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Gypenosides mediate cholesterol efflux and suppress oxidized LDL induced inflammation in retinal pigment epithelium cells

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Abstract

Age-related macular degeneration (AMD) is a predominant cause of visual deficit in aged population. Abnormal accumulation of cholesterol, including oxidized low-density lipoprotein (oxLDL), underneath the retinal pigment epithelium (RPE) cells contributes to the development of AMD. Gypenosides (Gyp) are glycosides extracted from *Gynostemma pentaphyllum* and have demonstrated protective effects against inflammation and oxidative stress. To determine the therapeutic potential of Gyp for AMD, we investigated its effect on cholesterol trafficking and metabolism and assessed the protective function of Gyp against oxLDL-induced damage in RPE cells. Cholesterol efflux to high-density lipoprotein (HDL) and human serum was significantly increased in RPE cells treated with Gyp when compared to untreated control cells. Expression of cholesterol metabolism (*CYP27A1*, *CYP46A1*) and trafficking (*TSPO*, *ABCA1* and *ABCG1*) genes was also markedly increased in Gyp-treated RPE cells. OxLDL-treated RPE cells had significantly increased cholesterol accumulation and lipid droplet formation. There were marked increases in reactive oxygen species (ROS) generation and proinflammatory cytokines via NF- κ B activation in RPE cells treated with oxLDL, while incubation with Gyp rectified these changes. These findings provide pharmacological evidence that Gyp has the potential to treat patients with early onset AMD by promoting cellular cholesterol removal from RPE cells and inhibiting inflammation and oxidative stress.

Keyword Gypenosides; cholesterol efflux; oxidized LDL; inflammation; retinal pigment epithelium

1. Introduction

Age-related macular degeneration (AMD) mainly affects people aged over 50 years old, leading to visual impairment and even blindness. Due to population ageing, the number of AMD patients is speculated to rise to 196 million by 2020 and to 288 million by 2040 (Wong et al., 2014). Abnormal extracellular focal deposits (referred as drusen) underneath the retinal pigment epithelium (RPE) cells at the macula are a major clinical feature of AMD. Pathological analyses of AMD patients' eyes showed unesterified and esterified cholesterol are enriched in drusen and Bruch's membrane (Pikuleva and Curcio, 2014). Although the process by which drusen forms is not understood, it has been suggested that dysfunction of cholesterol transport might be instrumental in the progression of AMD.

The reverse cholesterol transport (RCT) pathway is involved in the transport of surplus cholesterol from peripheral cells (including RPE cells) to the liver for excretion in bile (Ikonen, 2006). Cholesterol transporters and apolipoproteins that take part in the RCT process are expressed in human and mouse RPE/choroid, and in human primary RPE cells and a RPE cell line (ARPE-19) (Biswas et al., 2017; Storti et al., 2017; Zheng et al., 2012), while active cholesterol efflux has been reported in human RPE cells (Biswas et al., 2017; Ishida et al., 2006; Storti et al., 2017). Recently we found that the intracellular cholesterol trafficking protein, 18kDa translocator protein (TSPO), regulated cholesterol efflux from RPE and that deletion of TSPO caused intracellular cholesterol accumulation. Treatment with TSPO ligands (FGIN-1-27, Etifoxine and XBD173) upregulated expression of cholesterol transporter and metabolism genes (Biswas et al., 2017). Storti et al (2017) demonstrated that cholesterol efflux in RPE cells was predominantly mediated by ATP-binding cassette family A member 1 (ABCA1). By contrast, A2E, a major RPE lipofuscin fluorophore generated from the visual cycle, impaired cholesterol efflux and caused cholesterol and cholesteryl ester accumulation in RPE cells (Lakkaraju et al., 2007). Targeted knockout *ABCA1* and *ABCG1* in murine RPE cells led to intracellular lipid accumulation, abnormal RPE and retinal function, and increased inflammation and degeneration of RPE and photoreceptor cells. Single knockout of *ABCA1* or *ABCG1* in the murine RPE demonstrated that mice with only *ABCA1* knocked out exhibited similar pathology to that of double knockout mice (Storti et al., 2019). Population studies have also demonstrated that RCT

related genes (lipase C (*LIPC*), Cholesteryl ester transfer protein (*CETP*), Apolipoprotein E (*APOE*) and *ABCA1*) are linked to the development of this disease (Burgess and Smith, 2017; Chen et al., 2010; DeAngelis et al., 2017; Klaver et al., 1998; Neale et al., 2010; Yuan et al., 2018). This suggests that the RCT process may have a critical role to play in the pathogenesis of AMD. Strategies that promote the removal of cholesterol from RPE cells may therefore offer a means to treat early AMD patients.

Gynostemma pentaphyllum (Thunb) Makino is a long-established herbal medicine used in Asian countries for over 500 years. Li She-zhen (1578 A.D), a famous herbalist in China, first described the use of *G. pentaphyllum* for the treating a range of diseases including trauma, haematuria, oedema of the pharynx and the neck, pain, heat and tumours (Razmovski-Naumovski et al., 2005). The predominant bioactive component of *G. pentaphyllum* is Gypenosides (Gyp, Fig. 1A), which have demonstrated anti-oxidative, anti-inflammatory, antidiabetic and anti-tumorigenic activities. Gyp has also shown to possess anti-hyperlipidaemic activity by lowering serum cholesterol and triglyceride (Razmovski-Naumovski et al., 2005). We postulate that Gyp may play a similarly protective role in RPE cells. In the present study, we report that Gyp promote cholesterol efflux, reduce uptake and accumulation of oxidized LDL (oxLDL), and suppress oxLDL-induced inflammation in RPE cells.

2. Materials and methods

2.1 Cell culture

Human RPE cell line (ARPE-19 cells, ATCC[®] CRL-2302[™]) was cultured in DMEM-F12 medium (Lonza, UK) supplemented with streptomycin (100 µg/mL)/penicillin (100 IU/mL), sodium bicarbonate (1.2 g/L) and FBS (fetal bovine serum, 10%, v/v).

2.2 Cholesterol efflux and lipid analysis

Gyp (purity 98%) was bought from Xi'an Jiatian Biotech Co. Ltd, China. A Gyp concentration of 5 µg/ml was chosen based on our recent publication (Alhasani et al., 2018). Cellular cholesterol efflux was measured as previously described (Biswas et al., 2017). Briefly, RPE cells were cultured on 12-well plates. The following day, the cells were incubated with [³H]cholesterol (0.5µCi/mL) for 24h. The cells were then equilibrated with 0.1% (w/v) bovine serum albumin (BSA) for 1h and washed twice with serum free media. The cellular cholesterol efflux was initiated using different acceptors:

apolipoprotein A-I (ApoA-I, 10µg/mL), high-density lipoprotein (HDL, 10µg/mL) or human Serum (HS, 1%, v/v, Sigma H3667) with/without Gyp treatment for 24h. The basal labelled efflux was also measured when no acceptor was added. After the incubation, the media were collected and centrifuged at 1200 rpm for 10 min at 4°C to remove any debris. 200µl of media were collected into scintillation vials to which were added 2ml of scintillation fluid. The cells were washed with PBS and the cellular lipids were extracted using Hexane: Isopropanol (3:2). Similarly, extracted cellular lipids were collected into scintillation vials containing scintillation fluid. [³H]cholesterol was counted by the scintillation counter as counts per min (CPM), disintegrations per min (DPM) and Triple to Double Coincidence Ratio (TDCR) units. Cholesterol efflux was then calculated as an expression of the percentage of cholesterol efflux to each of the acceptors as follows: % efflux = (DPM Media/DPM Media + DPM Cells) X 100.

Incorporation of [¹⁴C] acetic acid (1 µCi/ml) into cholesterol, cholesteryl ester, fatty acid, phospholipid and triacylglycerol pools was analysed after 24h treatment with Gyp (5µg/ml) following our previous description (Biswas et al., 2017). The mass of total cellular cholesterol was measured using a cholesterol assay kit (Thermo Fisher Scientific UK) following the manufacturer's protocol. Lipid droplet formation was examined using Oil Red O (ORO) staining, as previously described (Xu et al., 2010).

To examine the effect of Gyp on lipid uptake and accumulation in ARPE-19 cells, we treated ARPE-19 cells with Gyp and oxLDL (or Dil-oxLDL) for 4h to assess lipid uptake or for 24h to assess intracellular lipid accumulation and analysed subsequent biochemical changes. Dil-oxLDL uptake and accumulation in the presence or absence of Gyp were assessed using fluorescence-activated cell sorting (FACS) and confocal microscopy, as previously described (Biswas et al., 2017).

2.3 Detection of intracellular reactive oxygen species (ROS)

Generation of intracellular ROS was quantified using fluorescence dye, DCFHDA (carboxy-2',7'-dichloro-dihydro- fluorescein diacetate, Sigma, UK). ARPE-19 cells were exposed to oxLDL (200 µg/ml) in the presence or absence of Gyp (5 µg/ml) for 4h or 24h. The cells were incubated with 10

µM DCFHDA for 30 min at 37°C. The intracellular fluorescence intensity was quantified using a microplate reader (Excitation, 485nm and Emission, 520nm).

2.6 Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded onto 12-well plates in complete media until confluence, then treated with oxLDL (200µg/ml) in the presence or absence of Gyp (5µg/ml) in serum free media for 4h or 24h. Media were collected and cell debris were removed by centrifugation, and IL-1β, IL6, IL8 and TNF-α were quantified via ELISA (Peprotech, UK) according to the manufacturer's protocol.

2.7 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA from treated and control cells was extracted using TRIZOL (Sigma, UK). cDNA was synthesised by using a cDNA synthesis kit (Thermo Fisher Scientific, UK). Expression of target genes was measured by qRT-PCR with SYBR Green Real-PCR Master Mixes (Thermo Fisher Scientific, UK). Sequences of primers used for this study are listed in Table 1.

2.8 Western blotting

Treated and control cells were washed with PBS, then lysed with RIPA buffer (Sigma, UK). Equal amounts of protein were loaded in SDS-PAGE (% of gel described in Figure legends). After electrophoresis proteins were transferred into a nitrocellulose membrane and blocked with blocking buffer, then incubated with primary antibody and second antibody respectively. Antibodies used in current study were listed in Table 2. Signals were detected using Li-cor Odyssey FC and analysed by Image Studio software.

2.9 Statistical Analysis

All experiments were repeated at least three times (described in the figure legends). Data were analysed using Prism software with the Wilcoxon Rank Sum test or Kruskal-Wallis test (followed by Dunn's multiple comparisons test). Data were displayed as mean ± standard deviation (SD).

3. Results

3.1 Gyp enhanced cholesterol efflux and reduced lipid content in RPE cells

To determine whether Gyp is involved in RPE cholesterol efflux, we analysed [³H]cholesterol efflux to ApoA-I, HDL and HS in ARPE-19 cells treated with Gyp (5 µg/ml) compared to vehicle control (0.1% DMSO, v/v). The efflux to HDL and HS was markedly increased by, respectively, 33% and

20% in cells treated with Gyp compared to untreated control cells (Fig. 1B). We also investigated if lipogenesis in ARPE-19 cells was influenced by Gyp. Treatment with Gyp (5 µg/ml) significantly reduced lipogenesis of phospholipids by 53%, triglycerides by 54% and free cholesterol by 28%; however, fatty acid and cholesterol ester were not changed in Gyp-treated cells (Fig. 1C). Further exposure to Gyp led to significantly reduced total cholesterol mass compared to control RPE cells (Fig. 1D).

3.2 Gyp upregulated expression of cholesterol trafficking and metabolism genes

Since Gyp exposure promoted cholesterol trafficking from RPE cells, we measured expression of cholesterol homeostasis genes in Gyp-treated and untreated RPE cells. Gyp significantly upregulated expression of LXRα (liver X receptor alpha, encoded by *NR1H3*), cholesterol trafficking and transporter genes (*TSPO*, *ABCA1* and *ABCG1*) and cholesterol metabolism genes (*CYP27A1* and *CYP46A1*) in ARPE-19 cells (Fig. 2A). We also measured LXRα, TSPO, ABCA1, ABCG1, CYP27A1 and CYP46A1 protein levels in Gyp-treated RPE cells. Gyp treatment led to significantly higher levels of LXRα, TSPO, ABCA1, ABCG1, CYP46A1 proteins compared to control cells, but the trend towards an increase in CYP27A1 protein level was not significant (Fig. 2B, C).

Previous studies demonstrated that geranylgeranyl diphosphate (GGPP), an LXRα antagonist, blocks LXRα activation and reduces the expression of ABC transporters (Aye et al., 2011; Gan et al., 2001; Li et al., 2014). Initially we assessed the toxicity of GGPP on ARPE-19 cells by treating cells with GGPP at different concentrations and found GGPP at a higher concentration (500 µM) exhibited toxic effects with a significant decrease in cell viability (Fig. 3A). Consequently, 200µM GGPP was used for further experiments. In order to determine if the action of Gyp on cholesterol homeostasis is dependent on LXRα activation, ARPE-19 cells were incubated with Gyp only or with Gyp and GGPP (200µM) for 24h. Again, Gyp-treated cells had a significant increase in cholesterol efflux to HDL and HS; GGPP treatment resulted in a notable decrease in cholesterol efflux to HDL and to HS, when compared to cells exposed to Gyp alone; the levels of cholesterol efflux to HDL and to HS were similar to that of control cells (Fig. 3B). Similarly Gyp-treated cells had a significant increase in ABCA1 and ABCG1 expression when compared to untreated cells; we also found a marked reduction

in ABCA1 and ABCG1 expression in APRE-19 cells co-treated with GGPP and Gyp when compared to cells treated with Gyp alone or untreated cells (Fig. 3C-E).

3.3 Gyp reduced lipid accumulation in RPE cells

Since Gyp treatment caused a rise in cholesterol efflux and upregulated expression of cholesterol genes, we therefore investigated whether Gyp can reduce cholesterol accumulation in oxLDL-fed RPE cells. We found that ARPE-19 cells that had been incubated with Gyp and Dil-oxLDL for 24h had notably decreased intracellular oxLDL when compared to cells incubated with Dil-oxLDL only (Fig. 4 B, D). We also examined whether increased intracellular accumulation of Dil-oxLDL is related to oxLDL uptake. ARPE-19 cells were incubated with Dil-oxLDL only or with Dil-oxLDL and Gyp for 4 hrs. The uptake of Dil-oxLDL, analysed by Confocal microscopy (Fig. 4A) and FACS (Fig. 4C), was significantly decreased compared to those cells that had not undergone Gyp treatment.

Given that Gyp exposure caused a decrease in oxLDL uptake and accumulation, we examined whether Gyp treatment affected lipid droplet formation. The RPE cells were exposed to oxLDL (200 $\mu\text{g}/\text{ml}$), with or without Gyp (5 $\mu\text{g}/\text{ml}$) for 4h and 24h, and ORO staining was performed to detect lipid droplets. The lipid droplet staining was noticeably decreased in Gyp-treated cells when compared to control cells with the treatment of 4 and 24 hrs (Fig. 5A and B). The amounts of ORO solubilised from cells treated with Gyp for 4h and 24h were significantly decreased by 20% and 14% respectively, when compared to control cells (Fig. 5C and 5D).

Early data showed that rodent RPE cells take up LDL and oxLDL primarily via LDLR (Tserentsoodol et al., 2006). We further examined whether Gyp regulates LDLR expression in RPE cells. A high dose of oxLDL (200 $\mu\text{g}/\text{ml}$) was applied to examine effects of Gyp on LDLR expression. In both 4 hrs and 24 hrs, LDLR expression was significantly increased in cells treated with oxLDL when compared to untreated cells; its expression was noticeably reduced by Gyp and oxLDL co-treatment compared to cells treated with oxLDL alone. However there was no significant change in LDLR expression between Gyp-oxLDL co-treated cells and untreated cells (Fig. 6A and B).

3.4 Gyp suppressed oxLDL-induced inflammation

Recent studies reported have demonstrated that oxLDL induces the production of ROS in endothelial cells and activates the NF- κ B pathway (Ji et al., 2015; Liang et al., 2017; Zhao et al., 2016). We also

found that oxLDL significantly increased ROS production in ARPE-19 cells, while the Gyp co-treatment caused a significant decrease in ROS level compared with cells exposed to oxLDL alone; the level of ROS in Gyp co-treated cells is similar to that of untreated control cells (Fig. 7). OxLDL-induced activation of the NF- κ B pathway results in increased secretion of inflammatory cytokines (Miller and Shyy, 2017). We investigated the NF- κ B pathway activation by detecting the level of NF- κ B p65 using Western blotting and immunostaining. We found total cellular NF- κ B p65 protein level was significantly increased in oxLDL-treated cells when compared to the controls (Fig. 8A). Immunostaining with anti-p65 antibody found oxLDL-treated cells had markedly increased nuclear p65 signals when compared untreated cells (Fig. 8B-C). Gyp co-exposure led to a markedly decreased NF- κ B p65 level (Fig. 8A-C). The levels of whole cellular and nuclear-localized NF- κ B p65 in Gyp co-treated cells were similar to that of untreated control cells. We also found that oxLDL significantly increased the levels of IL-1 β , IL-6, IL-8 and TNF α followed 4h and 24h treatment when compared to the controls (Fig. 8D-G). Gyp ameliorated the oxLDL-induced inflammatory response by causing marked decreases in the levels of these inflammatory cytokines, which were at similar levels to that of untreated control cells (Fig. 8D-G).

4. Discussion

Our current study examined the role of Gyp on cholesterol trafficking and metabolism and on oxLDL induced inflammation in RPE cells. We found Gyp enhanced cholesterol efflux, upregulated expression of cholesterol trafficking and metabolism genes, and suppressed oxLDL-induced oxidative stress and inflammation. Previous studies demonstrated treatment with Gyp in animal models reduces lipid accumulation. When high-fat-fed rats were treated with Gyp, total cholesterol, triglycerides and LDL-C (low-density lipoprotein cholesterol) in serum were significantly decreased when compared with untreated high-fat-fed rats (Gou et al., 2016; He et al., 2015; Yang et al., 2013); the treated rats also had markedly decreased levels of total cholesterol and triglycerides (Gou et al., 2016; Li et al., 2017; Yang et al., 2013), and notably reduced histopathological changes in the liver (He et al., 2015). Gyp also showed similarly protective effects in a fatty liver disease rat model (Qin et al., 2012). The underlying mechanisms for Gyp's function in lowering lipid are not fully understood. Our current study investigated the function of Gyp in lowering lipid deposition in the RPE cells. Gyp exposure

enhanced cholesterol efflux, reduced intracellular lipid accumulation and inhibited oxLDL-induced inflammation. These effects appear to be associated with upregulation of LXR α and downregulation of the NF- κ B signal pathway.

Liver X receptors (LXR) include LXR α and LXR β , which play an important part in cholesterol metabolism (Kidani et al., 2012; Zelcer et al., 2006). LXR α has a high level of expression in liver, intestine and white adipose and a low level of expression in lung, kidney, spleen and brown adipose tissue. All examined tissues express LXR β (Repa and Mangelsdorf, 2000). Previous reports showed retina and RPE had LXR α and LXR β expression (Biswas et al., 2017; Zheng et al., 2012). Both LXR α and LXR β can be activated by the same ligands, including endogenous oxysterols (22(R)-, 24(S)-, 27-hydroxycholesterol and 24(S), 25-epoxycholesterol) and synthetic agonists (T0901317, GW3965 and acetylpodocarpic dimer) (Huang et al., 2005; Kidani et al., 2012). Gyp has a similar structure to the oxysterols (Huang et al., 2005) and previous data showed GYP activated LXR α and LXR β in HEK293 cells, and upregulated ABCA1 and apoE expression in THP-1 macrophages (Huang et al., 2005). Our present study also demonstrated that Gyp treatment resulted in LXR α activation and upregulation of cholesterol trafficking genes (*ABCA1* and *ABCG1*) and cholesterol metabolism genes (*CYP27A1* and *CYP46A1*) (Fig. 2), which led to increased cholesterol efflux and decreased total cholesterol, phospholipid, triglycerides and free cholesterol (Fig. 1). Treatment with GGPP, an LXR α antagonist, reversed Gyp-induced changes in cholesterol efflux and expression of cholesterol trafficking genes (Fig. 3), also suggesting activation of LXRs by Gyp.

Gyp treatment also upregulated expression of LXR α and TSPO (Fig. 2). The latter is an outer mitochondrial protein that functions in mitochondrial cholesterol trafficking (Graham, 2015). TSPO can bind cholesterol at nanomolar affinity and functions cholesterol trafficking in mitochondrial, where cholesterol is metabolized by CYP27A1 to produce oxysterols (5-cholestanoic acid and 27-hydroxycholesterol) in macrophage and non-steroidogenic cells (Graham, 2015). Overexpression of TSPO in THP-1 macrophages triggered expression of LXR α , ABCA1, ABCG4 and APOE, and promoted cholesterol efflux (Taylor et al., 2014). TSPO-specific ligands also upregulated expression of cholesterol trafficking and metabolism genes in ARPE-19 cells (Biswas et al., 2017). The data suggest that upregulation of TSPO by Gyp may lead to increased expression of LXR α , cholesterol trafficking

genes and cholesterol metabolism genes, and subsequently to promote cholesterol efflux. However, the specific mechanism by which Gyp upregulate TSPO expression needs further investigation.

OxLDL can induce ROS production, switch on the NF- κ B signal pathway, and upregulate the expression of inflammatory cytokines (Rodríguez et al., 2010). Early data showed that oxLDL was localized in drusen and that internalization of oxLDL in RPE cells caused dysfunction of phagocytosis and led to apoptosis (Hoppe et al., 2001, 2004; Gordiyenko et al., 2004; Yamada et al., 2008). Recent data demonstrated that oxLDL promoted ROS generation and caused increased secretion of inflammatory cytokines in RPE cells (Biswas et al., 2017; Mao et al., 2017). Our current study showed that oxLDL treatment resulted in increased ROS production (Fig. 7), NF- κ B activation and high levels of secreted TNF- α , IL-1 β , IL-6 and IL-8 in RPE cells (Fig. 8). Gyp co-treatment reversed oxLDL-induced effects by reducing ROS production, inhibiting NF- κ B activation and decreasing inflammatory cytokine levels. Previous reports have shown that Gyp reduced IL-1 β or LPS-initiated NF- κ B activation in cell lines (Aktan et al., 2003; Wan and Zhao, 2017) and suppressed NF- κ B activation in an ischemia-reperfusion injury rodent model (Yu et al., 2016). Exposure to Gyp has also been reported to cause reduced secretion of inflammatory cytokines in β amyloid-exposed N9 microglia cells, in H₂O₂-treated ARPE-19 cells and in an ischemia-reperfusion induced rodent model (Alhasani et al., 2018; Cai et al., 2016; Ye et al., 2016). Our previous data showed that Gyp protected RPE cells against oxidative damage via the NRF2 pathway. Gyp-treated RPE cells had higher level of NRF2, which upregulated expression of antioxidant genes and downregulated expression of inflammatory cytokine genes (Alhasani et al., 2018). The current and previous findings suggest that Gyp could be therapeutically useful in the treatment of AMD, which is linked to both inflammation and oxidative stress (Datta et al., 2017).

In summary, we demonstrate *in vitro* evidence of protection by Gyp in RPE cells and suggest that Gyp offers therapeutic potential for prevention of AMD initiation and progression.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

LB performed the experiments. ZZ provided reagents. LB, AG and XS analysed the data and wrote the manuscript.

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474 **Table 1** The primers used for qRT-PCR

Genes	Forward primers 5'-3'	Reverse primers 5'-3'
<i>NR1H3</i>	CCCCATGACCGACTGATGTT	CGGAGGCTCACCAGTTTCATT
<i>ABCA1</i>	TGTCCAGTCCAGTAATGGTTCTGT	AAGCAGAGATATGGTCCGGAT
<i>ABCG1</i>	GAGGACGCGCTAGTGTTCTT	TGTGACATTGGCCTTTGTGTT
<i>CYP27A1</i>	GGCAAGTACCCAGTACGG	AGCAAATAGCTTCCAAGG
<i>CYP46A1</i>	GTACCTGTCCCAGGTCCTCA	CCAGGGAGAAGGGGAAGTAG
β - <i>ACTIN</i>	TCCACGAAACTACCTTCACTC	GTCATACTCCTGCTTGCTGAT

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476 **Table 2** Antibodies used for Western blotting

Antibodies	Species	Dilution	Manufacturer	Cat No.
LXR α	Rabbit	1:1000	Abcam	ab176323
ABCA1	Rabbit	1:250	Abcam	ab7360
ABCG1	Rabbit	1:1000	Abcam	ab52617
CYP27A1	Rabbit	1:1000	Abcam	ab64889
CYP46A1	Rabbit	1:1000	Novus	NBP2-16066
TSPO	Rabbit	1:2000	Abcam	ab109497
LDLR	Rabbit	1:500	Novus	NBP1-0670955
β -ACTIN	Mouse	1:2500	Novus	NB600-501
GAPDH	Mouse	1:2000	Santa Cruz	Sc-47724
2° Anti-mouse	Donkey	1:10000	LI-COR	925-68070
2° Anti-rabbit	Donkey	1:10000	LI-COR	926-32213

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Figures and legend

Figure 1 The effect of Gyp (5μg/ml) on cholesterol efflux and lipid profile from ARPE19 cells. (A) General structure for Gypenosides. R1 and R2: glucose, rhamnose; R3: glucose, xylose. (B) The efflux (24 h) of [³H]cholesterol to apoA-1 (10μg/ml), HDL (20μg/ml) or human serum (1%, v/v) in gypenosides (Gyp) treated and untreated ARPE-19 cells. (C) Effects of Gyp on [¹⁴C] acetate (1μCi/ml) incorporated into phospholipid, triacylglycerol, cholesteryl ester, free cholesterol and fatty acid pools expressed as percentages of the control. (D) The effect of Gyp on total cholesterol mass in treated and untreated ARPE-19 cells. All data from four independent experiments were presented as mean ±SD. NS: no significance; **p* < 0.05. The significance among means ±SD was determined using the Wilcoxon Rank Sum test (B-D).

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497 **Figure 2** Gyp (5µg/ml) treatment upregulated expression of cholesterol trafficking and metabolism
498 genes. **(A)** The expression of cholesterol transporters and metabolism genes in Gyp treated for 24
499 hours and control APRE-19 cells was measured by qRT-PCR and normalised to β-actin (n=4). **(B)**
500 Protein levels of cholesterol transporter and metabolism genes were examined by Western blotting
501 (8% gel for ABCA1 protein and 10% gel for other proteins) (n=4). **(C)** Quantification of the band
502 intensity of proteins presented in (C). Data presented in A and C were analysed by the Wilcoxon Rank
503 Sum test. NS: no significance. * $p < 0.05$.

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513 **Figure 3** The effect on LXRα antagonist, geranylgeranyl diphosphate (GGPP), on cholesterol
514 efflux and expression of cholesterol transporter gene was observed. (A) ARPE-19 cells were
515 treated with GGPP at different concentration for 24 hours, cell viability was measured using
516 an MTT assay; the viabilities were expressed as percentage of the control according to our
517 previous description (Alhasani et al., 2018). (B) GGPP (200μM) co-treatment caused
518 significantly decreased efflux (24h) of [³H] cholesterol to HDL and human serum (HS)
519 compared to cells treated with Gyp only. (C) The expression of ABCA1 and ABCG1 was
520 significantly decreased in GGPP co-treated cells compared to cells treated with Gyp alone.

521 (D) ABCA1, ABCG1 and β -actin proteins were detected by Western blotting. (E)
522 Quantification of ABCA1 and ABCG1 and normalization to β -actin. Experiments were
523 repeated four times, data were presented as mean $\pm$ SD and analysed with the Wilcoxon
524 Rank Sum test (A) or the Kruskal-Wallis test followed by Dunn's multiple comparison (B, C
525 and E). NS: no significance. * $p < 0.05$ and ** $p < 0.01$.

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Figure 4 Gyp ($5\mu\text{g/ml}$) treatment reduced the lipid accumulation in oxLDL-loaded RPE cells. (A, B) ARPE-19 cells were incubated with Gyp and DiI-oxLDL ($20\mu\text{g/ml}$) for 4 hrs or 24 hrs. The images from confocal microscope demonstrated reduced intake (4 hrs) and accumulation (24 hrs) of oxLDL in Gyp-treated cells compared to control cells. The fluorescence intensity was measured using Image J software. The fluorescence intensities were significantly reduced by 16% at 4hrs and by 20% at 24hrs respectively, in Gyp-treated cells compared to control cells. The data were presented as mean $\pm$ SD

and analysed by the Wilcoxon Rank Sum test ($n=4$). $*p < 0.05$. (C, D) FACS data also showed significant declined the fluorescent signal cells in Gyp-treated cells compared to control cells at both 4hrs and 24hrs with Kolmogoroff statistics ($p < 0.0001$).

Figure 5 Effects of Gyp (5 $\mu\text{g/ml}$) on lipid droplet formation on representative photomicrographs of oxLDL-laden RPE cells by optimized ORO staining (described in Materials and Methods). RPE cells were incubated with oxLDL (200 $\mu\text{g/ml}$) with the presence of Gyp (5 $\mu\text{g/ml}$) and with vehicle control for 4h and 24h then stained with ORO. The images were captured by EVOS cell imaging systems and presented for 4hrs (A) and 24hrs (B). The lipid droplets were dissolved in isopropanol and measured at the absorbance of 520nm. The absorbance was normalised by cell protein that was extracted using 0.5M NaOH. Gyp treatment resulted in markedly decreased lipid droplets by 19 % at 4hrs (C) and by 14 % at 24hrs (D) respectively. Experiments were repeated four times. The data were presented as mean $\pm$ SD and analysed with the Kruskal-Wallace test followed by Dunn's multiple comparison test (C, D). * $p < 0.05$ and ** $p < 0.01$.

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567 **Figure 6** Gyp (5 μ g/ml) suppressed oxLDL-induced LDLR expression. The expression of LDLR at
 568 4hrs (A) and 24hrs (B) treatment was examined by Western blotting. Quantification of LDLR band
 569 intensities at 4h and 24h treatment was normalized to β -actin. The data were presented as mean $\pm$ SD
 570 and analysed with Kruskal-Wallis test followed by Dunn's multiple comparison test (n=4). * p <0.05
 571 and ** p <0.01.

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Figure 7 Gyp reduced oxLDL induced cellular reactive oxygen species (ROS). When the RPE cells were incubated with oxLDL (200 μ g/ml), intracellular ROS was increased significantly in oxLDL-treated cells compared to control cells by 34% at 4hrs (A) and by 61% at 24hrs (n=5) (B). The data were presented as mean $\pm$ SD and analysed with Kruskal-Wallis test followed by Dunn's multiple comparison test. * p <0.05 and ** p <0.01.

Figure 8 Effects of Gyp on inflammation. (A) ARPE-19 cells were untreated or treated with oxLDL or oxLDL+Gyp for 24 hours, cell lysates were subjected to Western blotting. Total cellular NF-κB p65 protein was increased in oxLDL treated cells, Gyp co-treatment reversed the effect. Upper panel: Western blotting data; lower panel: quantification of band intensity (n=4). (B) Treated and control cells were subjected to immunostaining with primary antibody (rabbit anti-NF-κB p65 antibody) and

secondary antibody (Alexa Fluor 594 antibody) (n=4). (C) Quantification of nuclear-localized NF- κ B p65 red fluorescence signals. Fluorescence signals from 100 cells of treated and control samples were quantified using Image J software. Broken circle shows the edges of nuclei. (D-G) Gyp treatment resulted in reduced levels of inflammatory cytokines, IL-1 β , IL-6, IL-8 and TNF α (n=5). All data were presented as mean $\pm$ SD and analysed with Kruskal-Wallace test followed by Dunn's multiple comparison test. * $p < 0.05$.