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RESEARCH ARTICLE

Dapagliflozin prevents methylglyoxal-induced retinal cell death in ARPE-19 cells

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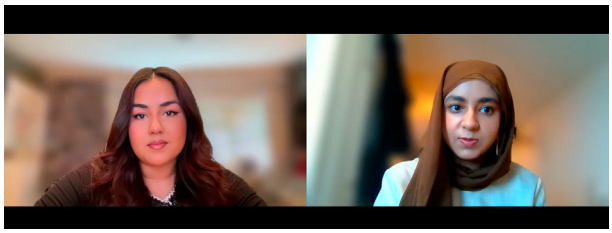
Diabetic macular oedema (DMO) is a sight-threatening complication of diabetes. Current research suggests methylglyoxal (MGO), an advanced glycation end product (AGE) and reactive oxygen species (ROS) precursor produced in states of chronic hyperglycaemia, may contribute to retinal damage in DMO. Dapagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, has shown antioxidant and anti-inflammatory properties in human brain neuronal cells. However, its protective effects in retinal cells remain unclear. This study investigates the potential protective role of current antidiabetics against MGO-induced cytotoxicity in human retinal pigment epithelial cells (ARPE-19), focussing on the NLRP3 and caspase-1 pathway. ARPE-19 cells were studied through four conditions: Control (untreated), MGO (1 nM and 1 mM), cotreatment of MGO (1 mM) with dapagliflozin (10 μM) to investigate cytotoxicity and cell viability through lactate dehydrogenase (LDH) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assays, respectively. Cells were further investigated using confocal microscopy to assess the presence and activation of NLRP3 and caspase-1 enzyme. MGO (1 mM) caused significant cytotoxicity by approximately 60%, which was reduced to 33% by dapagliflozin (10 μM), providing a significant level of protection to cells against MGO-induced cytotoxicity, in addition to a significant increase in cell viability from 60% to 83%, and reduction in NLRP3-independent caspase-1 activation and/or expression, associated with increased nuclear staining intensity reflecting potential nuclear condensation and pyroptosis. This study suggests dapagliflozin protects ARPE-19 cells from MGO-induced oxidative stress and inflammasome through reducing caspase-1 activation, underscoring its potential as a therapeutic approach for retinal inflammation and vascular dysfunction in DMO, which requires further clinical investigations.

Abbreviations
AGE, advanced glycation end product; anti-VEGF, antivascular endothelial growth factor; ARPE-19, human retinal pigment epithelial cells; A.U, arbitrary units; DAMPs, damage-associated molecular patterns; DMO, diabetic macular oedema; DPP-4, Dipeptidyl peptidase-4; GLP-1, glucagon like peptide-1; GSDMD, Gasdermin D; IL-18, Interleukin-18; IL-1β, Interleukin-1β; LDH, lactate dehydrogenase; MGO, Methylglyoxal; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide; NLRP3, nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain containing receptor 3; PRRs, pattern-recognition receptors; ROS, reactive oxygen species; SGLT2, sodium-glucose cotransporter 2; SH-SY5Y, human brain neuronal cells.



Introduction

Diabetes mellitus is a chronic metabolic disease characterised by uncontrolled elevation in blood sugar where fasting blood glucose concentration exceeds 7 mmol·L^{-1}. Globally, the diabetic population consists of 537 million adults with projections estimating an increase to 643 million by 2030 and 783 million by 2045, suggesting a 20–46% increase in the next 5–15 years [1,2]. Diabetes UK estimates 12.1 million adults in the United Kingdom are living with diabetes or prediabetes as of 2023–2024 [1]. Diabetes mellitus is classified into two main types: type 1 and type 2 diabetes. Type 1 diabetes accounts for approximately 10% of diabetes cases. It is an autoimmune condition where the body uncontrollably attacks the insulin-producing pancreatic beta-cells, resulting in insulin deficiency [3,4]. In contrast, type 2 diabetes accounts for approximately 90% of cases and occurs when pancreatic beta-cells are unable to produce levels of insulin required by the body, or where the body develops resistance against endogenous insulin [3,4]. Uncontrolled diabetes causes macrovascular complications, such as cardiovascular and peripheral vascular disease [5]. It can also result in microvascular complications such as retinopathy, nephropathy, in addition to neuropathic complications [5]. The risk of diabetes complications can be minimised through effective glycaemic control using antidiabetic medications such as metformin (biguanides), gliclazide (sulphonylureas), dapagliflozin (sodium glucose transport-2 (SGLT2) inhibitors), semaglutide (glucagon like peptide-1 (GLP-1) agonists), linagliptin (dipeptidyl peptidase-4 (DPP-4) inhibitors) and homologous insulin [2].

Retinopathy, particularly diabetic macular oedema (DMO), is among the most common complications in diabetes. DMO is a leading cause of vision impairment in diabetes and is estimated to occur in 5% of diabetic patients, with duration of diabetes playing a major risk factor; 3.2% of people living with diabetes for less than 10 years are affected, compared to 20% in people with diabetes for more than 20 years [6,7]. The pathophysiology of DMO is complex, involving oxidative stress, advanced glycation end products (AGEs) and inflammation [7]. Understanding the pathophysiology of DMO is important to help in finding therapeutic options for such a complex and challenging complication. The common pathway leading to DMO is the breakdown of the blood–retinal barrier, which exists to maintain homeostasis in the neural tissue. The inner BRB consists of tight junctions between retinal endothelial cells, surrounding basal lamina, pericytes, astrocytes and microglia [7]. The macula is a photoreceptor-rich region at the centre of the retina and hence plays pivotal roles in central vision. The retinal pigment epithelium is a single layer of cells found between photoreceptors and Bruch's membrane, composing the outer blood retinal barrier. It is formed by tight junctions between retinal pigment epithelial cells. When the integrity of the BRB is damaged, plasma leaks into interstitial spaces, causing oedema and fluid accumulation within and underneath the retina [8].

Current treatments for DMO primarily involve intravitreal injections of antivascular endothelial growth factor (anti-VEGF) agents such as ranibizumab or corticosteroids, which target vascular leakage and inflammation [9]. However, these therapies do not directly address the underlying metabolic dysregulation in diabetes that contributes to retinal cell damage, revealing the importance of finding new therapeutic targets to develop new treatments. Many studies have been conducted, investigating the pathophysiological role of hyperglycaemia in the development of retinopathy and diabetic macular oedema. One study suggested that the disruption in the BRB is caused by the release of inflammatory cytokines in states of chronic hyperglycaemia, some of the most implicated including: IL-1β, IL-6, IL-18 and VEGF-A [8].

IL-1β and other interleukins are released in retinal epithelial cells through the activation of the caspase-1 pathway due to the presence of AGEs, which bind and activate the retinal inflammasomes, triggering the conversion of pro-caspase-1 into caspase-1 [10,11]. Production of these inflammatory cytokines in the retinal epithelial cells will weaken tight junctions in the BRB causing increased vascular permeability, resulting in oedema. The chronic inflammation on these cells will cause cellular stress and oxidative damage, resulting in apoptosis [8,11]. AGEs are formed when reducing sugars nonenzymatically react with proteins, lipids or nucleic acids, and this process is accelerated in hyperglycaemia [12]. Methylglyoxal (MGO), an AGE precursor, could be implicated in the exacerbation of retinal damage in diabetes, contributing to endothelial dysfunction, pericyte loss and neuronal apoptosis seen

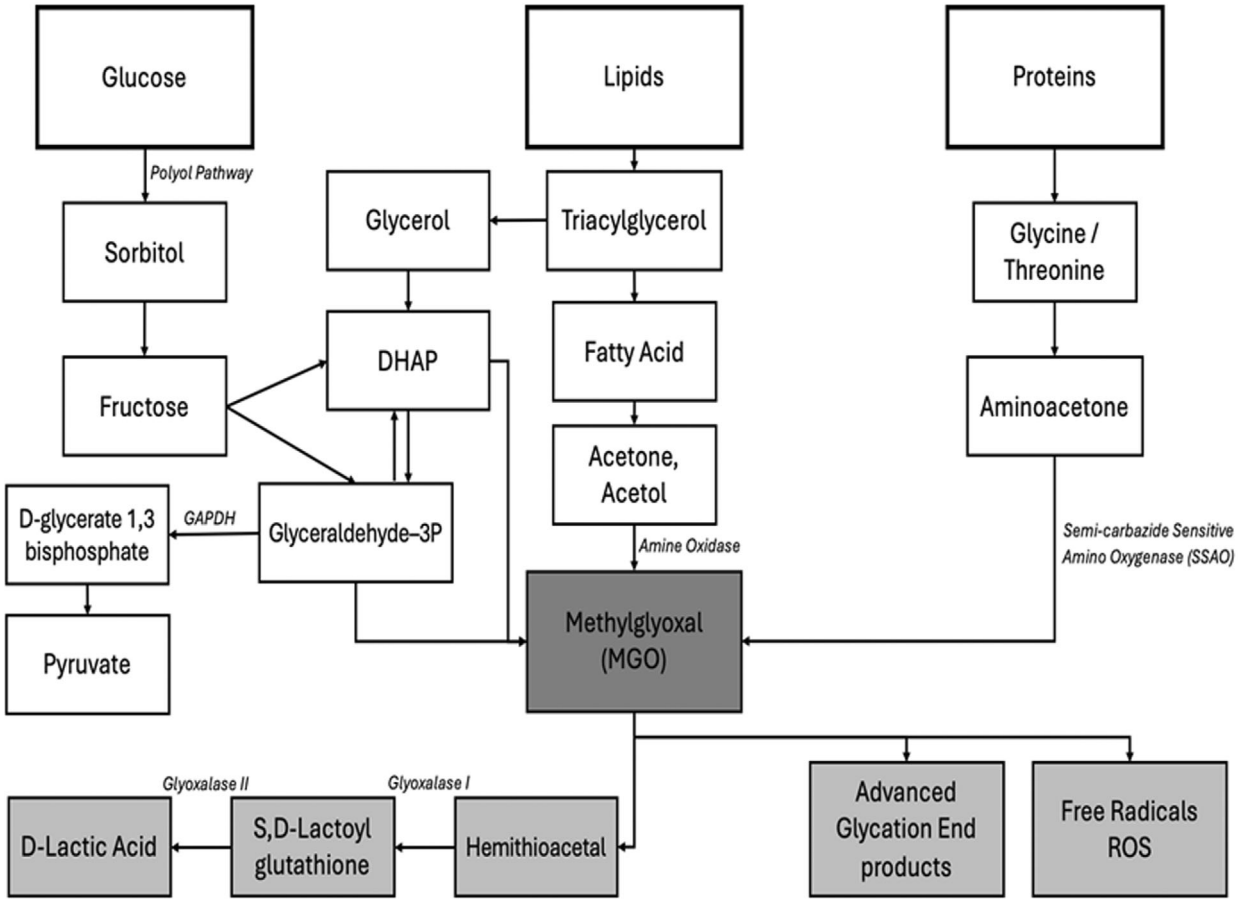


Fig. 1. Biochemical pathways of glucose, lipid and protein and metabolism of endogenous methylglyoxal (MGO), including the breakdown to D-lactic acid, Reactive Oxygen Species (ROS) and AGE's. Di-hydroxyacetone phosphate (DHAP); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [15–17].

in DMO [13]. This can be supported by the current evidence suggesting a pathophysiological association between methylglyoxal and hyperglycaemia in uncontrolled diabetes [14]. MGO is formed by various biochemical pathways and is present in all biological systems under normal physiological conditions, at a plasma concentration around 100–200 nM [15–17]. It is a highly reactive dicarbonyl by-product of glycolysis, where it arises from glyceraldehyde phosphate and dihydroxyacetone phosphate, as well as being formed through lipid peroxidation and threonine catabolism (Fig. 1). In diabetes, plasma concentration of MGO can be seen elevated 10-fold, due to elevations in glucose and therefore glycolysis [14].

Reactive oxygen species such as MGO can activate nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain containing receptor 3 (NLRP3) inflammasome, in addition to other multiple aetiological signals such as normal ageing, debris from dying cells, protein aggregates, lysosomal dysfunction and metabolic stress and reactive oxygen species [18]. The NLRP3 inflammasome can be activated through canonical and noncanonical pathways. The canonical pathway involves pattern-recognition receptors (PRRs) activation of the innate immune system in response to damage-associated molecular patterns (DAMPs), which are generated by endogenous stress, and trigger downstream inflammatory pathways to repair damaged tissues. PRRs oligomerise to form a complex structure called inflammasome such as NLRP3 [19]. NLRP3 contains a bipartite adaptor protein, which is known ASC containing a caspase-recruitment domain to facilitate the binding and recruitment of pro-caspase-1 to the inflammasome complex where it is activated into caspase-1. Caspase-1 cleaves and activates the cytokines pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into IL-1β and IL-18, respectively [20]. Active caspase-1 also cleaves

gasdermin D (GSDMD), which forms pores in the plasma membrane, thereby triggering plasma membrane rupture and hence the release of intracellular pro-inflammatory mediators such as IL-1β and IL-18, and nuclear condensation and damage and cell death (pyroptosis) [21,22]. In contrast, the noncanonical pathway is induced by direct cytosolic stimuli, which plays a pivotal role in the inflammatory disorders [23]. The noncanonical pathway involves the activation of caspase-4, −5 and −11 and hence the production of IL-1β. In addition to producing IL-1β, activated caspase-11 cleaves and activates GSDMD that yields pyroptosis that induces the NLRP3-dependent activation of caspase-1: canonical pathway [24–26].

There are a vast number of therapeutic treatments for the control and management of diabetes. Metformin is first-line treatment for Type 2 Diabetes and works by inhibiting gluconeogenesis by increasing activation of AMPK [27]. Studies have also shown metformin to have an anti-inflammatory effect, particularly through suppressing IL-1β and IL-6 [28]. Other therapeutics such as SGLT-2 inhibitors, such as dapagliflozin, work by inhibiting SGLT-2 cotransporters in the kidneys, therefore preventing the reabsorption of glucose, causing its excretion in the urine [29]. They also have anti-inflammatory effects on implicated interleukins and VEGF; therefore, some studies have hypothesised their protective effect in diabetic retinopathy [30]. Numerous studies have proven the role of metformin and SGLT-2 inhibitors in managing diabetes and its associated microvascular complications, such as DMO [31–34]. One study looked at the effect of metformin on MGO-induced retinal cell death; however, there was a lack of emphasis on the pathway through which cytotoxicity was induced, and inflammasome involvement in this pathway [35].

Accordingly, this research explores the cytotoxic effect of MGO on human retinal epithelial cells (ARPE-19) and the potential protective effect of dapagliflozin against MGO-induced cytotoxicity through the NLRP3 and caspase-1 pathway.

Methods

Cell culture

Mycoplasma-free human retinal pigment epithelial cells (ARPE-19) (RRID:CVCL_0145) (305025, 2BScientific; Cytion, Sioux Falls, SD, USA) were cultured in DMEM/F12 culture media (SH30271.01; Cytiva, Little Chalfont, Buckinghamshire, UK) with fetal bovine serum (10%) (10270-106; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and Penicillin Streptomycin (15140-122; Gibco). Cells were incubated at 37 °C in 5% CO_2 and 95% air. Once plated in 96-well plates and grown to approximately 90% confluent, cells were treated with four conditions: control (culture media only: untreated); MGO (MO252-25 mL; Sigma Millipore) in low physiological concentration (1 nM); high pathological concentration MGO (1 mM), and with MGO (1 mM) added with dapagliflozin (10 μM) (SML2804; Sigma Millipore, Gillingham, Dorset, UK). Cells' incubation and the concentration of dapagliflozin were adopted from our recent study in mycoplasma-free human brain neuronal cells (SH-SY5Y) (RRID:CVCL_0019) (CRL-2266; ATCC, Manassas, VA, USA) [17]. All cell lines used in this study were purchased from authenticated commercial cell banks (ATCC and Cytion) and have been authenticated within the past 3 years by the cell culture technical managers at the University of Brighton. Authentication was performed using short tandem repeat (STR) profiling for human cell lines, and all cell lines were confirmed to be free from cross-contamination and mycoplasma contamination prior to experimental use.

MTT assay

An MTT assay (475989; Sigma Millipore) was conducted to determine cell viability. Cells seeded in a 96-well plate were incubated for 48 h until confluent and then treated with 200 μL of fresh media with the following conditions: untreated (control), MGO (1 nM), MGO (1 mM) or MGO (1 mM) with dapagliflozin (10 μM) through which the cells were incubated overnight (18 h). In the next day, cells were then treated with 10 μL of MTT reagent and incubated for 2 h, after which 85 μL of solution was removed from each well, and 50 μL of DMSO was added to dissolve the formazan purple crystals. Readings were taken periodically (every 15 min) using a microplate reader (ASYS—UVM 340) at a wavelength of 540 nm.

LDH (lactate dehydrogenase) assay

An LDH assay (C20300; Invitrogen, Thermo Fisher Scientific) was conducted to determine levels of cell membrane damage. Cells seeded in a 96-well plate were incubated for 48 h until confluent and then treated with 200 μL of fresh media with the following conditions: untreated (control), MGO (1 nM), MGO (1 mM) or MGO (1 mM) with dapagliflozin (10 μM) through which the cells were incubated overnight (18 h). In the next day, cells' columns in the 96-well plate were alternately treated with 10 μL of lysis buffer and 10 μL of double distilled water to create spontaneous LDH release and Maximum LDH release duplicate series. They were then incubated for 45 min, after which 50 μL was taken from each well into a new 96-well plate and 50 μL of reaction mix was added. Absorbance readings were taken using the microplate reader periodically (every 15 min) at wavelengths of 490 and 680 nm.

Confocal microscopy

Cells were investigated through measuring caspase-1 intensity. After treating cells, they were fixed with 4% paraformaldehyde in PBS (p4417-100TAB; Sigma Millipore) and incubated for 1 h at room temperature (25°). Fixed cells were washed twice with PBS and then permeabilized with 0.5% TritonX-100 in PBS for 10 min. The cells were then washed with PBS and incubated with primary antibodies incubated in blocking buffer of PBS containing 2% FBS and 1% BSA. The primary antibodies were rabbit polyclonal antibody (19771-1-AP; Thermo Scientific, Thermo Fisher Scientific) and mouse monoclonal anti-caspase-1 antibody (M15-16215; Invitrogen). The cells were then incubated at 4° overnight. Afterwards, the cells were washed with PBS three times for 5 min per interval and incubated for 2 h at room temperature (25°) with anti-mouse and anti-rabbit fluorescence secondary antibodies (DK8828; Vectashield). Thereafter, cells were washed with PBS three times for 15 min before being stained with DAPI (H2000; Vectashield) to stain the nucleus of the cells. Cells were then stored at 4° in PBS until visualisation under the microscope. A laser scanning confocal microscope (LEICA TCS SP5, Version 2.7.3.9723, Wetzlar, Germany) was used to provide high-resolution detailed images of the cells, differentiated into different colours to show the nucleus in (blue) and caspase-1 (green) presence in each of the treatment groups, as well as overall cell viability. FIJI IMAGEJ (version 2.14.0/1.54F) was used to measure fluorescence intensity of caspase-1 in each treatment group. Moreover, the intensity of DAPI staining was measured to assess nuclear condensation as a sign of pyroptosis.

Enzyme-linked immunosorbent assay (ELISA)

Interlukin-18 (IL-18) was measured in the supernatant of the ARPE-19 cells using ELISA kit (BMS267-2; Invitrogen).

Data analysis

All data analysis was conducted using GRAPHPAD PRISM with statistical significance measured via a One-way ANOVA with Dunnett's multiple comparisons test.

Results

Light microscope images showed cell swelling when ARPE-19 cells were incubated with MGO (1 mM). However, such a morphological change was not observed when cells were co-incubated with dapagliflozin (10 μM) in the presence of MGO (1 mM) (Fig. 2).

LDH assay was conducted to understand the extent of cell death in each condition. It provides a measure of lactate dehydrogenase released from all cells, an enzyme released when there is cell membrane damage, with absorbance levels providing an indication of cell injury. Control untreated cells were referred to as 0% cytotoxicity. Cells treated with methylglyoxal MGO (1 mM) 59.06 ± 1.43% showed a significant difference compared to both: control 0.00 ± 0.0058% and MGO (1 mM) cotreatment with dapagliflozin (10 μM) 33.5 ± 7.99% (Fig. 3).

MTT assay was then conducted to assess cell viability in each condition. Control untreated cells were referred to as 100% viability. Cells treated with methylglyoxal MGO (1 mM) 61.78 ± 4.64% showed a significant difference compared to both: control 100 ± 1.54% and MGO (1 mM) cotreatment with dapagliflozin (10 μM) 83.69 ± 1.92% (Fig. 4).

Using the data gathered from the LDH and MTT assays, confocal microscopy was conducted to confirm, visibly, that 1 mM MGO was causing profound ARPE-19 cell damage, prevented by dapagliflozin (10 μM). Accordingly, we investigated whether NLRP3 is involved in the cytotoxic effects of MGO in retinal cells. As shown in Fig. 5, ARPE-19 retinal cells did not show a clear presence of NLRP3 inflammasome under any conditions. However, brain neuronal cells (SH-SY5Y) were used as a type of cell that is known to express NLRP3, showing a clear baseline presence of NLRP3 in untreated cells, which increases significantly with MGO (1 mM).

Since NLRP3 was absent in retinal cells, we further investigated whether the whole canonical cascade of NLRP3 inflammasome is absent. Accordingly, caspase-1 was investigated. As shown in Fig. 6, green staining shows caspase-1 and blue staining shows the cells' nuclei. ARPE-19 cells treated with MGO (1 mM) showed increased intensity of green staining of caspase-1 in these cells, in addition to increased intensity of blue staining of nuclei, indicating caspase-1 associated pyroptosis. Cells treated with dapagliflozin (10 μM) showed a profound decrease in green staining of caspase-1 and a decrease in the intensity of blue staining of nuclei.

Cells treated with methylglyoxal MGO (1 mM) showed a significant increase in caspase-1 intensity 26.99 ± 1.81 arbitrary units (A.U) compared to both: control 12.64 ± 1.60 A.U and MGO (1 mM) cotreatment with dapagliflozin (10 μM) 14.07 ± 0.86 A.U (Fig. 7).

Since pyroptosis involves nuclear condensation and cell death, therefore, the nuclear condensation was measured through measuring DAPI intensity (Fig. 8).

Cells treated with methylglyoxal MGO (1 mM) showed a significant increase in DAPI intensity 215.8 ± 7.85 arbitrary units (A.U) compared to both: control 161.1 ± 11.14 A.U and MGO (1 mM)

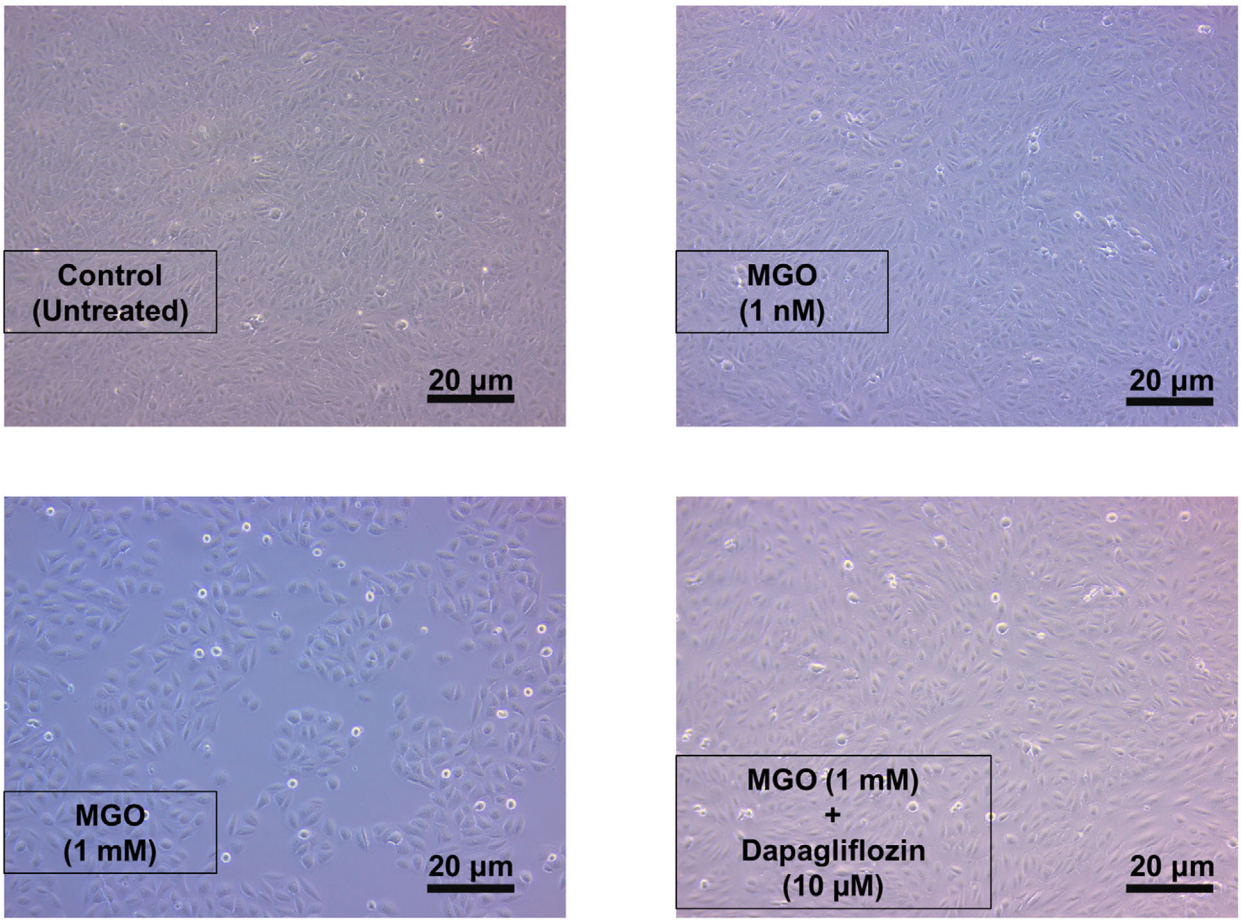


Fig. 2. ARPE-19 cells demonstrating cell swelling induced by MGO (1 mM) prevented by the cotreatment with dapagliflozin (10 µM) (20× magnification).

cotreatment with dapagliflozin (10 µM) 142.6 ± 10.21 A.U (Fig. 9).

Cells treated with methylglyoxal MGO (1 mM) showed a significant increase in IL-18 release 40 ± 8.33 pg·mL^{-1} compared to both: control 1.33 ± 3.71 pg·mL^{-1} and MGO (1 mM) cotreatment with dapagliflozin (10 µM) 8.67 ± 6.36 pg·mL^{-1} (Fig. 10).

Discussion

This study aimed to identify the effect of dapagliflozin as a potential therapeutic candidate that could complement existing treatments for DMO or work prophylactically to reduce its prevalence. The findings may offer insights into novel strategies for preventing retinal complications in diabetes and improving visual outcomes for affected individuals. It may also provide an investigation to confirm the pathway by which MGO causes DMO, as the involvement of NLRP3 inflammasomes in retinal cells is still under debate.

As shown in Fig. 3, a high concentration of methylglyoxal (1 mM) significantly increased the cytotoxicity of ARPE-19 cells. However, cotreatment with dapagliflozin provided significant protection against MGO-induced ARPE-19 cytotoxicity, exhibiting a protective effect by decreasing MGO-induced cytotoxicity by approximately 50% in ARPE-19. Such a cytotoxic effect of MGO (1 mM) is supported by a study, which has shown an approximately 75% reduction in cell viability when treated with MGO (1 mM) [36] and another recent study that has shown an approximately 50% reduction in cell viability when ARPE-19 cells were treated with glyoxal 1 mM [37]. However, in our preliminary studies, we have noticed a slight reduction in ARPE-19 cell viability when treated with MGO (1 µM) by 87.28 ± 10.28%; therefore, we applied a 1 nM concentration. Moreover, with the lack of knowledge regarding the exact MGO concentration in

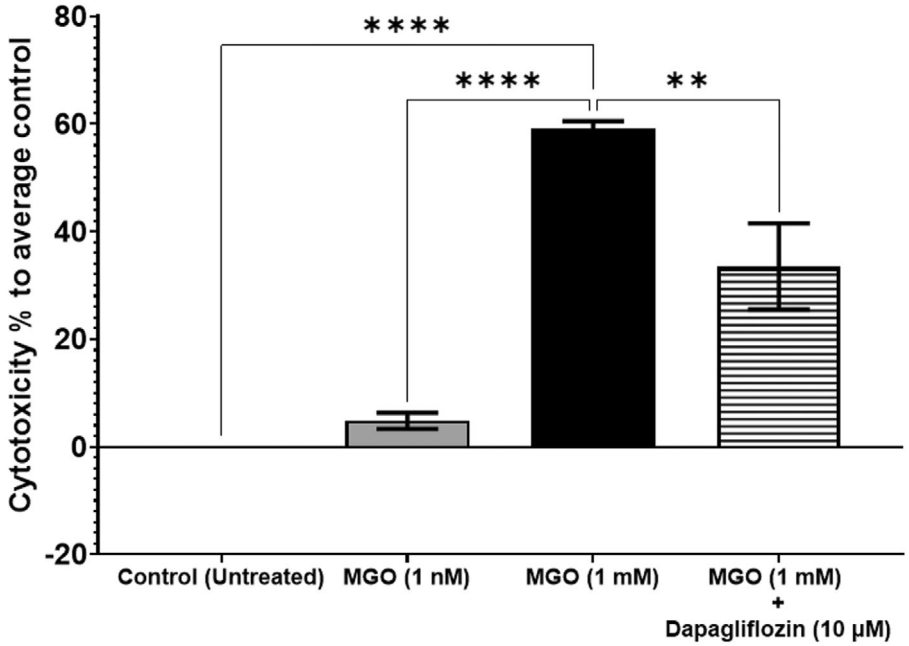


Fig. 3. Cytotoxicity from LDH assay in ARPE-19 cells. Cells treated with methylglyoxal MGO (1 mM) showed significant difference compared to both: control and MGO (1 mM) cotreatment with dapagliflozin (10 µM.**** representing significance when $P < 0.0001$ and ** representing significance when $P < 0.01$ analysed through one-way ANOVA using Dunnett's multiple comparisons test. Data presented as mean ± SEM ($n = 3$).

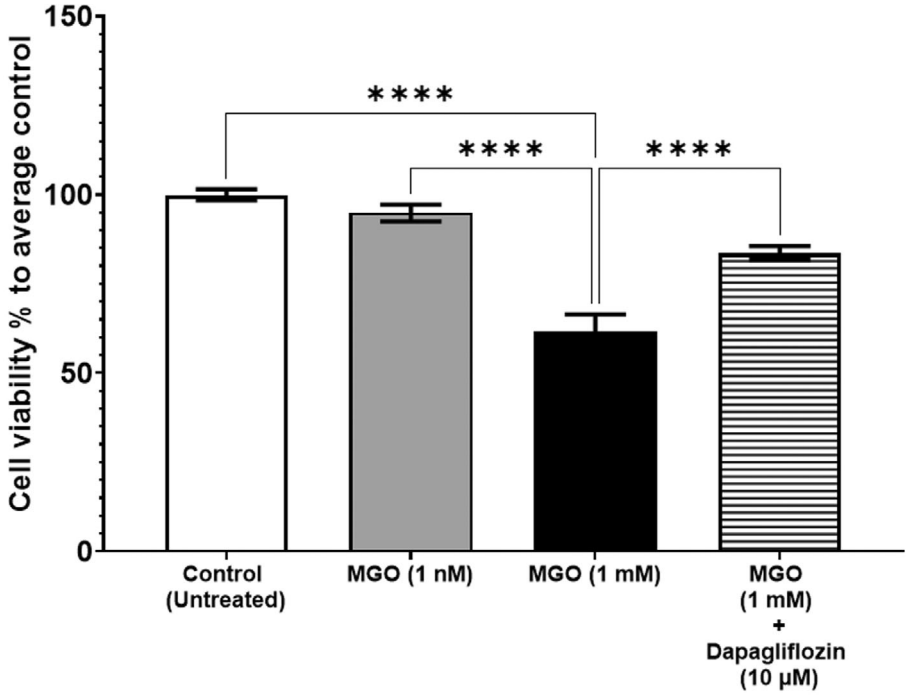


Fig. 4. Cell viability from MTT assay in ARPE-19 cells. Cells treated with methylglyoxal MGO (1 mM) showed significant difference compared to both: control and MGO (1 mM) cotreatment with dapagliflozin (10 µM).**** representing significance when $P < 0.0001$ analysed through one-way ANOVA using Dunnett's multiple comparisons test. Data presented as mean ± SEM ($n = 3$).

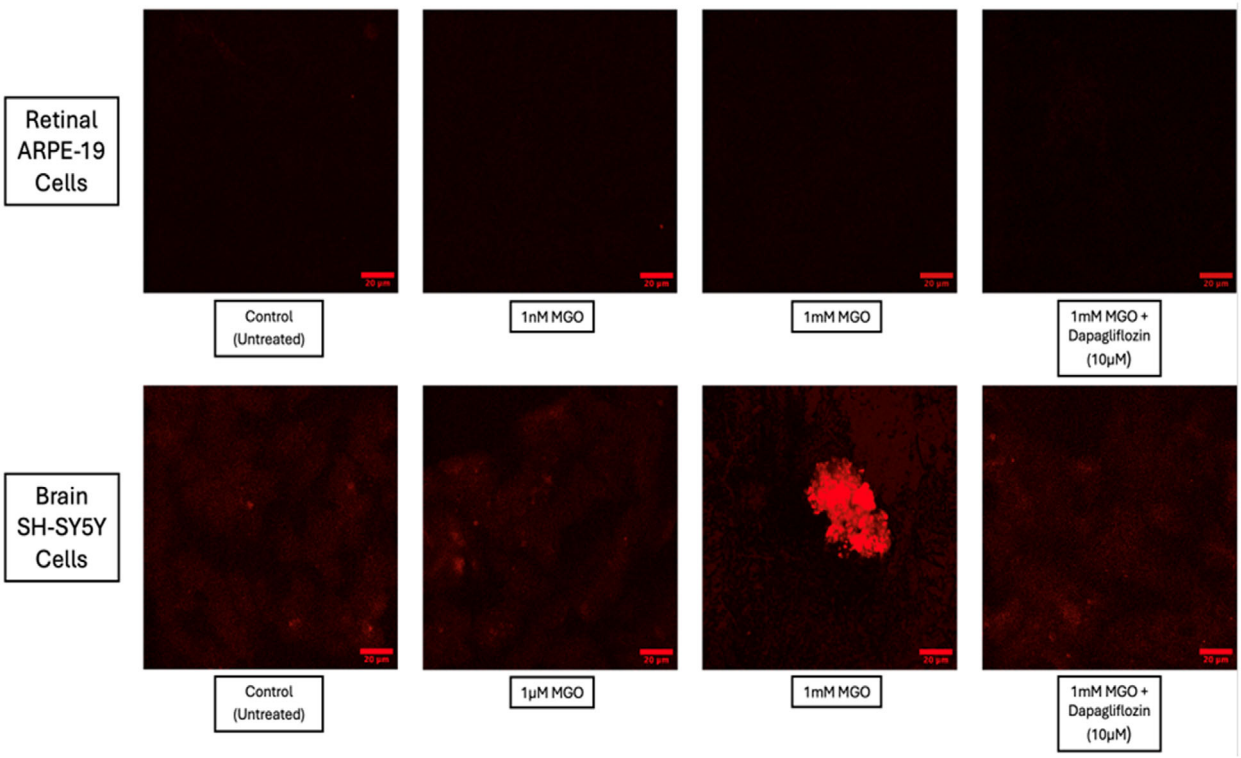


Fig. 5. Images from confocal microscopy of ARPE-19 cells and SH-SY5Y cells showing fluorescence emitted from the presence of NLRP3 inflammasome under four different conditions in both cells. ARPE-19 cells show no distinct fluorescence when looking for NLRP3 presence. The same antibody was used to identify NLRP3 in SH-SY5Y cells, and a distinct presence of NLRP3 can be identified, with a clear increase in fluorescence when applied with methylglyoxal (1 mM). SH-SY5Y cells were treated with a different lower concentration than ARPE-19 cells as a negative control condition.

retinal tissue in normal physiological conditions, the 1 nM concentration may represent a normal physiological concentration that is proportional to the 100 nM plasma concentration in physiological conditions [15]. Studies have shown that MGO, as a precursor to AGE formation, induces reactive oxygen species (ROS) production [38]. When produced in excess, ROS cause lipid peroxidation of the plasma membrane, increasing permeability and therefore LDH leakage, indicating significant cell membrane damage and death [39]. These data were further supported when conducting the MTT assay, which provided insight on cell viability under each of the conditions. As illustrated in Fig. 4, ARPE-19 cells treated with a high concentration of MGO (1 mM) showed approximately a 40% reduction in cell viability, an effect counteracted by dapagliflozin, which showed a cell viability of 83%, providing a significant increase in cell survival. The mitochondria serve as an indicator for viable cell presence, and the mitochondrial membrane potential (MMP) is vital for energy production and cell survival, as its depletion can result in a release of pro-apoptotic factors, triggering cell death [40]. The reduction in cell viability shown by MGO suggests MMP collapse, which could be stabilised by dapagliflozin, therefore protecting mitochondrial function. The mitochondria, and oxidative stress caused by MGO, could play a pivotal role in the cell death seen throughout the experiment. Thus, increased MGO leads to the production of ROS causing oxidative stress and hence inducing cell death [17]. Mitochondrial DNA (mtDNA) is highly susceptible to oxidative damage due to its limited repair mechanisms compared to nuclear DNA and its proximity to the electron transport chain, leading to defective oxidative phosphorylation and reduced ATP production [41]. Excess of ROS can damage the MMP and result in opening of the mitochondrial permeability transition pore (mPTP). This can cause the release of mtDNA into the cytoplasm, and Cytochrome C, triggering apoptosis [40,41]. Additionally, mitochondrial dysfunction caused by oxidative stress can trigger necrotic cell death, further releasing LDH. Dapagliflozin's ability to reduce cytotoxicity suggests a potential cytoprotective mechanism, possibly through mitochondrial preservation or reduced oxidative stress. These findings align with previous research, suggesting that high

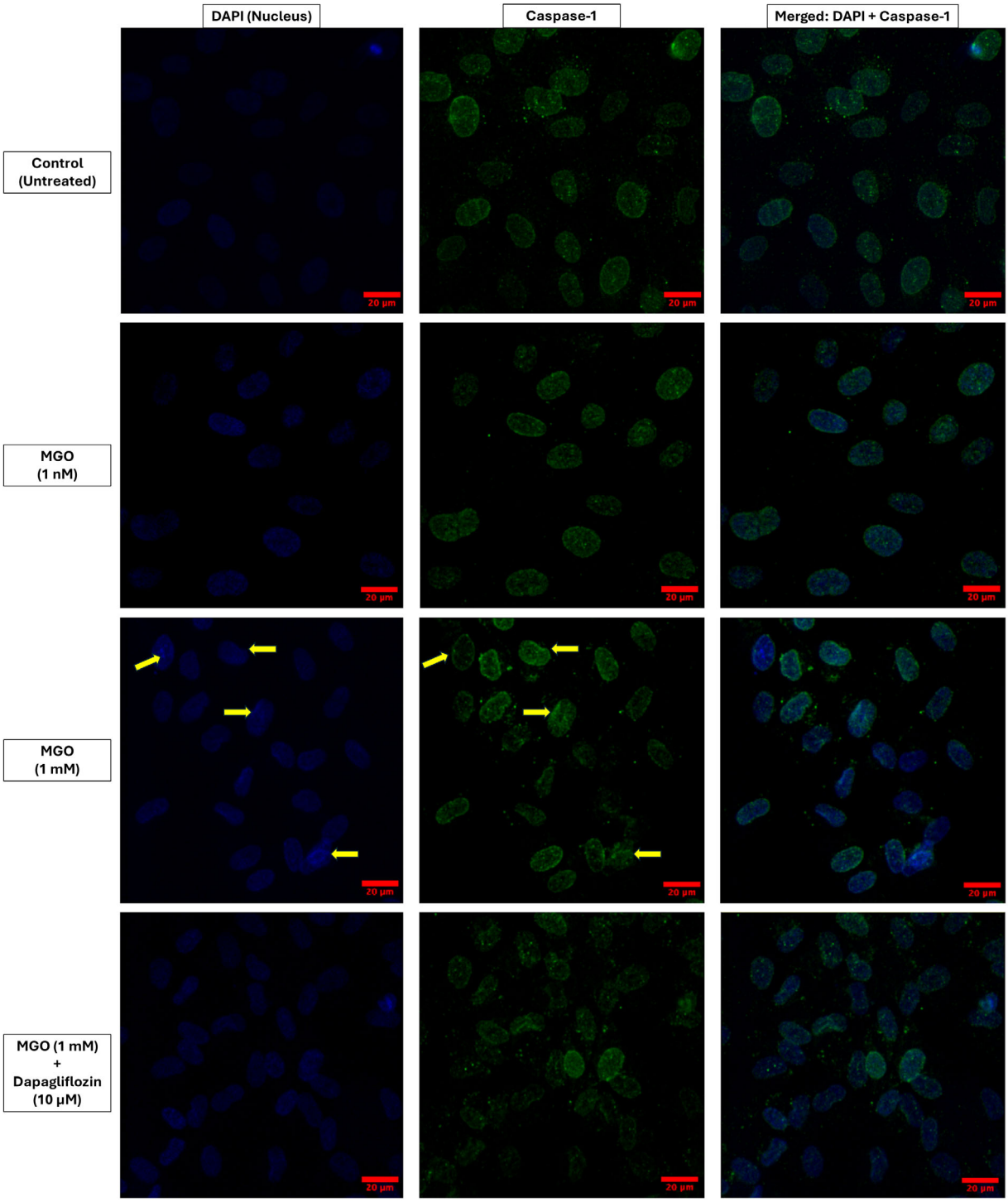


Fig. 6. ARPE-19 cells with different treatments under the confocal microscope (LEICA TCS SP5, Version 2.7.3.9723). Green stained cells have had mouse monoclonal anti-caspase-1 antibody (M15-16215 supplied from Invitrogen) applied to show caspase-1 presence in the cells, with intensity indicating concentration of active caspase-1 in the cell. Blue stained cells have had DAPI (H2000 supplied from Vectashield) applied and show the presence of the nucleus within the cells. The third column of images shows combined staining of caspase-1 and DAPI. Figure shows profound increase in caspase-1 intensity (green staining) when treated with MGO (1 mM) associated with increased intensity in DAPI (nucleus) staining as indicated by yellow arrows. Cells co-treated with dapagliflozin (10 µM) and MGO (1 mM) have decreased intensity of caspase-1 fluorescence with reduced intensity in DAPI (nucleus) staining.

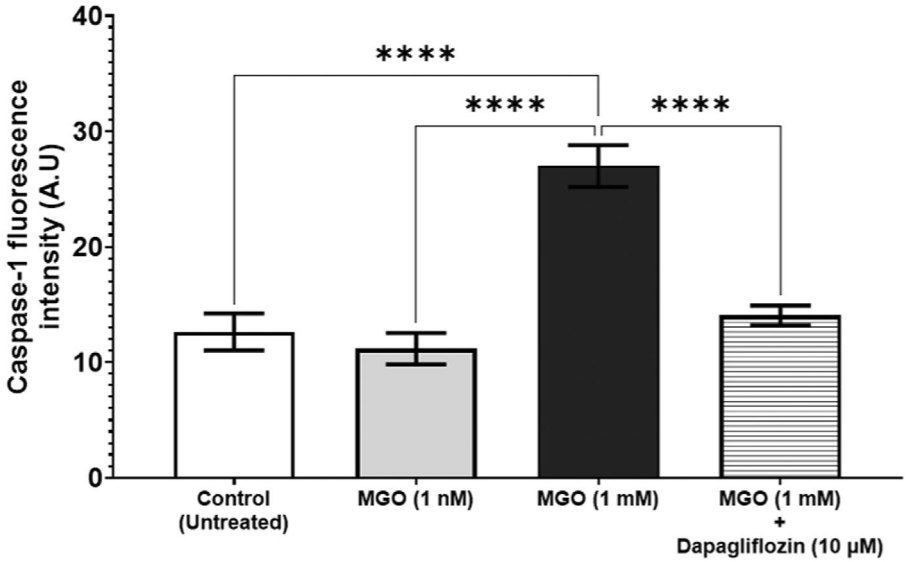


Fig. 7. Caspase-1 fluorescence intensity in ARPE-19 cells. Cells treated with methylglyoxal MGO (1 mM) showed a significant difference compared to both: control and MGO (1 mM) cotreatment with dapagliflozin (10 µM). **** representing significance when $P < 0.0001$ analysed through one-way ANOVA using Dunnett's multiple comparisons test. Data presented as mean ± SEM ($n = 4$).

concentrations of MGO contribute to retinal cell damage by induction of oxidative stress, mitochondrial dysfunction and apoptosis. However, the effect of MGO on cell proliferation needs further studies assessing the proliferation rate such as the BrdU assay. The focus on SGLT2i is based on recent approvals for empagliflozin and dapagliflozin for the treatment of heart failure [42,43]. Therefore, the effect of SGLT2i on retinal cells was conducted to investigate the possibility of applying this class of antidiabetics for DMO. However, other classes of antidiabetic drugs are still worth investigating for future studies. Interestingly, a study conducted in Alexandria investigated the effect of using dapagliflozin as an adjunct to anti-VEGF therapy for DMO [44].

Thereafter, confocal microscopy studies were conducted to investigate the association of NLRP3 in mediating such MGO (1 mM)-induced cytotoxicity. However, as shown in Fig. 5, NLRP3 was not detected in ARPE-19 cells, whilst it was detected in another cell line: human brain neuronal cells SH-SY5Y. The use of SH-SY5Y was based on a recent study that revealed the expression of NLRP3 in these cells [45], which were available in the lab during the time of experimentation. Whilst in previous studies, NLRP3 has been viewed as the primary inflammasome in diabetic retinal pathology, this study suggests there was no NLRP3 activation in ARPE-19 cells under hyperglycaemic conditions with high concentration MGO. This raises the possibility of alternate inflammasome presence and activation in retinal cells, which could be contributing to inflammation in the diabetic retina. One study found an absence of NLRP3 in various RPE models and suggested that previous detection of NLRP3 in retinal epithelium cells may have resulted from nonspecific antibody use [46]. Results from confocal microscopy support this argument, suggesting there may be other inflammasome involvement in the ARPE-19 cells. Thereafter, we investigated caspase-1 association with MGO (1 mM)-induced cytotoxicity. Accordingly, confocal microscopy confirmed the increased activation of caspase-1 in cells treated with MGO (1 mM), which is accompanied by an increase in DAPI nuclear staining intensity (Figs 7 and 8). Since DNA condensation is a sign of pyroptosis [47], therefore, increased DAPI staining may reflect DNA condensation (Figs 7 and 8), associated with caspase-1 increased intensity (Figs 5 and 6). However, when ARPE-19 cells were treated with MGO (1 mM) and dapagliflozin (10 µM), it led to a significant reduction in caspase-1 intensity and DAPI nucleus staining (Fig. 9), associated with increased IL-18 release (Fig. 10). This suggests that there is less caspase-1 activation found in cells treated with dapagliflozin and a reduction in inflammatory cytokine release: IL-18, reducing cell death and inflammation. Such an increase in IL-18 through an NLRP3-independent pathway was reported in a previous study where ARPE-19 cells' DNA damage induced NLRP3-independent IL-18 secretion in human RPE cells [48]. The activation of caspase-1 and the release of IL-18 may indicate the

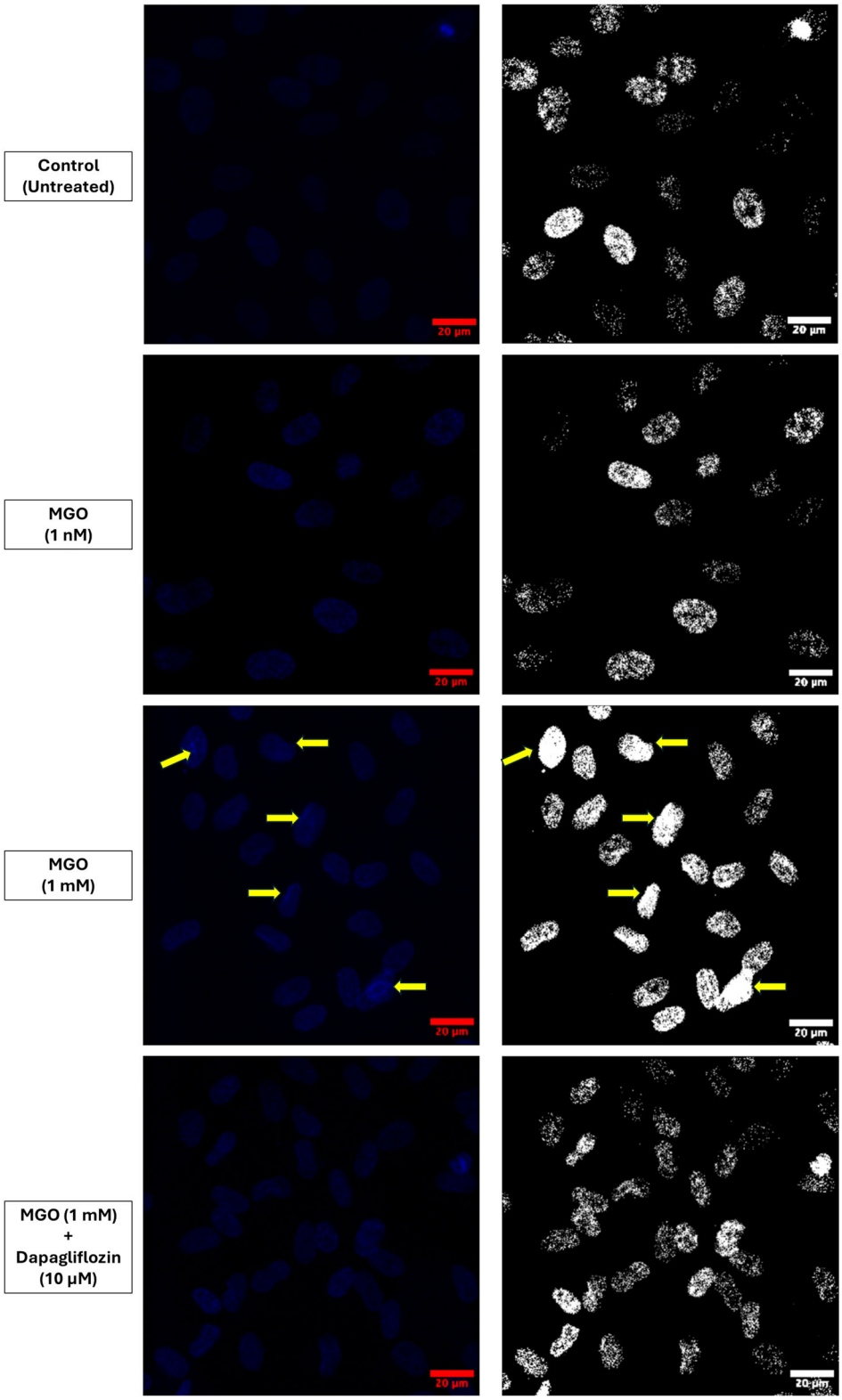


Fig. 8. Nuclear staining intensity in ARPE-19 cells with different treatments under the confocal microscope (LEICA TCS SP5, Version 2.7.3.9723). Blue stained nuclei with DAPI (H2000 supplied from Vectashield) showed profound high intensity, which was measured in greyscale as indicated by yellow arrows.

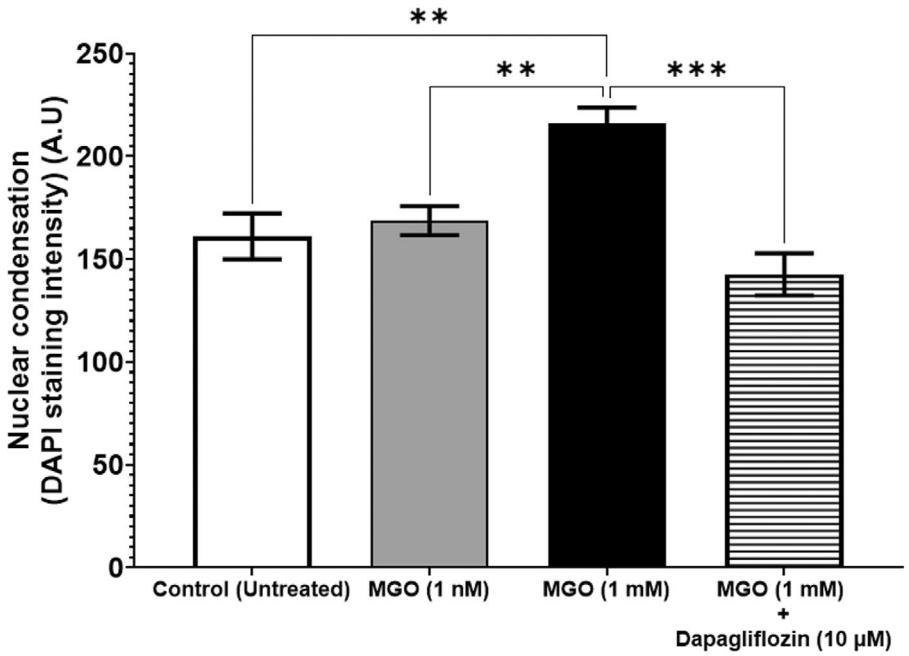


Fig. 9. DAPI fluorescence intensity in ARPE-19 cells. Cells treated with methylglyoxal MGO (1 mM) showed significant difference compared to both: control and MGO (1 mM) cotreatment with dapagliflozin (10 µM). *** representing significance when $P < 0.001$ and ** representing significance when $P < 0.01$ analysed through one-way ANOVA using Dunnett's multiple comparisons test. Data presented as mean ± SEM ($n = 4$).

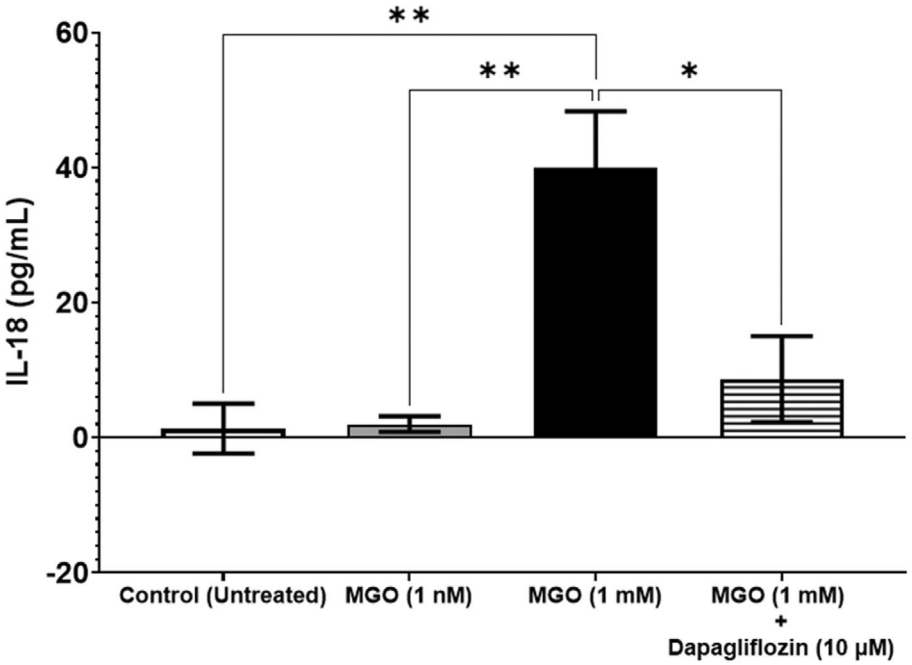


Fig. 10. Interlukin-18 (IL-18) release from ARPE-19 cells. Cells treated with methylglyoxal (MGO) (1 mM) showed significant difference compared to both: control and MGO (1 mM) cotreatment with dapagliflozin (10 µM). ** representing significance when $P < 0.001$ and * representing significance when $P < 0.05$ analysed through one-way ANOVA using Dunnett's multiple comparisons test. Data presented as mean ± SEM ($n = 3$).

involvement of GSDMD, which forms pores in the plasma membrane, thereby triggering plasma membrane rupture and hence the release of intracellular pro-inflammatory mediators such as IL-18, ionic/osmotic flux and cellular swelling (as shown in Fig. 2) and hence leading to pyroptosis [49,50].

Recent research on retinopathy of prematurity (ROP) highlighted the involvement of AIM2 inflammasome in retinal neovascularisation (RNV) [51]. The study demonstrated that excess ROS production impaired the autophagy-mediated degradation of AIM2, resulting in its accumulation and activation of the caspase-1 pathway, causing release of IL-1β and IL-18. This exacerbated pathological angiogenesis in the retina. Whilst ROP and DMO are distinct conditions driven by different triggers (hypoxia and hyperglycaemia-induced vascular leakage), both share common inflammatory pathways, particularly linked to ROS generation and IL-1β/IL-18 signalling. Hyperglycaemia is a major cause of oxidative stress in DMO; therefore, it is plausible that a similar ROS-mediated inflammasome activation may be occurring in retinal cells. The lack of NLRP3 involvement seen through confocal microscopy, especially compared to other cells, could suggest that the AIM2 inflammasome may potentially be involved in the inflammatory response of ARPE-19 cells under diabetic conditions, resulting in DMO. Previous studies showed AIM2 activation linked to oxidative stress and inflammatory cytokine release. Moreover, a previous study showed the AIM2 inflammasome is activated by numerous factors, the primary trigger being cytosolic DNA. It acts as a sensor for DNA in the cytoplasm, which has leaked from its primary source [52]. This could suggest a hypothesis for future investigation where the mtDNA may be released into the cytoplasm due to oxidative stress induced by MGO and hence may potentially contribute to activation of the AIM2 inflammasome, therefore triggering caspase-1 activation, inflammation and cell death in retinal cells.

Dapagliflozin is licenced for use in diabetes and heart failure, with its mechanisms of action not fully understood. Dapagliflozin's significant effects on ARPE-19 cell viability may be due to its ability to protect mitochondrial function by reducing ROS production. Studies have proposed that dapagliflozin does this through dampening the inhibitory effects of hyperglycaemia on Nrf2 signalling through AMPK activation [53]. Thus, under normal conditions, Nrf2 is bound to Keap1 and inactivated. However, under oxidative stress, Keap1 is unbound from Nrf2, allowing it to bind Antioxidant Response Elements (ARE's) in the nucleus and increase production of antioxidant enzymes such as superoxide dismutase (SOD), preventing excess superoxide formation ($O^{2\bullet-}$) [54]. Studies have shown that SGLT-2 inhibitors may activate the AMPK pathway, phosphorylating Nrf2 and releasing it from Keap1 to produce antioxidant enzymes and reduce ROS generation [55,56]. Other studies have suggested its role in NF-κB activation resulting in an anti-inflammatory response, though its role is not fully understood. Accordingly, dapagliflozin's protective effects may stem from mitochondrial preservation, oxidative stress reduction or modulation of alternate inflammatory pathways. Its ability to do so through the AMPK pathway could be confirmed in future study by comparing its activity to A769662, an AMPK Metabolic Activator [57]. This is because A769662 is used in research to directly activate AMPK by binding to its β-subunit, therefore helping to determine whether the effects of dapagliflozin are AMPK-dependent. The involvement of AIM2 as the primary inflammasome could be an investigation for further study, using techniques such as ELISA or western blotting to determine its presence. This can also be used to determine the involvement of IL-1β, IL-18 and VEGF as inflammatory markers in DMO.

Conclusion

To conclude, this study provides new insights into the protective effects of dapagliflozin against MGO-induced cytotoxicity in retinal cells. The results suggest that dapagliflozin may promote cell viability and reduce apoptosis through the NLRP3-independent caspase-1 pathway. These findings have important implications for developing potential therapeutic strategies for diabetic macular oedema and diabetic retinopathy, highlighting dapagliflozin as a promising candidate for further investigation in retinal protection in diabetic patients.

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Conflict of interest

The authors declare no conflict of interest.

Peer review

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Data accessibility

The data that support the findings of this study are available from the corresponding author [y.a.shamsaldeen@ljmu.ac.uk] upon reasonable request.

Author contributions

YAS conceived and designed the project. NT, ZQ, SKAF, MW, AC, RA and EM acquired the data. YAS, NT, ZQ and SKAF analysed and interpreted the data. YAS, NT and ZQ wrote the paper.

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