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Modulatory role of HMG-CoA reductase inhibitors and ezetimibe on LDL-AGEs-induced ROS generation and RAGE-associated signalling in HEK-293 Cells

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ABSTRACT

Aims: Advanced glycation end products (AGEs) trigger intracellular reactive oxygen species (ROS) generation, activation of receptor for AGEs (RAGE) expression/functionality and RAGE-associated signalling pathways which influence the diabetic-cum-atherosclerotic complications, whereas, the atherosclerosis progression is greatly influenced by hepatic β-Hydroxy-β-methyl-glutaryl-Co-A reductase (HMG-R) activity. The present report was premeditated to uncover the regulatory role of HMG-R inhibitors and ezetimibe (EZ) in attenuating the LDL-AGEs-induced pathogenicity via targeting cellular-ROS and RAGE-associated signalling in HEK-293 cells.

Main methods: The MTT assay was used to assess either the cytotoxic or cytoprotective impact of each HMG-R inhibitors, EZ, and LDL-AGEs, whereas, quantification of ROS was performed by DCFDA method. The qRT-PCR was used to detect the mRNA level of RAGE, neuropilin-1 (NRP-1) and other RAGE-associated genes like MMP-2, NF-κB, and TGFβ-1.

Key findings: The HMG-R inhibitors do not exert any cytotoxicity in HEK-293 cells, whereas, and LDL-AGEs negatively affected the cell viability of HEK-293 cells. However, viability of LDL-AGEs-treated HEK-293was markedly retained after simultaneous treatment with our test inhibitors. Further, DCFDA staining showed that LDL-AGEs-induced ROS was also suppressed upon treatment with our test inhibitors in HEK-293 cells. qRT-PCR analysis reflected that these inhibitors suppress the RAGE, NF-κB, TGFβ-1, and MMP-2 expression, whereas, the NRP-1 was up-regulated by these compounds in LDL-AGEs-exposed HEK-293 cells.

Significance: The above pharmacological effects signify that HMG-R inhibitors and EZ (alone or in combination) may implied in the treatment of AGEs-induced oxidative stress and tissue damage in diabetic complications via targeting intracellular-ROS, NRP-1 functionality and RAGE-associated genes i.e. NF-κB, TGFβ-1, and MMP-2.

1. Introduction

Advanced glycation end-products (AGEs) are formed non-enzymatically from successive redox reactions of reactive carbonyls and various biological macro molecules under diabetes [1,2]. Their accumulation leads to the pathogenesis of various diabetes-associated complications as AGEs intrude the cellular physiology attributing to the coproduction of reactive oxygen species (ROS) and reactive carbonyl species (RCS) as a consequence of glycoxidation [2,3]. AGEs-mediated pathologies under hyperglycemia are primarily accredited to the interaction of AGEs and their receptors (RAGE) which in turn triggers distinct signalling pathways [2,4]. Being a cell surface molecule, RAGE is associated with increased oxidative stress, cell growth, and inflammation and abundant level of RAGE was detected in diabetic patients with elevated blood-AGEs [2,5,6]. Studies related to the RAGE−/− mice, pharmacological blocking of RAGE, and genetic deletion of RAGE led the discovery of AGE-RAGE axis and its association to various clinical complications [2,4,7]. These studies have established RAGE as a promising therapeutic target in the management of diabetes and associated complications.

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RAGE is expressed in various cells including endothelial cells (ECs) and its transcription is regulated by nuclear factor-κB (NF-κB), which binds to the RAGE promotor [8,9]. On the other hand, neuropilin-1 (NRP-1) is a transmembrane receptor protein which mainly expresses in neuronal and renal cells [10]. NRP-1 plays regulatory role in various signalling pathways including those associated with AGEs-RAGE axis [11,12]. A recent report showed that AGEs accumulation suppresses the mRNA level of NRP-1 in differentiated podocytes [13]. Moreover, matrix metalloproteinase-2(MMP-2) has gained serious attention due to its ability to disintegrate type-I and -IV collagen and laminin which are the key constituents of tubular basement membrane and interstitium [14]. Furthermore, the increased AGEs-accumulation under hyperglycaemia stimulated the MMP-2 expression leading to renal matrix degradation and glomerular ECs hyper permeability [15,16].

Similarly, transforming growth factor-β1(TGF-β1), aprofibrotic cytokines, participates in the development and progression of diabetic glomerulosclerosis and renal fibrosis through the deposition of extracellular matrix components [17,18]. One of the study also showed direct correlation of high circulatory TGF-β1 level to diabetic retinopathy and HbA1c [19]. Further, AGEs-accumulation also leads to the high level of TGF-β1 levels in various cells which further raises the level of MMP-2 [20]. It has been observed that AGEs stimulate collagen IV expression through RAGE and TGF-β1-dependent pathways which ultimately triggers the expression of MMP-2 and MMP-9 [18,21].

Among various anti-glycating agent, aminoguanidine (AG), the standard drug, is being ignored nowadays due to its negative effects on cellular physiology [1–3]. Moreover, AGEs-induced pathogenesis of diabetic and vascular complications is greatly influenced by the function of HMG-CoA reductase (HMG-R), an enzyme reckoned for endogenous cholesterol production [22–24]. Statins, the HMG-R inhibitors, are well known for lowering the risk of atherosclerosis in diabetic patients [2]. Recently, we have screened the in-vitro anti-glycation potential of HMG-R inhibitors and ezetimibe (EZ) against D-ribose-induced AGEs formation [1]. Therefore, we hypothesized that the selected HMG-R inhibitors from our previous study may reduce the AGEs-induced renal pathologies. Thus, the present report was premeditated to uncover the regulatory role of HMG-R inhibitors and EZ in attenuating LDL-AGEs-induced pathogenicity via targeting cellular-ROS and RAGE-associated signalling in HEK-293 cells.

2. Material & methods

2.1. Reagents

Dulbecco's modified eagle medium (DMEM), Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) and other reagent grade chemicals were obtained from HiMedia®Laboratories Pvt. Ltd., Mumbai, India. TRIzol™ reagent and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) were procured from Invitrogen, USA. Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814) was supplied by Thermo Fisher Scientific Pvt. Ltd., Mumbai, India, whereas, SYBR®*Premix Ex Taq*™ II (TliRNaseH Plus) was obtained from DSS Takara Bio India Pvt. Ltd., India. HMG-R inhibitors (atorvastatin: AT and rosuvastatin: RT), ezetimibe (EZ) and aminoguanidine hydrochloride (AG) were purchased from Sigma Aldrich Co., USA. Purified Human LDL was procured from Calbiochem®, Merck, India. All the other chemicals/reagents used in this study were of analytical grade.

2.2. Preparation and characterization of LDL-AGEs

We have previously described the method of preparation and characterization of LDL-AGEs [1]. Briefly, 70 μg/mL of LDL was incubated at 37 °C with D-ribose (20 mM) in 100 mM phosphate buffer saline (pH 7.4; containing 0.05% sodium azide) for 21 days and characterized by various physical approaches i.e. UV–Vis and Fluorescence spectroscopy, circular dichroism (CD), FTIR as well as biochemical assays like estimation of carbonyl content (CC) and thiobarbituric acid to detect the hydroxy methyl furfural (HMF) content of the native and glycated LDL samples (data not shown) [1].

2.3. Cell culture

Human embryonic kidney cells (HEK-293) were obtained from National Centre for Cell Science (NCCS), Maharashtra, India. HEK-293 cells were cultured in DMEM supplemented with 100 mg/mL streptomycin (Himedia Lab. Pvt. Ltd., Mumbai, India) and 10% heat inactivated FBS. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Cytotoxicity and cytoprotective analysis (MTT assay)

The analysis of cytotoxicity of our test compounds and LDL-AGEs was determined by MTT assay as described previously [25]. Briefly, cells (5×10^3 cells/well) were seeded in 96–well tissue culture plates in complete high glucose DMEM medium, followed by incubation in 5% CO_2 for 24 h at 37 °C. To determine the cytotoxicity of test compounds and LDL-AGEs, exponentially growing HEK-293 cells (5×10^3 cells/well) were treated either with different concentrations (0–200 μM) of test compounds (AT, TT, RT, EZ, EZ-AT, EZ-RT, and AG) for 24and 48 h or with LDL-AGEs (0.78–200 μg/mL) only for 24 h. Following the drug and/or LDL-AGEs treatment, 10 μL MTT (prepared in PBS) was added to each well and the plates were incubated for 4 h at 37 °C. After incubation, the reaction mixture was carefully taken out and 200 μL of dimethyl sulfoxide (DMSO) was added to each well followed by gentle mixing using a pipette. The plates were kept on rocker shaker for 10 min at RT and then absorbance was recorded at490 nm using Multiwell Microplate Reader (Bio-Rad, USA). The untreated cells (neither treated with test compounds nor with LDL-AGEs) were designated as control. The cell survival/viability was expressed as the percentage (%) cell viability over the untreated control. Each experiment was performed in triplicate. On the other hand, to investigate the protective effects of above mentioned test drugs against LDL-AGEs-induced cytotoxicity in HEK-293 cells, we repeated the MTT assay in different sets of experiments in which test compounds (5, 10, 20, and 50 μM) and LDL-AGEs (200 μg/mL) were added to the cells simultaneously and incubated for 24 h prior to the addition of MTT.

2.5. Measurement of intracellular ROS level

Reactive Oxygen Species (ROS) generation was estimated by using 2′,7′-diclorodihydrofluorescein di-acetate (H2-DCFDA) method [26]. Briefly, HEK-293 cells (5×10^4 cells/well) were seeded in a 12-well plate and incubated for 24 h at 37 °C. The cells were treated with 5, 10, 20 and 50 μM of test compounds (AT, TT, RT, EZ, EZ-AT, EZ-RT, and AG) along with LDL-AGEs (200 μg/mL) simultaneously. Following the incubation for 24 h, the HEK-293 cells were incubated with 10 μM H2-DCFDA for 30 min at 37 °C. The excess H2-DCFDA dye was removed by washing and the images were captured through inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

2.6. Gene expression analysis

2.6.1. Extraction of cells and RNA isolation

In order to isolate the cellular RNA for gene expression analysis, the HEK-293 cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and incubated for 24 h at 37 °C and incubated with 50 μM test inhibitors and LDL-AGEs (200 μg/mL) simultaneously for 24 h. Following the incubation, media was discarded from the wells and cells were washed with 1.5 mL of chilled PBS followed by addition of 1 ml TRIzol™ Reagent. The cells were scrapped and the cell lysate was transferred into 1.5 mL centrifuge tube and left for incubation at RT for

5 min. 250 µL chloroform was added to each tube (containing cell lysate) following the incubation and tubes were shaken vigorously for about 15 s and incubated again at RT for 5 min. The tubes were centrifuged at 10,000 rpm for 15 min and the aq. phase (top most) was carefully allocated into another centrifuge tubes followed by addition of 550 µL isopropanol. The resulting mixture was incubated at RT for 5–10 min and centrifuged at 12,000 rpm for 20 min and discarded the supernatant. Thus obtained RNA pellet was washed with chilled ethanol (75%) and was dissolved in 50 µL of diethyl pyrocarbonate (DEPC) water and quantified by nanodrop.

2.6.2. Reverse transcription of the isolated RNA

Isolated RNA was subjected to reverse-transcription into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to the manufacturer's instructions. The reaction mixture (20 µL) having 2000 ng RNA and Master Mix (comprising MultiScribe™ Reverse Transcriptase, RNase inhibitor, reaction buffer, dNTPs mix, random primers, and RNase Free Water) was pipette out into a microcentrifuge tube and subjected to thermocycler with the program: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min.

2.6.3. qRT-PCR analysis

For gene expression analysis, specific primers were designed using the Primer Express Software (Version 3.0.1) and synthesized by Eurofins Genomics Pvt. Ltd., India. The primer sequences for all the genes used in this study have been listed in Table 1. Real-time PCR was performed using 10µlSYBR®*Premix Ex Taq*™ II (comprising of *TaKaRa Ex Taq* Hot-start DNA Polymerase, dNTP mix, Mg^{2+}, heat-resistant TliRNaseH, and SYBR Green I), 1 µL of each forward and reverse primer for each gene of interest, 5 µL of cDNA template and RNase free water to obtain desired reaction volume (20 µL). The reaction was performed on BioRadCFX 96™ Real Time System with thermal profiles: 10 min at 95 °C for activation of polymerase (1 cycle), 15 s at 95 °C for denaturation (40 cycles) and 1 min at 60 °C for annealing and 45 Sec at 72 °C for extension. Variation in the gene expression under the influence of test compounds and LDL-AGEs was calculated through $2^{-\Delta\Delta Ct}$ method and expressed as fold-change relative to β-actin, used as the house keeping gene.

2.7. Statistical analysis

The samples were taken in triplicate for all the biochemical measurements and data was represented as mean ± SEM. The statistical significance was evaluated by ANOVA through GraphPad Prism version 4.02 for Windows (GraphPad Software, San Di-ego, USA) [23,24]. The use of the variants of ANOVA (i.e. one-way or two-way ANOVA) for different experiments of our study has been mentioned in respective figure legends.

Table 1
Oligonucleotide primer sequences used for gene expression analysis.

Genes	Accession no	Direction	Oligonucleotide sequence (5′ → 3′)
RAGE	NM_001136	F	GCAGTAGTAGGTGCTCAAAACATCA
		R	GGGCCCCCTTACACTTCAG
MMP-2	NM_004530.5	F	GGACGGACTCCTGGCTCAT
		R	TCCATAGCTCATCGTCATCAAAA
NRP-1	NM_001024628.2	F	CAGATGTTGTGGTTGCAGTATTCC
		R	GCAGGCTTGATTCGGACAAA
TGFβ-1	NM_000660.6	F	AGTTCAAGCAGAGTACACACAGCAT
		R	AGAGCAACACGGGTTCAGGTA
NFkB-2	NM_001288724.1	F	GACGAGTGTGGTGAGCTTTCTG
		R	GAGTCTCCATGCCGATCCA
β-Actin	NM_001101.5	F	GCGCGGCTACAGCTTCA
		R	TCTCCTTAATGTCACGCACGATT

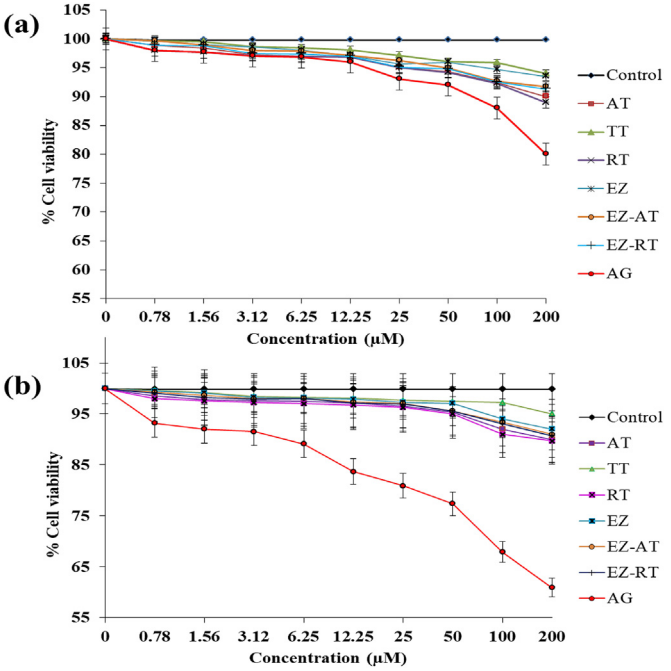


Fig. 1. MTT assay. Panel (a): HMG-R inhibitors and EZ did not affect the viability of HEK-293 cells after 24 h. HEK-293 cells (5×10^3 cells/well) were seeded in a 96-well culture plate and allowed to attach overnight and further treated with varying concentrations (0, 0.78, 1.56, 3.12, 6.25, 12.25, 25, 50, 100, and 200 µM) of HMG-R inhibitors and EZ for 24 h to assess their respective cytotoxic effects by MTT assay. The data (% cell viability) are mean ± SEM of three independent experiments. The level of statistical significance was determined by two-way ANOVA on GraphPad Prism software (version 4.02) for Windows. The level of significance for control v/s AT, TT, RT, EZ, EZ-AT, and EZ-RT at 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 µM was non-significant at $p > 0.05$. The level of significance for control v/s AT, TT, RT, EZ, EZ-AT, and EZ-RT at 100 and 200 µM was $p < 0.01$. The level of significance for control v/s AG at 0.78, 1.56, 3.12, 6.25, and 12.5 µM was non-significant at $p > 0.05$. The level of significance for control v/s AG at 25 and 50 µM was $p < 0.01$. The level of significance for control v/s AG 100 and 200 µM was $p < 0.001$. The level of significance for AT, TT, RT, EZ, EZ-AT, and EZ-RT at 0 v/s 0.78 µM, 0.78 v/s 1.56 µM, 1.56 v/s 3.12 µM, 3.12 v/s 6.25 µM, 6.25 v/s 12.5 µM was non-significant at $p > 0.05$.The level of significance for AT, TT, RT, EZ, EZ-AT, and EZ-RT at12.5 v/s 25 µM and 25 v/s 50 µM was $p < 0.01$. The level of significance for 50 v/s 100 µM and 100 v/s 200 µM was $p < 0.001$. The level of significance for AG at 0.78 to 12.5 was non-significant at $p > 0.05$. The level of significance for AG at 12.5 v/s 25 and 25 v/s 50 µM was < 0.01. The level of significance for AG at 50 v/s 100 and 100 v/s 200 µM was < 0.001.
Panel (b): HMG-R inhibitors and EZ did not affect the viability of HEK-293 cells after 48 h. HEK-293 cells (5×10^3 cells/well) were seeded in a 96-well culture plate and allowed to attach overnight and further treated with varying concentrations (0–200 µM) of HMG-R inhibitors and EZ for 48 h to assess their respective cytotoxic effects by MTT assay. The data (% cell viability) are mean ± SEM of three independent experiments. The level of statistical significance was determined by two-way ANOVA on GraphPad Prism software (version 4.02) for Windows. The level of significance for control v/s AT, TT, RT, EZ, EZ-AT, and EZ-RT at 0.78–50 µM was non-significant at $p > 0.05$. The level of significance for control v/s AT, TT, RT, EZ, EZ-AT, and EZ-RT at 100 and 200 µM was $p < 0.01$, whereas, the effect of AG at 0.78–6.25 µM was non-significant v/s control at $p > 0.05$. The level of significance for control v/s AG at 12.5–200 µM was $p < 0.001$. The effect of AT, TT, RT, EZ, EZ-AT, and EZ-RT at 0.78–50 µM was non-significant at $p > 0.05$ in same treatment groups. The level of significance of AT, TT, RT, EZ, EZ-AT, and EZ-RT at 50 v/s 100 and 100 v/s 200 was significant at $p < 0.01$. The effect of AG v/s AG at 0.78–6.25 µM was non-significant at $p > 0.05$ in same treatment group. The effect of AG v/s AG at 6.25 v/s 12.5, 12.5 v/s 25, 25 v/s 50, 50 v/s 100, and 100 v/s 200 µM was significantly different at $p < 0.001$ in same treatment group.

3. Results

3.1. HMG-R inhibitors and EZ are non-toxic to HEK-293 cells

The results from our MTT assay with different concentrations of HMG-R inhibitors and EZ (0.78–200 μM) against HEK-293 cells showed that HEK-293 cells retained their viability up to 90.0%, 94.0%, 89.0%, 93.49%, 91.69%, and 91.29% in AT, TT, RT, EZ, EZ-AT and EZ-RT treated cells, respectively, at 200 μM, when compared to control HEK-293 cells (99.79%) after 24 h. Reference standard (AG) also did not show any remarkable change in the cell viability after 24 h of incubation (Fig. 1.a). Similarly, prolonged incubation (48 h) with above mentioned drugs (identical concentration range) also did not influence the viability of HEK-293 cells (89.89%, 95.0%, 89.67%, 92.0%, 91%, and 90.69%, respectively). In contrast, treatment with different doses of AG for 48 h showed a markedly diminished cell viability (up to 60.90%), when compared to control cells (Fig. 1.b). Thus, the results of this study have established that HMG-R inhibitors and EZ did not show considerable cytotoxic effects onHEK-293 cells.

3.2. LDL-AGEs negatively affect HEK-293 cells viability

Effect of LDL-AGEs on HEK-293 cells was determined by treating/incubating the cells with varying concentrations of the LDL-AGEs (ranging from 0.78 to 200 μg/mL) for 24 h at 37 °C. The treatment of cells with the above mentioned concentrations of LDL-AGEs resulted in marked reduction in the cell viability up to 97.1%, 92.23%, 86.4%, 78.45%, 67.68%, 52.34%, 42.97%, 24.71%, and 12.77% in 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μg/mL LDL-AGEs treated cells, respectively, as compared to control cells. The least viability of HEK-293 cells was reported in 200 μg/mL LDL-AGEs treated cells. As a result of this experiment, we found that LDL-AGEs negatively affected the HEK-293 cell viability in a concentration dependent manner. Hence, for further experiments, we used 200 μg/ml LDL-AGEs (Fig. 2).

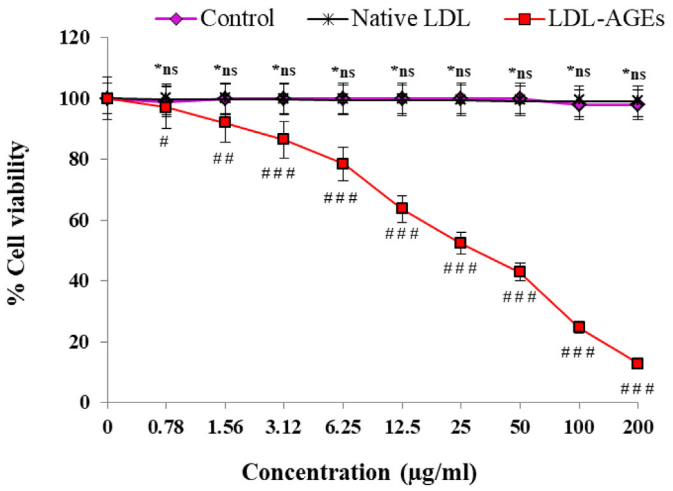


Fig. 2. LDL-AGEs are highly cytotoxic to HEK-293 cells. HEK-293 cells (5×10^3 cells/well) were seeded in a 96-well culture plate and allowed to attach overnight and further treated with 0, 0.78, 1.56, 3.12, 6.25, 12.25, 25, 50, 100, and 200 μg/mL of LDL-AGEs for 24 h to determine their cytotoxic effects on HEK-293 cells via MTT assay. The data (% cell viability) are mean ± SEM of three independent experiments. The level of statistical significance was determined by two-way ANOVA on GraphPad Prism software (version 4.02) for Windows. There was no-significant difference between the viability of control cells and native-LDL (0.78–200 μg/mL) treated cells ($^{ns*}p > 0.05$). The level of significance for Control v/s LDL-AGEs at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 μg/mL was $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#}p < 0.001$, respectively. The level of significance among all the doses (0.78–200 μg/mL) of LDL-AGEs was $p < 0.001$ (symbols not represented in figure).

3.3. HMG-R inhibitors and EZ restore the cell viability of LDL-AGEs treated HEK-293 cells

To assess the cytoprotective effect of AT,TT,RT,EZ,EZ-AT, and EZ-RT on cell viability upon LDL-AGEs treatment for 24 h, HEK-293 cells were treated with LDL-AGEs (200 μg/mL) in presence or absence of varying concentrations (5–50 μM) of test compounds and AG, used as a reference drug. The results from MTT assay evident that the LDL-AGEs markedly decreased the viability of HEK-293 cells. This down fall in the viability of HEK-293 cells was remarkably enhanced in the presence of all the test compounds mentioned above in a concentration dependent manner. The maximum cell viability of95.98%, 97.45%, 95.12%, 97.0%, 96.0%, and 96.0% was observed in 50 μM AT, TT, RT, EZ, EZ-AT, and EZ-RT treated HEK-239 cells, respectively, when compared to only LDL-AGEs treated cells. In contrast, AG also increased the cell viability up to 75.12% when compared to only LDL-AGEs treated cells, but not as much as compared to the above test compounds (Fig. 3).

3.4. HMG-R inhibitors and EZ suppressed LDL-AGEs-induced ROS generation in HEK-293 cells

As a result of H2-DCFDA staining, it was observed that the degree of oxidative stress, evident by ROS generation, was markedly increased by 89.77% upon exposure to LDL-AGEs as compared to control HEK-293 cells. This rise in the LDL-AGEs-induced ROS generation was markedly restored upon the treatment with our test compounds in HEK-293 cells in a concentration dependent manner. The maximum inhibition of the ROS generation was reported at 50 μM dose of each test compound which diminished the ROS generation by 83.92%, 87.80%, 81.20%, 85.81%, 85.50%, and 84.43% in AT, TT, RT, EZ, EZ-AT, and EZ-RT treated cells, respectively, when compared to LDL-AGEs-treated HEK-293 cells. In contrast, AG, reference anti-glycating agent, also down-regulated the LDL-AGEs-induced ROS generation (−57.87%), when compared to LDL-AGEs-treated HEK-293 cells but this diminution in intracellular ROS content was not up to that of caused by our test inhibitors (Fig. 4).

3.5. HMG-R inhibitors and EZ down-regulate the expression of RAGE and its transcriptional activator NFkB-2 in LDL-AGEs treated HEK-293 cells

Receptor of AGE (RAGE), when interacts with AGEs, triggers rapid ROS generation and the up-regulation of inflammatory pathways via RAGE-associated signal transduction. Therefore, cellular levels of RAGE expression were analyzed in the present study to elucidate whether RAGE expression responded concomitantly to the LDL-AGEs treatment and we reported that incubation with LDL-AGEs resulted in a significant ($p < 0.001$) up-regulation in RAGE mRNA expression (from 1.16 to 1.87 folds) in HEK-293 cells, while the treatment with 50 μM test compounds (AT, TT, RT, EZ, EZ-AT, and EZ-RT) showed a marked down-regulation up to 1.21, 1.07, 1.45, 1.08,1.14,and 1.17 folds relative to control cells, respectively, which was significantly lower than the RAGE mRNA level found in LDL-AGEs treated cells. In contrast, AG exerted lesser effect on down-regulation of RAGE mRNA expression which was found to be up to 1.45 folds relative to control HEK-293 cells (Fig. 5.a).

On the other hand, the expression of NFκB-2, the transcriptional inducer of RAGE, was found to be significantly ($p < 0.001$) up-regulated by 1.73 folds relative to the control cells, when HEK-293 cells were exposed to LDL-AGEs (200 μg/mL) only for 24 h. However, this up-regulation of NFκB-2 expression in LDL-AGEs (200 μg/mL) exposed HEK-293 cells was suppressed up to 1.18, 0.94, 1.44, 0.96, 1.02 and 1.05 folds of control in 50 μM AT,TT, RT, EZ, EZ-AT, and EZ-RT treated cell, respectively, which was significantly lower than the NFκB-2 mRNA level found in LDL-AGEs treated cells. Reference anti-glycating agent

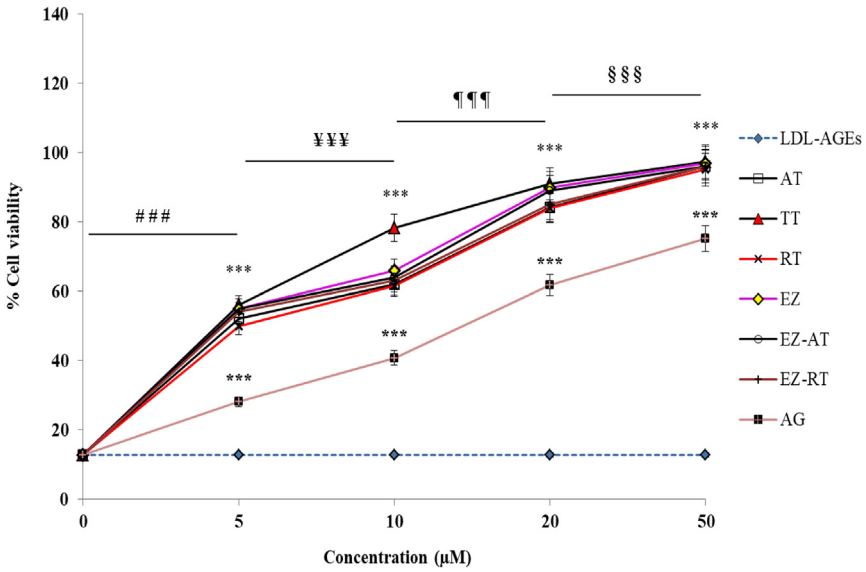


Fig. 3. HMG-R inhibitors and EZ protect HEK-293 cells against LDL-AGEs-induced cytotoxicity. Exponentially growing HEK-293 cells (5×10^3 cells/well) were seeded in a 96-well culture plate and left overnight followed by treatment with 200 μg/mL of LDL-AGEs and varying concentrations of HMG-R inhibitors and EZ (0, 5, 10, 20, and 50 μM) simultaneously and incubated for 24 h to perform MTT assay. The data (% cell viability) are mean ± SEM of three independent experiments. The level of statistical significance was determined by two-way ANOVA on GraphPad Prism software (version 4.02) for Windows. The level of significance for LDL-AGEs v/s different concentrations (5, 10, 20, and 50 μM) of AT, TT, RT, EZ, EZ-AT, EZ-RT, and AG was $^{***}p < 0.001$. The level of significance for 0 μM v/s 5 μM of all test inhibitors was $^{###}p < 0.001$; 5 μM v/s 10 μM at $^{¥¥¥}p < 0.001$; 10 μM v/s 20 μM at $^{¶¶¶}p < 0.001$; 20 μM v/s 50 μM at $^{§§§}p < 0.001$.

AG also showed a slight down-regulation (up to 1.505 folds of control) of NFκB-2 expression (Fig. 5.b).

3.6. HMG-R inhibitors and EZ down-regulate the expression of MMP-2 and its transcriptional activator TGFβ-1 in LDL-AGEs-treated HEK-293 cells

Further, the expression of MMP-2 was also assessed to investigate whether the above mentioned test compounds halt the LDL-AGEs-triggered inflammatory response in HEK-293 cells or not. As a result, we depicted that the level of MMP-2 was found to be significantly ($p < 0.001$) up-regulated up to 1.794 folds in the LDL-AGEs-exposed HEK-293 cells, when compared to control HEK-293 cells. However, treatment with 50 μM AT, TT, RT, EZ, EZ-AT, and EZ-RT suppressed the MMP-2 mRNA expression up to 1.27, 1.07, 1.28, 1.08, 1.09, and 1.26 folds of control, respectively. In contrast, AG showed slight down-regulation in MMP-2 mRNA expression (up to 1.51 folds of control) which was comparatively closer to the MMP-2 mRNA level found in LDL-AGEs-exposed cells (Fig. 6.a).

Moreover, our qRT-PCR analysis clearly depicted that the exposure to LDL-AGEs resulted in a significant ($p < 0.001$) up-regulation of 1.76 folds in TGFβ-1mRNA expression in HEK-293 cells, when compared to control HEK-293 cells. However, treatment with 50 μM AT, TT, RT, EZ, EZ-AT, and EZ-RT significantly down-regulated the TGFβ-1mRNA expression in LDL-AGEs treated HEK-293 cells (up to 1.21, 1.13, 1.39, 1.2, 1.14, and 1.16 folds of control, respectively). Treatment with AG also down-regulated the TGFβ-1 mRNA expression (up to 1.51 folds of control) but this down-regulation in TGFβ-1 expression by AG was not comparable to the inhibition observed in HMG-R inhibitors treated HEK-293 cells (Fig. 6.b).

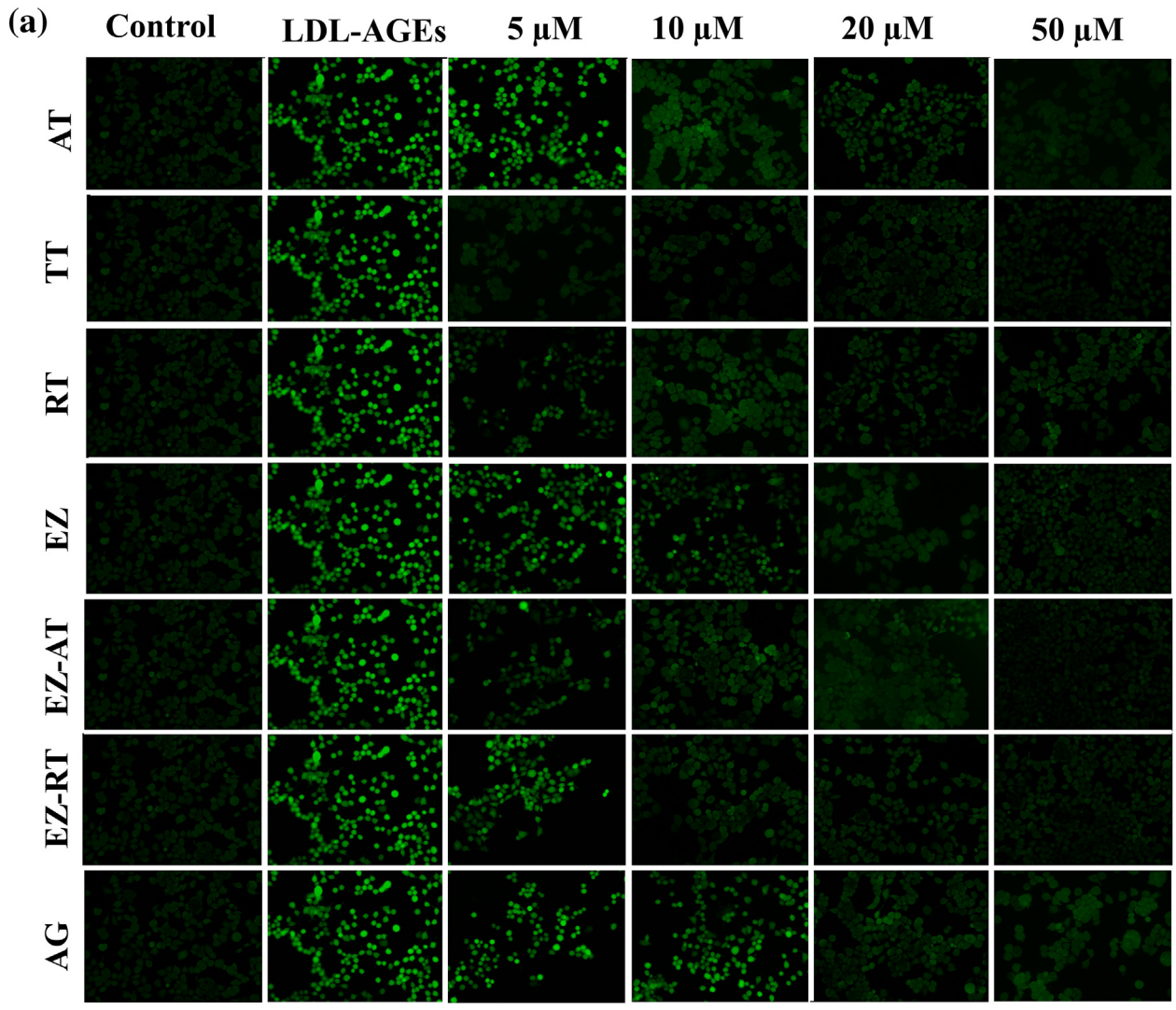
3.7. HMG-R inhibitors and EZ up-regulated the expression of NRP-1 in LDL-AGEs treated HEK-293 cells

Considering the potential regulatory role of NRP-1 in the growth and development of various cells including ECs, neural cells and renal podocytes we analyzed the impact of LDL-AGEs-exposure on NRP-1 expression in HEK-293 cells and their modulation by subsequent treatment with HMG-inhibitors and EZ. In this order, our qRT-PCR analysis showed that the exposure to LDL-AGEs significantly ($p < 0.001$) reduced the expression of NRP-1mRNA in HEK-293 cells. However, treatment with 50 μM AT, TT, RT, EZ, EZ-AT, and EZ-RT significantly up-regulated the NRP-1 mRNA expression up to 1.61, 1.69, 1.61, 1.68, 1.67 and 1.65 folds, respectively, in LDL-AGEs treated HEK-293 cells, when compared to untreated LDL-AGEs-exposed cells. AG treated HEK-293 cells showed slightly lesser increase in Nrp-1 expression (up to 1.51 folds) in LDL-AGEs-exposed HEK-293 cells, when compared to the effect of our test inhibitors on NRP-1 expression (Fig. 7).

4. Discussion

The current study was attempted to delineate the modulatory role of the HMG-R inhibitors alone as well as in combination with EZ on LDL-AGEs-induced cytotoxicity, intracellular ROS generation and mechanistic action of these inhibitors on LDL-AGEs-induced cellular signalling in HEK-293.Assessment of cytotoxicity of therapeutic agents is a most crucial factor in the field of drug development and disease targeting through chemical agents. In this order, MTT assay, used to determine the cell viability, is the most frequently implied in vitro cytotoxicity assessment method [27]. In the present study, we analyzed the modulatory effects of different HMG-R inhibitors and EZ on the viability of HEK-293 cells and found that these compounds (up to 200 μM) showed no significant cytotoxic effects on HEK-293 cells when used for 24 and 48 h, however, AG showed noticeable cytotoxicity on HEK-293 cells when incubated for 48 h. In contrast, LDL-AGEs also showed a marked impact on the HEK-293 cells viability, even exposed only for 24 h. This negative effect of LDL-AGEs on HEK-293 cells may be attributed either to their ability to cause oxidative stress leading to cellular-DNA-damage and subsequent cell death or to enhanced AGE-RAGE signalling due to the presence of neoantigenic epitopes on the LDL-AGEs, which are well reckoned to be produced upon glycoxidation process and to elicit immunogenic response [1,2]. Moreover, the treatment with HMG-R inhibitors and EZ markedly restored the viability of LDL-AGEs-exposed HEK-293 cells when compared to only LDL-AGEs treated cells. Such protective impact of HMG-R inhibitors and EZ was possibly achieved due to their strong anti-radical as well as anti-glycation potential [1,2,22].

Furthermore, ROS also functions as the initiator of signal transduction and is known to be induced by the activation of AGE-RAGE axis [2,28]. Similarly, the results from our DCFDA staining assays demonstrated that LDL-AGEs significantly stimulated the ROS generation in HEK-293 cells which may be attributed to the enhanced AGEs-RAGE interaction and subsequent stimulation of distinct signalling pathways leading to the progression of various diabetic complications, particularly nephropathy [2]. However, the higher level of ROS in LDL-AGEs-exposed HEK-293 cells was diminished significantly when treated with



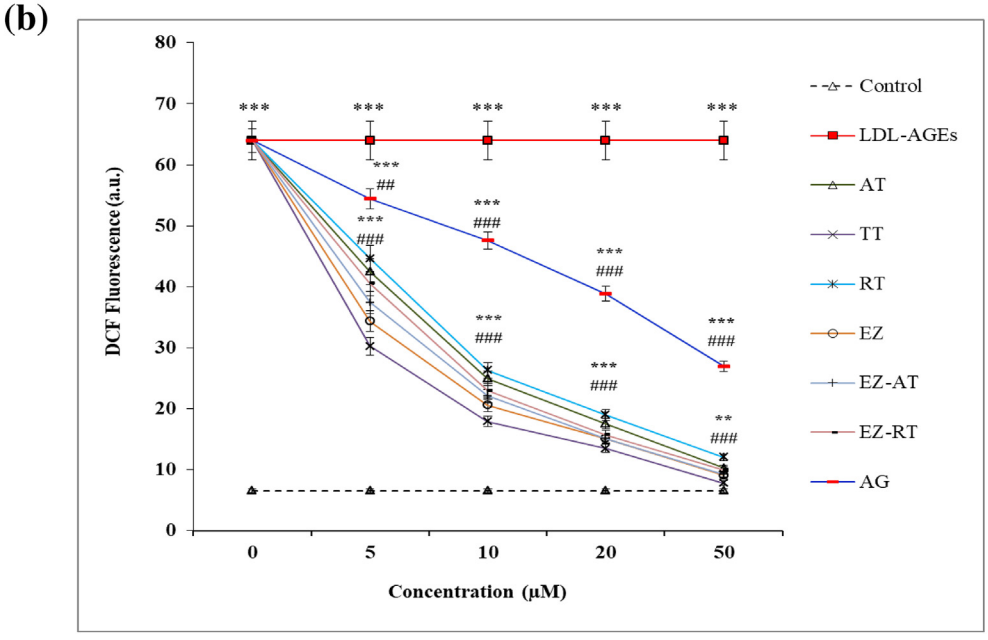


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Fig. 4. Protective effect of HMG-R inhibitors and EZ on LDL-AGEs-induced ROS generation in HEK-293 cells. Panel a: Representative micrographs for DCF fluorescence analyzed by fluorescence microscopy. To The determination of protective role of HMG-R inhibitors and EZ against LDL-AGEs-induced intracellular ROS generation in HEK-293 cells was performed via DCFH-DA method. Briefly, DCFH-DA staining was applied to HEK-293 cells to detect the changes in intracellular ROS level after 24 h treatment with LDL-AGEs (200 μg/mL) in presence/absence (5, 10, 20, and 50 μM) of test compounds (AT, TT, RT, EZ, EZ-AT, EZ-RT, and AG). Panel b: The line diagram is showing the ROS level in HEK-293 cells. The DCF fluorescence (a.u.) has been represented as mean ± SEM of three independent experiments. The level of statistical significance was determined by two-way ANOVA on GraphPad Prism software (version 4.02) for Windows. Significantly different from control HEK-293 cells at $^{***}p < 0.001$. Significantly different from control HEK-293 cells at $^{**}p < 0.01$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#\#}p < 0.001$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#}p < 0.01$. The level of significance between all doses (0 v/s5, 5 v/s 10, 10 v/s 20, and 20 v/s 50 μM) of each test inhibitor was $p < 0.001$ (Not shown in figure to avoid the misinterpretation of the data).

HMG-R inhibitors and EZ. This protective effect of HMG-R inhibitor and EZ treatment may further be accredited to their ability to quench the glycoxidative stress-induced ROS generation [1,29].

In last few decades, the researchers have concentrated on distinct mechanistic aspects of AGEs-RAGE interaction in different vascular complications of diabetes mellitus and the receptor-mediated mechanism demonstrating the role of AGEs in the pathogenesis of these chronic complications have been most widely accepted [2,30]. RAGE is a well-characterized receptor of AGEs and expresses in various cells including HEK-293 cells [30,31]. On the other hand, the accumulation of AGEs up-regulates the mRNA expression as well as protein level of RAGEs (both; soluble and membrane bound) [32]. This augmented expression of RAGE also facilitates the AGEs-induced signalling cascades in glomerular podocytes [12]. Similarly, the results from our gene expression analysis also depicted an up-regulation in the RAGE mRNA expression in LDL-AGEs-exposed HEK-293 cells. Moreover, the treatment with HMG-R inhibitors and EZ markedly suppressed the RAGE expression in LDL-AGEs-induced HEK 293 cells.

It has been also postulated that AGEs-RAGE interaction stimulates intracellular oxidative stress response characterized by increased activation of transcription factors such as NF-κB [32]. The translocation of this transcription factor to the nucleus is well known to up-regulate the expression of RAGE via targeting the RAGE promotor [33]. High levels of NF-κB as well as increased NF-κB signalling have been linked to the enhanced inflammatory responses [2,32]. Our study also reported an up-regulation in the mRNA expression of NF-κB in LDL-AGEs-exposed HEK-293cells. This augmented expression of NF-κB was suppressed significantly by our test inhibitors in a dose dependent manner which advocates the protective role of these compounds in targeting AGEs-induced diabetic complications, particularly microvascular complications.

Moreover, pathogenesis of diabetes has been linked to chronic inflammatory responses, whereas, the production of various cytokines and chemokines are believed to contribute positively in the progression of diabetic nephropathy (DN) [34]. Among distinct cytokines/inflammatory mediators, TGF-β1 is a well-established causal factor for the progression of DN [34,35]. We also reported an up-regulation of TGF-β1 mRNA expression in LDL-AGEs-exposed HEK-293 cells and that

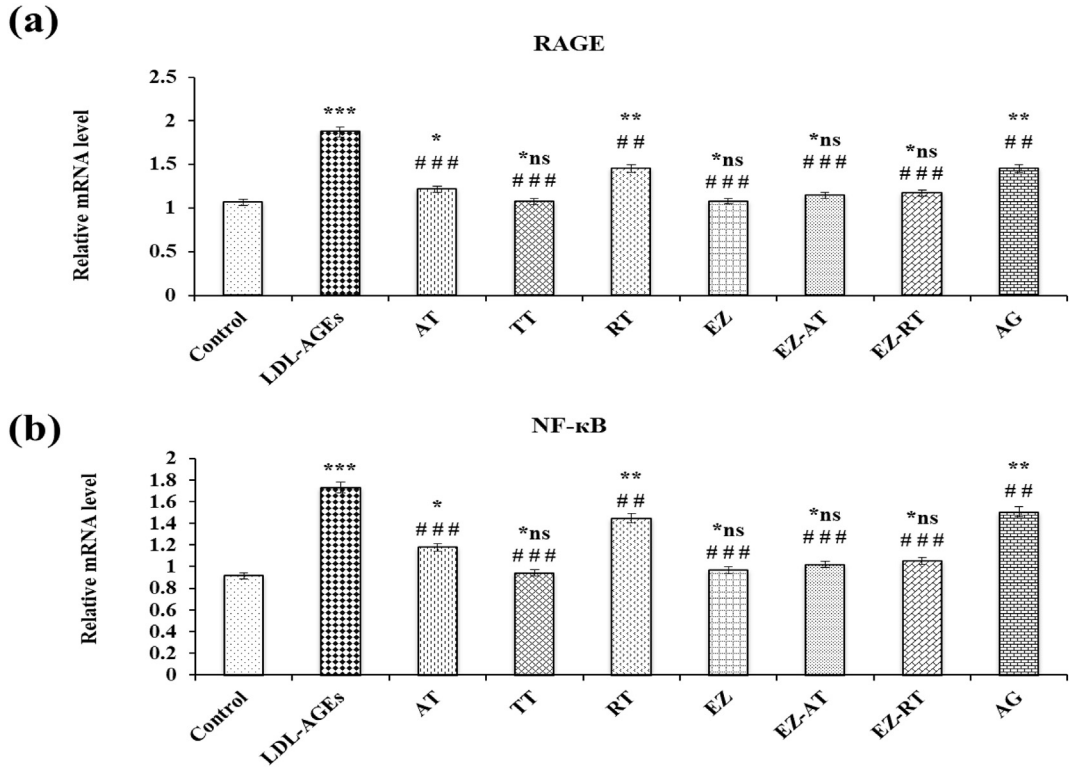

Fig. 5. HMG-R inhibitors and EZ down-regulate expression of RAGE (Panel a) and its transcriptional activator NFkB-2 (Panel b) in LDL-AGEs-induced HEK-293 Cells. HEK-293 cells were seeded in a 24-well plate at a density of 1.2×10^6 cells/well and incubated for 24 h at 37 °C for adherence. Following the incubation, HEK-293 cells were treated with LDL-AGEs (200 μg/mL) in presence or absence of test compounds (50 μM HMG-R inhibitors and EZ) and incubated for 24 h. After incubation for 24 h, cells were extracted for mRNA isolation, cDNA synthesis and qRT-PCR analysis. Values are expressed as mean ± SEM of three independent experiments. The level of statistical significance was determined by one-way ANOVA on GraphPad Prism software (version 4.02) for Windows. Significantly different from control HEK-293 cells at $^{***}p < 0.001$. Significantly different from control HEK-293 cells at $^{**}p < 0.01$. Significantly different from control HEK-293 cells at $^{*}p < 0.05$. No significant difference from control HEK-293 cells at $^{ns}p > 0.05$. Significantly different from LDL-AGEs treated HEK-293 cells at$^{\#\#\#}p < 0.001$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#}p < 0.01$.

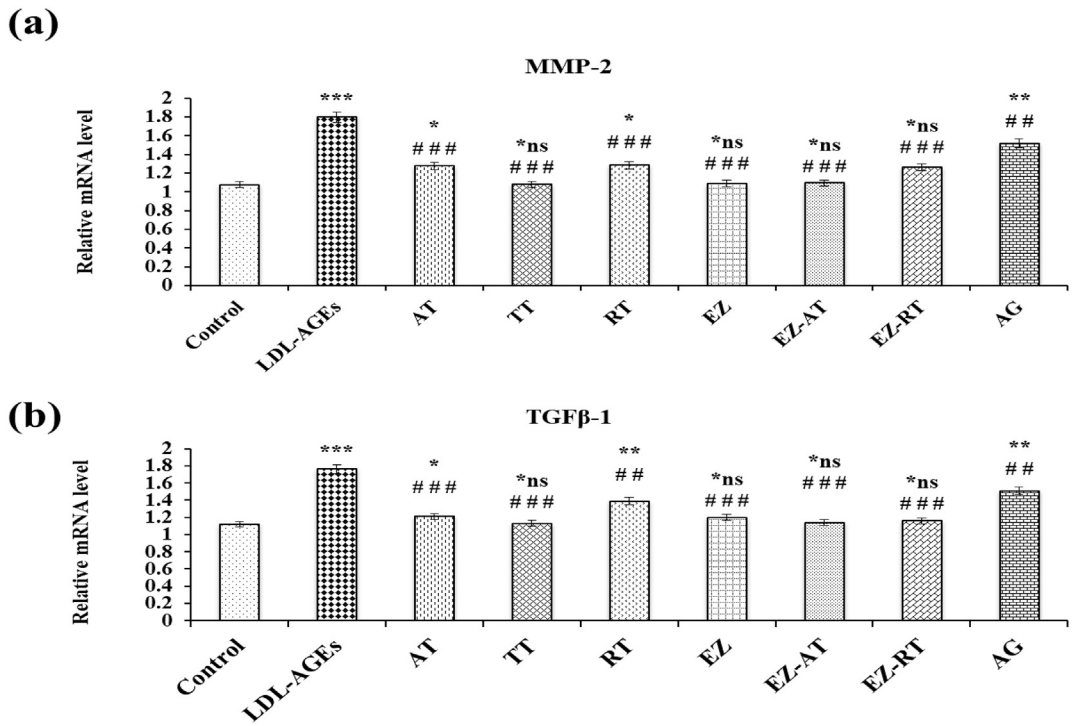

Fig. 6. HMG-R inhibitors and EZ down-regulate the expression of MMP-2 (Panel a) and its transcriptional activator TGFβ-1 (Panel b) in LDL-AGEs-induced HEK-293 Cells. The strategies for the HEK-293 cells culture and treatment with LDL-AGEs and our test compounds (HMG-R inhibitors and EZ) were same as described in Fig. 5. Values are expressed as mean ± SEM of three independent experiments. The level of statistical significance was determined by one-way ANOVA on GraphPad Prism software (version 4.02) for Windows. Significantly different from control HEK-293 cells at $^{***}p < 0.001$. Significantly different from control HEK-293 cells at $^{**}p < 0.01$. Significantly different from control HEK-293 cells at $^{*}p < 0.05$. No significant difference from control HEK-293 cells at $^{*ns}p > 0.05$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#\#}p < 0.001$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#}p < 0.01$.

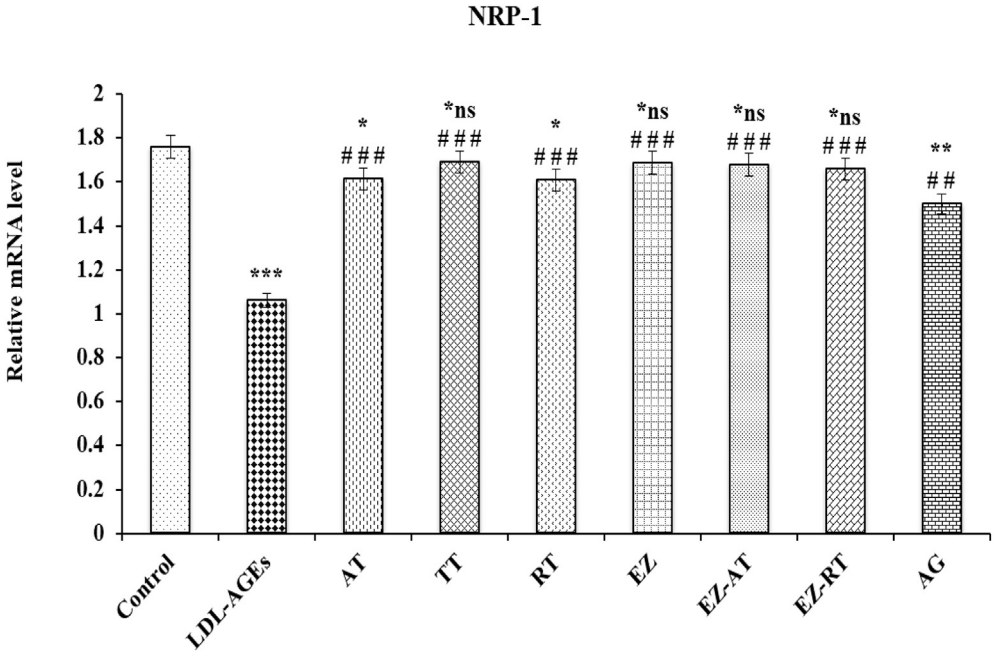

Fig. 7. HMG-R inhibitors and EZ up-regulate NRP-1 expression in LDL-AGEs-induced HEK-293 Cells. The strategies for the HEK-293 cells culture and treatment with LDL-AGEs and our test compounds (HMG-R inhibitors and EZ) were same as described in Fig. 5. Values are expressed as mean ± SEM of three independent experiments. The level of statistical significance was determined by one-way ANOVA on GraphPad Prism software (version 4.02) for Windows. Significantly different from control HEK-293 cells at $^{***}p < 0.001$. Significantly different from control HEK-293 cells at $^{**}p < 0.01$. Significantly different from control HEK-293 cells at $^{*}p < 0.05$. No significant difference from control HEK-293 cells at $^{*ns}p > 0.05$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#\#}p < 0.001$. Significantly different from LDL-AGEs treated HEK-293 cells at $^{\#\#}p < 0.01$.

may be attributed to the LDL-AGEs-triggered ROS generation which is a well reckoned factor responsible for the induction of TGF-β-1 mRNA level in different types of renal cells [35,36]. Meanwhile, treatment with HMG-R inhibitors and EZ significantly down-regulated the TGF-β1 expression in LDL-AGEs-treated HEK-293 cells and these beneficial effects were possibly achieved due to the ability of our test compounds to diminish the ROS generation as well as down-regulation of RAGE expression and associated signalling.

On the other hand, MMP -2 is a prime target in DN and chronic kidney disease (CKD) as it contributes to the establishment of DN and CKD via degradation of the extracellular matrix in various renal tissues/cells [37]. The expression of MMP-2 is stimulated under CKD and renal fibrosis due to co-stimulation of TGF-β, a well reckoned activator of MMP-2 transcription [37,38]. Further, AGEs are also known to

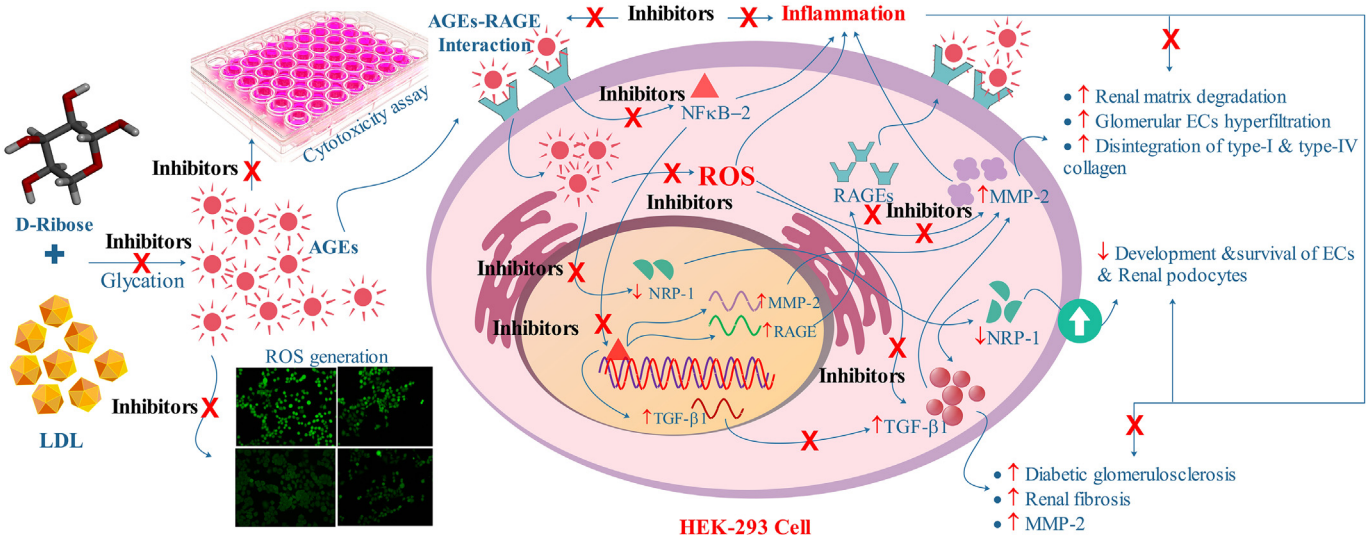

Fig. 8. Overall regulatory mechanisms of HMG-R inhibitors and EZ against LDL-AGEs-induced alterations in HEK-293 cells. HMG-R inhibitors and EZ protect LDL-AGEs-induced alterations in HEK-293 cells via inhibiting/slowing down the process of glycation; inhibition of intracellular ROS generation; inhibition of LDL-AGEs induced death of HEK-293 cells (cytotoxicity);interruption of AGEs-RAGE interaction; inhibition of AGEs-RAGE signalling; inhibition of NFκB-2 translocation to nucleus; blocking the NFκB-2 signalling leading to reduced RAGE expression as NFκB-2 over-stimulates the expression of RAGE via binding to RAGE promotor; suppression of TGF-β1 expression thereby reduction in diabetic glomerulosclerosis and renal fibrosis; down-regulation of MMP-2 expression leading to reduced renal matrix degradation, glomerular ECs hyperfiltration, and disintegration of type-I & type-IV collagen; inhibition of ROS-mediated MMP-2 and TGF-β1signaliing; inhibition of inflammation and inflammation-induced signalling cascades responsible for HEK-293 cell degradation; and up-regulation of NRP-1 which is essential for development and survival of ECs and renal podocytes.

participate in CKD development by enhanced glomerular hyperpermeability due to the disruption in tight junctions [16]. Similar to these findings, we also reported an up-regulation of MMP-2 mRNA expression in LDL-AGEs-induced HEK-293 cells that may be attributed to the LDL-AGEs-triggered over-expression of RAGE and subsequent signalling which in turn activated the transcription of TGF-β and NF-κB in HEK-293 cells. The expression of both these transcription factors is known for the activation of MMP-2. This over-expression of MMP-2 in LDL-AGEs-exposed HEK-293 cells was significantly down-regulated by treatment with HMG-R inhibitors and EZ in our study which was possibly achieved by their potent anti-inflammatory properties and pointing towards the implication of these compounds in DN, CKD, and end stage renal disease (ESRD). These results are well in agreement with a recent study that also reported the efficacy of these compounds/statins in the management of CKD and ESRD [39].

Moreover, the expression of NRP-1 has been linked to the major functions i.e. migration, growth rate, and survival in various cells including neuronal cells, renal podocytes and ECs via targeting p53/caspase axis [40,41].NRP-1 mediated functions of the cell are controlled by vascular endothelial growth factor (VEGF) as NRP-1 acts as a receptor for VEGF [40]. Further, Bondeva et al. [42] showed that BSA-AGEs down-regulate the NRP-1 expression in differentiated podocytes. In the same context, we analyzed the impact of LDL-AGEs exposure onNRP-1 mRNA expression HEK-293 cells as these cells as well as renal podocytes are the preferential targets of AGEs-associated RAGE signalling. In this attempt, we observed a significant down-regulation of NRP-1 expression in LDL-AGEs-treated HEK-293 cells and our findings are well justified by previously published studies [12]. However, this compromised expression of NRP-1 in LDL-AGEs-treated HEK-293 cells was augmented by the treatment with our test HMG-R inhibitors and EZ. This ameliorative effect of these compounds on NRP-1 expression in AGEs-induced toxic environment shows their potent pharmacological ability to counter act the diabetes and associated complications i.e. DN, CKD, and ESRD. To sum up, the HMG-R inhibitors and EZ protect LDL AGEs-induced alterations in HEK-293 cells which is because of their antioxidant and anti-glycation potential that basically inhibits intracellular ROS generation as well as LDL-AGEs-induced toxicity to HEK-293 cells. Moreover, they also prevented the AGE-RAGE-interaction and associated signalling via down-regulation of NF-κB which ultimately suppressed the RAGE expression (Fig. 8). Our inhibitors also down-regulated the NF-κB-mediated expression of inflammatory cytokine (i.e. MMP-2) and TGFβ-1 and their signalling in order to protect against LDL-AGEs-induced aberrations to HEK-293 cells as both of these are reckoned to trigger inflammatory cascades in renal cells. In addition, HMG-R inhibitors and EZ also up-regulated the expression of NRP-1 which is essential for the development and survival of various renal cells including ECs and renal podocytes. These beneficial pharmacological effects of HMG-R inhibitors and EZ advocate their implications in targeting diabetes/AGEs-induced glomerulosclerosis and renal fibrosis, renal matrix degradation, glomerular ECs hyperfiltration, and disintegration of type-I & type-IV collagen.

5. Conclusions

Conclusively, the result from our cytotoxicity assay/MTT assay evident no significant impact of HMG-R inhibitors and EZ on viability of HEK-293 cells after 24 and 48 h, whereas, LDL-AGEs negatively affected the HEK-293 cell viability. Moreover, this study for the first time infers that HMG-R inhibitors and EZ prevent LDL-AGEs or glycoxidative stress-induced cytotoxicity and cellular damage via diminished ROS generation and down-regulation of RAGE expression and other mediators of RAGE-associated signalling i.e. NF-κB, TGFβ-1, and MMP-2. In contrast, these compounds also augmented the expression of NRP-1 as it was down-regulated upon incubation with LDL-AGEs. These pharmacological effects signify that HMG-R inhibitors (TT, AT & RT) and EZ may implied in the treatment of AGEs-induced oxidative stress and tissue damage in diabetic complications, particularly CKD and ESRD, via targeting intracellular-ROS, NRP-1 functionality and RAGE-associated signalling.

Conflict of interest

Authors declare that they have no conflict of interest.

Declaration of competing interest

The authors declare no competing financial interests.

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