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ORIGINAL ARTICLE

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The effect of quercetin on adipogenesis, lipolysis, and apoptosis in 3T3-L1 adipocytes: The role of SIRT1 pathways

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Abstract

Background: Lipotoxicity, caused by adipocyte triglyceride over-accumulation, contributes to obesity-related comorbidities such as hypertension, type 2 diabetes, coronary heart disease, respiratory dysfunction, and osteoarthritis. This study focuses on determining how sirtuin-1 (SIRT-1) mediates quercetin's (QCT) effect on 3T3-L1 adipocytes. Key aspects of this study include preventing adipogenesis, inducing lipolysis, and stimulating adipocyte apoptosis.

Methods: 3T3-L1 adipocytes underwent treatment with varying QCT doses, lipopolysaccharide (LPS), and the SIRT-1 inhibitor EX-527, followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide [MTT] assay for cell viability assessment. Furthermore, quantitative real-time polymerase chain reaction measured mRNA expression levels of adipogenesis markers (*fatty acid synthase* [*FASN*] and *peroxisome proliferator-activated receptor gamma* [*PPARγ*]), lipolysis markers (*adipose triglyceride lipase* [*ATGL*] and *hormone-sensitive lipase* [*HSL*]), and apoptosis markers (*B-cell lymphoma2 [Bcl-2], Bcl-2 Associated -X-protein [BAX]* and *Caspase-3*).

Results: The data showed that LPS + QCT significantly reduced cell viability in a dose- and time-dependent manner, unaffected by LPS + QCT + EX-527. Treatment with LPS + QCT did not affect *FASN* and *PPARγ* expression but significantly increased *ATGL* and *HSL* mRNA expression compared with LPS alone. Interestingly, EX-527 reversed the effects of LPS + QCT on lipogenesis and lipolysis markers completely. QCT enhanced apoptosis in a SIRT-1 independent pattern.

Conclusion: The data suggest that QCT suppresses adipogenesis while increasing lipolysis via SIRT-1. However, QCT's effects on apoptosis appear to be independent

Abbreviations: AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; DMEM.F12, Dulbecco's Modified Eagle's Medium F-12; FASN, fatty acid synthase; FBS, fetal bovine serum; FOXO, Forkhead family of transcription factors; GLP-1, glucagon-like peptide 1; HDACs, histone deacetylases; HSL, hormone-sensitive lipase; IL-18, interleukin-18; IL-1β, interleukin-1 beta; IL-6, interleukin-6; LPS, lipopolysaccharide; mTOR, mechanistic target of rapamycin; QCT, quercetin; ROS, reactive oxygen species; SIRT, sirtuin; TNF-α, tumor necrosis factor alpha.

Mohammad Hasan Maleki and Sara Abdizadeh Javazm both contributed equally to this work.

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of SIRT-1. These findings provide further evidence for QCT's effects on adipocytes, particularly its interaction with SIRT-1.

KEYWORDS
adipogenesis, apoptosis, obesity, quercetin, SIRT-1

1 | INTRODUCTION

An imbalance between energy intake and expenditure leads to the accumulation of excess fat, resulting in obesity. Lipotoxicity, arising from the excessive storage of triglycerides in adipocytes, contributes to obesity-related comorbidities such as hypertension, type 2 diabetes, cancer, coronary heart disease, respiratory dysfunction, and osteoarthritis.[1] In obesity, adipose tissue has an excess of larger adipocytes, which are caused by fat buildup inside these cells, resulting in hypertrophy.[2] Additionally, some variables have been associated with low-grade systemic and adipose tissue inflammation. These variables include altered adipose tissue resident immune cell phenotype,[3] increased pro-inflammatory cytokine production and endoplasmic reticulum stress,[4] hypoxia, an increase in reactive oxygen species (ROS), excessive apoptosis,[5] and increased lipopolysaccharide (LPS) levels.[6] In response to a high-fat diet, intestinal Gram-negative bacteria upregulate the production of LPS, which can potentially exacerbate inflammation, contributing to a more severe inflammatory response.[7,8] Under the influence of LPS, adipocytes may become susceptible to Caspase-4/5/11 activation, initiating pyroptosis, a highly inflammatory form of programmed cell death. Notably, large adipocytes, characterized by higher metabolic activity, exhibit greater LPS vulnerability than their smaller counterparts.[9]

Fatty acid synthase (FASN) serves as a marker indicative of fat production, whereas hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) play pivotal roles in fat breakdown.[10,11] FASN actively participates in lipogenesis by utilizing Nicotinamide Adenine Dinucleotide Phosphate (NADPH), acetyl-CoA, and malonyl-CoA. At the same time, ATGL is responsible for breaking down triglycerides into energy-yielding fatty acids, a crucial process for maintaining an overall energy balance.[12] On the other hand, HSL is indispensable for full hormone-stimulated lipolysis and acts as the rate-limiting factor for the hydrolysis of diacylglycerol and cholesteryl in adipose tissue.

Mammals possess three isoforms of PPARs: PPARα, PPARβ/δ, and peroxisome proliferator-activated receptor gamma (PPARγ).[13] While these isoforms share similarities, they exhibit distinct tissue distribution patterns, ligand specificity, and biological roles. PPARγ, predominantly located in adipose tissue, plays a crucial role in regulating the differentiation and proliferation of adipocytes.

Furthermore, PPARγ is implicated in the pathophysiology of various disorders, including diabetes, atherosclerosis, cancer, and obesity. Several studies have demonstrated that down-regulation of PPARγ diminishes adipogenesis and mitigates obesity.[14] Apoptosis, a well-known energy-dependent cell death process, is crucial in controlling adipose mass. This process involves pro-apoptotic proteins such as Bcl-2 Associated -X-protein (BAX), Bad, and Bid and anti-apoptotic proteins including Bcl-2 and Bcl-XL.[15]

Several phytochemicals have been extensively studied for their effects on adipocytes, including the inhibition of adipogenesis, stimulation of lipolysis, inhibition of preadipocyte maturation into mature adipocytes,[16] reduction of energy intake, and promotion of energy expenditure.[17] While medications exhibiting these characteristics are being explored as potential treatments for obesity prevention, it is important to recognize that their impact on adipose tissue can have dual effects. For example, excessive stimulation of cellular apoptosis and lipolysis may contribute to inflammation and insulin resistance.[18] In contrast, stimuli of adipogenesis such as glucagon-like peptide 1 (GLP-1) were found to improve insulin resistance by recruitment of the new adipocytes, which display high insulin sensitivity. On the contrary, factors promoting adipogenesis, such as GLP-1, have been observed to enhance insulin resistance through the recruitment of newly formed adipocytes characterized by heightened insulin sensitivity.[19] In fact, additional rigorous studies are imperative to conclusively ascertain the clinical efficacy of these phytochemicals and it should be determined if their effects on these pathways are controlled.[16,20]

Among these phytochemicals, a number of research conducted in recent years have focused on Quercetin (QCT) as a flavonol abundant in foods such as onions, grapes, berries, cherries, broccoli, and citrus fruits and is a potent anti-obesity agent with antioxidant and anti-inflammatory properties.[21] QCT has been demonstrated to display lipolytic and anti-adipogenic characteristics in addition to lowering cell proliferation, causing cell cycle arrest and apoptosis in both in vitro and in vivo investigations with a range of cell lines including 3T3-L1 pre-adipocytes.[22]

QCT appears to suppress pro-adipogenic signals, perhaps leading to decreased adipose tissue mass via apoptosis. Recent research also underscores the connection between QCT and the activation of sirtuin-1 (SIRT-1).[23] SIRTs, belonging to the NAD-dependent class III histone deacetylases (HDACs), are crucial in regulating redox and metabolic stress. Specifically, SIRT-1 is an intracellular nutritional sensor that monitors cellular NAD + levels. It becomes active in response to rising NAD + levels due to calorie restriction and oxidative stress. Conversely, reduced NAD + levels during hypoxia can deactivate SIRT-1.[24] Recent studies have uncovered a link between the anti-oxidant and anti-inflammatory effects of QCT and SIRT activity.[25] However, it remains unclear whether the effects of QCT on increasing apoptosis, reducing adipogenesis, and stimulating lipolysis are SIRT-dependent. Determining how SIRT-1 influences all of QCT's effects on adipose tissue is the main objective of the current study.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

The 3T3-L1 pro-adipocytes (derived from mouse embryonic fibroblasts) used in this study were obtained from the Pasteur Institute in Tehran, Iran. QCT (CAS# 520-33-2, high performance liquid chromatography (HPLC) 98%), SIRT-1 inhibitor (EX-527) (CAS# 49843-98-3), LPS (#L2630), insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) were all acquired from Sigma-Aldrich (St. Louis).

2.2 | Cell culture and differentiation

The study employed 3T3-L1 cell lines to examine the impact of LPS, QCT, and the SIRT-1 inhibitor (EX-527) on adipocytes. 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) were added to Dulbecco's Modified Eagle's Medium F-12 (DMEM.F12) (Biosera), which was used for growing 3T3-L1 pre-adipocyte cells at 37°C with 5% CO^2. Pre-adipocytes were differentiated 48 h after achieving 70% confluency by adding 10 μg/ml insulin, 1 μg dexamethasone, and 0.5 mM IBMX to DMEM-F12 (differentiation media). The cells were incubated with 1 μg/mL insulin in DMEM-F12 after 48 h of differentiation. Media was changed to differentiation one (10% FBS/DMEM-F12) 2 days later. For 15 days, the media was replaced every 2 days.[26]

2.3 | Cell viability test and study design

The cytotoxicity of LPS, QCT, and EX-527 was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In 96-well plates with 10^4 cells per well, differentiated 3T3-L1 cells were treated with various concentrations of QCT (0–250 μM), LPS (50–1000 ng/mL), and EX-527 (2–100 μM) for 24 and 48 h. Each well received 20 μL of 5 mg/mL MTT (Solarbio), which was then incubated at 37°C for 4 h. Following the removal of the cell supernatant, 100 μL of dimethyl sulfoxide (DMSO) was added. A Synergy Microplate Reader (BioTek) was used to measure the spectrophotometric absorbance at 570 nm following the 20-minute incubation period of DMSO. The cytotoxicity of the medicines in combination was assessed using the MTT test based on the optimal treatment dose for QCT (100 μM), LPS (200 ng/mL), and EX-527 (10 μM), and the effective time was determined.

3T3-L1 cells were pretreated with EX-527 (10 μM) for 4 h, then LPS (200 ng/mL) was added, and cells were incubated for 2 h (without medium change). Finally, the cells were co-treated with QCT (100 μM) for 24 and 48 h and MTT experiments were performed as previously described.

2.4 | Adipogenesis measurement by Oil Red O staining method

Pre-adipocytes (24,000 cells/well) were cultured in 24-well plates using the pre-adipocyte medium for 72 h until they reached 100% of confluence. Subsequently, distinct culturing media were applied to each well: DMEM.F12 for pre-adipocyte cells, differentiation media as previously mentioned for adipocyte cells (positive control), differentiation media with QCT (100 μM), LPS (200 ng/mL), EX-527 (10 μM), QCT (100 μM) + LPS (200 ng/mL) and QCT (100 μM) + LPS (200 ng/mL) + EX-527 (10 μM). The medium of each well was refreshed every 48 48-h until pre-adipocytes cultured in differentiation media turned into adipocytes. Afterward, the total lipid contents in adipocytes were measured using the Oil Red O staining method. For this purpose, the differentiated adipocytes were fixed with 10% formalin for 30 min, cleansed with phosphate-buffered saline (PBS), stained with 0.5% (w/v) Oil Red O solution at 37°C for 1 h, and photographed by an inverted microscope (Axio Vert.A1 FLLED, ZEISS®). To determine the accumulative lipid content, the absorbed dye in adipocytes was extracted with 100% isopropanol and quantified by detecting the optical absorbance at a wavelength of 510 nm via the microplate reader. The data was expressed as the relative lipid content of the untreated differentiated adipocytes.[27]

2.5 | Lipolysis measuring by glycerol assay

Adipocytes (density 24,000 cells/well) were placed in 24-well plate for 12 h, and cultured with IBMX for positive control, phenol red-free DMEM for differentiated adipocytes treated with QCT (100 μM), LPS (200 ng/mL), EX-527 (10 μM), QCT (100 μM) + LPS (200 ng/mL) and QCT (100 μM) + LPS (200 ng/mL) + EX-527 (10 μM), and followed with 24 h incubation at 37°C in humidified atmosphere of 5% CO^2. Afterward, glycerol content release measurement represented the lipolysis state of adipocytes evaluated by free glycerol reagent according to the manuscript protocol (Sigma®, St. Louis) to explain briefly that for inactivation of cellular released enzymes, the supernatant of each well was heated at 70°C for 10 min. Afterward samples were exposed to free glycerol reagent in the ratio of 1:1 v/v and incubated for 15 min at room temperature and optical absorbance was achieved at wavelength of 540 nm via a microplate reader. The lipolytic effect was demonstrated as released glycerol content (μM) into the control.[28]

2.6 | Quantitative real-time PCR

After pretreatment with EX-527 (10 μM) for 4 h, 3T3-L1 cells were exposed to LPS (200 ng/mL) for 2 h before the medium was changed. After 24 h of co-treatment with QCT (100 μM), total RNA was extracted with Trizol-Reagent (Yekta-Tajhiz-Azma). The purity and

concentration of the extracted RNA were calculated using the ratio of absorbance at 260 and 280 nm measured using a Nano-Drop spectrophotometer. The purity of the RNA was also evaluated using gel electrophoresis. Reverse transcription was carried out with a cDNA synthesis kit (Yekta- Tajhiz-Azma). *SIRT-1*, *ATGL*, *HSL*, *PPARγ*, *FASN*, *Caspase-3*, *Bcl-2*, and *BAX* mRNA expression was examined by real-time RT-PCR on the ABI 7500 Real-time PCR equipment with SYBR Green master mix (Ampliqon). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as a housekeeping gene. The sequences of the PCR primers are shown in Table 1. Comparative Ct ($2^{-\Delta\Delta ct}$) was used to determine the relative amount of comparing mRNA. There were three independent analyses of each sample.

2.7 | Statistical analysis

The data are presented as means ± standard deviations. One-way analysis of variance (ANOVA) and Tukey's post hoc test were used for the statistical assessment of the groups' differences. For the statistical evaluation, SPSS (version 24.0), and GraphPad PRISM (version 9.4.1) were used for drawing the graphs. When the *p*-value was less than 0.05, statistical significance was declared.

3 | RESULTS

3.1 | Cell viability tests

The cytotoxic effects of QCT, LPS, and EX-527 were assessed using the MTT assay. Different concentrations of QCT (5–250 μM), LPS (50–2000 ng/mL), and EX-52 (2–100 μM) were used to treat the cells. Figure 1A–C indicate how QCT, LPS, and EX-527 dramatically reduced 3T3-L1 cell viability in a time- and dose-dependent manner. In Figure 1A, it is evident that LPS led to a notable increase in cell death after 24 and 48 h. Statistical analysis revealed significance ($p < 0.01$) for the 200 ng/mL LPS in 24h and $p < 0.001$ for concentrations ≥400 ng/mL at both 24 and 48-h intervals. Figure 1B shows that QCT treatment significantly reduced the viability of 3T3-L1 cells ($p < 0.001$ for concentrations ≥100 μM (24 h) and $p < 0.001$ for concentrations ≥50 μM (48 h). Similarly, the treatment with EX-527 increased cell death in a time-dose dependent manner, as shown in Figure 1C ($p < 0.001$ for concentrations ≥5 μM (24 h), $p < 0.001$ for concentrations ≥2 μM (48 h)). After 24 and 48 h of treatment with 100 μM QCT and 200 ng/mL LPS, the viability of the 3T3-L1 cells was dramatically reduced in comparison to LPS alone ($p < 0.001$). As depicted in Figure 1D, 24 h or 48 h treatment of 3T3-l1 adipocytes with LPS (200 ng/mL) significantly reduced cell viability compared to control ($p < 0.001$). Treating with LPS (200 ng/mL) + QCT (100 M) reduced the cell viability of 3T3-l1 adipocytes significantly more than LPS (200 ng/mL) ($p < 0.001$) after 24 h or 48 h. Administration of LPS (200 ng/mL) + EX-527 (10 M) + QCT (100 M) for 24 h or 48 h diminished cell viability of 3T3-l1 adipocytes markedly more than LPS (200 ng/mL) ($p < 0.001$). After 24 h or 4 h, there is no discernible change in cell viability between LPS (200 ng/mL) + QCT (100 M) and LPS (200 ng/mL) + EX-527 (10 M) + QCT (100 M) Finally, 24 h as the optimal exposure time was chosen for the next step based on the results of the MTT experiment (Figure 1D).

TABLE 1 The sequence of designed primers.

Description	Sequences	Accession number
PPARγ	5′-CACAATGCCATCAGGTTTGG	NM_011146
	3′-GCTGGTCGATATCACTGGAGATC	
ATGL	5′-GGAATGGCCTACTGAACCAA	NM_001163689
	3′-GCAATTGATCCTCCTCTCCA	
HSL	5′-GCATGGATTTACGCACAATG	NM_010719
	3′-AGTTGGTTCTAGCCCCAGTG	
FASN	5′-GTGGACATGGTCACAGAG	NM_007988.3
	3′-CATAGCTGACTTCCAACAGC	
Caspase-3	5′-AAGGAGCAGCTTTGTGTGTG	NM_009810.3
	3′-GGCAGGCCTGAATGATGAAG	
Bcl-2	5′-TCTGGTTGGGATTCCTACGG	NM_009741.3
	3′-AGGAGGGTTTCCAGATTGGG	
BAX	5′-CAAGAAGCTGAGCGAGTGTC	NM_007527.3
	3′-GTCCACGTCAGCAATCATCC	
GAPDH	5′-GACAAAATGGTGAAGGTCGGTG	NM_008084
	3′-TGATGTTAGTGGGGTCTCGCTC	

3.2 | Quercetin inhibit adipogenesis in a SIRT-1 dependent pattern

As shown in Figure 2A–C, 3T3-L1 pre adipocytes were differentiated into adipocytes and stained with Oil Red. Moreover, PPARγ gene expression augmented more than 10-fold ($p < 0.0001$) in differentiated adipocytes compared to pre-adipocytes (Figure 2D). Inhibitory effects of LPS and QCT on cell differentiation were evaluated by measuring intracellularly accumulated lipid in adipocytes using Oil Red O staining. According to Figure 2E, QCT and LPS could significantly diminish lipid contents compared to untreated adipocytes (*p*-value <0.0001). EX-527 significantly neutralized the anti-adipogenesis effects of QCT and LPS (*p*-value <0.0001).

3.3 | Quercetin could not attenuate adipogenesis-related genes after SIRT-1 inactivation

FASN and *PPARγ* mRNA level was detected by RT-PCR after 24 h. As shown in Figure 3A, a significant downregulation of *PPARγ* was observed in LPS-treated cells compared to controls ($p < 0.05$). A similar pattern was seen after QCT was applied to differentiated adipocytes (3T3-L1 cells). Co-treatment of QCT with LPS and EX-527 significantly increased expression of *PPARγ* compared with the

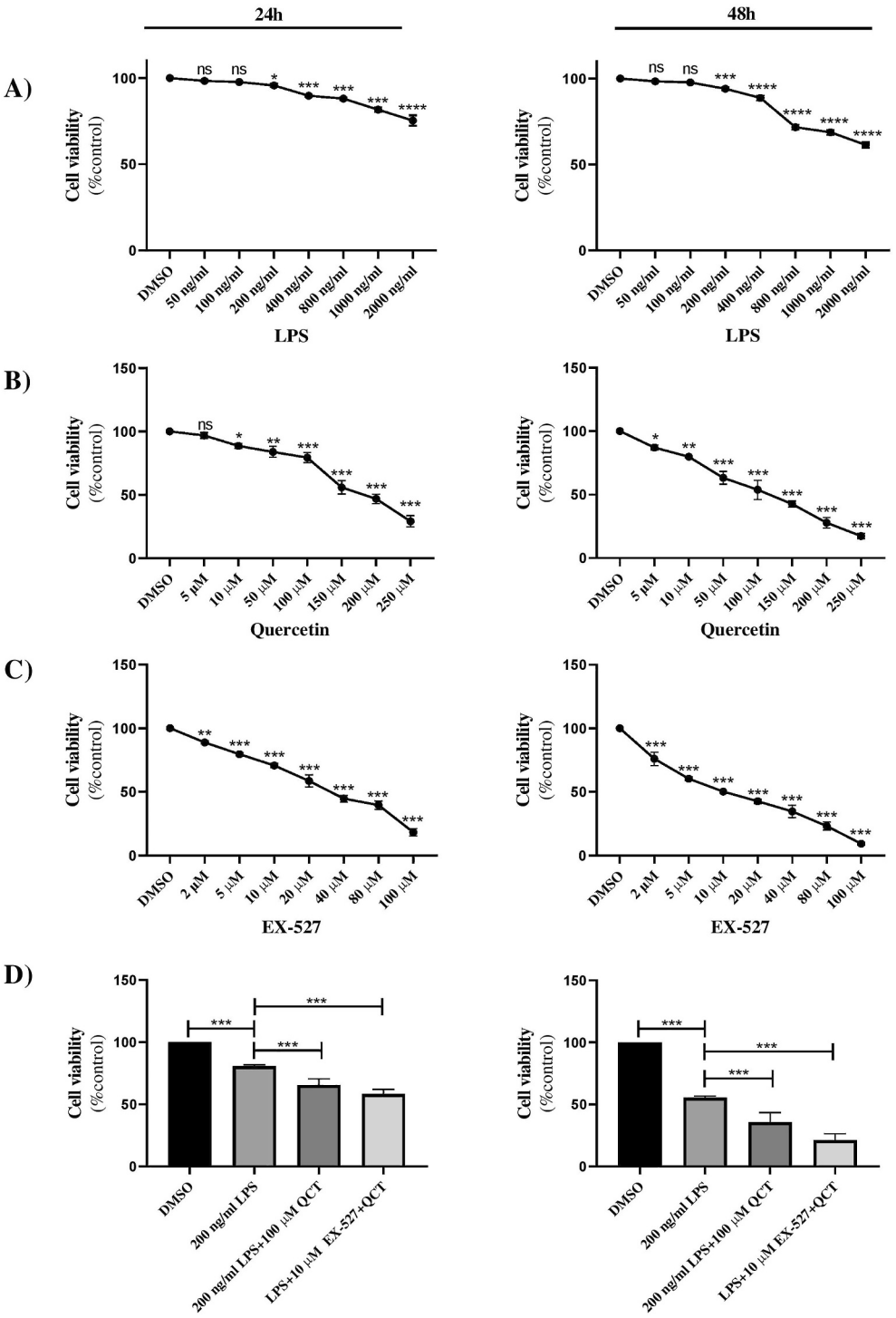

FIGURE 1 Effects of QCT, LPS, EX-527 and their combination on the growth of 3T3-L1 cells. The cells were exposed to different concentrations of QCT (A), LPS (B), EX-527 (C) and a combination of them (D) for 24 and 48 h. Cell viability was measured by MTT assay. Data are reported as mean ± SD of three independent assays ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; QCT, quercetin's.

combination of QCT and LPS ($p < 0.0001$). Therefore, EX-527 suppressed the anti-adipogenesis effect of QCT, which indicates that QCT acts via SIRT-1 ($p < 0.0001$). Similarly, a remarkable reduction was observed in the mRNA level of *FASN* after LPS treatment compared with control ($p < 0.01$) (Figure 3B). Co-treatment of QCT with LPS and EX-527 noticeably enhanced the expression level of *FASN* mRNA compared with the combination of QCT and LPS ($p < 0.0001$). Accordingly, EX-527 induces adipogenesis by upregulating the expression of *PPARγ* and *FASN*, an effect that QCT cannot reverse when SIRT-1 is suppressed.

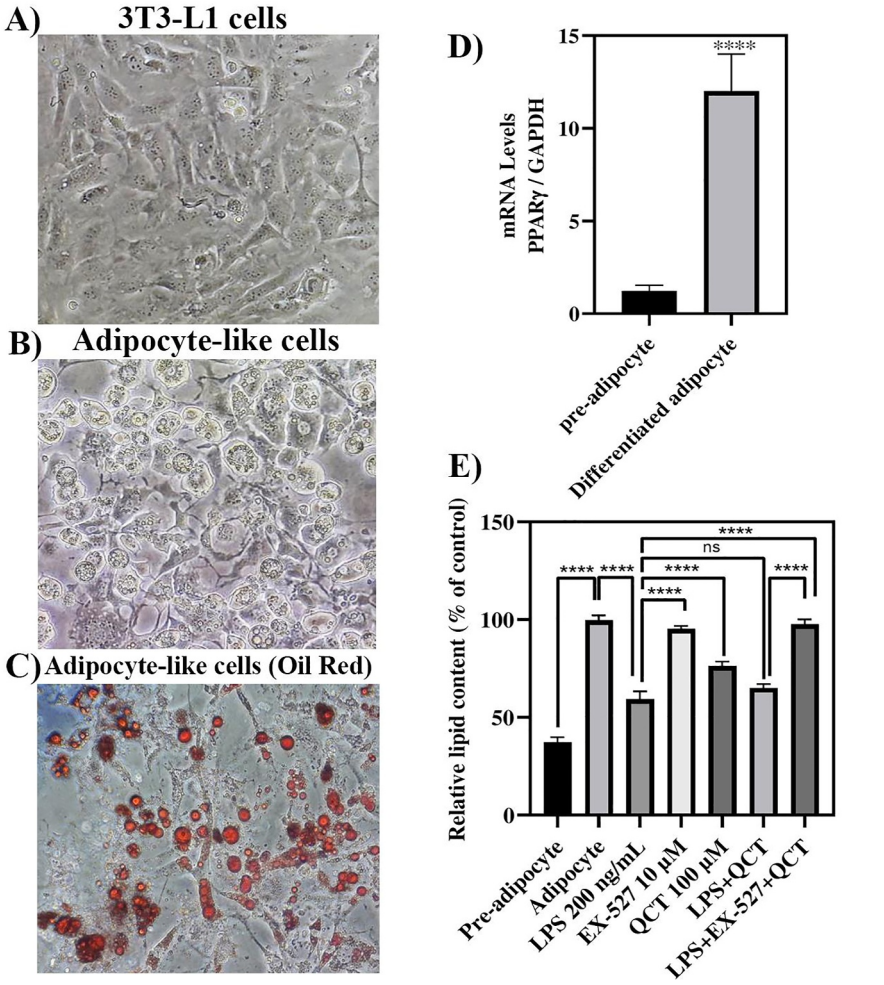

FIGURE 2 Quercetin inhibits adipogenesis in a SIRT-1 dependent pattern. (A), (B), and (C) indicate the differentiation process. Figure (D) illustrates the detected mRNA expression of *peroxisome proliferator-activated receptor gamma* in differentiated adipocytes compared to pre-adipocytes. Figure (E) shows adipogenesis measurements in 3T3-L1 cells treated with LPS (200 ng/mL), EX-527 (10 µM), QCT (100 µM), LPS (200 ng/mL) + QCT (100 µM), LPS (200 ng/mL) + QCT (100 µM) + EX-527 (10 µM). Data are reported as mean ± SD of three independent assays ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). LPS, lipopolysaccharide; QCT, quercetin's; SIRT-1, sirtuin-1.

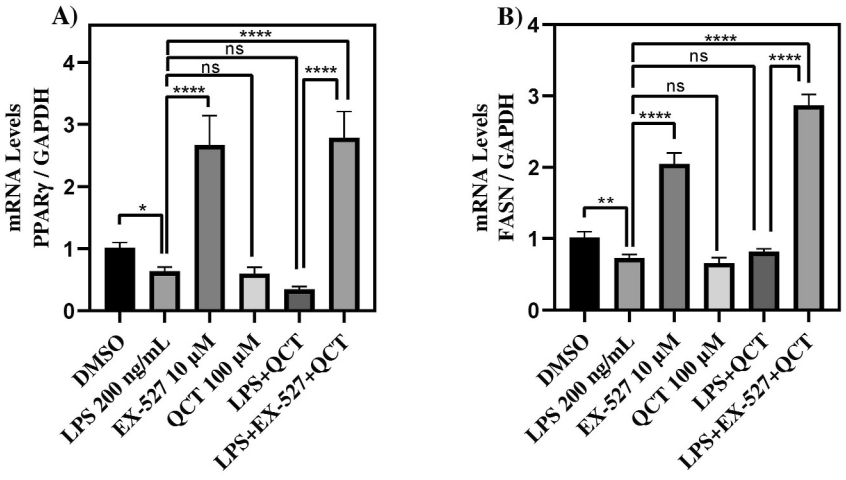

FIGURE 3 Quercetin stimulates adipogenesis-related genes SIRT-1 dependently. 3T3-L1 cells were treated with LPS (200 ng/mL), EX-527 (10 µM), QCT (100 µM), LPS (200 ng/mL) + QCT (100 µM), LPS (200 ng/mL) + QCT (100 µM) + EX-527 (10 µM) for 24 h. The *mRNA* expression of *peroxisome proliferator-activated receptor gamma* (A) and *fatty acid synthase* (B) was determined by RT-PCR. Data are reported as mean ± SD of three independent assays ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). LPS, lipopolysaccharide; QCT, quercetin's; SIRT-1, sirtuin-1.

3.4 | Quercetin stimulates lipolysis in a SIRT-1 dependent pattern

As shown in Figure 4, increased glycerol release was observed after QCT (100 μM) or LPS or LPS + QCT administration compared to untreated adipocytes ($p < 0.001$). Administration of EX-527 did not change the lipolysis state of adipocytes compared to untreated adipocytes, albeit it significantly prevented the lipolysis effects of LPS ($p < 0.001$) or QCT ($p < 0.05$) or LPS + QCT ($p < 0.001$).

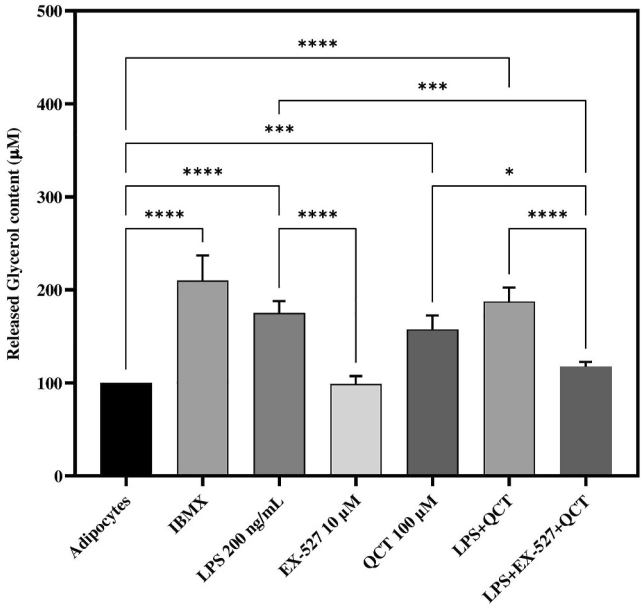

FIGURE 4 *Quercetin induced lipolysis in 3T3-L1 adipocyte SIRT-1 dependently*. Figure 4 shows lipolysis measured by glycerol assay in 3T3-L1 cells treated with LPS (200 ng/mL), EX-527 (10 μM), QCT (100 μM), LPS (200 ng/mL) + QCT (100 μM), LPS (200 ng/mL) + QCT (100 μM) + EX-527 (10 μM). Data are reported as mean ± SD of three independent assays (n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). LPS, lipopolysaccharide; QCT, quercetin's; SIRT-1, sirtuin-1.

3.5 | Quercetin upregulates HSL and ATGL mRNA expression in a SIRT-1 dependent pattern

The mRNA levels of *HSL* and *ATGL* were evaluated by q-RT-PCR to examine the effect of SIRT-1 inhibitor (EX-527) in combination with QCT on the expression of genes involved in lipolysis. Figure 5A shows that LPS treatment considerably (albeit not statistically) reduced the mRNA expression level of *HSL* compared with control. QCT could significantly up-regulate the expression level of *HSL* even after LPS treatment compared with LPS alone ($p < 0.0001$). As shown in Figure 5B, *ATGL* mRNA levels were significantly increased after induction of 3T3-L1 cells with LPS compared with controls ($p < 0.0001$). QCT alone or in combination with LPS significantly increased *ATGL* mRNA expression ($p < 0.0001$ and $p < 0.05$, respectively).

However, EX-527 completely abrogated the effect of QCT in the LPS + EX-527+ QCT group and inhibited lipolysis-related gene expression, including *HSL* (Figure 5A) and *ATGL* (Figure 5B), compared with co-treatment of the LPS and QCT groups, indicating that SIRT-1 activity can be necessary for increasing lipolysis by QCT.

3.6 | Quercetin induces apoptotic related genes expression in a SIRT-1 independent pattern

As shown in Figure 6, the induction of 3T3-L1 cells by LPS markedly increased mRNA expression of apoptotic related genes as

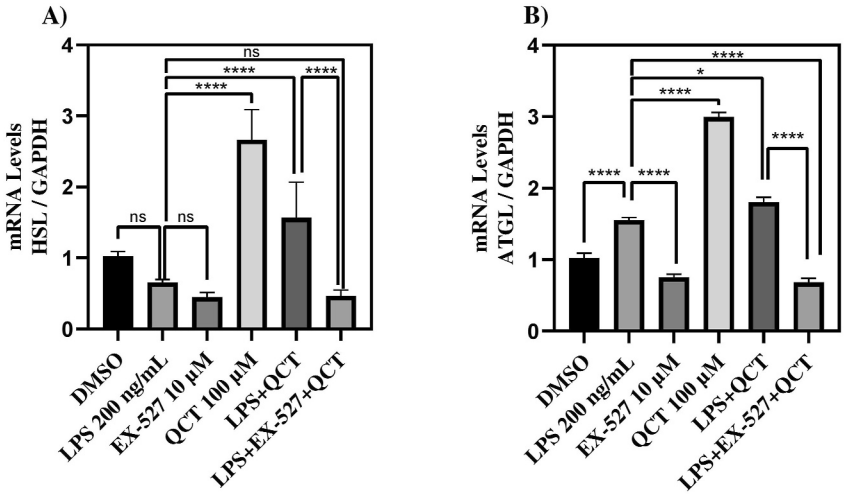

FIGURE 5 Quercetin upregulates HSL and ATGL mRNA expression in a SIRT-1 dependent pattern. 3T3-L1 cells were treated with LPS (200 ng/mL), EX-527 (10 μM), QCT (100 μM), LPS (200 ng/mL) + QCT (100 μM), LPS (200 ng/mL) + QCT (100 μM) + EX-527 (10 μM) for 24 h. The *mRNA* expression of *HSL* (A) and *ATGL* (B) was determined by RT-PCR. Data are reported as mean ± SD of three independent assays ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; LPS, lipopolysaccharide; QCT, quercetin's; SIRT-1, sirtuin-1.

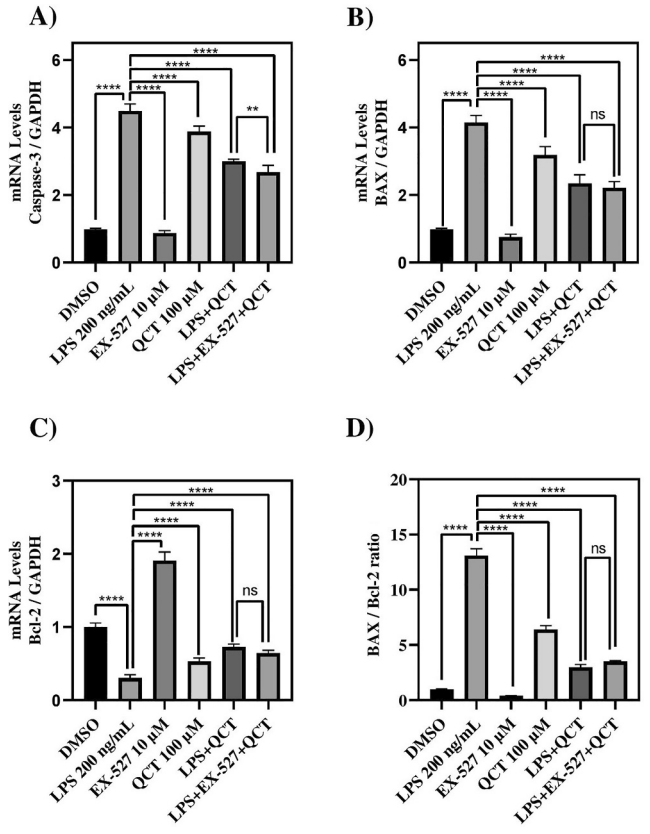

FIGURE 6 Quercetin induces apoptotic-related gene expression in a SIRT-1 independent pattern. 3T3-L1 cells were treated with lipopolysaccharide (LPS) (200 ng/mL), EX-527 (10 µM), quercetin's (QCT) (100 µM), LPS (200 ng/mL) + QCT (100 µM), LPS (200 ng/mL) + QCT (100 µM) + EX-527 (10 µM) for 24 h. The *mRNA* expression of *Caspase-3* (A), *BAX* (B), and *Bcl-2* (C) was determined by RT-PCR. The *BAX/Bcl-2* ratio was also determined (D). Data are reported as mean ± SD of three independent assays ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). BAX, Bcl-2 Associated -X-protein; LPS, lipopolysaccharide; QCT, quercetin's; SIRT-1, sirtuin-1.

demonstrated by prominent increase in Caspase-3 (Figure 6A). and *BAX* (Figure 6B) expression ($p < 0.0001$). Moreover, the expression of *Bcl-2* as an anti-apoptotic factor (Figure 6C) declined significantly following LPS treatment. QCT also remarkably up-regulated mRNA expression of *Caspase-3* (Figure 6A) and *BAX* (Figure 6B) and down-regulation of *Bcl-2* (Figure 6C) compared with LPS alone. However, lower augmentation of *Caspase-3* and *BAX* and lower downregulation of *Bcl-2* expression was detected compared with LPS alone ($p < 0.0001$), which indicates that LPS causes more severe apoptosis activation compared with QCT alone. Co-treatment of LPS and QCT attenuated mRNA expression of *Caspase-3* and *BAX* and up-regulation of *Bcl-2*, which indicates that QCT modulates severe apoptosis activation resulting from LPS treatment. In comparison with LPS it was also observed a significant increase in the expression of *Caspase-3* and *BAX* ($p < 0.0001$) and a remarkable decrease in *Bcl-2* expression after co-treatment with LPS + QCT and LPS + QCT + EX-527 ($p < 0.0001$). These findings confirm that QCT can provoke apoptosis even after SIRT-1 inhibition.

4 | DISCUSSION

The key discovery of the study suggests that QCT's impact on SIRT-1 might be necessary for its dual ability to inhibit adipogenesis and enhance lipolysis while also promoting apoptosis. The research specifically compared QCT's effects on adipocytes' adipogenesis, lipolysis, and apoptosis in 3T3-L1 cells induced by LPS, a common model for studying obesity-related biology.[29] QCT treatment demonstrated a reduction in adipogenesis accompanied by lowering the mRNA expression of FASN and PPARγ, which are essential regulators of lipid metabolism and adipocyte differentiation. Additionally, it increased the mRNA expression of lipolysis-related enzymes such as HSL and ATGL. Notably, all these effects were blocked in the presence of a SIRT-1 inhibitor. QCT may cause apoptosis, as evidenced by the study's findings of a decrease in anti-apoptotic Bcl-2 and an increase in pro-apoptotic markers (BAX and Caspase-3). However, it was found that QCT-induced cell death could occur independently of SIRT-1, suggesting the involvement of additional signaling pathways in 3T3-L1 cells.

Because QCT may prevent adipogenesis and cause cell death in 3T3-L1 cells, it has potential as a natural obesity treatment. Nevertheless, little is known about the precise molecular signaling pathway having these effects.[30,31] Since QCT has been identified as a direct activator of SIRT-1,[32] it is hypothesized that suppressing SIRT-1 activity with EX-527 could be key to unveiling QCT's anti-obesity effects. SIRT-1 has been found to play a crucial role in regulating lipid metabolism by suppressing adipogenesis and preventing triglyceride buildup in cultured adipocytes.[33,34]

Accepted wisdom posits that the two key enzymes, HSL and ATGL, govern lipolysis, and research suggests that SIRT-1, by modulating ATGL, could regulate lipolysis in adipocytes.[35] Numerous studies have demonstrated that LPS induces lipolysis and contributes to insulin resistance.[36,37] While increased lipolysis in obesity diminishes fat accumulation, it may exacerbate insulin sensitivity due to elevated free fatty acids. This study reveals that exposing cells to LPS treatment results in an upregulation of ATGL mRNA expression, corroborating previous findings on LPS-induced lipolysis in 3T3-L1 cells.[38]

However, the data concerning HSL expression when treated with LPS indicated a slight reduction that was not statistically significant compared to the control group. The study findings suggest that mRNA levels may not be a reliable predictor of HSL activation, and future studies should contemplate analyzing HSL at the protein level to directly measure HSL phosphorylation and localization, both of which are regulated at the post-translational level.[39,40]

In contrast to LPS, QCT has been demonstrated to enhance insulin sensitivity, and the study results indicate that it also upregulates the expression of genes (ATGL, HSL) associated with lipolysis. This discrepancy can be elucidated by the hypothesis that free fatty acids resulting from lipolysis may serve as substrates for beta-oxidation, and this process might be stimulated by QCT rather than LPS.[41] Increased lipolysis can potentially lead to elevated levels of free fatty acids in circulation, resulting in issues such as insulin resistance. The

beneficial effects of QCT might arise from concurrent enhancement of both lipolysis and beta-oxidation of fatty acids.[42] This effect is not observed in relation to LPS, indicating that increasing lipolysis alone may not be a suitable approach for obesity treatment.

On the other hand, as per the preceding results, QCT inhibits the expression of pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1 beta (IL-1β), interleukin-18 (IL-18) and tumor necrosis factor alpha (TNF-α),[43] suggesting its interference with the lipolysis-inflammation-insulin resistance loop induced by LPS treatment. The lipolytic effects of QCT were attenuated when EX-527 inhibited SIRT-1. These findings suggest that QCT stimulates lipolysis through a SIRT-1-dependent mechanism.

Additionally, the study revealed that QCT effectively inhibited cell differentiation and adipogenesis. Consistent with previous research, QCT demonstrated the potential to inhibit adipocyte development, enhance lipolysis, and influence crucial molecular factors such as PPARγ, FASN, FOXO1 (Forkhead family of transcription factors), HSL, and ATGL. This effect could be attributed to its ability to activate AMPK (AMP-activated protein kinase) through the SIRT-1-AMPK pathway.[44–46] U R Kuppusamy revealed that QCT stimulates adipocyte lipolysis by synergizing with epinephrine on the beta-adrenergic receptor.[44]

On the other hand, the findings showed that QCT induced apoptosis both in the presence and absence of the EX-527. Specifically, this study investigated the effects of QCT on the mRNA expression levels of key genes associated with apoptosis, including Caspase-3, BAX, and Bcl-2, with or without EX-527 administration.

Consistent with the current study, several pieces of research have confirmed that QCT can induce apoptosis in adipocytes.[47,48] They revealed that QCT-induced apoptosis was associated with BAX and Caspase-3 activation in 3T3-L1 pre-adipocytes. Additionally, it is noted that QCT causes cell death by inhibiting ERK1/2 and c-Jun N-terminal kinase (JNK) phosphorylation and by activating the apoptotic pathway in mature adipocytes.[49]

In another investigation by Chin-Lin Hsu, it was observed that QCT induces a dose- and time-dependent increase in apoptotic cells.[50] These findings highlight that QCT significantly reduces cell proliferation and triggers apoptosis in 3T3-L1 adipocytes. However, the specific involvement of SIRT-1 in the apoptosis process of 3T3-L1 cells treated with QCT remains unexplored. Research suggests that SIRT-1 influences cellular energy metabolism, apoptosis, and autophagy by modulating PPAR, AMPK, and the mechanistic target of rapamycin (mTOR).[51,52]

SIRT1 activation in different cell types can lead to two distinct outcomes. Firstly, it regulates the expression and activity of the FOXO to prevent cell apoptosis.[53] Secondly, it can activate the caspase family, leading to cell death.[54] It is noteworthy that based on the study results, the influence of QCT on the apoptotic pathway seems to be independent of SIRT-1.

Prior studies have proposed that the inflammation induced by LPS might instigate an excessive death of adipose cells through a mechanism known as pyroptosis, distinct from apoptosis. However, QCT, known for its anti-inflammatory properties, has been found to counteract this harmful outcome.

In summary, the potential effects of QCT on adipocyte cells involve impeding differentiation and adipogenesis while promoting lipolysis through increased SIRT-1 expression. This process includes inducing apoptosis even in the presence of SIRT-1 inhibition in 3T3-L1 adipocytes, suggesting the potential involvement of alternative pathways independent of SIRT-1.

While the study sheds light on the potential effects of QCT dependent SIRT-1 in the context of adipogenesis, lipolysis pathways some limitations should be noted. Further research is necessary to determine the precise signaling pathways involved in the interactions between QCT and SIRT-1. Considering that a hypertrophic condition provides a better model of obesity in 3T3-L1 adipocytes, further investigation is warranted. While the results revealed that QCT-induced apoptosis appears to be independent of SIRT-1, more detailed investigations into the apoptotic pathways involved are necessary. In the present study, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify the levels of mRNA expression of apoptosis markers; additional validation of the reported effects might be obtained by using Western blot analysis as well as Annexin-PI assay. Additionally, measuring the protein profile in each signaling pathway offers a more comprehensive overview and can confirm the results. Additional investigations are needed to elucidate the underlying mechanisms and confirm its efficacy in vivo.

5 | CONCLUSION

In conclusion, the findings reveal that QCT diminishes adipogenesis and promotes lipolysis in a SIRT-1-dependent manner. The suppression of adipogenesis by QCT and LPS involves the downregulation of FASN and PPARγ mRNA expression, while the upregulation of HSL and ATGL expression accompanies the stimulation of lipolysis. However, the observation of QCT-induced apoptosis persists even in the presence of SIRT-1 inhibitors, indicating that the impact of QCT on apoptosis operates independently of SIRT-1. These results suggest a crucial role for SIRT-1 in mediating QCT lipolysis induction and anti-adipogenic effects in 3T3-L1 adipocytes.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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