

Original Research

Differential Modulation of Oxidative Stress and VEGF-A Secretion by Saffron Repron® Pretreatment in ARPE-19 Cells Exposed to High Glucose

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Academic Editor: Shikun He

Submitted: 18 June 2026 Revised: 15 September 2026 Accepted: 29 September 2026 Published: 20 October 2026

Abstract

Background: Hyperglycemia contributes to diabetic retinopathy by promoting oxidative stress, inflammatory signaling, retinal pigment epithelial (RPE) dysfunction, and vascular endothelial growth factor A (VEGF-A) secretion. Saffron Repron® (sR), selected (and patented) according to its chemical composition, has been shown to activate neuroprotective responses. This study investigated whether sR pretreatment modulates high glucose-induced oxidative damage, antioxidant and inflammatory signaling, VEGF-A secretion, and paracrine endothelial activation in ARPE-19 cells. **Methods:** ARPE-19 cells were pretreated with sR (40 µg/mL for 24 h), washed, and exposed to high-glucose (HG) (25 mM) for 24 or 96 h. Cell viability and cytotoxicity were assessed using colorimetric assays, reactive oxygen species (ROS) using a luminescence assay, superoxide dismutase 2 (SOD2), nuclear factor erythroid 2–related factor 2 (NRF2), KEAP1, nuclear factor kappa B (NF-κB), and IκBα expression by western blotting, catalase activity by spectrophotometry, and VEGF-A secretion by bioluminescent immunoassay. Human microvascular endothelial cells (HMEC-1) were exposed to ARPE-19 conditioned media for 24 h, after which viability and ROS levels were evaluated. Data were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. **Results:** sR alone did not substantially affect basal ARPE-19 viability or VEGF-A secretion. HG increased cytotoxicity and ROS levels at 24 h and reduced viability, while increasing the cytotoxicity/viability ratio and ROS levels at 96 h. sR reduced HG-induced cytotoxicity, restored viability at 96 h, and normalized ROS levels. However, SOD2 expression and catalase activity were reduced by HG and/or sR, indicating that sR-mediated ROS reduction was not associated with induction of these antioxidant defenses. Total NRF2 expression and the NRF2/KEAP1 ratio remained unchanged, although sR prevented HG-induced KEAP1 upregulation at 24 h. HG altered the NF-κB/IκBα balance and increased VEGF-A secretion at both time points; sR did not prevent increase in VEGF-A at 24 h but significantly reduced the HG-induced increase at 96 h. Conditioned media from HG-treated ARPE-19 cells increased HMEC-1 viability and ROS levels, whereas media from sR-pretreated HG cells attenuated these effects. **Conclusions:** Pretreatment with sR protects ARPE-19 cells from HG-induced cytotoxicity and oxidative stress primarily by reducing ROS levels rather than by inducing NRF2/SOD2-associated antioxidant responses. sR pretreatment also attenuated the pro-oxidative paracrine effects of HG-exposed ARPE-19 RPE model on endothelial cells, supporting further investigation of its potential protective effects in models of hyperglycemic retinal stress.

Keywords: ARPE-19 cells; Saffron Repron®; diabetic retinopathy; oxidative stress; NADPH oxidases; vascular endothelial growth factor A

1. Introduction

Hyperglycemia is a major cause of retinal damage in diabetic retinopathy, one of the most common complications of diabetes, by promoting oxidative stress, inflammation, and vascular dysfunction within the retinal microenvironment [1,2,3]. The burden of the disease is substantial: pooled data from population-based studies indicate that diabetic retinopathy affects approximately 34.6% (95% CI 34.5–34.8%) of people with diabetes aged 20 to 70 years, with proliferative disease in 6.96% and sight-threatening retinopathy in 10.2%, corresponding to roughly 28 mil-

lion people worldwide [4]. Beyond its vascular component, diabetic retinopathy (DR) is increasingly conceptualized as a disease of the neurovascular unit, in which neurons, glial elements, vascular cells, and the retinal pigment epithelium (RPE) are functionally interconnected [5], and retinal dysfunction has been documented in patients with no or minimal microvascular retinopathy, suggesting that early neurodegeneration may precede overt microvascular lesions [6]. RPE plays a crucial role in maintaining retinal homeostasis by contributing to the outer retinal barrier, supporting photoreceptor function, regulating oxida-



tive balance, and secreting trophic and angiogenic factors [7,8]. Among these, vascular endothelial growth factor A (VEGF-A) is particularly relevant [9]. Under physiological conditions, VEGF-A contributes to choroidal vascular maintenance and retinal integrity [10]. However, sustained or dysregulated VEGF-A production under diabetic or hyperglycemic conditions may promote vascular permeability, inflammatory responses, and pathological angiogenesis, thereby contributing to retinal vascular alterations and disease progression. In the diabetic retina, VEGF-A — secreted mainly by retinal neurons and glia — activates the endothelium through VEGFR2 binding and is implicated in vascular hyperpermeability and pathological neovascularization [11].

Several studies have demonstrated that chronic exposure to high-glucose (HG) conditions affects RPE function, leading to excessive reactive oxygen species (ROS) production, mitochondrial dysfunction, and activation of pro-inflammatory signaling pathways [12,13,14]. In ARPE-19 cells, HG has been shown to promote ROS generation and apoptosis through ROS-mediated inhibition of the PINK1/Parkin mitophagy pathway [15], while prolonged hyperglycemic exposure induces oxidative stress, DNA damage, and premature senescence, together with a decrease in the antioxidant protein superoxide dismutase 2 (SOD2) [16]. Oxidative stress represents a key pathogenic mechanism linking hyperglycemia to retinal degeneration [17,18]. Increased ROS levels may activate redox-sensitive transcription factors, including nuclear factor kappa B (NF- κ B), which promotes the expression of inflammatory and pro-angiogenic mediators, including VEGF-A. In contrast, nuclear factor erythroid 2-related factor 2 (NRF2) is a major regulator of cellular antioxidant defense, regulating the expression of detoxifying and antioxidant enzymes such as SOD2 [19]. Consistent with this role, NRF2 signaling has been identified as an essential protective system in the RPE, where it also contributes to the maintenance of mitochondrial function and metabolism [20]. Interestingly, NRF2 and NF- κ B signaling pathways are functionally interconnected and may compete for shared transcriptional coactivators, such as CBP/p300, thereby influencing the balance between antioxidant protection and inflammatory activation [21].

Natural compounds, either administered as dietary supplements or incorporated into topical formulations, have attracted increasing interest as adjunctive strategies for ocular diseases because of their potential antioxidant, anti-inflammatory, and cytoprotective properties [22,23,24]. Saffron (*Crocus sativus*) is among the best-characterized natural products for its anti-inflammatory and cytoprotective properties [25]. Experimental and clinical evidence has suggested a potential protective role of saffron-derived compounds in retinal diseases, including diabetic retinopathy (DR), age-related macular degeneration (AMD), retinitis pigmentosa, and glaucoma [25,26,27], increasing the

interest in saffron-based formulations as neuroprotective agents in retinal pathologies. Accordingly, a growing number of human studies have investigated the effects of saffron and its constituents in ocular diseases, including diabetic maculopathy [28]. In particular, saffron bioactive compounds, such as crocins and crocetin, have been reported to counteract oxidative stress, mitochondrial dysfunction, and retinal degeneration in several experimental models [29,30,31,32]. Of note, preclinical evidence indicates that the crocin fraction exerts cytoprotective, antioxidative, anti-inflammatory and blood flow-enhancing effects in retinal tissue, in part through activation of PI3K/Akt and inhibition of NF- κ B signaling [33]. A well-defined ratio among molecules is linked to a patented batch known as Saffron Repron® (sR) and offers the maximum protection against neurodegeneration [34]. sR slows down retinal degeneration in both genetically and induced degenerative processes [35,36].

We previously demonstrated that saffron protects ARPE-19 cells against oxidative stress and inflammatory damage [37,38]. However, the mechanisms underlying the persistence of this protection after saffron withdrawal remain incompletely defined, particularly under HG conditions. Unlike studies in which saffron or its constituents remain present during the damaging stimulus, here we investigated whether a transient 24-h pretreatment with sR, followed by complete compound removal, is sufficient to confer sustained protection against subsequent HG exposure. Beyond evaluating cytotoxicity, ROS generation, inflammatory signaling, and VEGF-A secretion, we specifically examined whether the persistent redox protection induced by sR is associated with modulation of ROS-generating NADPH oxidase activity and changes in overall cellular antioxidant capacity, rather than with activation of the canonical NRF2/SOD2 antioxidant response. Finally, we assessed whether these changes modify the paracrine effects of HG-exposed ARPE-19 cells on microvascular endothelial cells. This integrated approach was designed to distinguish among reduced ROS-generating activity, increased total antioxidant capacity, and induction of specific endogenous antioxidant pathways as potential mechanisms underlying the sustained protective effects of sR.

2. Materials and Methods

2.1 Cell Culture

The human retinal pigment epithelial cell line ARPE-19 (ATCC CRL-2302; RRID: CVCL_0145) and the human dermal microvascular endothelial cell line HMEC-1 (ATCC CRL-3243; RRID: CVCL_0307) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines are authenticated by ATCC using short tandem repeat (STR) profiling, and their identity was cross-checked against the Cellosaurus database. The cell cultures used in this study were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma

Detection Kit (Lonza, Basel, Switzerland) and tested negative. All cell lines were maintained at 37 °C in a 5% CO₂ atmosphere. ARPE-19 cells were grown in DMEM/F12 1:1 medium (Life Technologies Italia, Milan, Italy) supplemented with 10% fetal bovine serum and 2 mM glutamine (Euroclone, Milano, Italy) at 37 °C in 5% CO₂ up to confluence. Afterward, cells were seeded in multi-well plates, allowed to reach confluence, and then cultured for 7–9 days in DMEM/F12 supplemented with 3% FBS to promote monolayer differentiation [39]. Then, cells were pre-treated or not with 40 µg/mL Saffron Repron® (sR, gently gifted by Hortus Novus srl) for 24 hours. Afterward, cells were extensively washed to remove sR and incubated for 24 or 96 hours in the presence or absence of 25 mM glucose. Accordingly, the experimental conditions were as follows: cells cultured under standard conditions (CTR), cells pre-treated with sR (sR CTR), cells exposed to HG, and cells pretreated with sR before HG exposure (sR HG). HMEC-1 cells were grown in MCDB131 medium (Life Technologies Italia, Milan, Italy) supplemented with 10% fetal bovine serum and 10 mM glutamine (Euroclone, Milano, Italy) up to confluence. Then, conditioned media collected from ARPE-19 cells treated with or without sR and HG were added to HMEC-1 cells for 24 h. At the end of the treatment, viability and ROS production were evaluated.

2.2 Cell Viability Assays

To evaluate cell proliferation, ARPE-19 cells were seeded on 96-well plates (10×10^3 cells/well) and cultured as described. Viable cells were determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbance was recorded using a microplate reader (Tecan Trading AG, Männedorf, Switzerland) at the wavelength of 490 nm. Results were expressed as a percentage of absorbance in each treatment compared to the absorbance value of the control (CTR, 100%).

2.3 Cell Cytotoxicity Assay

To evaluate HG-induced cell cytotoxicity, ARPE-19 cells were seeded on 96-well plates (10×10^3 cells/well) and cultured as described. Cytotoxicity was determined using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, CytoTox 96® Non-Radioactive Cytotoxicity Assay quantitatively measures the amount of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, released LDH in culture supernatants with a 30-minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (iodonitrotetrazolium violet; INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Absorbance was recorded using a microplate reader (Tecan Trading AG, Männedorf, Switzerland) at the wavelength of 490 nm. Results were expressed

as a percentage of absorbance in each treatment compared to absorbance value of the control (CTR, 100%).

2.4 Evaluation of Intracellular ROS

The levels of ROS were assessed using a Reactive Oxygen Species Assay Kit (Promega, Madison, WI, USA), adhering to the manufacturer's protocol. In brief, cells were seeded into 96-well culture plates at 10×10^3 cells/well and treated as previously described. Then, substrate solution was added to each well. Subsequently, the media samples were transferred to a separate plate and combined with an equal volume of Detection solution. The relative luminescence units were recorded using a plate-reading luminometer (Glomax, Promega, Madison, WI, USA). Results were expressed as a percentage of luminescence compared to control (CTR, 100%).

2.5 Cell Lysis and Subcellular Fractionation

At the end of the experiments, a set of cells was processed for subcellular fractionation using the Subcellular Protein Fractionation Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, various cellular compartments were isolated by sequential addition of different extraction buffers to the cell pellet. Cytosolic, nuclear soluble, and chromatin-bound protein extracts were collected, stored at –80 °C, and then used for immunoblotting analysis. GAPDH, HDAC2, and H3 expression was evaluated by western blot analyses (described below) as markers of the cytoplasmic, nuclear soluble, and chromatin-bound fractions, respectively.

2.6 Western Blot Analysis

At the end of the experiments, a batch of ARPE-19 cells was lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Pierce, Rockford, MD, USA), and protein concentrations were determined using the BCA Protein Assay Kit. Thirty micrograms of total cell proteins and subcellular compartments were separated on 4–20% SDS-PAGE gradient gels (Life Technologies Italia, Milan, Italy) and transferred onto nitrocellulose using the iBlot system 3 (Life Technologies Italia, Milan, Italy). Filters were blocked in Protein Free T20 Blocking Buffer (Pierce Biotechnology, Rockford, IL, USA) and incubated overnight at 4 °C with primary antibodies specific to NRF2 (D1Z9C, cat 12721, dil 1/1000), Keap-1 (D6B12, cat 8027, dil 1/1000), NF-κB p65 (cat. 6956, dil 1/1000) and IKBα (cat. 9242, dil 1/1000), H3 (D1H2, cat. 4499, dil 1/2000), GAPDH (14C10, cat. 2118, dil 1/5000), HDAC2 (3F3, cat. 5113, dil 1/1000) and β-ACT (8H10D10 cat. 3700, dil 1/10000) from Cell Signaling Technology (Beverly, MA, USA) and SOD2 (cat A19576, dil 1/2000) from AbClonal Germany GmbH (Düsseldorf, Germany). Secondary specific horseradish-peroxidase-linked antibodies Anti-rabbit IgG (cat 7074, dil 1/3000) and Anti-mouse IgG (cat 7076, dil 1/2000) (Cell Signaling Technology, Bev-

erly, MA, USA) were added and left for 1 h at room temperature. Bound antibodies were detected using the enhanced chemiluminescence lighting system (LiteAbloT EX-TEND, Euroclone, Milan, Italy), according to the manufacturer's instructions. Bands of interest were quantified by densitometry using Alliance LD2 with Uvitec 1D software (v18.07, UVITEC Ltd., Cambridge, UK). The results were expressed as a percentage of CTR (defined as 100%).

2.7 VEGF-A Secretion

To evaluate VEGF-A secretion, ARPE-19 cells were seeded in a 96-well plate and incubated as previously described. VEGF-A release was evaluated using the bioluminescent assay Lumit® VEGF-A (Human) Immunoassay (Promega, Madison, WI, USA). Briefly, medium was transferred from the cell culture plate to another assay plate, incubated with a pair of anti-human VEGF-A monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies recognize and bind to released VEGF-A, the complementary LgBiT and Sm-BiT are brought into proximity, thereby reconstituting the NanoBiT® enzyme and generating luminescence in the presence of the Lumit® substrate. The luminescence generated is directly proportional to the amount of VEGF-A present in the sample. Secretion was normalized to the cell content of the corresponding well. Results were shown as pg/mL.

2.8 Catalase Activity Assay

Catalase activity was assessed as a marker of antioxidant defense capacity. For each assay, 50 µg of total protein was used. Enzymatic activity was determined spectrophotometrically by following the decomposition of H₂O₂ at 240 nm. The reaction mixture contained 100 mM Tris-HCl buffer, pH 7.4, and 5 mM H₂O₂.

2.9 NADPH Oxidase Activity Assay

NADPH oxidase (NOX) activity was evaluated spectrophotometrically in cell homogenates by measuring the superoxide-dependent reduction of cytochrome c at 550 nm. The reaction mixture contained 60 µM cytochrome c, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1 mM NADPH. Parallel reactions were performed in the presence or absence of 300 U/mL superoxide dismutase (SOD). After incubation for 5 min at 37 °C, samples were centrifuged at 800 ×g for 5 min, and the absorbance of the supernatant was measured at 550 nm. NOX activity was calculated as the SOD-inhibitable cytochrome c reduction, determined from the difference between the absorbance measured in the absence and presence of SOD. Enzymatic activity was normalized to the protein concentration of each sample and expressed as mU/mg of protein.

2.10 Total Antioxidant Capacity Evaluation

Total antioxidant capacity was evaluated in cell lysates using the Total Antioxidant Capacity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK187), according to the manufacturer's instructions. The assay is based on the reduction of Cu²⁺ to Cu⁺ by antioxidants present in the sample, followed by colorimetric detection at 570 nm. Antioxidant capacity was determined using Trolox as the reference antioxidant and expressed as Trolox equivalents.

2.11 Scratch Assay

HMEC-1 cells (2 × 10⁴ cells/well) were seeded in a 12-well plate. Confluent cell layers were scratched with a pipette tip, and cells were extensively washed to remove detached cells. Conditioned media collected from ARPE-19 cells cultured for 24 h and 96 h under different conditions were added to the wounds. Images of the damaged areas were taken at 10× magnification using a bright-field microscope immediately after the scratches (T0) and after 6 h of incubation (T1). Quantification of cell migration rate was based on the distance between the edges of the scratch and expressed as a percentage of closure in comparison to T0.

2.12 Statistical Analysis

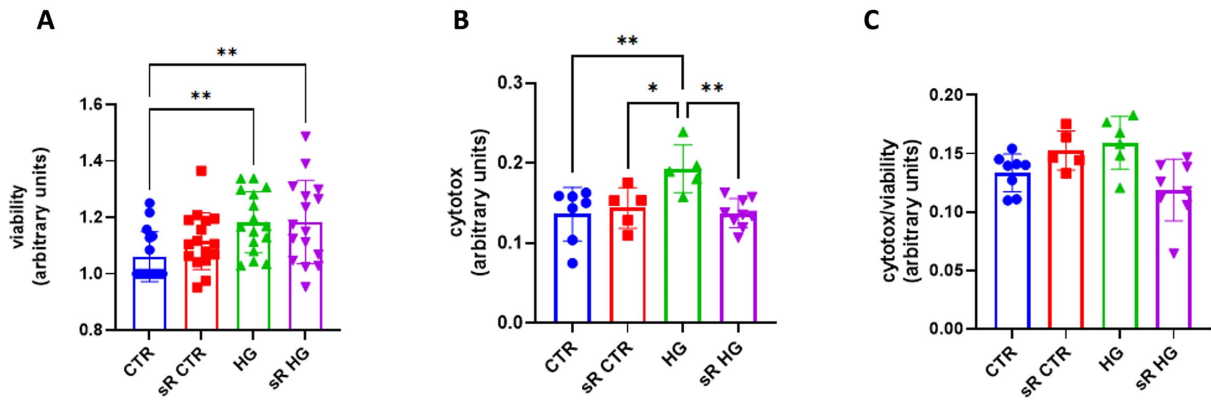
Quantitative results represent the mean ± standard deviation (SD) from at least 3 independent experiments. Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Statistical comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical significance was defined as *p* < 0.05.

3. Results

3.1 sR Pretreatment Preserves ARPE-19 Cell Viability and Reduces Cytotoxicity and ROS Production Under Hyperglycemic Conditions

Cell proliferation and cytotoxicity were evaluated after 24 h and 96 h of exposure of ARPE-19 cells to HG with or without sR pretreatment (Fig. 1). sR pretreatment alone did not affect cell proliferation. Conversely, after 24 h, HG significantly increased cell proliferation compared with CTR cells, and a similar increase was observed in the sR-HG group, indicating that sR pretreatment did not prevent the proliferative effect induced by HG (Fig. 1A). However, although HG exposure also significantly increased cytotoxicity compared with CTR, sR pretreatment markedly reduced HG-induced cytotoxicity, indicating an early cytoprotective effect (Fig. 1B). In addition, the cytotoxicity/proliferation ratio remained unchanged (Fig. 1C). After 96 h, HG exposure significantly decreased cell viability compared with CTR, whereas sR pretreatment restored viability to the levels of untreated cells and reduced LDH release in cells cultured with HG (Fig. 1D,E). Consistently, the cytotoxicity/viability ratio was significantly increased

24h



96h

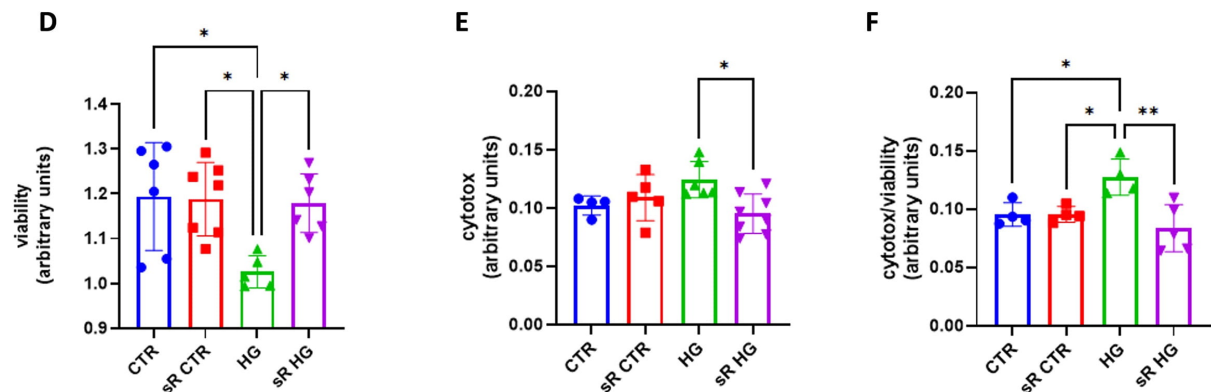


Fig. 1. Effects of HG and sR pretreatment on ARPE-19 cell viability, cytotoxicity, and cytotoxicity/viability ratio. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG). Cell viability (A,D), cytotoxicity (B,E), and the cytotoxicity/viability ratio (C,F) were evaluated after 24 h (A–C, upper panels) and 96 h (D–F, lower panels) of HG exposure. Data are shown as individual values with bars representing the mean $\pm$ SD from at least 4 experiments. One-way ANOVA with Tukey's test (* p < 0.05 and ** p < 0.01). sR, Saffron Repron®; SD, standard deviation; ANOVA, analysis of variance.

in HG-treated cells compared with CTR (Fig. 1F) but this increase was reduced when HG exposure was preceded by sR treatment (Fig. 1F).

Then, ROS production was evaluated to investigate whether HG-induced cytotoxicity was associated with oxidative stress. ROS levels were normalized to the cell number in each well and expressed relative to CTR cells (Fig. 2). After 24 h of treatment, intracellular ROS levels were significantly increased in cells exposed to HG (Fig. 2A), and this effect was counteracted by the sR pretreatment. After 96 h, ROS production was further increased in cells exposed to HG compared with CTR (Fig. 2B), indicating that prolonged hyperglycemic exposure promotes oxidative stress in ARPE-19 cells. Conversely, sR pretreatment restored ROS levels to values comparable to those observed in CTR cells, suggesting a protective effect against long-term HG-induced oxidative damage.

3.2 sR Pretreatment Modifies Cellular Redox Balance Independently of SOD2 and Catalase Induction

To investigate whether the reduction in ROS induced by sR pretreatment was associated with changes in endogenous antioxidant defenses, SOD2 expression and catalase activity were first evaluated. Under control glucose conditions, sR pretreatment significantly reduced SOD2 expression at both 24 h and 96 h (Fig. 3A,E, respectively). HG exposure also reduced SOD2 expression at both time points; however, under HG conditions, sR pretreatment did not significantly further modify SOD2 levels compared with HG alone (Fig. 3A,E). A similar pattern was observed for catalase activity: sR pretreatment reduced catalase activity under control glucose conditions, whereas no significant additional effect of sR was detected in HG-exposed cells at either 24 h or 96 h (Fig. 3B,F, respectively). Thus, although sR markedly reduced ROS accumulation under HG conditions, this effect was not associated with increased SOD2 expression or catalase activity, suggesting the involvement of additional mechanisms regulating cellular redox balance.

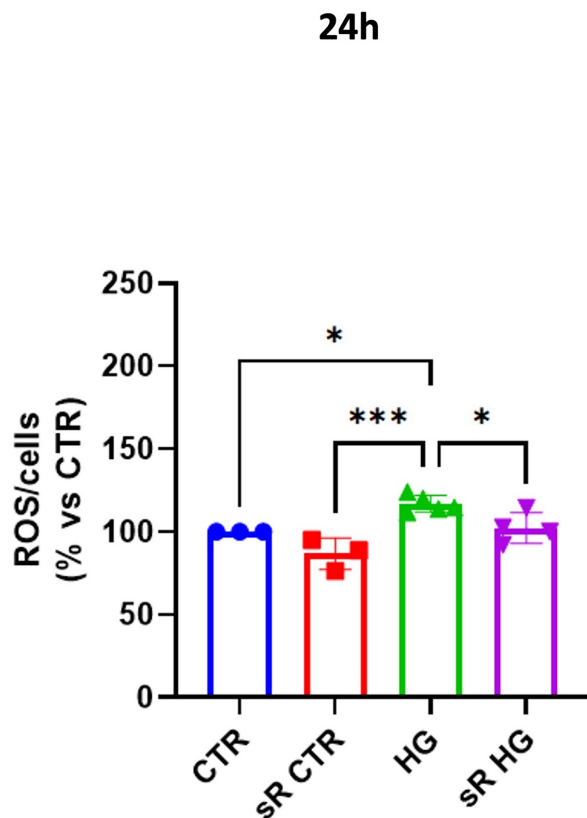
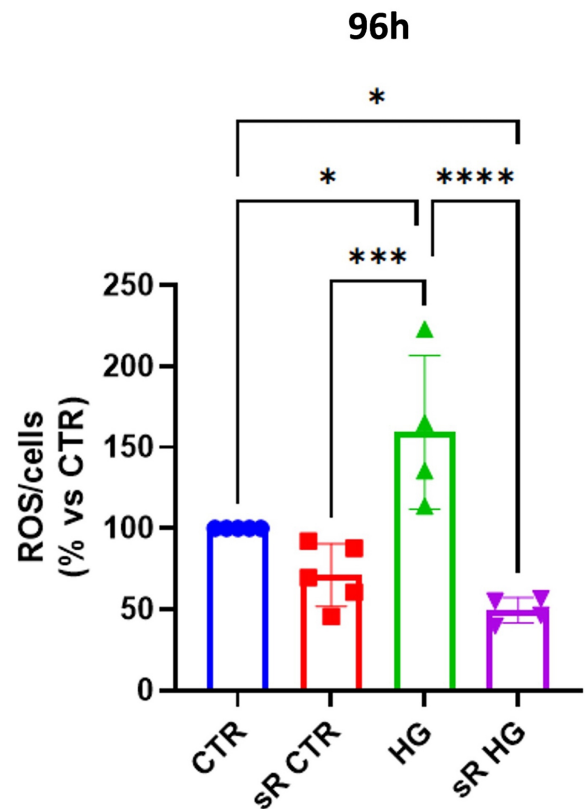
A**B**

Fig. 2. sR pretreatment prevents ROS accumulation induced by hyperglycemia. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG). Intracellular ROS levels were normalized to cell number and expressed as a percentage relative to CTR cells. ROS production was evaluated after 24 h (A) and 96 h (B) of HG exposure. Data are presented as individual values with bars showing the mean $\pm$ SD. One-way ANOVA with Tukey's test from at least 4 experiments, * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$. ROS, reactive oxygen species.

To further investigate the mechanism underlying this apparent dissociation, NOX activity and total antioxidant capacity were assessed. sR pretreatment significantly reduced NOX activity under both control and HG conditions at 24 h and 96 h (Fig. 3C,G, respectively). In parallel, sR pretreatment increased total antioxidant capacity, measured as Trolox-equivalent antioxidant capacity, at both time points (Fig. 3D,H). These findings indicate that the reduction in ROS observed after sR pretreatment was associated with both decreased pro-oxidant activity and an increased overall antioxidant capacity of the cells. Thus, although sR did not induce SOD2 expression or catalase activity, it promoted a broader shift in cellular redox balance characterized by reduced ROS-generating activity and increased total antioxidant capacity.

In addition, we investigated whether sR pretreatment modulated the NRF2/KEAP1 pathway in ARPE-19 cells exposed to HG. After 24 h, total NRF2 expression was not significantly modified by either HG exposure or sR pretreatment (Fig. 4A). In contrast, KEAP1 expression was

significantly increased in HG-treated cells compared with CTR and sR CTR, whereas sR pretreatment prevented this HG-induced increase, restoring KEAP1 levels close to control values (Fig. 4B). Despite these changes in KEAP1 expression, the NRF2/KEAP1 ratio was not significantly different among experimental groups (Fig. 4C). After 96 h, total NRF2 expression remained unchanged across conditions (Fig. 4D), while KEAP1 expression was reduced in sR-pretreated HG cells compared with the indicated control condition (Fig. 4E), again without a significant change in the NRF2/KEAP1 ratio (Fig. 4F).

3.3 sR Pretreatment Does not Affect NF- κ B Expression

To investigate whether sR pretreatment modulated the inflammatory response, we first analyzed total NF- κ B and its inhibitor I κ B α (Fig. 5). At 24 h, total NF- κ B protein levels did not differ significantly among the experimental groups (Fig. 5A), whereas I κ B α expression was significantly increased by HG (Fig. 5B). Although sR pretreatment showed a tendency to reduce the HG-induced increase

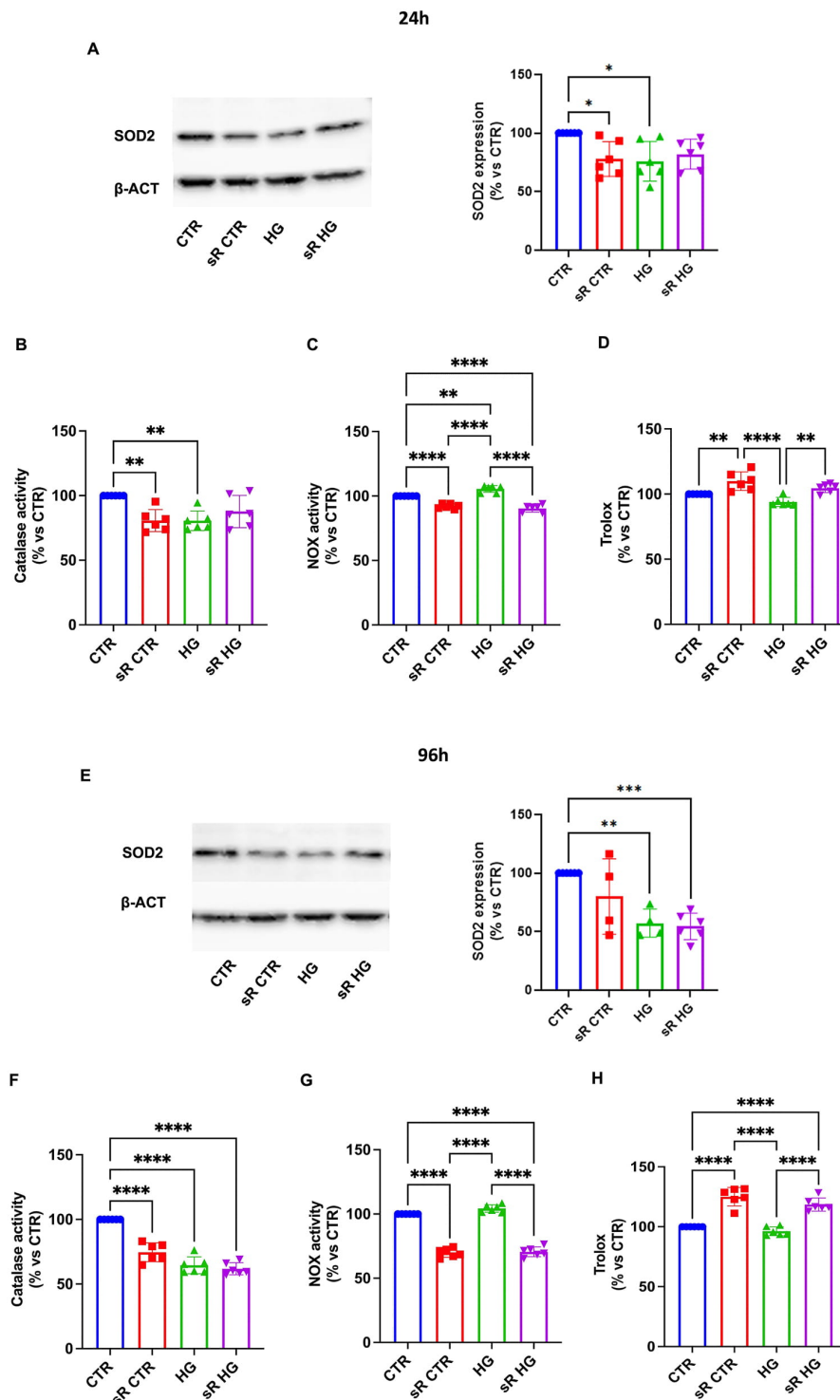


Fig. 3. sR pretreatment modulates the cellular redox balance in ARPE-19 cells. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG). SOD2 protein expression was evaluated by Western blotting after 24 h (A) and 96 h (E) of HG exposure. Representative Western blots and the corresponding densitometric quantification, normalized to β -actin, are shown. Catalase activity was assessed after 24 h (B) and 96 h (F). NADPH oxidase (NOX) activity was evaluated after 24 h (C) and 96 h (G). Total antioxidant capacity, expressed as Trolox-equivalent antioxidant capacity, was determined after 24 h (D) and 96 h (H). Results are expressed as percentages relative to CTR (CTR, 100%). Values represent the mean $\pm$ SD from at least 4 independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test; significant comparisons are indicated by brackets: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

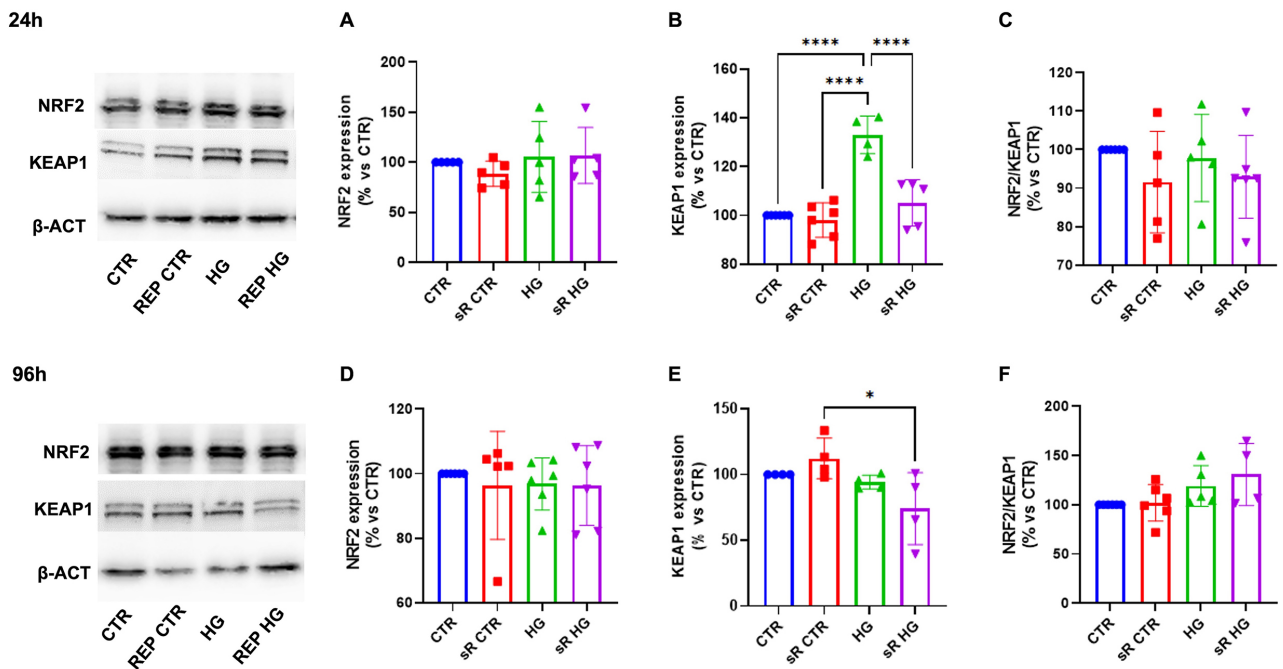


Fig. 4. Expression of NRF2 and KEAP1. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG). Western blot analysis of protein expression of NRF2 (A,D), KEAP1(B,E), and their ratio (C,F) in ARPE-19 cells cultured for 24 h and 96 h, respectively. In the left part of the panel, representative images of western blots were shown. In the right part of the panel, the respective densitometric quantification of relative bands was graphed. The results are expressed as a percentage compared to control (CTR, 100%). Values represent the mean $\pm$ SD from at least 4 independent experiments. One-way ANOVA with Tukey's test: * $p < 0.05$, and **** $p < 0.0001$.

in I κ B α , this difference was not statistically significant. Consequently, the NF- κ B/I κ B α ratio was significantly reduced in HG-exposed cells irrespective of sR pretreatment (Fig. 5C). After 96 h, total NF- κ B expression remained unchanged across experimental conditions (Fig. 5D), whereas I κ B α showed a tendency to decrease in HG- and sR HG-treated cells compared with CTR (Fig. 5E), resulting in an increased NF- κ B/I κ B α ratio (Fig. 5F). Thus, analysis of total NF- κ B and I κ B α abundance did not demonstrate a significant effect of sR pretreatment on either protein.

3.4 sR Pretreatment Modulates Subcellular Distribution of NRF2 and NF- κ B

Since total cellular NRF2 abundance does not necessarily reflect NRF2 activation, we next examined its subcellular distribution to determine whether HG and/or sR pretreatment affected NRF2 nuclear localization and chromatin association. NRF2 was predominantly localized in the chromatin-bound fraction under all experimental conditions at both 24 h and 96 h (Fig. 6). After 24 h, the relative distribution of NRF2 among the cytosolic and chromatin bound subcellular compartments was not modulated by the experimental treatments (Fig. 6). However, after 24 h, the localization of NRF2 in the nuclear soluble fraction was significantly reduced by HG and sR HG. After 96 h, differences were observed among the chromatin-bound

fractions, with the highest NRF2 levels detected in the control cells progressively decreased in cells treated with sR or HG and became significantly reduced in sR HG-treated cells (Fig. 6).

Since NF- κ B activation primarily depends on its subcellular redistribution rather than on changes in total protein levels, we next assessed NF- κ B distribution in the cytosolic, nuclear soluble, and chromatin-bound fractions. At 24 h and 96 h, NF- κ B was predominantly detected in the cytosolic fraction, whereas lower levels were observed in the chromatin-bound fractions (Fig. 6). However, after 24 h NF- κ B localization in the nuclear soluble fraction was significantly reduced by sR and HG (Fig. 6). Under high-glucose conditions, sR promotes NF- κ B retention in the soluble nuclear compartment at both time points analyzed. After 96 h of treatment, the difference in distribution between the cytosolic and nuclear compartments was attenuated, and the localization in the chromatin bound fraction was generally increased (Fig. 6).

3.5 Hyperglycemia Promotes Sustained VEGF-A Secretion Despite sR Pretreatment

Oxidative stress has been reported to promote VEGF-A secretion in ARPE-19 cells, contributing to pro-angiogenic and inflammatory responses [40,41]. Therefore, to assess whether HG-induced oxidative stress was

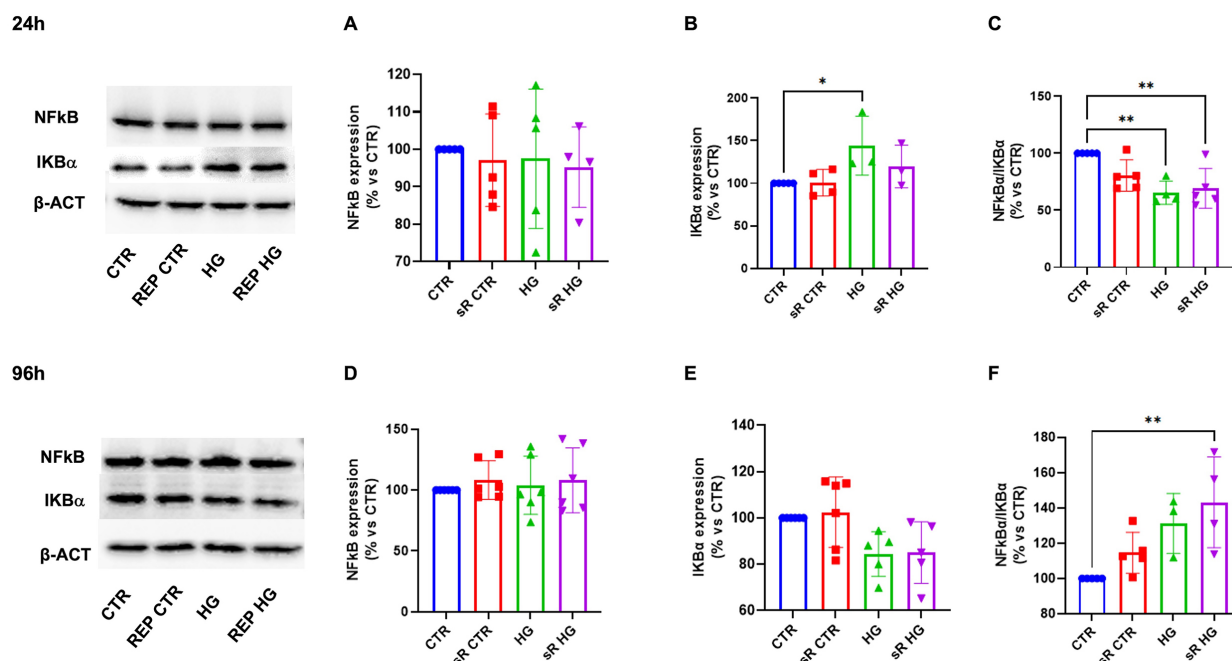


Fig. 5. Balance between NF- κ B and I κ B α was differently affected by HG at different experimental times. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG). Western blot analysis of protein expression of NF- κ B (A,D), I κ B α (B,E), and their ratio (C,F) in ARPE-19 cells cultured for 24 h and 96 h, respectively. In the left part of the panel, representative images of western blots were reported. In the right part of the panel, the respective densitometric quantification of relative bands was graphed. The results are expressed as a percentage compared to control (CTR, 100%). Values represent the mean $\pm$ SD from at least 4 independent experiments. One-way ANOVA with Tukey's test. * $p < 0.05$, and ** $p < 0.01$ vs CTR.

associated with functional changes in angiogenic signaling, VEGF-A secretion was evaluated (Fig. 7). Pretreatment with sR did not significantly affect basal VEGF-A secretion. In contrast, HG significantly increased VEGF-A secretion in ARPE-19 cells at both 24 h and 96 h compared with CTR conditions (Fig. 7A,B). sR pretreatment did not prevent the HG-induced increase in VEGF-A secretion at 24 h (Fig. 7A), whereas at 96 h it reduced VEGF-A secretion compared with HG-treated cells, although this reduction was not statistically significant (Fig. 7B).

3.6 Effects of ARPE-19 Secretome on Endothelial Cell Activation

To investigate whether secretory products from ARPE-19 cells treated as previously described may have paracrine effects on endothelial cells, we exposed HMEC-1 cells for 24 h to the conditioned media collected from ARPE-19 cells and evaluated cell viability and ROS production (Fig. 8).

Conditioned media from HG-treated ARPE-19 cells significantly increased HMEC-1 proliferation compared with control conditioned media at both 24 h and 96 h (Fig. 8A,C). Conditioned media from ARPE-19 cells pretreated with sR restored proliferation values close to those observed in control cells at both time points.

At 24 h, conditioned media from both sR- and HG-treated ARPE-19 cells significantly increased ROS generation in HMEC-1 cells compared with CTR (Fig. 8B,D). sR pretreatment significantly decreased HG-induced ROS production. When HMEC-1 cells were cultured with conditioned media collected after 96 h, the increase in ROS was significant only in cells exposed to HG-conditioned media (Fig. 8B,D).

To investigate whether the changes in the RPE secretory phenotype were associated with functional effects on endothelial cells, a scratch-wound migration assay was performed in HMEC-1 cells exposed to conditioned media collected from RPE cells under the different experimental conditions. When HMEC-1 cells were cultured with conditioned media collected after 24 h, the conditioned media from HG-treated RPE cells induced a marked, but not significant, increase in endothelial wound closure compared with control conditioned media, with approximately 60% closure compared with 30% in the control group (Fig. 9, upper panel). Conditioned media from sR-treated RPE cells induced an intermediate increase in wound closure, whereas the response to conditioned media from sR + HG-treated cells was lower than that observed with HG alone, reaching approximately 45–50% closure. A similar overall pattern was observed when HMEC-1 cells were cultured with con-

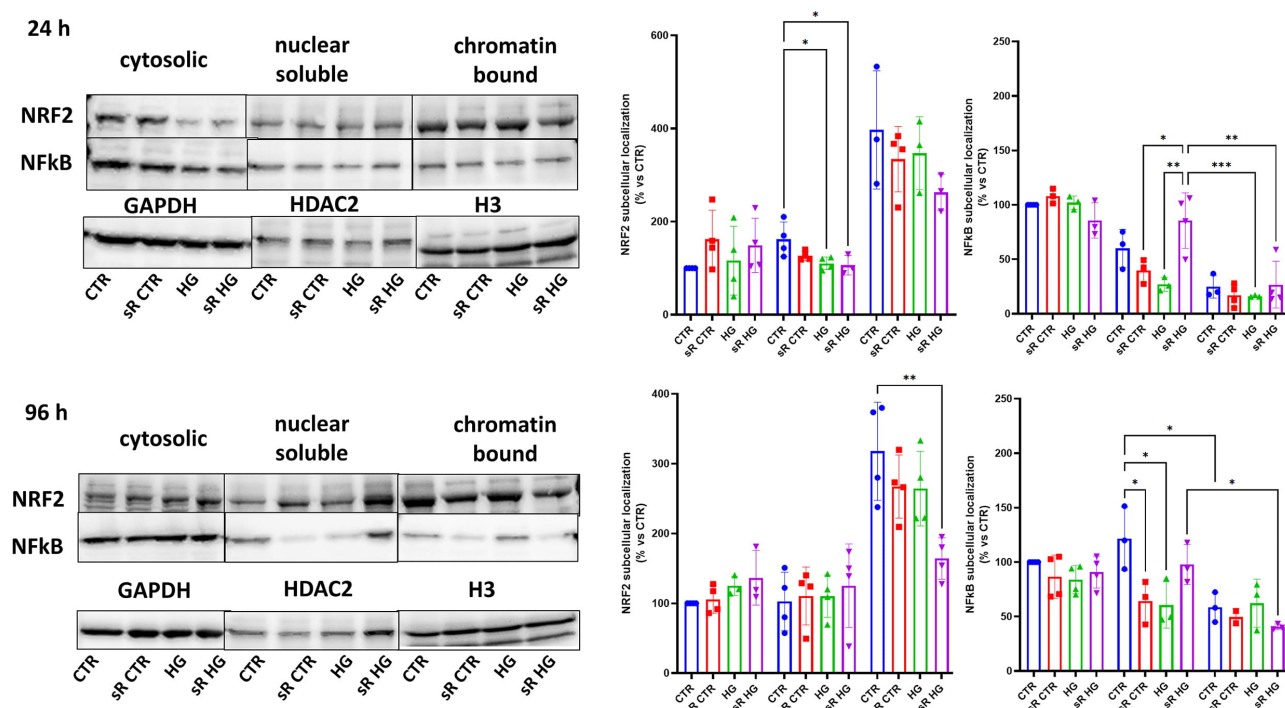


Fig. 6. Subcellular distribution of NRF2 and NF- κ B. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG) respectively for 24 h (upper panel) and 96 h (lower panel). Western blot analysis of protein expression of NRF2 and NF- κ B in cytoplasmic fraction, nuclear soluble fraction and chromatin-bound fraction. The same membrane was excised at the level of the molecular weight representing 80 kDa and 50 kDa, and the fragments were immunoblotted, respectively, for NRF2 (100 kDa) and H3 (17 kDa); then the upper part of the membrane was reblotted with NF- κ B (65 kDa) and HDAC2 (60 kDa) and the lower part with GAPDH (37 kDa). Each panel reports a representative Western blotting signal and quantification of densitometries of Western blot bands normalized against the specific housekeeping protein for each fraction. All panels are representative of at least 3 individual experiments, and data are expressed as mean $\pm$ SD compared to cytosolic (CTR, 100%). One-way ANOVA with Tukey's test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For clarity, only selected statistically significant comparisons considered most relevant to the interpretation of the data are shown.

ditioned media collected after 96 h (Fig. 9, lower panel). In this case, conditioned media from HG-treated RPE cells significantly increased endothelial wound closure, reaching approximately 60%, compared with about 20% in the control condition. Treatment with conditioned media from RPE cells treated with sR alone resulted again in an intermediate response, whereas exposure to conditioned media from sR HG-treated cells resulted in lower endothelial migration compared with HG alone.

4. Discussion

In this study, we show that a 24 h sR pretreatment protects ARPE-19 cells from HG-induced cytotoxicity and oxidative imbalance. HG exposure induced a time-dependent detrimental effect on ARPE-19 cells, characterized by an early increase in cell proliferation associated with increased cytotoxicity, followed by a marked reduction in cell viability after prolonged exposure. This pattern is consistent with an initial adaptive response to metabolic stress that becomes insufficient over time, leading to progressive

glucotoxic damage. This time-dependent pattern is consistent with previous reports in ARPE-19 cells, in which prolonged HG exposure induces oxidative stress, DNA damage and premature senescence [16], and promotes ROS generation and apoptosis through ROS-mediated inhibition of PINK1/Parkin-dependent mitophagy [15]. sR pretreatment did not substantially affect cells under control conditions, but reduced HG-induced cytotoxicity and restored cell viability after prolonged HG exposure, suggesting that its protective action becomes evident mainly under stress conditions, confirming previous results [42]. Notably, these effects were still detectable after 96 hours of exposure to HG levels, despite sR being administered only as pretreatment, suggesting the possible establishment of a protective adaptation that may help cells to counteract potential hyperglycemic stress even after sR withdrawal.

The cytoprotective effect of sR was closely associated with reduced ROS accumulation. HG significantly increased intracellular ROS production at both 24 h and 96 h, whereas sR pretreatment counteracted this effect and re-

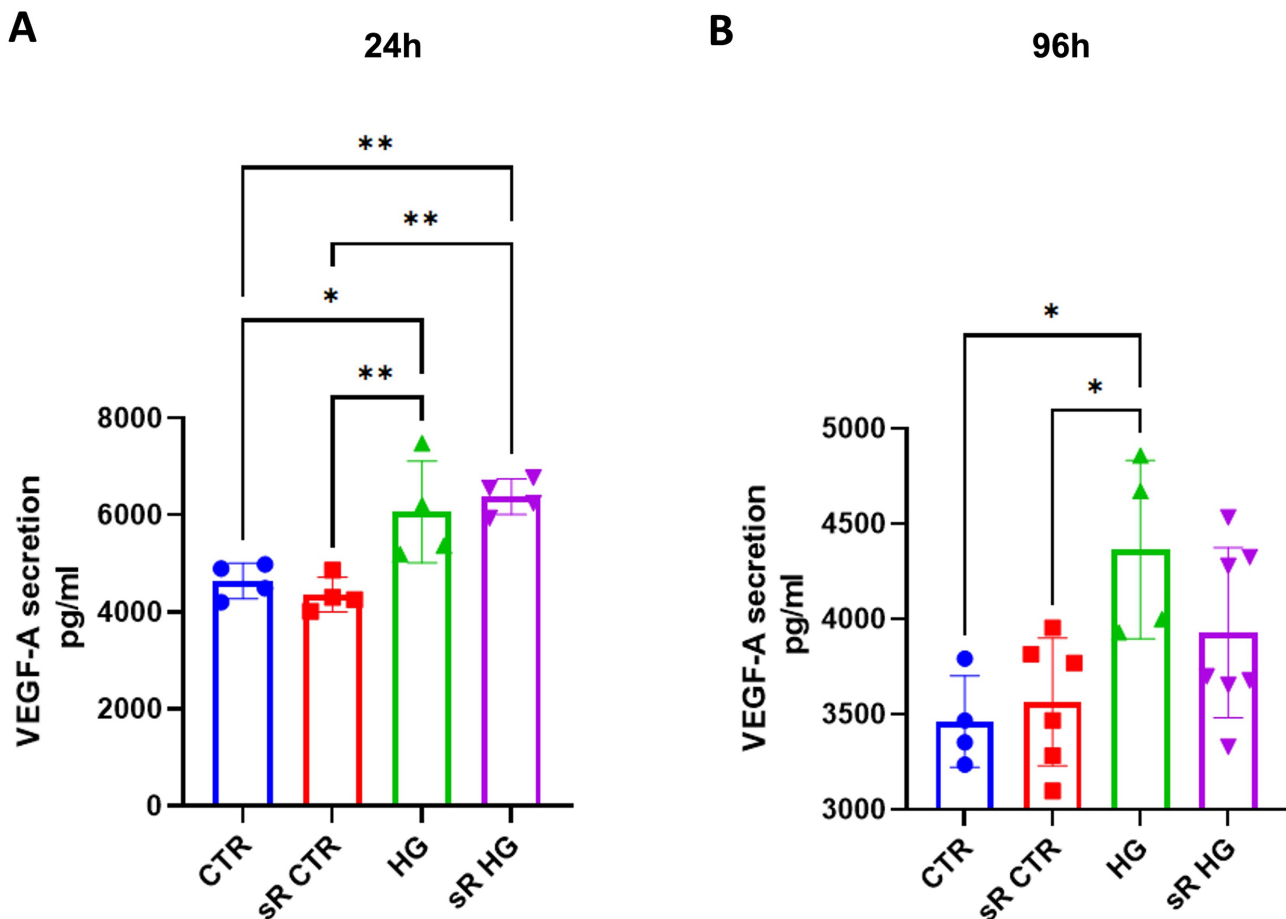


Fig. 7. Effects of sR pretreatment on HG-induced VEGF-A secretion in ARPE-19 cells. ARPE-19 cells were cultured under control conditions (CTR), pretreated with sR (sR CTR), exposed to high glucose (HG), or pretreated with sR before HG exposure (sR HG). VEGF-A secretion was evaluated after 24 h (A) and 96 h (B) of HG exposure. Data are presented as individual values with bars showing the mean $\pm$ SD from at least 4 experiments. One-way ANOVA with Tukey's test $*p < 0.05$, and $**p < 0.01$.

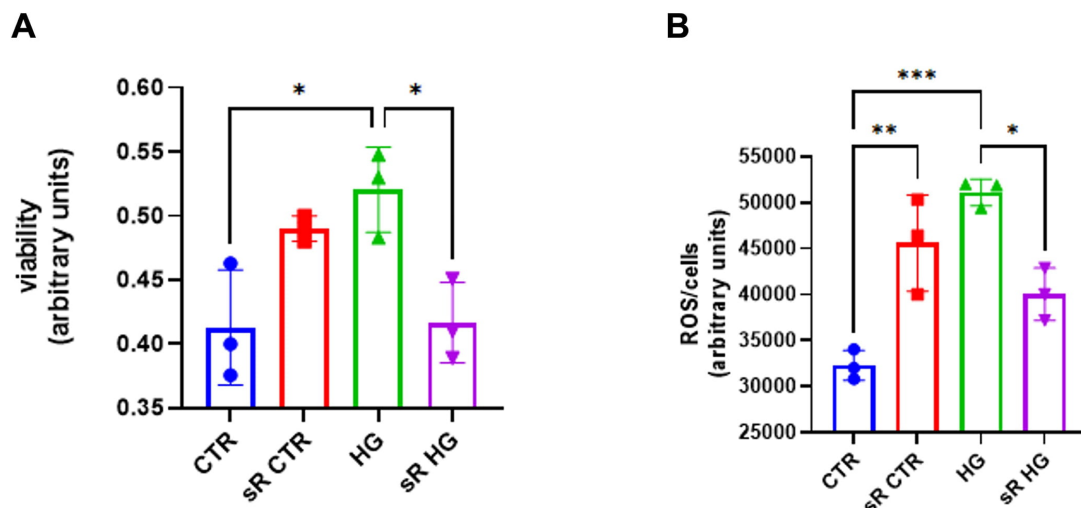
stored ROS levels close to those observed in control cells. These findings indicate that oxidative stress represents a major component of HG-induced damage in ARPE-19 cells and that sR limits this redox imbalance. Oxidative stress is indeed a well-established driver of RPE degeneration, in close interplay with inflammatory signalling [20]. However, the analysis of endogenous antioxidant defences suggests that this protection does not depend on induction of antioxidant enzymes. Indeed, HG exposure reduced SOD2 expression, particularly after prolonged treatment, and a similar reduction was observed for catalase activity. Surprisingly, sR pretreatment did not restore SOD2 expression or catalase activity. Therefore, the reduction in ROS observed after sR pretreatment cannot be explained by increased SOD2- or catalase-dependent detoxification, suggesting that additional mechanisms affecting ROS generation and/or the overall antioxidant capacity of the cells are involved.

This apparent dissociation between ROS reduction and antioxidant enzyme expression is an important finding of the study. In the context of HG exposure, reduced

SOD2 expression and catalase activity may reflect an impaired adaptive antioxidant response, consistent with the concept that chronic hyperglycaemic stress can compromise endogenous antioxidant defences [43,44], including the reduction in SOD2 expression induced by prolonged HG exposure in ARPE-19 cells [16]. Similarly, hyperglycaemia has been reported to affect NRF2-dependent antioxidant responses and the expression of antioxidant enzymes such as SOD2, HO-1, and catalase in ARPE-19 cells [45].

The additional assessment of NOX activity and total antioxidant capacity provides further insight into the mechanisms underlying the sR-mediated reduction in ROS. sR pretreatment markedly reduced NOX activity under both control and HG conditions, indicating that its protective effect is associated with a lower ROS-generating capacity. This finding is particularly relevant because NADPH oxidases represent an important source of ROS under hyperglycaemic conditions and may contribute to the establishment and amplification of cellular oxidative stress. Consistent with our findings, previous studies have reported that saffron and its bioactive constituents can modulate NADPH

24h



96h

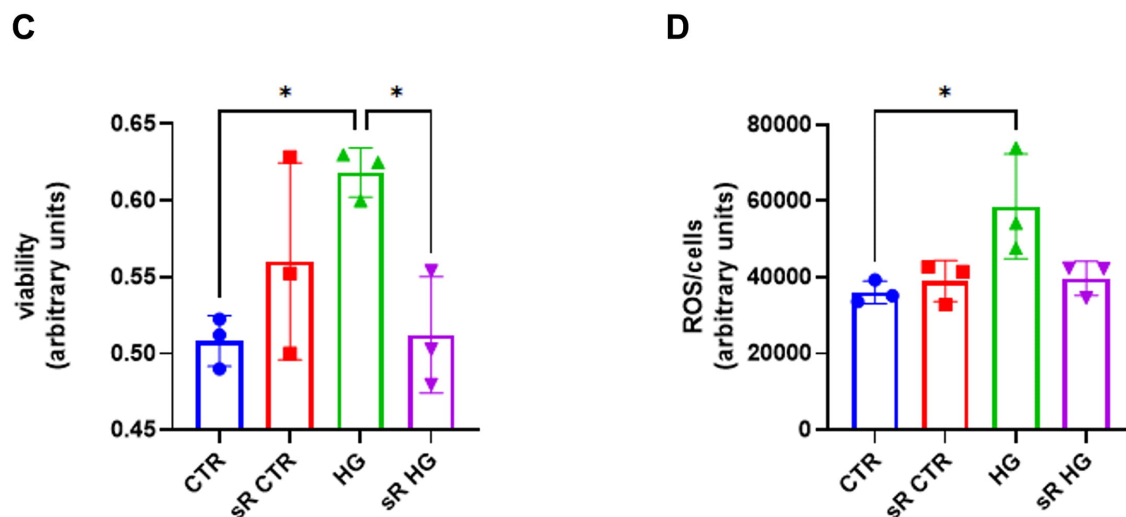


Fig. 8. sR pretreatment prevents damage to endothelial cells. Effects of conditioned media collected from ARPE-19 cells on cell viability and intracellular ROS levels of HMEC-1 cells. Cell viability (A,C) and ROS (B,D) were evaluated in HMEC-1 cells exposed to media collected from ARPE-19 cells treated for 24 or 96 h. Values represent the mean $\pm$ SD from 3 independent experiments. One-way ANOVA with Tukey's test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

oxidase-dependent ROS generation. In particular, saffron extract and its major constituents have been associated with reduced activation of NADPH/NADH oxidase-dependent oxidative pathways [46], while crocin has been shown to attenuate hyperglycaemia-induced oxidative stress in endothelial cells [47]. In addition, the carotenoid crocetin interferes with NADPH oxidase activation by inhibiting PKC ζ -mediated p47phox phosphorylation, membrane translocation of p47phox, and NOX2 complex assembly [48]. In parallel, sR pretreatment increased total antioxidant capacity at both experimental time points. Thus, despite the absence of SOD2 or catalase induction, sR-treated cells ex-

hibited an enhanced overall capacity to counteract oxidative stress. Taken together, these observations suggest that sR does not act primarily by inducing individual antioxidant enzymes but rather promotes a broader shift in cellular redox balance, combining decreased pro-oxidant activity with increased total antioxidant capacity. The latter may reflect changes in multiple enzymatic and non-enzymatic antioxidant components that are not individually resolved by the present assay. This interpretation is consistent with the complex composition of sR, which contains multiple bioactive compounds [34], including crocins, for which cytoprotective and antioxidative effects have been documented in

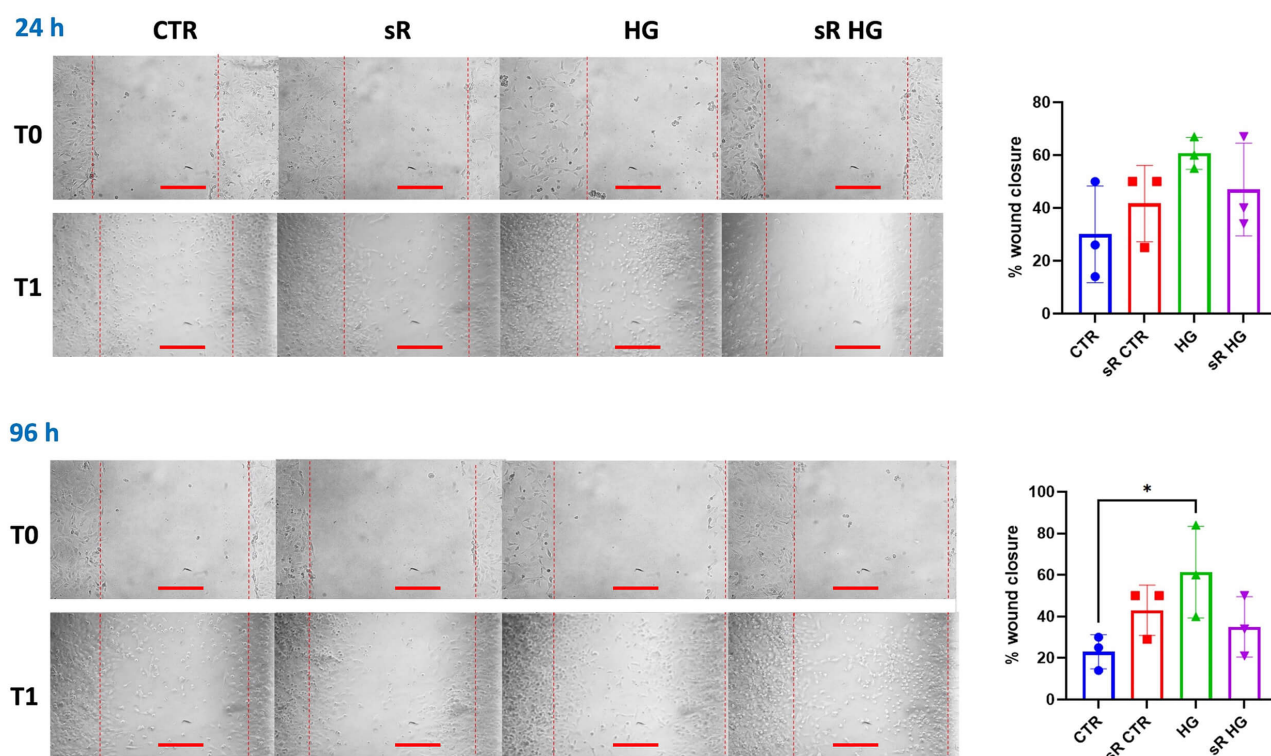


Fig. 9. Conditioned media from HG-treated RPE cells increases endothelial cell migration. Representative images of the damaged areas immediately after the scratches (T0) and after 6 h (T1) incubation with conditioned media collected from ARPE-19 cells treated for 24 or 96 h. The bar scale corresponds to 10 μ m. Values represent the mean $\pm$ SD from 3 independent experiments. One-way ANOVA with Tukey's test ($*p < 0.05$).

retinal tissue [33,34]. Such intrinsic redox-buffering activity may contribute during the pretreatment phase by limiting oxidative burden and potentially reducing the consumption of endogenous antioxidant resources. However, because sR was completely removed before HG exposure, direct scavenging alone is unlikely to account for the persistence of protection for up to 96 h. Rather, the sustained reduction in NOX activity together with the increased total antioxidant capacity supports the establishment of a longer-lasting cellular redox adaptation following sR pretreatment. The mechanisms operating *in vivo* and in humans are likely more complex, and it has been proposed that the synergistic activity of the different chemical components of saffron contributes to the induction of a resilience response [27]. Thus, in our experimental model, sR appears to reduce the oxidative burden imposed by HG through mechanisms that extend beyond the classical induction of compensatory antioxidant enzymes and persist after compound withdrawal.

The analysis of the NRF2/KEAP1 axis provides further insight into the nature of the antioxidant response induced by sR. Total NRF2 expression remained largely unchanged at both 24 h and 96 h, despite changes in KEAP1 abundance under specific experimental conditions. These findings should not be interpreted as a discrepancy, since NRF2 signaling is not determined simply by an inverse re-

lationship between the total cellular abundance of NRF2 and KEAP1. Rather, pathway activation critically depends on NRF2 stabilization and redistribution to the nucleus, where its association with chromatin enables transcriptional regulation of antioxidant-response genes. Therefore, measurements of total NRF2 expression alone provide limited information on its functional activation. To address this point more directly, we analyzed the subcellular distribution of NRF2, including its nuclear and chromatin-associated fractions. Our results indicate that NRF2 was predominantly localized in the chromatin-bound fraction, suggesting that a substantial proportion of NRF2 may participate in the transcriptional regulation of cellular stress-response genes. Interestingly, despite its predominant localization in the chromatin-bound fraction, exposure to sR and HG resulted in a time-dependent reduction in chromatin-associated NRF2. This finding is consistent with the observed decrease in SOD2 expression. Since NRF2 transcriptional activity is highly dependent on its interaction with specific regulatory elements, cofactors, and the cellular redox environment, the reduction in SOD2 expression may reflect a selective remodeling of the antioxidant response. The subcellular distribution data therefore provide a functional complement to measurements of total NRF2 abundance. In particular, changes in the chromatin-

associated fraction allow NRF2 activation to be interpreted in terms of transcriptional engagement rather than protein expression alone. When these findings are considered together with SOD2 expression, catalase activity, NOX activity, and total antioxidant capacity, they indicate that the redox protection afforded by sR cannot be simply ascribed to induction of the canonical NRF2-dependent antioxidant program. Rather, sR appears to induce a broader redox adaptation in which modulation of ROS-generating activity and overall antioxidant capacity may coexist with a condition-dependent regulation of NRF2 signaling.

The inflammatory response showed a partially distinct behavior from the redox response. Total NF- κ B levels remained relatively stable, whereas I κ B α expression changed over time during HG exposure. Importantly, however, sR pretreatment did not significantly modify the total abundance of either NF- κ B or I κ B α , indicating that these measurements alone do not support attenuation of NF- κ B signaling by sR. Since NF- κ B activation critically depends on its nuclear translocation and chromatin engagement, we therefore examined its subcellular distribution. Our results indicate that NF- κ B was predominantly localized in the cytosolic fraction under all experimental conditions at both 24 h and 96 h. However, the relative distribution of NF- κ B in the nuclear fractions increased over time. Despite the comparable NF- κ B distribution observed across the subcellular compartments, under HG conditions sR retained NF- κ B predominantly in the nuclear soluble fraction, thereby limiting its recruitment to chromatin and potentially reducing NF- κ B transcriptional activity. Together, these findings suggest that sR may modulate the balance between NRF2- and NF- κ B-dependent transcriptional responses through differential regulation of their nuclear compartmentalization. The analysis of NF- κ B redistribution provides a more functionally informative assessment of pathway activation than total protein abundance alone. Although phosphorylation of p65 was not assessed, redistribution of NF- κ B toward the nucleus and, particularly the chromatin-associated fraction is associated with the potential transcriptional activity of the pathway.

Consistently, HG increased VEGF-A secretion in ARPE-19 cells, supporting the presence of a pro-angiogenic response under hyperglycaemic conditions [40,41,49]. sR pretreatment did not affect the early HG-induced increase in VEGF-A secretion at 24 h but significantly attenuated this response after 96 h. Moreover, sR alone did not alter basal VEGF-A secretion, indicating that the treatment does not substantially affect angiogenic signaling under control conditions. Overall, these findings indicate that VEGF-A secretion is not fully normalized by sR pretreatment and that the pro-angiogenic response induced by HG is only partially sensitive to ROS modulation. This is consistent with the possibility that VEGF-A release under hyperglycemic stress is regulated not only by oxidative stress, but also by additional pathways such as inflam-

matory signalling, metabolic stress responses, and other glucose-dependent mechanisms. A comparable multiplicity of hyperglycaemia-driven mechanisms has been described in diabetic vascular disease, where endothelial dysfunction arises from the convergence of oxidative, inflammatory and metabolic signals [50].

The experiments performed with HMEC-1 cells provide an important functional extension of these observations. Conditioned media from HG-treated ARPE-19 cells increased endothelial cell proliferation and ROS production, suggesting that HG-exposed ARPE-19 RPE model releases soluble mediators capable of promoting endothelial activation and oxidative stress. Importantly, conditioned media from sR-pretreated ARPE-19 cells attenuated these effects, restoring endothelial proliferation closer to control levels and reducing ROS generation. These data suggest that sR pretreatment modifies the secretory impact of HG-exposed ARPE-19 cells on endothelial cells, even though VEGF-A secretion was not completely suppressed. Therefore, the protective effect of sR at the paracrine level may depend on a broader remodelling of the ARPE-19 RPE model secretome rather than on VEGF-A modulation alone. The enhanced endothelial migration following exposure to HG-conditioned media provides functional evidence that hyperglycemia can shift the RPE secretome toward a pro-angiogenic phenotype. However, the fact that sR+HG reduces migration despite VEGF-A remaining relatively high suggests that restoring cellular resilience may involve more than simply suppressing VEGF-A and that short-term sR exposure may partially preserve the ability of RPE cells to maintain a less pro-angiogenic secretory environment. The concomitant increase in endothelial ROS and migratory activity suggests also the possibility that oxidative signaling contributes to the endothelial response to the pro-angiogenic RPE secretome. Altogether these findings indicate that the RPE secretome can modulate endothelial function beyond simply affecting cell survival. Overall, these data support the concept that preservation of RPE resilience under metabolic stress may have consequences beyond RPE cell function itself, potentially limiting the ability of the stressed RPE to promote endothelial activation and pathological vascular responses. This paracrine dimension is physiologically relevant *in vivo*, where the diabetic retina behaves as an integrated neurovascular unit in which ARPE-19 cells-derived mediators influence endothelial function and vascular integrity [5], and where hyperglycaemia is known to alter endothelial junctional organization and vascular permeability [11].

Taken together, these findings indicate that the persistent protection induced by sR reflects a coordinated remodeling of cellular redox homeostasis rather than the simple induction of a single antioxidant pathway. This response is characterized by a marked reduction in ROS accumulation associated with decreased NOX activity and increased total antioxidant capacity, despite the absence of SOD2 or

catalase induction. The persistence of these changes after complete removal of sR further suggests that the protective effect cannot be explained solely by transient direct scavenging, but is consistent with the establishment of a sustained redox adaptation that limits ROS-generating activity while maintaining a greater overall capacity to counteract oxidative stress.

5. Limitations

Several aspects should be considered when interpreting the present findings. First, the study was conducted using ARPE-19 cells, a well-established and reproducible *in vitro* model for investigating retinal pigment epithelium-related responses. Nevertheless, this cell line does not fully recapitulate the phenotypic, structural, and functional complexity of primary human RPE. Validation of the main findings in primary human RPE or human iPSC-derived RPE would therefore further strengthen their translational relevance. Second, the experiments were performed using a single concentration of sR selected on the basis of previous studies employing the same pretreatment conditions in ARPE-19 cells. Accordingly, the present study was designed to investigate the persistence and mechanisms of the protective response rather than to establish a concentration–response relationship under HG conditions. Third, an osmotic control was not included. Therefore, a contribution of increased osmolarity to some of the effects observed under HG conditions cannot be completely excluded. However, previous studies using ARPE-19 cells exposed to comparable HG concentrations and including mannitol as an osmotic control reported that mannitol did not reproduce the cellular alterations induced by HG [51,52,53,54]. These observations support the interpretation that the changes observed in our experimental setting are predominantly attributable to HG exposure rather than to osmotic stress. Nevertheless, the inclusion of a dedicated osmotic control in future studies would allow this distinction to be addressed more directly.

In addition, while the assessment of NADPH oxidase activity and total antioxidant capacity provides further mechanistic insight into the reduction in ROS observed after sR pretreatment, the specific NOX isoforms involved and the individual components contributing to the overall antioxidant capacity were not addressed. Similarly, mitochondrial ROS production was not directly evaluated. Finally, the experiments using conditioned media from ARPE-19 cells provide evidence of a functional paracrine effect on endothelial cells; however, additional vascular endpoints, such as endothelial permeability and three-dimensional angiogenic responses, were beyond the scope of the present study. Future studies addressing these aspects may help further define the mechanisms and biological relevance of the protective effects observed.

6. Conclusions

In conclusion, sR pretreatment protects ARPE-19 cells from HG-induced oxidative and cytotoxic damage mainly by reducing ROS burden rather than by inducing NRF2/SOD2 antioxidant response. Although sR does not completely abolish HG-induced inflammatory and pro-angiogenic signalling, it limits the deleterious paracrine effects of HG-exposed ARPE-19 cells on endothelial cells. These findings support a protective role of sR in a cellular model during the early phase of hyperglycemic retinal stress. However, as these observations were obtained *in vitro*, further *in vivo* studies are needed to confirm their translational relevance.

Availability of Data and Materials

The data used to support the findings of this study are included within the article. All data reported in this paper will also be shared by the lead contact upon request.

Author Contributions

AP and SR designed the research study; AP and SR wrote the manuscript; AP, MB and AG performed the research; IP, SR, and DM analyzed the data; IP provided help and advice on designing the research study; DM and SR supervised. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

We gratefully acknowledge Dr Maria Anna Maggi of the Hortus Novus Srl Italy for having tested and provided Saffron Repron®. Doctor Maria Maggi is co-inventors of the following international patent: “Compositions based on saffron for the prevention and/or treatment of degenerative eye disorders”, 2015 (W02015/145316) and is owned by Hortus Novus srl, to which is linked a mark (Repron® TM) that attests the quality of ophthalmic saffron.

Funding

This research received no external funding.

Conflicts of Interest

The authors declare no conflicts of interest. Although products provided by Hortus Novus Srl were used in this study, the judgments in data interpretation and writing were not influenced by this relationship.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT-3.5 to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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