JOURNAL OF PHARMA INSIGHTS AND RESEARCH ISSN NO. 3048-5428

RESEARCH ARTICLE

Synergistic Attenuation of Letrozole-Induced Granulosa Cell Dysfunction by Berberine and Resveratrol via Mitigation of Oxidative Stress, Inflammatory Pathways, and Steroidogenic Impairment

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Publication history: Received on 24th May 2026; Revised on 29th June 2026; Accepted on 1st July 2026

Article DOI: 10.69613/da5ptm63

Abstract: Polycystic ovary syndrome is a complex endocrine and metabolic disorder caused by hyperandrogenism, systemic low-grade inflammation, intraovarian oxidative stress, and impaired steroidogenesis. Granulosa cells serve as central targets of ovarian dysfunction, where suppressed aromatase activity and elevated reactive oxygen species disrupt follicular maturation. Evaluation of combined phytotherapy using berberine and resveratrol in a letrozole-induced human granulosa cell (KGN) model reveals profound cytoprotective and restorative mechanisms. Pharmacological inhibition of aromatase by letrozole (1 μM, 24 h) induces structural degeneration, reduces cell viability to 63.8%, elevates intracellular reactive oxygen species and lipid peroxidation, depletes superoxide dismutase and reduced glutathione, and promotes the hypersecretion of tumor necrosis factor-alpha, interleukin-6, and interleukin-1 beta. Concurrently, steroidogenesis shifts toward hyperandrogenism, marked by elevated testosterone and reduced estradiol levels. Monotherapy with either berberine or resveratrol significantly mitigates cellular damage; however, co-administration of both compounds demonstrates superior synergistic protection. Combined treatment restores cell viability to 92.5%, normalizes endogenous antioxidant defenses, suppresses pro-inflammatory cytokine secretion to baseline levels, and re-establishes the physiological estradiol-to-testosterone ratio. These findings demonstrate that dual targeting of metabolic and cellular defense pathways via berberine and resveratrol effectively reverses granulosa cell injury and steroidogenic dysfunction, establishing a strong theoretical foundation for combination botanical interventions in managing polycystic ovary syndrome pathology.

Keywords: Polycystic ovary syndrome; Berberine; Resveratrol; Granulosa cells; Steroidogenesis.

1. Introduction

Polycystic ovary syndrome (PCOS) is a primary endocrine condition affecting individuals of reproductive age globally, with clinical estimates indicating a prevalence between 6% and 18% depending on the diagnostic criteria applied [1, 2]. The clinical spectrum of PCOS encompasses heterogeneous manifestations including persistent anovulation, hyperandrogenism, polycystic ovarian morphology, and metabolic dysregulation [3]. Affected individuals frequently present with concurrent insulin resistance, systemic dyslipidemia, metabolic syndrome, and elevated long-term risks for type 2 diabetes mellitus and cardiovascular diseases [4, 5]. At the intraovarian level, granulosa cells fulfill essential roles in guiding oocyte development, maintaining follicular structural integrity, and regulating endocrine signaling through gonadotropin-responsive cascades [6]. Physiology dictates that follicle-stimulating hormone (FSH) binds to its corresponding receptor on granulosa cells, upregulating cytochrome P450 aromatase (CYP19A1) activity. Aromatase catalyzes the rate-limiting enzymatic conversion of theca-derived androgens, such as testosterone and androstenedione, into 17β-estradiol and estrone [7]. In PCOS, disrupted intraovarian paracrine signaling, intrinsic enzymatic alterations, and hyperandrogenic microenvironments impair granulosa cell differentiation and suppress CYP19A1 expression [8]. Consequent accumulation of intraovarian androgens leads to premature follicular arrest, follicular atresia, and the formation of multiple subcortical cystic structures characteristic of the diseased ovary [9].

Intraovarian redox imbalance is recognized as a major driver in the progression of PCOS [10]. Reactive oxygen species (ROS), including superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (· OH), are natural byproducts of aerobic cellular respiration and steroidogenesis. Under non-pathological conditions, endogenous antioxidant mechanisms comprising enzymatic factors like superoxide dismutase (SOD) and catalase alongside non-enzymatic molecules such as reduced glutathione (GSH) neutralize ROS to preserve cellular homeostasis [11]. In contrast, diseased granulosa cells undergo excessive ROS production

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coupled with a severe decline in endogenous antioxidant defenses [12]. Uncontrolled accumulation of ROS initiates lipid peroxidation of polyunsaturated fatty acids within cell membranes, yielding toxic aldehyde intermediates such as malondialdehyde (MDA), which compromise membrane fluidity, disrupt organelle integrity, and trigger apoptosis [13].

Redox imbalance operates in a self-amplifying feed-forward loop with chronic low-grade inflammation within the ovarian microenvironment [14]. Elevated ROS concentrations activate redox-sensitive transcription factors, particularly nuclear factor kappa B (NF-ϰ B), which translocates to the nucleus and upregulates the transcription of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) [15]. These cytokines impair insulin signaling cascades in granulosa and theca cells, further enhance androgen secretion, and suppress CYP19A1 transcription [16]. Consequently, targeting both oxidative stress and cytokine-mediated inflammation is essential for restoring functional folliculogenesis.

Cellular metabolic signaling networks regulate energy homeostasis, redox balance, and survival in granulosa cells [17]. Adenosine monophosphate-activated protein kinase (AMPK) functions as a metabolic sensor, responding to elevated AMP/ATP ratios by restoring cellular energy homeostasis [18]. Activation of AMPK stimulates fatty acid oxidation and glucose uptake while suppressing energy-consuming anabolic processes. In ovarian tissues, AMPK signaling enhances antioxidant enzyme expression and protects granulosa cells against apoptotic stimuli [19].

Sirtuin-1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, acts as a master regulator of cellular stress responses, mitochondrial biogenesis, and inflammatory suppression [20]. SIRT1 deacetylates key downstream substrates, including peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) and nuclear factor erythroid 2-related factor 2 (Nrf2), thereby promoting mitochondrial repair and inducing phase II antioxidant enzymes [21]. Cross-talk between AMPK and SIRT1 establishes a regulatory network: AMPK activation increases intracellular NAD+ concentrations to stimulate SIRT1 activity, whereas SIRT1 deacetylates liver kinase B1 (LKB1) to phosphorylate and activate AMPK [22]. Functional impairment of this dual signaling axis contributes significantly to metabolic and steroidogenic disturbances in PCOS granulosa cells [23].

Conventional therapeutic options for PCOS focus primarily on symptom management via oral contraceptives, insulin sensitizers, or ovulation induction agents, which often carry side-effect profiles or incomplete efficacy regarding intraovarian pathology [24]. Naturally derived bioactive phytocompounds offer multi-target therapeutic potential. Berberine, a quaternary isoquinoline alkaloid isolated from *Berberis* species, possesses metabolic, anti-inflammatory, and antioxidant properties [25]. Berberine directly activates AMPK, improves insulin receptor sensitivity, modulates lipid metabolism, and attenuates NF-ϰ B-mediated cytokine release [26, 27].

Resveratrol, a naturally occurring stilbenoid polyphenol found in grapes and berries, exhibits potent antioxidant and cytoprotective activities [28]. Resveratrol serves as a direct activator of SIRT1, enhancing mitochondrial efficiency, scavenging free radicals, and downregulating pro-inflammatory mediators [29, 30]. It is known that berberine and resveratrol engage distinct yet synergistic nodes within the interconnected AMPK/SIRT1 axis, co-administration represents a promising therapeutic approach. Combining these phytochemicals may achieve enhanced protection against oxidative stress, cytokine production, and steroidogenic dysregulation in granulosa cells compared to individual treatments. The present work evaluates the synergistic protective effects of berberine and resveratrol in a human ovarian granulosa cell (KGN) model of PCOS established through letrozole-induced aromatase inhibition.

2. Materials and Methods

2.1. Reagents and Cell Culture

Letrozole (purity ≥ 98%), berberine chloride (purity ≥ 98%), and resveratrol (purity ≥ 99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), penicillin-streptomycin solution (10,000 U/mL penicillin, 10,000 μg/mL streptomycin), trypsin-EDTA (0.25%), and phosphate-buffered saline (PBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Biochemical determination kits for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ROS, MDA, SOD, and GSH were acquired from Abcam (Cambridge, UK). Enzyme-linked immunosorbent assay (ELISA) kits for human TNF-α, IL-6, IL-1β, testosterone, and 17β-estradiol were procured from R&D Systems (Minneapolis, MN, USA).

Human ovarian granulosa (KGN) cells, an immortalized human granulosa cell line retaining functional FSH receptor expression and aromatase activity, were obtained from the National Centre for Cell Science (NCCS), Pune, India. KGN cells were cultured in DMEM/F-12 medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin in a humidified incubator held at 37°C under an atmosphere of 5% CO_2. Culture media were refreshed every two days, and cells were passaged upon reaching 75-80% confluence using 0.25% trypsin-EDTA.

2.2. Induction of In Vitro PCOS Phenotype

To mimic the intraovarian hyperandrogenic and endocrine dysregulated environment of PCOS, KGN cells were exposed to letrozole, a non-steroidal competitive aromatase inhibitor. Optimization experiments determined that exposure to 1 μM letrozole for 24 h consistently inhibited aromatase activity, suppressed estradiol synthesis, elevated extracellular testosterone levels, generated intracellular oxidative stress, and impaired cellular morphology without inducing absolute toxicity.

2.3. Experimental Grouping and Phyto-Intervention Protocol

Logarithmically growing KGN cells were seeded into multi-well culture plates or culture dishes at established densities and assigned to five distinct treatment groups:

1. Control Group: Cells cultured in standard growth medium containing 0.1% dimethyl sulfoxide (DMSO) vehicle for 24 h.
2. Letrozole Group (PCOS Model): Cells exposed to 1 μM letrozole for 24 h.
3. Letrozole + Berberine Group: Cells pre-treated with berberine (10 μM) for 2 h prior to exposure to 1 μM letrozole for 24 h.
4. Letrozole + Resveratrol Group: Cells pre-treated with resveratrol (15 μM) for 2 h prior to exposure to 1 μM letrozole for 24 h.
5. Letrozole + Berberine + Resveratrol Group: Cells pre-treated with a combination of berberine (10 μM) and resveratrol (15 μM) for 2 h prior to exposure to 1 μM letrozole for 24 h.

Concentrations for berberine and resveratrol were selected based on preliminary concentration-response toxicity screens confirming maximum non-cytotoxic efficacy.

2.4. Determination of Cell Viability

Granulosa cell viability was quantified using the MTT colorimetric assay. KGN cells were seeded into 96-well plates at a density of 1 × 104 cells per well and allowed to adhere overnight. Following designated treatments, culture media were removed, and 100 μL of fresh medium containing 0.5 mg/mL MTT reagent was added to each well. Plates were incubated at 37°C for 4 h in the dark. Formazan crystals formed by metabolically active cells were solubilized in 150 μL DMSO per well with gentle shaking for 10 min. Absorbance was recorded at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was calculated as a percentage relative to untreated control cells.

2.5. Measurement of Oxidative Stress Markers and Antioxidant Status

Intracellular ROS production was evaluated fluorometrically using the 2',7'-dichlorofluorescin diacetate (DCFH-DA) cellular ROS assay kit according to manufacturer instructions. Briefly, treated cells were washed with PBS and incubated with 10 μM DCFH-DA at 37°C for 30 min in the dark. Fluorescence intensity was measured at excitation/emission wavelengths of 485/535 nm.

Lipid peroxidation was assessed by measuring MDA levels using the thiobarbituric acid reactive substances (TBARS) assay. Cell lysates were reacted with thiobarbituric acid under high temperature (95°C) for 45 min, and pink chromogen absorbance was measured at 532 nm. Values were normalized to total cellular protein content determined by the Bradford assay and expressed as nmol/mg protein.

SOD activity was determined based on the inhibition of superoxide anion-mediated nitroblue tetrazolium reduction. One unit of SOD activity was defined as the amount of enzyme producing 50% inhibition of reduction, expressed as U/mg protein. Reduced glutathione (GSH) content was measured spectrophotometrically at 412 nm following reaction with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) and expressed as μmol/mg protein.

2.6. Assessment of Steroidogenic Hormones

Culture media supernatants were collected following 24 h treatment periods, centrifuged at 1,000 × g for 10 min at 4°C to remove cellular debris, and stored at -80°C. Quantitative determination of testosterone and 17β-estradiol concentrations was performed using specific ELISA kits according to manufacturer protocols. Minimum detectable thresholds were 5.0 pg/mL for testosterone and 2.5 pg/mL for estradiol. Absorbances were measured at 450 nm, and hormone concentrations were derived from standard curves and expressed as pg/mL. The androgen-to-estrogen balance was evaluated by calculating the testosterone/estradiol ratio.

2.7. Quantification of Pro-inflammatory Cytokines

The secretion of pro-inflammatory cytokines into cell culture supernatants was quantified using human-specific sandwich ELISA kits for TNF-α, IL-6, and IL-1β. Standards and samples were added to pre-coated microtiter plates and incubated for 2 h at room temperature. Following automated washing steps, biotinylated secondary antibodies and horseradish peroxidase-conjugated streptavidin were added sequentially. Color development was initiated with 3,3',5,5'-tetramethylbenzidine substrate, stopped with 2 N H_2SO_4, and read at 450 nm. Cytokine concentrations were calculated from calibration curves and expressed as pg/mL.

2.8. Statistical Analysis

Data were derived from three independent experimental replicates performed in triplicate and expressed as Mean ± Standard Error of the Mean (SEM). Statistical evaluations were conducted using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Normal distribution of data was verified using the Shapiro-Wilk test. Differences among multiple experimental groups were analyzed using one-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. A value of $p < 0.05$ was designated as statistically significant.

3. Results

3.1. Characterization of Letrozole-Induced Cellular Morphology and Phenotype

Exposure of KGN human granulosa cells to 1 μM letrozole for 24 h induced marked structural degeneration and phenotypic alterations characteristic of cellular distress. Untreated control cells displayed regular polygonal morphology, intact plasma membranes, consistent cell-to-cell adhesion, and formed a uniform confluent monolayer (92.4 ± 2.1% confluence). In contrast, letrozole treatment disrupted cellular integrity, resulting in cell body shrinkage, cytoplasmic condensation, membrane blebbing, and loss of monolayer continuity (54.7 ± 2.8% confluence, $p < 0.05$).

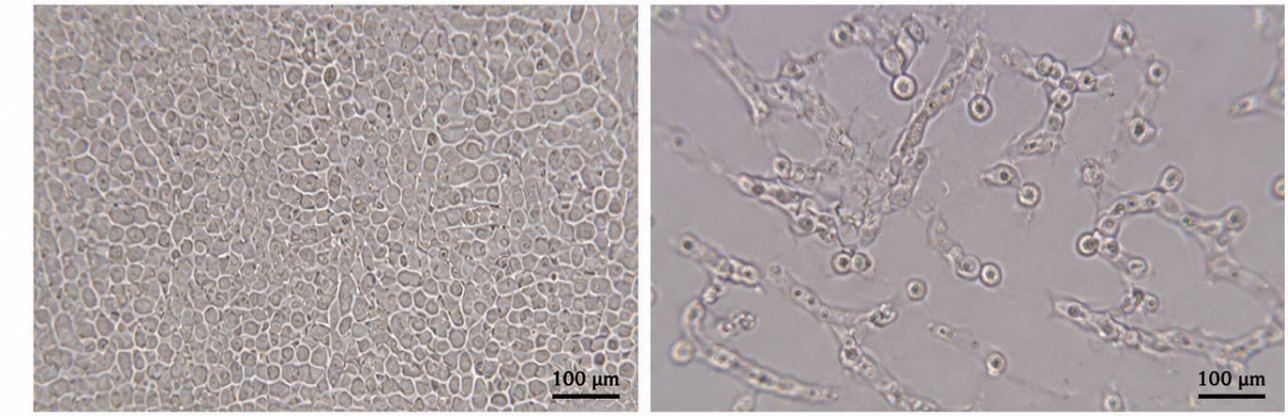

Figure 1. Morphological changes in KGN granulosa cells following 24 h treatment with 1 μM letrozole. Phase-contrast micrographs show dense, adherent control monolayers compared to shrunken, detached letrozole-treated cells.

Quantitative morphometric analysis confirmed significant structural changes following letrozole treatment, including reductions in mean cell area and circularity index alongside a fivefold increase in detached, floating cells.

Table 1. Quantitative morphometric parameters of KGN cells in control and letrozole-treated groups.

Parameter	Control (Untreated)	Letrozole (1 μM)	Statistical Significance
Monolayer Confluence (%)	92.4 ± 2.1	54.7 ± 2.8	$p < 0.05$ vs. Control
Mean Cell Area (μm²)	1213.6 ± 45.2	678.3 ± 38.7	$p < 0.05$ vs. Control
Circularity Index (0 - 1)	0.78 ± 0.03	0.42 ± 0.02	$p < 0.05$ vs. Control
Detached Cells (%)	4.8 ± 0.7	22.6 ± 1.3	$p < 0.05$ vs. Control

Values expressed as Mean ± SEM (n = 3 independent experiments). Statistical comparison performed using Unpaired Student's t-test.

3.2. Cytoprotective Effects of Berberine and Resveratrol Monotherapy and Combination on Granulosa Cell Viability

Assessment of cell viability using the MTT assay showed that 1 μM letrozole exposure reduced KGN cell viability to 63.8 ± 3.1% relative to untreated controls (100.0 ± 3.5%, $p < 0.001$). Pre-treatment with berberine (10 μM) or resveratrol (15 μM) individually significantly improved cell survival to 78.4 ± 2.9% ($p < 0.01$) and 80.6 ± 3.2% ($p < 0.01$), respectively.

Dual pre-treatment with both berberine and resveratrol restored cell viability to 92.5 ± 2.4%, demonstrating significantly greater protection than either monotherapy ($p < 0.001$ vs. letrozole; $p < 0.05$ vs. individual treatments).

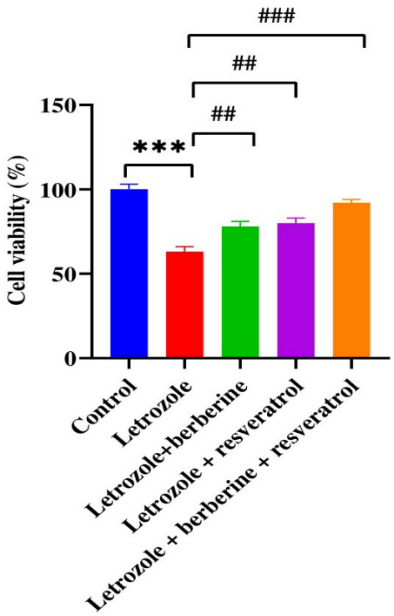

Figure 2. Effect of berberine (10 μM), resveratrol (15 μM), and their combination on cell viability in letrozole-treated KGN granulosa cells. Values indicate Mean ± SEM (n = 3). $^{***}p < 0.001$ vs. Control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Letrozole.

3.3. Attenuation of Intracellular Oxidative Stress and Restoration of Antioxidant Capacities

Letrozole treatment induced intracellular redox imbalance in KGN cells. Relative ROS production increased to 192 ± 7.5% of control values (100 ± 4.2%, $p < 0.05$), accompanied by a severe increase in MDA concentration from 1.8 ± 0.2 nmol/mg protein to 4.9 ± 0.4 nmol/mg protein ($p < 0.05$). Concurrently, endogenous enzymatic SOD activity decreased from 18.6 ± 1.1 U/mg protein to 8.2 ± 0.7 U/mg protein, and non-enzymatic GSH levels dropped from 9.4 ± 0.5 μmol/mg protein to 4.1 ± 0.3 μmol/mg protein ($p < 0.05$).

Monotherapy with berberine or resveratrol significantly attenuated ROS generation and MDA levels while partially restoring SOD activity and GSH content. Combination treatment with berberine and resveratrol produced superior antioxidant effects, lowering ROS levels to 112 ± 4.9% and MDA content to 2.1 ± 0.2 nmol/mg protein, while increasing SOD activity to 17.2 ± 1.0 U/mg protein and GSH content to 8.8 ± 0.4 μmol/mg protein, values comparable to non-treated controls.

Table 2. Modulation of oxidative stress parameters and antioxidant status across experimental groups.

Treatment Group	ROS (% of Control)	MDA (nmol/mg protein)	SOD (U/mg protein)	GSH (μmol/mg protein)
Control	100 ± 4.2	1.8 ± 0.2	18.6 ± 1.1	9.4 ± 0.5
Letrozole (1 μM)	192 ± 7.5*	4.9 ± 0.4*	8.2 ± 0.7*	4.1 ± 0.3*
Letrozole + Berberine	145 ± 6.2#	3.3 ± 0.3#	13.5 ± 0.8#	6.8 ± 0.4#
Letrozole + Resveratrol	138 ± 5.8#	3.1 ± 0.2#	14.1 ± 0.9#	7.1 ± 0.5#
Letrozole + Berberine + Resveratrol	112 ± 4.9#@	2.1 ± 0.2#@	17.2 ± 1.0#@	8.8 ± 0.4#@

*Values indicated as Mean ± SEM (n = 3). *p < 0.05 vs. Control; #p < 0.05 vs. Letrozole; @p < 0.05 vs. individual treatment groups (One-way ANOVA with Tukey's post hoc test).*

3.4. Reversal of Letrozole-Induced Steroidogenic Dysfunction and Hormonal Imbalance

Aromatase inhibition by letrozole altered steroidogenic output in KGN cells. Extracellular testosterone concentrations increased significantly from a baseline of 42.5 ± 3.2 pg/mL to 96.8 ± 5.1 pg/mL ($p < 0.05$). Conversely, 17β-estradiol production declined from 118.4 ± 6.3 pg/mL to 54.7 ± 4.2 pg/mL ($p < 0.05$). Consequently, the testosterone-to-estradiol ratio increased from 0.36 in controls to 1.77 in letrozole-treated cells, confirming hyperandrogenic status.

Single-agent treatment with berberine or resveratrol reduced testosterone levels (71.6 ± 4.3 pg/mL and 68.4 ± 4.0 pg/mL, respectively) and enhanced estradiol secretion (82.3 ± 5.0 pg/mL and 87.1 ± 4.8 pg/mL, respectively). Combined treatment with berberine and resveratrol demonstrated the most pronounced effect, reducing testosterone levels to 48.9 ± 3.5 pg/mL and elevating estradiol levels to 108.6 ± 5.4 pg/mL. This treatment restored the testosterone-to-estradiol ratio to 0.45, near physiological control levels.

Table 3. Concentrations of steroidogenic hormones and testosterone/estradiol ratios in culture media.

Treatment Group	Testosterone (pg/mL)	Estradiol (pg/mL)	Testosterone / Estradiol Ratio
Control	42.5 ± 3.2	118.4 ± 6.3	0.36
Letrozole (1 μM)	96.8 ± 5.1*	54.7 ± 4.2*	1.77
Letrozole + Berberine	71.6 ± 4.3#	82.3 ± 5.0#	0.87
Letrozole + Resveratrol	68.4 ± 4.0#	87.1 ± 4.8#	0.79
Letrozole + Berberine + Resveratrol	48.9 ± 3.5#@	108.6 ± 5.4#@	0.45

*Data presented as Mean ± SEM (*n = 3*). *p < 0.05 vs. Control; #p < 0.05 vs. Letrozole; @p < 0.05 vs. individual treatment groups.*

3.5. Suppression of Pro-inflammatory Cytokine Production

Letrozole exposure significantly upregulated pro-inflammatory cytokine secretion in KGN cells. TNF-α levels rose from 28.4 ± 2.1 pg/mL to 74.2 ± 4.6 pg/mL, IL-6 from 18.7 ± 1.5 pg/mL to 62.5 ± 3.9 pg/mL, and IL-1β from 12.3 ± 1.1 pg/mL to 41.8 ± 2.7 pg/mL ($p < 0.05$ for all). Pre-treatment with berberine or resveratrol monotherapy significantly attenuated cytokine production. However, co-administration of berberine and resveratrol achieved near-complete suppression of the inflammatory response, reducing TNF-α to 31.7 ± 2.4 pg/mL, IL-6 to 21.4 ± 1.9 pg/mL, and IL-1β to 14.6 ± 1.3 pg/mL ($p < 0.05$ vs. single treatments).

Table 4. Quantification of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in KGN cell supernatants.

Treatment Group	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)
Control	28.4 ± 2.1	18.7 ± 1.5	12.3 ± 1.1
Letrozole (1 μM)	74.2 ± 4.6*	62.5 ± 3.9*	41.8 ± 2.7*
Letrozole + Berberine	49.3 ± 3.2#	39.6 ± 2.8#	25.4 ± 2.0#
Letrozole + Resveratrol	46.8 ± 3.0#	36.9 ± 2.5#	23.8 ± 1.8#
Letrozole + Berberine + Resveratrol	31.7 ± 2.4#@	21.4 ± 1.9#@	14.6 ± 1.3#@

*Data presented as Mean ± SEM (*n = 3*). *p < 0.05 vs. Control; #p < 0.05 vs. Letrozole; @p < 0.05 vs. individual treatment groups.*

4. Discussion

Establishing reliable cell models is vital for elucidating molecular mechanisms driving PCOS and screening novel therapeutic agents. Competitive inhibition of cytochrome P450 aromatase (CYP19A1) using letrozole blocks androgen conversion into estrogens, mimicking intraovarian hyperandrogenism and follicular arrest observed clinically [31]. In human KGN granulosa cells, letrozole-mediated aromatase inhibition suppresses estradiol synthesis, increases androgen levels, alters cell morphology, and elevates oxidative distress [32]. The morphological degeneration observed here characterized by cell shrinkage, detachment, and reduced viability reflects granulosa cell dysfunction during follicular atresia in polycystic ovaries [33].

Granulosa cell survival is essential for supporting oocyte maturation and maintaining follicular structural integrity. The pronounced reduction in viability following letrozole exposure stems from mitochondrial impairment, intracellular ROS generation, and inflammatory signaling [34]. Monotherapy with either berberine or resveratrol significantly attenuated letrozole-induced cytotoxicity, whereas co-administration restored cell viability to near-control levels.

These cytoprotective effects likely involve complementary metabolic regulatory mechanisms. Berberine activates AMPK by increasing cellular AMP levels, restoring energy homeostasis and promoting cell survival [26]. Resveratrol acts primarily as a direct

activator of SIRT1 deacetylase, promoting mitochondrial biogenesis and resistance to metabolic stress [29]. Because AMPK and SIRT1 engage in positive cross-talk where AMPK increases intracellular NAD+ to fuel SIRT1 activity, and SIRT1 deacetylates LKB1 to sustain AMPK activation simultaneous treatment engages both pathways [22]. This dual activation enhances mitochondrial protection and energy regulation, explaining the superior cytoprotections observed relative to single-agent treatments.

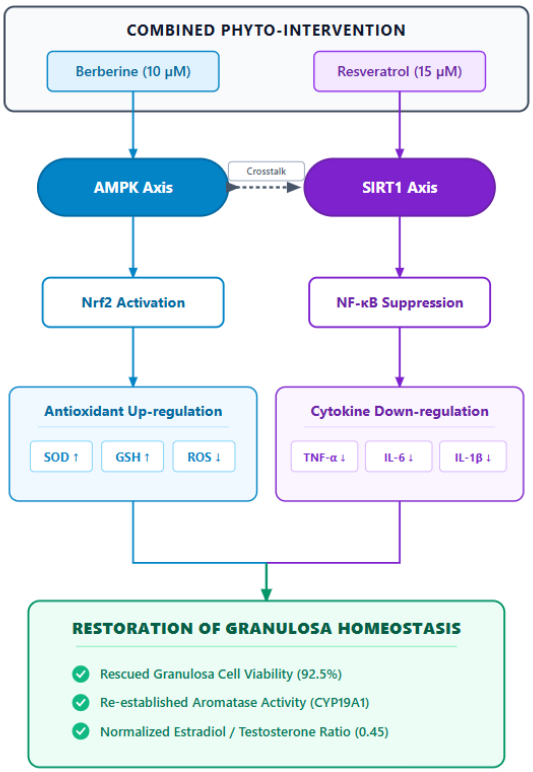

Figure 3. Synergistic Mechanism of Berberine and Resveratrol in Granulosa Cells

Oxidative stress plays a central role in PCOS pathophysiology, causing membrane damage, protein modification, and mitochondrial breakdown in granulosa cells [10, 12]. In this study, letrozole exposure significantly increased intracellular ROS and MDA levels while depleting SOD activity and GSH pools. Elevated lipid peroxidation destabilizes cell membranes and generates reactive aldehyde intermediates that perpetuate metabolic injury [13].

Co-administration of berberine and resveratrol lowered ROS and MDA levels to baseline while restoring endogenous SOD and GSH concentrations. Berberine upregulates phase II antioxidant responses through Nrf2 nuclear translocation, increasing transcription of SOD, GSH-Px, and heme oxygenase-1 (HO-1) [27]. Resveratrol directly scavenges free radicals and stimulates SIRT1-dependent deacetylation of PGC-1α and FoxO3a, enhancing mitochondrial antioxidant defenses [29]. Combined intervention simultaneously targets free radical production and endogenous antioxidant capacity, demonstrating greater efficacy against redox imbalance than monotherapy.

Steroidogenic dysfunction characterized by excessive androgen accumulation and deficient estrogen synthesis defines the endocrine profile of PCOS [7]. In letrozole-treated KGN cells, aromatase inhibition increased extracellular testosterone while significantly reducing 17β-estradiol secretion, elevating the androgen-to-estrogen ratio.

Combined treatment with berberine and resveratrol reversed this imbalance, reducing testosterone accumulation and restoring 17β-estradiol synthesis. Berberine modulates steroidogenic enzyme transcription and improves insulin sensitivity, indirectly regulating androgen production [26]. Resveratrol modulates estrogen receptor sensitivity and rescues CYP19A1 transcript expression under conditions of metabolic stress [28]. Reversal of the testosterone-to-estradiol ratio demonstrates that combined treatment helps overcome competitive aromatase inhibition, promoting functional endocrine recovery in granulosa cells.

Chronic low-grade intraovarian inflammation contributes to impaired folliculogenesis, altered steroidogenesis, and insulin resistance in PCOS [14, 15]. Elevated ROS levels activate NF-ϰ B transcription factors, promoting the expression of TNF-α, IL-6, and IL-1β

[16]. In agreement with these mechanisms, letrozole exposure significantly increased pro-inflammatory cytokine concentrations in KGN cell supernatants.

Co-administration of berberine and resveratrol suppressed TNF-α, IL-6, and IL-1β secretion, bringing cytokine concentrations back to near-baseline levels. Berberine inhibits Iκ Bα phosphorylation and prevents NF-κ B p65 nuclear translocation [25]. Resveratrol enhances SIRT1-mediated deacetylation of the NF-κ B p65 subunit at lysine 310, suppressing its transcriptional activity [30]. Combined treatment breaks the feed-forward cycle connecting oxidative stress and inflammation in granulosa cells by simultaneously blocking NF-κ B activation through distinct molecular steps.

5. Conclusion

Co-administration of berberine and resveratrol effectively mitigates letrozole-induced damage in human KGN granulosa cells. Dual treatment restores cell viability, reduces intracellular ROS generation and lipid peroxidation, and recovers endogenous antioxidant defenses including superoxide dismutase and glutathione. Concurrently, combination therapy suppresses pro-inflammatory cytokine secretion (TNF-α, IL-6, IL-1β) and normalizes the estradiol-to-testosterone balance. These results indicate that combining berberine and resveratrol produces superior multi-target protection compared to individual monotherapies, highlighting combination phytotherapy as a potential therapeutic approach for managing polycystic ovary syndrome pathology.

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