Resveratrol Restores LINE-1 Methylation Levels by Modulating SIRT1 and DNMTs Functions in Cellular Models of Age-Related Macular Degeneration

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Abstract: The role of epigenetic alterations in the pathogenesis of age-related macular degeneration (AMD) has been pending so far. Our study investigated the effect of oxidative stress and inflammation on DNA methyltransferases (DNMTs) and Sirtuin 1 (SIRT1) functions, as well as on long interspersed nuclear element-1 (LINE-1) methylation, in human retinal pigment epithelial (ARPE-19) cells. Therefore, we evaluated whether treatment with resveratrol may restore changes in LINE-1 methylation by modulating DNMTs and SIRT1 functions. Cells were treated with 25 mU/ml glucose oxidase (GOx) or 10 µg/ml lipopolysaccharide (LPS) to mimic oxidative or inflammatory conditions, respectively. Oxidative stress decreased DNMT1, DNMT3a, DNMT3b and SIRT1 expression (p-values <0.05), as well as total DNMTs (-28.5%; p<0.0001) and SIRT1 (-29.0%; p<0.0001) activities. Similarly, inflammatory condition decreased DNMT1 and SIRT1 expression (p-values<0.05), as well as total DNMTs (-14.9%; p=0.007) and SIRT1 (-20.1%; p<0.002) activities. Interestingly, GOx- and LPS-treated cells exhibited lower LINE-1 methylation compared to controls (p-values<0.0001). We also demonstrated that treatment with 10 µM resveratrol for 24 hours counteracted the detrimental effect on LINE-1 methylation via increasing DNMTs and SIRT1 functions in cells upon oxidative and inflammatory conditions. However, further studies should explore the perspectives of resveratrol as a suitable strategy for the prevention and/or treatment of AMD.

Keywords: retinal degeneration; DNA methylation; epigenetics; oxidative stress; inflammation

1. Introduction

Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries, with a prevalence that ranges from 2% to 20% among elderly people [1]. Overall, the pathological process of AMD leads to the progressive destruction of the neurosensory macular area, involving retinal pigment epithelium (RPE), Bruch's membrane and choroid [2]. While the early stages of AMD are characterized by the aberrant pigmentation of the RPE and the accumulation of extracellular deposits of lipid, cellular debris, and proteins (i.e. “drusen”), the advanced stages may manifest as non-exudative or exudative AMD: the first is characterized by the geographic atrophy of RPE and thinning of the retina; the second is characterized by the development of choroidal neovascularization (CNV) which negatively affects central vision [3,4]. AMD is one of the most investigated multifactorial diseases since several socio-demographic (age and race) [5], environmental (cigarette smoking, light exposure and nutrient intake) [6-10] and genetic risk factors...
[11-14] can act together leading to a chronic condition of inflammation and oxidative stress [15]. Moreover, a typical gene–environment interaction has been also proposed [16], with retinal cells showing altered gene expression in response to exogenous and endogenous exposures [17]. Given this scenario, epigenetic mechanisms, especially DNA methylation and histone modifications, might modulate the interaction between genetic factors and environmental exposures [18], affecting both gene expression and genome stability [19,20]. However, the significance of epigenetic alterations in the pathogenesis of AMD has been pending so far.

The methylation process is carried out by DNA methyltransferases (DNMTs), out of which only DNMT1, DNMT3A and DNMT3B are catalytically active [21]. In mammals, the methylation process almost occurs at short DNA sequences (i.e. CpG islands) which typically contain around 5-10 CpGs per 100 bp. Up to 80% of CpG islands is localized in non-coding regions scattered throughout the genome (e.g. satellite repeat, short interspersed nuclear element, and long interspersed nuclear element-1 - LINE-1) that mainly contribute to the global methylation status [21]. LINE-1 sequences, accounting for ~18% of human genome, are widely used as a surrogate marker of global methylation in aging and age-related disease [22-25].

Sirtuin 1 (SIRT1), one of the seven mammalian homologs (SIRT1–SIRT7) of yeast silent information regulator 2, is a NAD+-dependent histone deacetylase with multiple roles in aging, apoptosis, DNA repair, inflammation, and oxidative stress [26]. Although DNA methylation and histone deacetylation are distinct biochemical processes that control gene expression, SIRT1 regulates the activities of DNMT1, the enzyme responsible for maintenance of DNA methylation [27].

Resveratrol (2,3,4′-trihydroxystilbene), a flavonoid associated with the cardiovascular benefits of red grapes and wine, has been shown to significantly increase SIRT1 activity through allosteric interaction, increasing SIRT1 affinity for both NAD+ and the acetylated substrate [28,29]. More recently, due to its antioxidant, anti-inflammatory, and anti-angiogenic properties, resveratrol has been also proposed as a candidate for the treatment of ocular diseases [30].

The present study investigated the effect of oxidative stress and inflammation on retinal DNMTs and SIRT1 functions, as well as on LINE-1 methylation levels, in RPE cells. Therefore, we evaluated whether treatment with resveratrol may restore changes in LINE-1 methylation by modulating DNMTs and SIRT1 functions.

2. Results

2.1. Oxidative stress and inflammatory condition affect cell viability in ARPE-19 cells

Human retinal pigment epithelial (ARPE-19) cells were treated with glucose oxidase (GOx) to mimic a condition of oxidative stress through the continuous production of H2O2, which leads to reactive oxygen species (ROS) production and a cytotoxic effect of less than 50%. Consistently with a previous study [31], compared to untreated cells, treatment with 25 mU/ml GOx for 24 hours reduced cell viability by 35.8% (p=0.004) increasing ROS production by 50.1% (p<0.001). Similarly, ARPE-19 cells were treated with lipopolysaccharide (LPS; type Escherichia coli, serotype 0127:B8) to mimic an inflammatory condition [32] which leads to a cytotoxic effect of less than 50%. Consistently with a previous study [33], treatment with 10 µg/ml LPS for 24 hours reduced cell viability by 24.2% (p=0.035). Interestingly, treatment with 10 µg/ml LPS for 24 hours significantly increased ROS production by 32.6% than in untreated cells (p=0.004). According to these results, treatments of ARPE-19 with 25 mU/ml GOx or 10 µg/ml LPS for 24 hours were applied for further experiments.

2.2. Oxidative stress affects LINE-1 methylation by modulating DNMTs and SIRT1 functions

To determine whether oxidative stress may affect DNA methylation process, we firstly evaluated DNMTs functions in ARPE-19 cells treated with 25mU/ml GOx for 24 hours. Compared to untreated cells, GOx treatment decreased DNMT1, DNMT3a and DNMT3b expression levels (FC=0.63, FC=0.47 and FC=0.46, respectively; p-values <0.05). Accordingly, total DNMTs activity was reduced by 28.5%
in GOx treated cells than in untreated ones (p<0.0001). Since DNMTs functions, especially DNMT1, are regulated by SIRT1 [27], we hypothesised that GOx treatment might also affect SIRT1 expression and activity. Interestingly, we demonstrated that GOx treatment decreased SIRT1 expression (FC=0.53; p=0.002) and activity (-29.0%; p<0.0001) compared to untreated cells. To evaluate the effect of these changes on global DNA methylation, we measured methylation levels of LINE-1, a surrogate marker of global DNA methylation. In line with reduced DNMTs and SIRT1 functions, LINE-1 methylation levels were lower in GOx treated cells compared to untreated ones (69.6% ± 0.1 vs. 72.6% ± 0.1; p<0.0001).

2.3. Inflammatory condition affects LINE-1 methylation by modulating DNMTs and SIRT1 functions

To determine whether inflammatory condition may affect DNA methylation process, we firstly evaluated DNMTs functions in ARPE-19 cells treated with 10 µg/ml LPS for 24 hours. Previous studies reported that treatment with LPS of RPE cells increased the expression of proinflammatory cytokines IL-6 and IL-8 [32,33]. Our study added to the current knowledge, demonstrating that LPS treated cells exhibited lower DNMT1 expression level (FC=0.50; p=0.004), while DNMT3A and DNMT3B expression seemed to be unaffected. Consistently, treatment with LPS reduced total DNMTs activity by 14.9 % (p=0.007). Compared to untreated cells, we also showed that LPS treatment decreased both SIRT1 expression (FC=0.57; p=0.003) and activity (-20.1%; p=0.002). In line with these results, treated cells exhibited lower LINE-1 methylation levels compared to untreated ones (69.7% ± 0.4 vs. 72.6% ± 0.1; p<0.0001).

2.4. Resveratrol ameliorates viability and ROS production in cells upon oxidative and inflammatory conditions

We also aimed at demonstrating the antioxidant and anti-inflammatory effect of resveratrol against GOx- and LPS-induced changes in ARPE-19 cells. Firstly, we determined viability of cells exposed to various concentrations of resveratrol (1–10 µM) for 24 hours. In line with a previous study [34], we showed that treatment with 1-10 µM resveratrol for 24 hours did not affect viability of ARPE-19 cells. Similarly, resveratrol treatment (1-10 µM) of control cells did not induce changes in ROS production. However, Figure 1 shows that treatment with 10 µM resveratrol for 24 hours was able to ameliorate cell viability and to alleviate ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions.

Figure 1. Cell viability and ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions. (a) MTT assay showed that treatment with 25 mU/ml GOx or 10 µg/ml LPS for 24 hours reduced cell viability by 35.8% (p=0.004) and 24.2% (p=0.035), respectively. (b) The determination of ROS using DCFDA demonstrated higher ROS production in GOx- and LPS-treated cells compared to
controls (50.1%, p<0.001; 32.6%, p=0.004; respectively). Resveratrol restores viability (a) and ROS production (b) in cells upon oxidative and inflammatory conditions.

2.4. Resveratrol restores LINE-1 methylation via activating DNMTs and SIRT1 in cells upon oxidative and inflammatory conditions

Finally, we evaluated whether resveratrol may restore changes in LINE-1 methylation via modulating DNMTs and SIRT1 functions. We demonstrated that treatment with 10 µM resveratrol for 24 hours restored both the expression and activity of DNMTs (Figure 2) and SIRT1 (Figure 3) in ARPE-19 cells upon oxidative stress condition. Similarly, resveratrol increased DNMT1 expression and total DNMTs activity (Figure 4), as well as SIRT1 expression and activity (Figure 5) in cells upon inflammatory condition. In line with these results, resveratrol also counteracted the detrimental effect on LINE-1 methylation in cells exposed to GOx and LPS.

![Figure 2](image1.png)

**Figure 2.** DNMTs expression and activity in ARPE-19 cells upon oxidative stress. (a)(b)(c) Analysis of gene expression showed that treatment with 25 mU/ml GOx for 24 hours downregulated DNMT1, DNMT3A and DNMT3b expression levels (FC=0.63, FC=0.47 and FC=0.46, respectively; p-values <0.05). (d) Analysis of total DNMTs enzymatic activity using a using colorimetric assay confirmed that total DNMTs activity was reduced by 28.5% in GOx treated cells compared to controls (p<0.0001). Treatment with 10 µM resveratrol for 24 hours restores DNMTs functions in cells upon oxidative.

![Figure 3](image2.png)

**Figure 3.** SIRT1 expression and activity in ARPE-19 cells upon oxidative stress. (a) Analysis of gene expression showed that treatment with 25 mU/ml GOx for 24 hours downregulated SIRT1 expression level (FC=0.53; p=0.002). (d) Analysis of SIRT1 enzymatic activity using a using fluorimetric assay
confirmed that total SIRT1 activity was reduced by 29.0% in GOx treated cells compared to controls (p<0.0001). Treatment with 10 µM resveratrol for 24 hours restores SIRT1 functions in cells upon oxidative stress.

Figure 4. DNMTs expression and activity in ARPE-19 cells upon inflammatory condition. (a) Analysis of gene expression showed that treatment with 10 µg/ml LPS for 24 hours downregulated DNMT1 expression level (FC= 0.50; p=0.004), (b)(c) while DNMT3A and DNMT3B expression seemed to be unaffected. (d) Analysis of total DNMTs enzymatic activity using a using colorimetric assay confirmed that total DNMTs activity was reduced by 14.9 % in LPS treated cells compared to controls (p=0.007). Treatment with 10 µM resveratrol for 24 hours restores DNMTs functions in cells upon inflammatory condition.

Figure 5. SIRT1 expression and activity in ARPE-19 cells upon inflammatory condition. (a) Analysis of gene expression showed that treatment with 10 µg/ml LPS for 24 hours downregulated SIRT1 expression level (FC=0.57; p=0.003). (d) Analysis of SIRT1 enzymatic activity using a using fluorimetric assay confirmed that total SIRT1 activity was reduced by 20.1% in LPS treated cells compared to controls (p=0.002). Treatment with 10 µM resveratrol for 24 hours restores SIRT1 functions in cells upon inflammatory condition.
3. Discussion

The functional mechanisms during retinal aging are not clearly elucidated and the discovery of both genetic and environmental risk factors begs the question as to whether there is an interaction in the pathogenesis of AMD. In general, this hypothesis has been raised by genome-wide association studies that have failed to explain the incomplete genetic heritability in complex diseases such as AMD [35]. Although it is becoming evident that epigenetic mechanisms - including DNA methylation and histone modification - might explain how interactions between genetics and the environment result in particular phenotypes, the extent to which DNA methylation contributes to AMD is not currently clarified. Evidence that methylation of repetitive elements changes over time points out LINE-1 methylation as a surrogate marker of global methylation in aging and age-related disease [22-25]. Efforts to understand the mechanisms underpinning the multifactorial nature of AMD have led us to explore DNA methylation process in RPE cells upon oxidative and inflammatory conditions, two of the major causes of retinal degeneration [36]. To our knowledge, the present work demonstrated for the first time that oxidative stress and inflammatory conditions reduced LINE-1 methylation in RPE cells by modulating DNMTs and SIRT1 functions, some of the main enzymes involved in epigenetic mechanisms. Particularly, decreased LINE-1 methylation leads to genomic instability and plays a crucial role in the development of chronic degenerative disease. In patients with AMD, the degeneration of RPE layer is a progressive process with severe consequences on visual pigment regeneration, synthesis and remodeling of the interphotoreceptor matrix, transport of nutrients, ions and waste products, absorption of light via the pigmentation, and adhesion to the retina [2]. Interestingly, the effect of oxidative stress and inflammatory conditions on retinal LINE-1 methylation and DNMTs functions was similar, suggesting it as a convergence point during the pathogenesis of AMD. However, while inflammatory condition seemed to affect only DNMT1 expression – the maintenance DNMT – oxidative stress also reduced mRNA levels of de novo DNMTs (i.e. DNMT3a and DNMT3b), which in turn enable key epigenetic modifications for cellular differentiation, transcriptional regulation, heterochromatin formation, X-inactivation, imprinting and genome stability [37]. In support of the interplay between inflammation and oxidative stress, we also observed that RPE cells treated with LPS to mimic an inflammatory condition with increased expression of proinflammatory cytokines [32,33], also exhibited increased ROS production compared to untreated cells. In fact, a chronic low-level inflammation status might be exacerbated over time by the accumulation of oxidation products, which in turn cause tissue damage and impairment of central vision [36].

Our study is not the only one investigating how oxidative stress and inflammation can influence epigenetic mechanisms in AMD, though findings are partially inconclusive. Oxidative stress occurs when ROS levels exceeds the detoxifying capacity of antioxidants or molecular chaperones [38]. A previous study, comparing DNA methylation between AMD patients and age-matched controls, revealed that glutathione S-transferase isoforms mu1 (GSTM1) and mu5 (GSTM5) undergo epigenetic repression in AMD RPE/choroid via promoter hypermethylation, which in turn decreased mRNA and protein levels [39]. These enzymes play an important role in the detoxification of electrophilic compounds, including products of oxidative stress, by conjugation with glutathione; reduced activity of GSTM1 and GSTM5 could affect protection from genome-damaging oxidants with increased vulnerability to oxidative insults. Genome-wide differences in DNA methylation between three pairs of twins (both monozygotic and dizygotic) with discordant AMD were assessed by Wei et al. [40]. Their results, further validated in discordant siblings for AMD as well as in an AMD case–control cohort, reported a significantly decreased level of interleukin 17 receptor C (IL17RC) promoter methylation in AMD patients, which in turn led to increased expression of its protein and mRNA in peripheral blood and in the affected retina [40]. The IL17RC gene encodes for an essential subunit of the IL-17 receptor complex that modulates activity of proinflammatory IL-17A and IL-17F. Although these findings were not confirmed in a subsequent study [41], the putative epigenetic mechanism by which proinflammatory stimuli could promote AMD pathology should be investigated.

Recently, several lines of evidence suggested that SIRT1, a NAD+-dependent histone deacetylase, protects RPE cells against apoptosis and counteracts changes in RPE functions induced
by oxidative stress and chronic inflammations. In fact, SIRT1 might be involved in the AMD pathogenesis via modulating cell senescence, DNA damage repair and apoptosis [26]. Previous studies demonstrated that SIRT1 expression significantly decreased with increasing age in retinal stem cells, and that it was down-regulated in human AMD retina compared to non-AMD donors [42]. Consistently, we reported for the first time that RPE cells upon oxidative and inflammatory conditions exhibited decreased SIRT1 expression and activity compared to untreated cells. Since SIRT1 regulates the activities of DNMTs, especially DNMT1 [27], this result partially explains how oxidative stress and inflammation might affect DNA methylation mechanism. Interestingly, SIRT1 also attenuated changes induced by Amyloid beta (Aβ), a known constituent of drusen which induces chronic inflammation [43]. In fact, treatment with a SIRT1 agonist (i.e. SRT1720) restored Aβ-induced upregulation of IL-6, IL-8, and matrix metalloproteinase-9 (MMP-9); this inhibitory effect was abolished in SIRT1 knockdown cells [43]. In addition, a mutual effect between SIRT1 and nuclear factor-kappa B (NF-κB) has been established: if, on one hand, SIRT1 inhibits the activation of NF-κB-mediated inflammatory pathway, on the other hand, NF-κB signaling and inflammatory response can suppress SIRT1 activity [44]. Whilst it appears evident that SIRT1 is crucial for maintaining integrity and function of RPE cells under oxidative stress and chronic inflammation, results about the role of SIRT1 during neovascularization are controversial [45,46]. In fact, a previous study, observing higher SIRT1 expression level in choroidal neovascularization (CNV) membranes than in healthy donor eyes, demonstrated that SIRT1 inhibition with nicotinamide decreased the secretion of proangiogenic factors, such as VEGF-A, platelet-derived growth factor BB and angiogenin in ARPE-19 cells [47]. By contrast, others revealed that treatment with resveratrol, a natural SIRT1 activator, restored VEGF secretion in RPE cells upon oxidative and inflammatory conditions [48]. Recent literature reported the anti-aging effect of resveratrol in cutaneous, endothelial and corneal wound healing [49-51]. In addition to antioxidant and anti-inflammatory properties, resveratrol stimulates SIRT1 activity through allosteric interaction and increases SIRT1 affinity for both NAD+ and the acetylated substrate [28,29]. This is consistent with another study indicating that resveratrol downregulated VEGF expression and inhibited hypoxic-induced choroidal vascular endothelial cell proliferation, via modulating the SIRT1 pathway [52]. However, there is also evidence that resveratrol might inhibit angiogenesis both in vivo and in vitro by a SIRT1-independent pathway [53]. The present study adds to the current knowledge, demonstrating that treatment with 10 µM resveratrol for 24 hours not only ameliorated cell viability and ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions, but also counteracted the detrimental effect on LINE-1 methylation via increasing DNMTs and SIRT1 functions. However, further studies should be performed to elucidate controversies regarding the role of SIRT1 in AMD pathogenesis in general, and in CNV in particular. Moreover, it is worth investigating whether the protective effect of resveratrol on RPE cells relies on SIRT1 activation rather than on its antioxidant and anti-inflammatory properties.

4. Materials and Methods

4.1. Cell culture and treatments

ARPE-19, purchased from the American Type Culture Collection (Manassas, VA), were maintained in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (Gibco BRL), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco BRL). Cells between 6-10 passages were used in all experiments and incubated at 37 °C and 5% CO2. Medium was changed every 48 hours.

To mimic conditions of oxidative stress and inflammation, cells reaching 80%–90% of confluence were starved in serum-free DMEM and treated with 25 mU/ml glucose oxidase (GOx) or 10 µg/ml LPS (type Escherichia coli, serotype 0127:B8; Sigma Chemical) for 24 hours, respectively. The concentrations of GOx and LPS were chosen according to previously published studies that used the same cell line [31,33]. To investigate whether resveratrol might restore changes induced by treatment with GOx and LPS, treated and untreated cells were also incubated with 10 µM resveratrol for 24
hours. This concentration was chosen according to results of cell viability after incubation with increasing concentrations (1–10 µM) of resveratrol for 24 hours.

4.2. Determination of cell viability

After treatment with GOx, LPS and resveratrol, alone or in combination, we evaluated cell viability by using the Thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, treated and untreated cells were seeded at a density of 2.0×10^4 cells/well in a 96-well plate and incubated for 24 hours. Then, cells were incubated with MTT (1.6 mg/ml) at 37 °C for 4 h. After removing the solution, cells were re-suspended in 100 µl of dimethyl sulfoxide and optical density was read at 540 with an optional reference wavelength of 670 nm. Cell viability was reported as percentage of control.

4.3. Determination of ROS

Intracellular ROS levels were determined using the Abcam cellular ROS detection assay kit according to manufacturer’s instructions (Abcam plc, Cambridge, UK). Briefly, cells were seeded at a density of 2.0×10^4 cells/well in a dark, clear bottom 96-well microplate. Cells were rinsed with 100 µL/well of 1X Buffer and stained by adding 100 µL/well of the redox-sensitive fluoroprobe 2',7'–dichlorofluorescin diacetate (DCFDA) for 45 minutes at 37°C in the dark. After removing DCFDA, 100 µL/well of 1X Buffer were added and fluorescence was measured at Ex/Em=485/535 nm. ROS production was reported as percentage of control.

4.4. Nuclear protein extraction

Nuclear proteins were extracted using the Nuclear Extraction Kit according to manufacturer’s instructions (Abcam plc, Cambridge, UK). Briefly, cell pellet (2×10^6 cells) was obtained by trypsinization and centrifugation of cells at 70-80% of confluence following standard protocols. Cell pellet was re-suspended in 200 µL of pre-extraction buffer and incubated on ice for 10 minutes. After centrifugation, nuclear pellet was re-suspended in 400 µL of extraction buffer and incubated on ice for 15 minutes. Finally, the suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C and the supernatant was transferred into a new vial to measure the protein concentration of the nuclear extract. Nuclear proteins quantification was performed by the Qubit fluorometer (Invitrogen) using the Qubit Protein Assay Kit according to manufacturer’s instructions.

4.5. DNMTs activity quantification

Total DNMTs activity was quantified using the colorimetric DNMTs Activity Quantification Kit (Abcam plc, Cambridge, UK) according to manufacturer’s instructions. Briefly, 7.5 ng of nuclear extracts were diluted in 50 µl/well of reaction solution and incubated at 37°C for 120 min, including blank and positive control. After removing the reaction solution, each well was rinsed with wash buffer for three times, and 50 µl/well of the diluted capture antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 60 min. After removing the capture antibody, each well was rinsed with wash buffer for three times, and 50 µl/well of the diluted detection antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the detection antibody, each well was rinsed with wash buffer for four times, and 50 µl/well of the enhancer solution were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the enhancer solution, each well was rinsed with wash buffer for five times, and 100 µl/well of the developer solution were added. Finally, the plate was covered with aluminium foil and incubated at room temperature for 10 min, away from direct light. When the positive control turned to medium blue, 100 µl/well of stop solution were added to stop the reaction. OD was read within 2-10 min at 450 nm with an optional reference wavelength of 655 nm. DNMTs activity was reported as percentage of control.
4.6. SIRT1 activity quantification

SIRT1 activity was quantified using a Sirt1 activity assay kit (Abcam plc, Cambridge, UK) according to manufacturer’s instructions. The reaction mixture containing 30 µL ddH2O, 5 µL fluoro-substrate peptide, 5 µL NAD, 5 µL developer, and 7.5 ng nuclear extract was mixed thoroughly, and the fluorescence intensity was measured at Ex/Em= 350-450 nm for 30 to 60 minutes at 1-2 min interval. SIRT1 activity was reported as percentage of control.

4.7. Quantitative real-time polymerase chain reaction (qPCR)

Total cellular RNA was extracted using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to single-stranded cDNA using the SuperScript III Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocols. mRNA levels were determined by qPCR with TaqMan Gene Expression Assays (Life Technologies, Monza, Italy) using the QuantStudio™ 7 Flex System (Applied Biosystems, Foster City, CA, USA). Specific primers were used to detect DNMT1 (assay no. Hs00945875_m1), DNMT3a (Hs01027162_m1), DNMT3b (Hs00171876_m1), and SIRT1 (Hs01009006_m1). Data were normalized to the values of GAPDH expression (Hs02758991_g1). Relative RNA quantification was performed using the 2^(-ΔΔCT) method [54].

4.8. LINE-1 methylation analysis

DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Milan, Italy) and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies, Monza, Italy) according to the manufacturer’s protocols. Methylation analysis of three CpG sites in the LINE-1 promoter (GeneBank accession no. X58075) was performed by pyrosequencing of bisulfite-converted DNA using PyroMark Q24 instrument (Qiagen, Milan, Italy), as previously reported [55,56]. Briefly, 20 µg of DNA extracted from each sample were converted by bisulfite treatment using the Epitect Bisulfite kit (Qiagen, Milan, Italy). Converted DNA was eluted in 20 µl of Elution buffer and stored at -80°C until used. A reaction volume of 25 mL was amplified by polymerase chain reaction (PCR), using the PyroMark PCR Kit (Qiagen, Milan, Italy), according to the manufacturer’s instructions. Briefly, each reaction mixture contained 12.5 µl of PyroMark PCR Master Mix 2X, 2.5 µl of CoralLoad Concentrate 10X, 2 µl of the forward primer (5’-TTTTGAGTTAGGTGGGATATA-3’) and the reverse-biotinylated primer (5’-biotin-AAAATCAAAAAATTCCCTTTC-3’) (0.2 µM for each) and 1.5 µl of bisulfite-converted DNA. HotStart PCR cycling conditions were 1 cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The bisulfite PCR product was purified and made single stranded using the Pyrosequencing Vacuum PrepTool (Biotage, Inc., Charlottesville, VA, USA). The biotinylated single-stranded product was annealed to the pyrosequencing primer (5’ AGTTAGGTGGGATATA-3’) and then subjected to sequencing using an automatically generated nucleotide dispensation order. The pyrogram was analysed using allele quantification mode to determine the proportion of methylated and unmethylated cytosines. LINE-1 methylation level was reported as the average of the three specific CpG sites.

4.9. Statistical analysis

All experiments were performed in triplicate for three times. Results were reported as MD ± SE unless otherwise indicated. Differences were assessed by one-way repeated measures analysis of variance (ANOVA), or by unpaired student’s t-test for comparison of two groups. All the analyses were conducted using GraphPad Version 6.0 with a significance level of 0.05.

5. Conclusions
In conclusion, we demonstrated for the first time that oxidative stress and inflammatory conditions negatively affect LINE-1 methylation in RPE cells via modulating DNMTs and SIRT1 functions. However, treatment with resveratrol counteracts this detrimental effect on LINE-1 methylation, by restoring the expression and activity of DNMTs and SIRT1. Further studies should be encouraged to explore the perspectives of resveratrol as a suitable strategy for the prevention and/or treatment of AMD.

Author Contributions: Conceptualization, AM, MM and AA; Methodology, AM and FG; Investigation, AM, FG, MB.; Resources, MM and FG; Data Curation, AM and MB; Writing-Original Draft Preparation, AM, MB and AA; Writing-Review & Editing, AM, MM, FG, GB, MB and AA; Supervision, AA and MM.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

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<th>Acronym</th>
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<td>AMD</td>
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<td>Aβ</td>
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<td>ARPE-19</td>
<td>Human retinal pigment epithelial</td>
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<td>Choroidal neovascularization</td>
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