated for each compound across a range of concentrations. The median-effect parameters, including the median-effect dose (Dm, equivalent to IC_{50}) and the slope (m), were obtained from these curves using the median-effect equation: $F_a/F_u = (D/D_m)^m$, where F_a represents the fraction affected and $F_u = 1 - F_a$. These parameters were derived automatically by CompuSyn through linear regression of the median-effect plot [log(F_a/F_u) versus log(D)]. Based on these parameters, the theoretical doses required to achieve a given effect level (Dx_1 and Dx_2) for every single agent were calculated by the software. For combination treatments, the actual doses (D_1 and D_2) producing the same effect level were obtained experimentally. The CI was then calculated using the equation: $CI = (D_1/Dx_1) + (D_2/Dx_2)$. The selected concentrations were based on preliminary experiments to ensure minimal cytotoxicity (> 90% viability) while producing measurable anti-inflammatory effects. The combinations were prepared at fixed ratios as follows: NAC:curcumin (3 mM:10 µM), NAC:CoQ10 (3 mM:20 µM), curcumin:CoQ10 (10 µM:20 µM), and ternary combination at the same ratios. Serial dilutions were prepared to generate dose–response curves covering a fraction affected (Fa) range of approximately 0.20–0.80 for accurate CI estimation. RAW 264.7 macrophages were pretreated with test compounds for 1 h, followed by stimulation with LPS (100 ng/mL) for 24 h. Supernatants were collected and analyzed for nitric oxide (Griess assay), TNF-α and IL-6 (ELISA), and MDA (TBARS assay). CI values were interpreted as follows: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

2.10 Statistical analysis

The data are presented as mean ± standard deviation (SD) of three independent biological replicates (n = 3). Cell viability, NO, TNF-α, IL-6, and MDA were analyzed as continuous variables. The assessment of the normality of data distribution was done by the Shapiro-Wilk test. Since data met the parametric assumptions, comparisons between groups (control,

LPS, NAC, curcumin, and CoQ10) were done with one-way analysis of variance (ANOVA). In case an overall significant effect was observed, the Tukey post hoc test was used to compare each treatment group with the LPS control. A p-value of less than 0.05 was considered to be statistically significant. Statistical tests were conducted with GraphPad Prism (version X).

The values of percentage inhibition were not statistically evaluated independently but only compared descriptively. In the case of combination experiments, the Chou-Talalay median-effect method of computation of CI values in CompuSyn software (CompuSyn Inc., USA) was used. The single-agent dose-response curves were used to obtain median-effect parameters (D_m = IC_{50} and m = slope). The values of CI were determined for each biomarker at various levels of Fa, where CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism. Values of percentage inhibition for each of the biomarkers were derived against the LPS-treated control.

3. RESULTS AND DISCUSSION

The outcomes of the use of individual agents demonstrated their efficacy at the chosen working concentrations (NAC [3 mM], curcumin [10 µM], CoQ10 [20 µM]). Statistical analysis showed that NAC and curcumin significantly reduced NO, TNF-α, and IL-6 levels compared to the LPS control ($p < 0.05$), whereas CoQ10 produced moderate but still significant reductions. All treatments maintained cell viability above 90%, indicating that the observed effects were not due to cytotoxicity. CoQ10 exhibited a comparatively stronger effect in reducing lipid peroxidation, while NAC and curcumin were more effective in suppressing inflammatory mediators. These endpoint-specific differences informed the selection of fixed-ratio combinations for subsequent combination index analysis. The single-agent results are summarized in Table 1.

3.1 Dose–response analysis and dose selection

Dose–response analysis demonstrated a clear concentration-dependent inhibition of inflammatory and oxidative stress markers for NAC, curcumin, and CoQ10 in LPS-stimulated RAW 264.7 macrophages (Figures 1(A-C)).

For NAC (Figure 1(A)), increasing concentrations (0.5–5 mM) resulted in progressive reductions in nitric oxide (NO), TNF-α, IL-6, and MDA levels, with maximal inhibition observed at 5 mM. However, cell viability declined at this concentration (86.4 ± 2.7%), whereas concentrations up to 3 mM maintained viability above 90%, with substantial but submaximal inhibition.

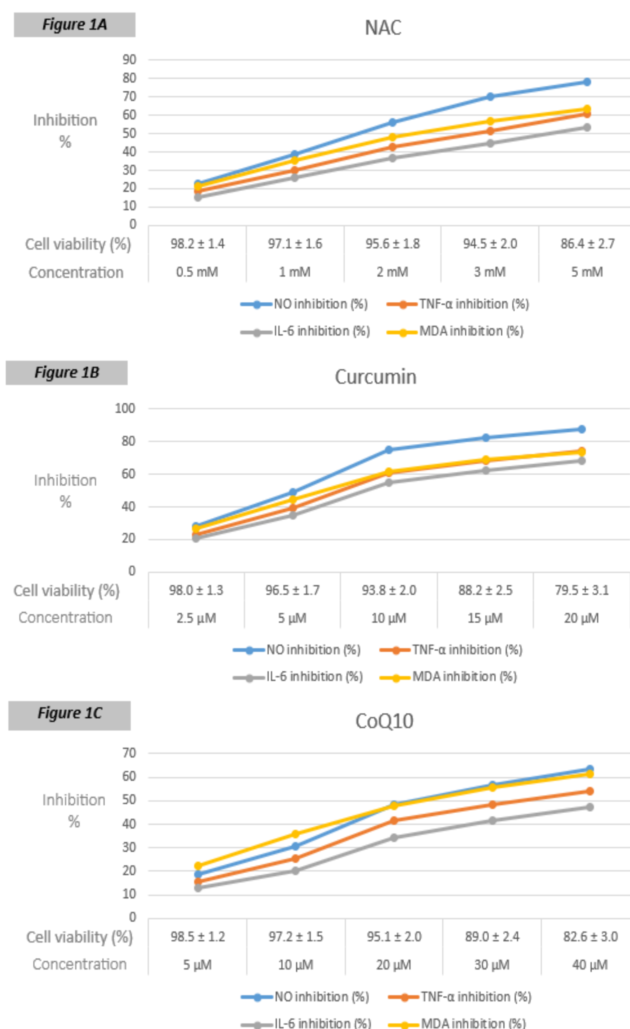

Figure 1. Concentration-dependent dose–response curves of (A) N-acetylcysteine (NAC), (B) curcumin, and (C) coenzyme Q10 (CoQ10)

Table 1. Effects of single agents on cell viability, inflammatory mediators, and oxidative stress in LPS-stimulated RAW 264.7 macrophages

Treatment	Viability (%)	NO (µM)	% Inhibition vs LPS (NO)	TNF-α (pg/mL)	% Inhibition vs LPS (TNF-α)	IL-6 (ng/mL)	% Inhibition vs LPS (IL-6)	MDA (nmol/mg)	% Inhibition vs LPS (MDA)
Control	100.0 ± 1.5^a	0.90 ± 0.05^a	N/A	38 ± 3^a	N/A	0.10 ± 0.01^a	N/A	0.80 ± 0.06^a	N/A
LPS	91.8 ± 2.5^b	6.20 ± 0.40^d	0.0%	820 ± 45^d	0.0%	1.90 ± 0.12^d	0.0%	4.20 ± 0.28^d	0.0%
NAC (3 mM)	94.5 ± 2.0^b	1.86 ± 0.12^b	70.0%	395 ± 25^c	51.7%	1.05 ± 0.08^c	44.7%	1.80 ± 0.12^b	57.1%
Curcumin (10 µM)	93.8 ± 2.0^b	1.55 ± 0.10^c	75.0%	320 ± 20^b	61.0%	0.85 ± 0.07^b	55.3%	1.60 ± 0.10^b	61.9%
CoQ10 (20 µM)	95.1 ± 2.0^b	3.22 ± 0.20^c	48.1%	480 ± 30^c	41.5%	1.25 ± 0.09^c	34.2%	2.20 ± 0.15^c	47.6%

Data are presented as mean ± SD (n = 3). Percentage inhibition was calculated relative to the LPS-treated group. Values are not applicable (N/A) for the unstimulated control group. NO: Nitric oxide; TNF-α: tumor necrosis factor-alpha; IL-6: interleukin-6; MDA: malondialdehyde; LPS: lipopolysaccharide. Different superscript letters within the same column indicate statistically significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey's post hoc test.

Curcumin (Figure 1(B)) exhibited a strong dose-dependent inhibitory effect across the tested range (2.5–20 μM), with pronounced reductions in NO and cytokine levels at higher concentrations. Although maximal inhibition was observed at 20 μM, cell viability decreased below 80% (79.5 ± 3.1%), whereas 10 μM maintained acceptable viability (> 90%) while producing robust inhibitory effects.

CoQ10 (Figure 1(C) showed a moderate but consistent concentration-dependent effect (5–40 μM), particularly on MDA and NO levels. At higher concentrations (30–40 μM), inhibitory effects increased; however, a reduction in cell viability was observed (82.6 ± 3.0% at 40 μM). The concentration of 20 μM maintained > 90% viability with moderate inhibitory activity.

Based on the observed dose–response trends, the concentrations producing approximately 50% inhibition were within the mid-range of tested doses, corresponding to ~1.6–2.0 mM for NAC (NO inhibition), ~5–7 μM for curcumin (NO/TNF-α inhibition), and ~20–25 μM for CoQ10 (MDA inhibition). These values are presented as approximate estimates rather than formally calculated IC_{50} values.

Based on these findings, 3 mM NAC, 10 μM curcumin, and 20 μM CoQ10 were selected as optimal working concentrations, as they provided measurable yet submaximal inhibition while maintaining cell viability above 90%, making them suitable for subsequent single-agent and combination index analyses.

3.2 Cell viability influenced by test solutions

The MTT assay showed high cell viability in all the treatments. The mean viability in control wells not stimulated was 100.0 ± 1.5%, and the LPS-stimulated vehicle control slightly declined to 91.8 ± 2.5%. Treatment wells had a high viability above 90%: NAC (3 mM) was 94.5 ± 2.0%, curcumin (10 μM) was 93.8 ± 2.0%, and CoQ10 (20 μM) was 95.1 ± 2.0%. These findings suggest that the concentrations used did not cause overt cytotoxicity and that the decrease in inflammatory or oxidative endpoints would not be caused by cell death.

3.3 Stimulation of nitric oxide production

LPS significantly increased nitrite accumulation to 6.20 ± 0.40 μM compared with the unstimulated control. All treatments significantly reduced NO production relative to the LPS group (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). NAC (3 mM) and curcumin (10 μM) produced strong inhibition (70.0% and 75.0%, respectively), while CoQ10 (20 μM) showed a moderate reduction (48.1%). Curcumin demonstrated a significantly greater reduction in NO levels compared with NAC ($p < 0.05$), whereas both NAC and curcumin were significantly more effective than CoQ10 ($p < 0.05$).

3.4 Effects of test solutions on pro-inflammatory cytokines

LPS significantly induced pro-inflammatory cytokine release (TNF-α = 820 ± 45 pg/mL; IL-6 = 1.90 ± 0.12 ng/mL). All treatments significantly reduced cytokine levels compared with the LPS group (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Curcumin exhibited the strongest inhibitory effect on cytokine production (TNF-α: 61.0%; IL-6: 55.3%), followed by NAC (TNF-α: 51.7%; IL-6: 44.7%), while CoQ10 showed comparatively lower inhibition. Curcumin significantly reduced TNF-α and IL-6 levels compared with NAC ($p < 0.05$), and both agents were significantly more effective than CoQ10 ($p < 0.05$).

3.5 Influence of test solutions on lipid peroxidation

The results of LPS on MDA revealed that NAC (3 mM) was 1.80 ± 0.12 nmol/mg (57.1% inhibition), curcumin (10 μM) was 1.60 ± 0.10 nmol/mg (61.9% inhibition), and coQ10 (20 μM) was 2.20 ± 0.15 nmol/mg (47.6% inhibition). All treatments significantly reduced MDA levels relative to the LPS group (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Curcumin showed the greatest reduction in MDA levels, followed by NAC, whereas CoQ10 demonstrated a comparatively lower but still significant effect. Curcumin significantly reduced MDA levels compared with CoQ10 ($p < 0.05$), while the difference between curcumin and NAC was not statistically significant ($p > 0.05$). Both NAC and curcumin were significantly more effective than CoQ10 ($p < 0.05$). These findings indicate that while all agents attenuate LPS-induced lipid peroxidation, CoQ10 primarily exerts its effects through membrane and mitochondrial antioxidant mechanisms.

3.6 Combination testing

Fixed-ratio combinations (NAC: curcumin [3 mM:10 μM]; NAC: CoQ10 [3 mM: 20 μM]); curcumin: CoQ10 [10 μM: 20 μM] were performed, and Fa and CI were calculated by the Chou-Talalay method. Table 2 shows CI values of NO, TNF-α, MDA, and IL-6, percent of inhibition. The Chou-Talalay analysis showed that all NAC-based combinations exhibited CI values that were below 1.0, indicating synergistic to near-additive effects. The ternary NAC + curcumin + CoQ10 combination had the greatest synergism with NO, TNF-α, MDA, and IL-6 (CI 0.65–0.72) with 75-92% inhibition of the respective markers. This was followed by the NAC + curcumin pair (CI 0.70–0.78; 65–87% inhibition). Conversely, only weak synergy or near additive effects (CI 0.82–0.98) with slightly smaller inhibition (≈ 42–60%) were observed with NAC + CoQ10 and curcumin + CoQ10.

Table 2. Combination index (CI) and percentage inhibition of inflammatory and oxidative stress markers for pairwise and ternary treatments in LPS-stimulated RAW 264.7 macrophages

Pair	CI (NO)	CI (TNF-α)	CI (MDA)	CI (IL-6)	% NO Inhibition (%)	% TNF Inhibition (%)	% MDA Inhibition (%)	% IL-6 Inhibition (%)
NAC + Curcumin	0.73	0.70	0.78	0.72	87.1	72.0	65.0	70.0
NAC + CoQ10	0.95	0.98	0.82	0.96	50.0	42.0	55.0	45.0
Curcumin + CoQ10	0.92	0.90	0.88	0.91	60.0	50.0	58.0	54.0
Ternary (N + C + Q)	0.68	0.65	0.72	0.66	92.0	80.0	75.0	78.0

CI values (Chou–Talalay method) indicate synergism (< 1), additivity (= 1), or antagonism (> 1). Percentage inhibition was calculated vs LPS; control values were excluded. Abbreviations: NAC, N-acetylcysteine; CoQ_{10}, coenzyme Q10; CI, combination index; NO, nitric oxide; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; MDA, malondialdehyde; LPS, lipopolysaccharide.

The present comparative research proves that NAC and curcumin show a synergistic anti-inflammatory effect on LPS-stimulated RAW 264.7 macrophages, whereas CoQ10 mainly decreases the process of lipid peroxidation. The fixed-ratio of Chou-Talalay results demonstrates that NAC + curcumin is the most effective synergistic duo in the inhibition of NO and TNF-α. The observed effects are likely to be explained by mechanical complementarity between glutathione replacement (NAC), NF-κB inhibition (curcumin), and mitochondrial protection (CoQ10).

The effects of NAC, curcumin, and CoQ10 in LPS-stimulated RAW 264.7 macrophages were different yet complementary. At the experimental concentrations (3 mM NAC, 10 µM curcumin, 20 µM CoQ10), both NAC and curcumin caused a significant decrease in NO and pro-inflammatory cytokines TNF-α and IL-6; however, CoQ10 caused more subtle changes in these mediators but had a definite inhibitory effect on the lipid peroxidation indicated by MDA. All treatment groups had cell viability greater than 90%, indicating that lower mediator levels reflected biochemical modulation rather than cytotoxicity.

LPS increased the concentration of nitrite up to about 6.2 µM, which is an indication of high induction of iNOS in RAW 264.7 cells. NAC and curcumin inhibited NO by approximately 70-75%, and CoQ10 inhibited NO by approximately 48%. The profound effect of NAC is consistent with its capability to restore intracellular thiols, support glutathione (GSH) generation, and scavenge reactive species. Through these mechanisms, NC inhibits redox-sensitive iNOS expression and NO secretion, which have been observed in stimulated macrophages [33]. A high effect of curcumin on NO is consistent with other studies, which indicate that NF-κB and MAPK signalling are inhibited, and iNOS expression is suppressed in RAW 264.7 cells activated with LPS [34]. The relatively weak effect of CoQ10 on NO is consistent with its major effect as a membrane and mitochondrial antioxidant and not as a direct transcriptional inhibitor.

In the case of cytokines, the greatest reduction in TNF-α and IL-6 was observed with curcumin, followed by NAC, while CoQ10 showed less pronounced changes. In RAW 264.7 macrophages, curcumin was observed to inhibit the LPS-stimulated TNF-α and IL-6 production by regulating SOCS-1/-3 and p38 MAPK, which provides a direct transcriptional inhibition of the expression of inflammatory genes [34]. The primary mechanism of NAC is to restore redox homeostasis and attenuate NF-κB activation, explaining its intermediate effect on cytokine production. At 20 µM, CoQ10 appears to play a role largely at the antioxidant level. CoQ10 exerts its antioxidant effects primarily through stabilization of mitochondrial membranes and inhibition of electron leakage from the respiratory chain, thereby reducing reactive oxygen species (ROS) generation and downstream NF-κB activation. This finding agrees with clinical and experimental evidence demonstrating that CoQ10 consistently improves oxidative stress biomarkers more than cytokines [35].

LPS raised the levels of MDA to approximately 4.20 nmol/mg protein. NAC and curcumin reduced MDA by an average of 57-62%, and CoQ10 reduced MDA by an average of 48%. MDA is a stable product of lipid peroxidation and a delicate measure of oxidative damage in membranes [29]. The NAC and CoQ10 decreases of MDA are compatible with thiol-based GSH-refilling and mitochondrial membrane-protective measures, respectively. According to the meta-analyses, CoQ10 supplementation profoundly lowers the levels of circulating MDA and enhances the total antioxidant potential of the human organism in adults, which confirms the trend of the current MDA values [35]. These profiles of individual agents can be modeled as three nodes: (i) NAC is a redox buffer and restricts the activation by ROS/RNS [36]; (ii) curcumin is a transcriptional brake on NF-κB/MAPK-regulated genes [34]; and (iii) CoQ10 stabilizes mitochondrial electron transport and prevents peroxidation of lipid membranes [35]. This multi-level targeting of oxidative stress and inflammatory signaling provides a mechanistic basis for the enhanced efficacy observed in combination treatments, particularly in macrophage-mediated inflammation. Since these agents affect distinct but interconnected pathways, their combination is expected to produce additive or synergistic effects, as confirmed by the Chou–Talalay analysis [33].

The values of CI of NO, TNF-α, IL-6, and MDA were found to be less than one, which indicates that the interactions between them were synergistic to nearly additive. The strongest synergy was observed in the NAC + curcumin pair and the ternary NAC + curcumin + CoQ10 combination, while weaker synergy (CI values closer to 1) was observed in NAC + CoQ10 and curcumin + CoQ10 combinations. The relatively weaker synergy observed in NAC + CoQ10 combinations may be attributed to overlapping antioxidant mechanisms without sufficient complementary modulation of transcriptional pathways, in contrast to NAC + curcumin, which simultaneously targets redox balance and inflammatory signaling pathways. Previous studies similarly report that NAC and curcumin act synergistically in reducing oxidative stress and inflammatory signaling due to their complementary mechanisms [37].

Reports on CoQ10-based combinations have been more mixed with less consistent cytokine effects, which is consistent with the low synergies of CoQ10-based binary combinations [38]. The enhanced activity of the ternary NAC + curcumin + CoQ10 combination suggests that multi-level inhibition of macrophage activation can be achieved through simultaneous modulation of thiol redox balance, inflammatory transcription pathways, and mitochondrial function. These findings highlight the therapeutic potential of combination antioxidant strategies in inflammatory conditions, where targeting multiple interconnected pathways may yield superior efficacy compared to single-agent approaches. Further mechanistic investigations, including analysis of NF-κB activation, intracellular ROS levels, mitochondrial membrane potential, and GSH/GSSG ratios, would assist in localizing the main site of action of each of the agents and further streamline the design of subsequent combination regimens.

However, certain translational limitations should be considered, particularly the poor bioavailability of curcumin, which may restrict its clinical applicability. Future studies should explore advanced delivery systems, such as nanoformulations or liposomal carriers, to enhance its therapeutic potential.

4. LIMITATIONS

This study has several limitations. First, it was conducted in vitro using RAW 264.7 macrophages, which may not fully represent in viv*o* inflammatory conditions. Second, fixed-dose combinations were used rather than equi-effective dosing strategies. Third, molecular pathways such as NF-κB activation and ROS generation were not directly measured.

Future studies should include in vivo validation and mechanistic assays.

5. CONCLUSIONS

The study's findings suggest that NAC, curcumin, and CoQ10 reduce oxidative stress and inflammatory responses in LPS-stimulated macrophages with unique but complementary mechanisms. NAC improves the cellular redox situation by offering thiol groups and restoring glutathione. Curcumin blocks inflammatory signaling by inhibiting NF-κB and MAPK pathways. CoQ10 preserves mitochondrial integrity and restricts lipid peroxidation. With these agents, synergy is observable when the NAC and curcumin are used together as a pair and in the ternary combination, showing greater effects on NO, TNF-κB, IL-6, and MDA reductions in targeted compounds than when this is done in isolation. This implies that the concomitant attack on several redox-inflammatory checkpoints can be associated with additive or synergistic effects without being cytotoxic. Such combinations can be used as adjunctive treatment of disorders that are caused by oxidative stress and overactivation of macrophages, including rheumatoid arthritis, atherosclerosis, and metabolic inflammation. Further molecular and *in vivo* studies are required to verify their mechanisms and evaluate their clinical applicability potential.

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