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Assessment of potential cellular toxicity of methylglyoxal on primary human epidermal keratinocytes

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ABSTRACT

Skin is the outermost layer of the human body, which is important to prevent damage by infection, ultraviolet radiation, and toxic materials. The impermeable barrier of the skin has to be maintained via skin regeneration to preserve its essential function. However, skin regeneration declines due to decreased cell proliferation during skin aging. In this study, we investigated the role of methylglyoxal (MGO) as a precursor of advanced glycation end-products (AGE), which have been reported as an emerging threat of early aging in the skin. Previous studies reported a reduction in survival of the HaCaT cell line exposed to 500 µM MGO. However, studies on primary keratinocytes are still limited. We evaluated the viability and proliferation of primary human epidermal keratinocytes (HEKa) under the exposure of MGO. We also employed H_2DCFDA assay to quantify the generation of intracellular ROS due to MGO. Our study revealed a biphasic response in which lower doses of MGO (25 and 50 µM) increased viability, while the cellular toxicity began at higher concentrations of 100, 200, and 300 µM. The proliferation assay also suggests an overall reduction of primary epidermal keratinocytes exposed to 100 and 200 µM MGO after 24 and 48 hours of culture. Increasing ROS concentrations were also observed in cells treated with 100 and 200 µM MGO. Our data suggested that a lower concentration of MGO was required to induce cellular toxicity in primary human epidermal keratinocytes compared to the HaCaT cell line reported by another study, indicating different sensitivity to MGO.

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1. Introduction

Human skin is the first layer of defense to protect the internal organs against the threat of parasitic infections and chemical intoxication and to prevent cellular damage by ultraviolet radiation (Zhang and Duan, 2018). Healthy and youthful skin also represents physical aesthetics and contributes to the level of self-esteem and emotional well-being (Costeris et al., 2021; Humphrey et al., 2021). To preserve skin health, the skin needs to maintain its regenerative capacity (Nanba et al., 2021). The balance of skin regeneration is manifested by symmetric and asymmetric divisions of keratinocytes in the basal layer of the epidermis (Pastushenko et al., 2015; Poulson and Lechler, 2012). The main objective of the asymmetric division is to undergo terminal differentiation to form an impermeable barrier of stratum corneum, which protects the dermis and hypodermis from environmental stressors (Poulson and Lechler, 2012; Zhong et al., 2022). This property maintains the level of water, which is important for skin hydration (Wang et al., 2020; Zhang and Duan, 2018).

In symmetric division, basal keratinocytes have the competence to proliferate to produce two identical daughter cells to increase the number of progenitor cells in the basal layer of the epidermis (Doi et al., 2020). The proliferation rate of basal keratinocytes is an essential element during skin repair, in addition to its ability to migrate and differentiate (Piipponen et al., 2020). However, previous studies have observed reduced proliferation capacity of basal keratinocytes during skin aging (Ho and Dreesen, 2021). A relationship between the decline in the proliferation rate of keratinocytes and skin aging was reported by Gilhar et al. (Gilhar et al., 2004). This research noted the fall in the proliferation rate of keratinocytes, supported by elevated levels of cells that underwent apoptosis in subjects with older age compared to younger subjects (mean age of 70.7 years vs 23.4 years, respectively) (Gilhar et al., 2004). Similar findings were also reported by Rübe et al., who observed a significant decline in the proliferation of basal keratinocytes in the skin of older subjects (Rübe et al., 2021).

In addition to persistent sun exposure, inhalation of harmful pollutants, and diet (Bahraman et al., 2021; Choi et al., 2016; Percoco et al., 2021), high levels of sugar consumption have become an emerging threat that potentially manifests the aging signs at a younger age (Quondamatteo, 2014). Food with high sugar is a major source of exogenous advanced glycation end products (AGE), which have been reported to be elevated due to increasing age. Accumulation of AGE causes collagen crosslinking, which leads to

reduced skin flexibility. Glycated collagen is also more resistant to degradation and interferes with the regeneration of collagen (Chen et al., 2022; Gkogkolou and Böhm, 2012; Zeng et al., 2019).

In addition to being supplied exogenously, the precursor of AGE can also be synthesized internally in the form of methylglyoxal (MGO) (Sheng et al., 2020). MGO is a by-product of the cellular glycolysis pathway in which the concentration is elevated due to increasing glucose (hyperglycemia) (Nickel et al., 2021). MGO is the predominant precursor of AGE and is classified as a reactive dicarbonyl species. Due to its highly reactive nature, MGO may form interactions with protein and DNA to form AGE (Waqas et al., 2022). According to a report, glycation of MGO produced more than 90% Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), which could bind to the receptor of advanced glycation end product (RAGE) to induce signaling cascade of inflammation, differentiation, and cell death (Nickel et al., 2021).

The cellular toxicity effect of MGO in skin cells has been reported previously. A study on fibroblast samples from diabetic and normal subjects suggested that exposure to MGO increased the level of intracellular reactive oxygen species (ROS), elevated secretion of interleukin-6 (IL6), and reduced glyoxalase (Glo) I activity (Nickel et al., 2021). In another study, the treatment of 500 μM MGO was shown to reduce the cell viability of the HaCaT cell line to almost 50% (Cepas et al., 2021).

Previous reports on skin research were focused on the HaCaT cell line due to its immortalized properties. Although they have long been recognized as a powerful tool in toxicological research, cell lines are genetically altered and may cause a change in their response to stimuli, cellular function, and phenotype (Kaur and Dufour, 2012). Therefore, the use of primary cells is urgently required to obtain more representative data. In the present study, we assessed the cellular toxicity effect of MGO on the proliferation rate of primary adult human epidermal keratinocytes (HEKa) as a more representative cell model for studying skin aging due to MGO deposition. We also compared our result with the HaCaT cell line reported by another study.

2. Materials and methods

2.1. Materials

Methylglyoxal 40% (Cat. No. M0252) was purchased from Sigma-Aldrich (Darmstadt, Germany). EpiLife medium with 60 μM calcium (Cat. No. MEPI-500-CA), primary human epidermal keratinocytes (HEKa) (Cat. No. C-005-5C), human keratinocyte growth supplement (HKGS, Cat. No. S0015), trypsin/EDTA solution (Cat. No. R001100), defined trypsin inhibitor (Cat. No. R007100) and antibiotic-antimycotic (100X) (Cat. No. 15240096) were purchased from Gibco (New York, USA). Promega cell titer 96-aqueous non-radioactive cell proliferation assay (Cat. No. G5421) was purchased from Promega (Wisconsin, USA). Reactive oxygen species (ROS) detection assay kit (ab287839) was purchased from Abcam (Massachusetts, USA).

2.2. Methods

2.2.1. Cell culture

The experiment was conducted at the Molecular Biology and Proteomics Core Facilities, Indonesian Medical Education and Research Institute, Universitas Indonesia, from October to November 2023. The primary adult human epidermal keratinocyte (HEKa) was grown in a humidified incubator under 5% CO_2 at 37°C using EpiLife medium enriched with human keratinocyte growth supplement (HKGS) and antibiotic-antimycotic solution to ensure maximum cell doubling capacity (Lecci et al., 2021). Culture management was performed by passaging once the population reached approximately 70% confluence to prevent basal keratinocyte differentiation and reduced proliferation (Leone et al., 2013).

2.2.2. Viability assay

The toxicity of methylglyoxal (MGO) was performed using the Promega CellTiter 96-aqueous non-radioactive cell proliferation assay kit following the manufacturer's instruction (Liu et al., 2018). The assay was conducted to determine the toxic effect of MGO on HEKa within the concentration range of 25, 50, 100, 200, and 300 μM for 24 hours (Wang et al., 2022). Briefly, the assay was initiated by culturing keratinocytes in a clear 96-well plate at a cell density of 10^4 cells/well. The survival of keratinocytes was determined by the formation level of soluble formazan in cultured media at the absorbance of 490 nm after incubating with MGO for 24 hours. The impact of MGO on the viability of HEKa was compared to cells treated with complete media and cells exposed with 6.25 μM of H_2O_2 as positive control.

2.2.3. Intracellular ROS measurement

The level of intracellular ROS was determined by H_2DCFDA assay, which can detect the presence of hydroxyl, peroxyl, and other classes of reactive oxygen species (Berliner and Parinandi, 2020). The assay was performed by seeding the cells at 3 x10^4 cells/well in a black 96-well plate and then left overnight to allow cell attachment. The culture was washed with 100 μL Abcam ROS assay buffer before being treated with 0, 100, and 200 μM of MGO for 24 hours. Another washing step was performed before transferring 100 μL of 1x ROS label to each well and then placed in the incubator for 45 minutes. The concentration of ROS was quantified with fluorometric readings at Ex. 495/Em. 529 nm using ThermoFisher Varioskan™ Lux Multimode Microplate Reader. The intensity of each well was normalized against the absorbance of formazan readings at 490 nm to minimize the variable of cell number. The effect of MGO in stimulating ROS generation was compared to cells treated with complete media and cells exposed with 6.25 μM of H_2O_2 as positive control.

2.2.4. Proliferation assay

Proliferation capacity of HEKa was measured under the presence of 100 and 200 μM of MGO for 24 and 48 hours of culture (Wang et al., 2022). The proliferation assay was conducted using the same colorimetric-based assay according to the manufacturer's protocol (Liu et al., 2018). The culture was prepared on a clear 96-well plate in a starting concentration of 10^4 cells/well and incubated for 24 hours for cell attachment. The treatment was performed by switching the culture media, whether with control or media containing 100 and 200 μM of MGO. The proliferation capacity was determined by measuring the soluble formazan at 490 nm after 24 and 48 hours of incubation (Liu et al., 2018).

2.2.5. Morphological evaluations

The morphological changes of keratinocytes were also observed to compare the effect of MGO between the experimental groups. The impact of MGO on the morphology of HEKa was observed with Nikon Eclipse Ti2 inverted microscope. The evaluation was also conducted on control and cells treated with 6.25 μM of H_2O_2 as reference.

2.2.6. Data analysis

Experiments in this study were conducted during cell passages 4-6 to maintain the consistency of the culture population. Experiments are performed with three replicates with background correction. Data analysis and visualization were performed with GraphPad Prism 9.5.1. One-way ANOVA was used to test for significant differences between each treatment and the control with minimum $p<0.05$.

3. Results and discussion

3.1. Results

3.1.1. MGO-induced biphasic response of HEKa

The measurement of cell viability was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based assay kit which was reduced to soluble formazan by dehydrogenases from viable cells. The concentration of viable cells is accurately correlated with the level of soluble formazan in culture media with optimum absorbance at 490 nm (Kamiloglu et al., 2020). Low-dose exposure of MGO on HEKa at concentrations 25 and 50 μM elevated the absorbance by 56.2% and 52% against the control, respectively. This result should indicate an increase in cell viability in HEKa exposed to lower doses of MGO (Fig. 1).

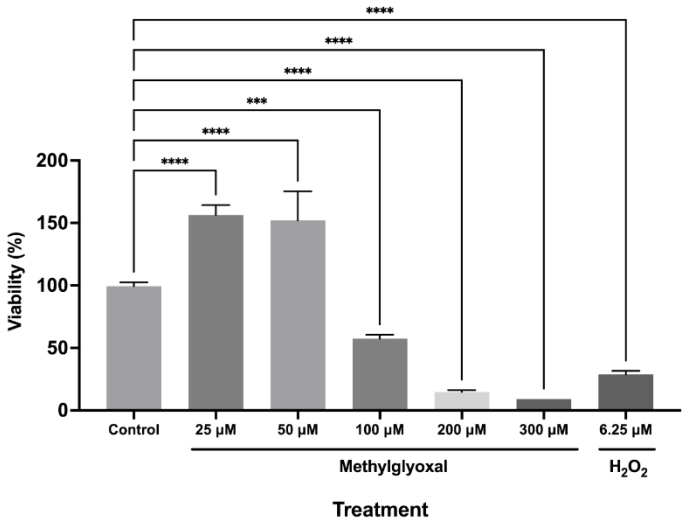

Fig. 1. Determination of HEKa viability with MTS assay. Note biphasic responses of HEKa during low and higher exposures with MGO. One-way ANOVA was performed to determine statistical significance. Quantitative data are expressed as mean values with ±SD represented by error bars; n=3, *p<0.05.

Cellular toxicity was observed on HEKa exposed to MGO at a starting concentration of 100 μM, which reduced the viability to 57.3%. The decline in viability was also monitored on HEKa treated with MGO at concentrations 200 and 300 μM. HEKa treated with 200 μM showed a viability of 14.6%, while the viability on 300 μM dropped to 9% compared to control. The decline in viability manifests an increased toxicity of MGO in concentrations of 100, 200, and 300 μM. Therefore, these data suggest a biphasic response, in which low doses of MGO induced elevation in cell viability, while higher doses induced a toxic effect on HEKa *in vitro* (Fig. 1).

3.1.2. MGO stimulates the production of intracellular ROS

Quantitative measurement of intracellular ROS showed significant increases in ROS concentration on cells exposed to 100 and 200 μM MGO, as this elevation was also noted on cells treated with H_2O_2 as the positive control (Fig. 2). This finding may indicate that reduced viability from the previous assay was accompanied by a significant generation of ROS. In addition to the detection of hydroxyl, this assay would indicate the presence of peroxyl and other classes of ROS, which may facilitate the cellular death mechanism in HEKa.

3.1.3. MGO delayed cellular proliferation of HEKa

The proliferation rate of HEKa under the exposure of MGO was quantified at the checkpoint of 24 and 48 hours. The rate of proliferation was measured by the formation level of soluble formazan inside the culture media. A standard curve was made by culturing cells in a 96-well plate at cell densities of 2.5 x 10^3, 5 x 10^3, 7.5 x 10^3, 1 x 10^4, 1.25 x 10^4, 1.5 x 10^4, 3 x 10^4, and 4 x10^4. The data was analyzed to generate a sigmoidal standard curve using four four-parameter logistics (4PL) regression models with an r^2 value of 0.9962 (Fig. 3). Therefore, the absorbance obtained during the experiment would significantly reflect the number of cells and ideal for interpolating the cell number during the subsequent proliferation assay.

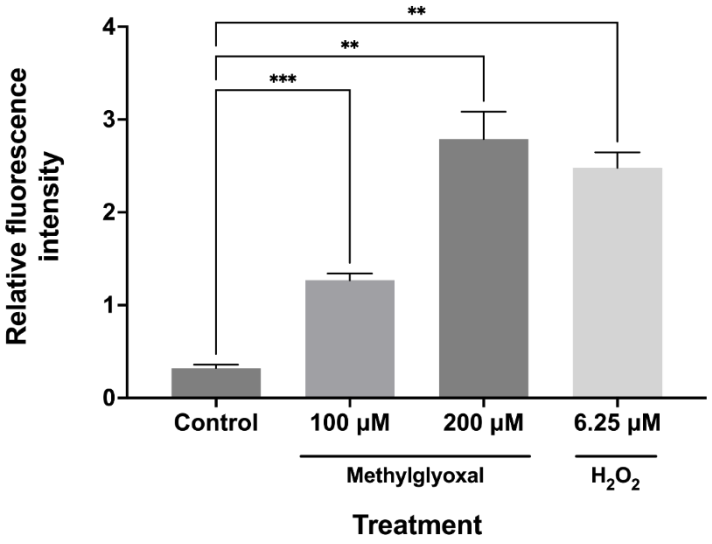

Fig. 2. Measurement of intracellular reactive oxygen species (ROS) using H_2DCFDA assay. Increased ROS generation in cells treated with 100 and 200 μM MGO. The fluorescence intensity was normalized against the absorbance of formazan at 490 nm to account for the number of viable cells. One-way ANOVA was performed to determine statistical significance. Quantitative data are expressed as mean values with ±SD represented by error bars; n=3, *p<0.05.

Exposure of MGO on HEKa led to an overall decline in proliferation after 24 and 48 hours of culture compared to control. At 24 hours, cells exposed both with 100 and 200 μM MGO significantly lost the proliferation capacity by 74%. However, there was no significant difference in proliferation level between cells treated with 100 and 200 μM MGO after 24 hours of culture (Fig. 3).

After a culture duration of 48 hours, we observed a recovery of the proliferation capacity of HEKa exposed to 100 μM MGO to approximately 63% compared to cells treated with complete media. We also noted a significantly lower recovery of proliferation on HEKa treated with 200 μM MGO, which only reached 37.8%. Therefore, this study provides evidence of a higher toxic effect of 200 μM MGO, which significantly delayed the proliferation capacity of HEKa.

In contrast to cells exposed to 6.25 μM H_2O_2, we did not observe noticeable changes in cell shape and cell density on HEKa exposed to 100 and 200 μM MGO after 24 hours of incubation (Fig. 4). This finding would suggest a different regulated cell death (RCD) mechanism, including a possibility of terminal differentiation from basal keratinocytes to corneocytes, considering minimum changes in observed morphology.

3.2. Discussion

Our findings in this study revealed a biphasic effect of HEKa exposed to different concentrations of methylglyoxal (MGO). HEKa incubated with 25 and 50 μM demonstrated an elevation in cell viability by 56.2% and 52%, respectively. Cellular toxicity was

observed at higher concentrations of 100, 200, and 300 μM, in which the viability was reduced to 57.3%, 14.6%, and 9%, respectively.

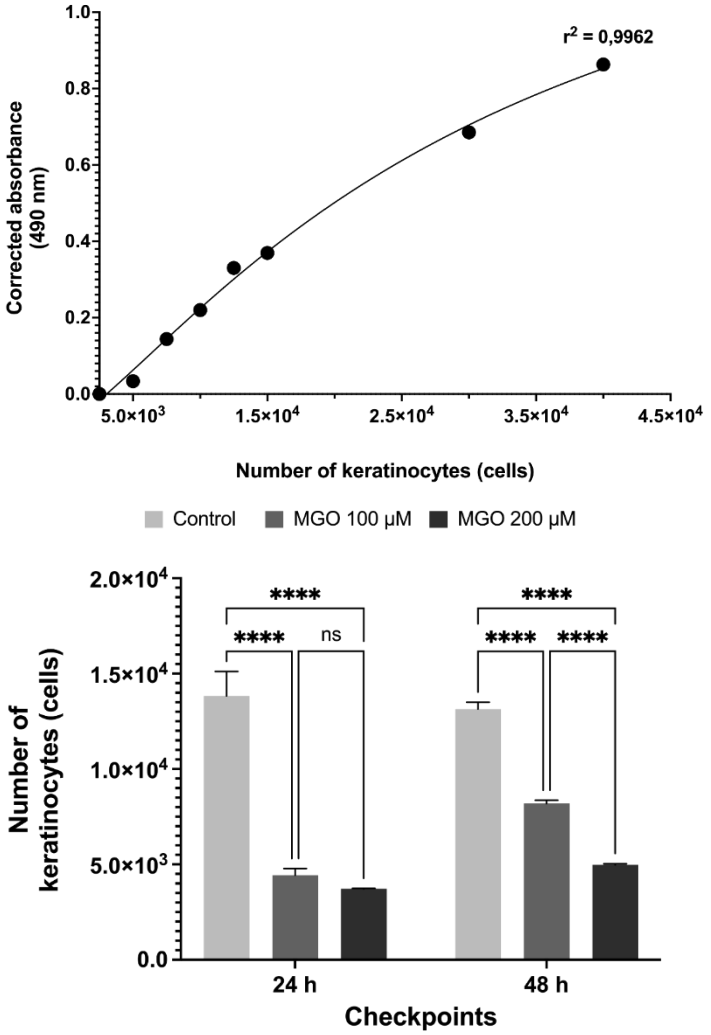

Fig. 3. Observation of significant delayed of cell proliferation due to 100 and 200 μM MGO exposure. The number of cells was estimated by standard curve. Quantitative data are expressed as mean values with ±SD represented by error bars; $n=3$, $^*p<0.05$.

Previous research to study the toxicity of MGO in HEKa has been limited. However, a similar biphasic response was observed in the HaCaT and HUVEC cell lines reported by another study (Blázquez-Castro and Stockert, 2015). A report from Blázquez-Castro and Stockert (2015) showed a biphasic response of the HaCaT cell line treated with low and higher doses of H_2O_2. HaCaT exposed to lower concentrations of H_2O_2 (10^{-6} to 10^{-5} M) showed a significant increase in cell proliferation and survival after 24 hours of culture. However, the cytotoxicity effect of H_2O_2 began to show at higher concentrations (10^{-4} to 10^{-3} M) (Elmhiri et al., 2014). In another study, the proliferation rate of HUVEC was also elevated due to a lower dose of H_2O_2 (10 μM), while a significant reduction in cell proliferation was observed in higher concentrations (200-500 μM) compared to control (Mu et al., 2010).

A distinct physiological response between primary adult human epidermal keratinocytes (HEKa) and its HaCaT cell line, as reported by another study. In the present research, a significant reduction of cell viability to 57.3% in HEKa exposed to only 100 μM MGO after 24 hours of treatment. In another study, an approximately 50% reduction in cell viability was observed on the HaCaT cell line after exposure to a higher dose (500 μM MGO) after 48 hours of incubation (Cepas et al., 2021). Another body of evidence also suggests similar findings, in which exposure to a higher dose of MGO (400 μM) only resulted in a 38.3% reduction of cell viability in the HaCaT cell line after 12 hours of culture (Sheng et al., 2020). This result indicates a higher sensitivity of HEKa to experimental stimuli by MGO compared to the HaCaT cell line, and therefore may provide a comparable cellular model of *in vivo* system.

In this study, we also revealed an overall reduced proliferation of HEKa exposed to 100 and 200 μM MGO after 24 and 48 hours of culture. In 24 hours, the proliferation of primary epidermal keratinocytes was significantly decreased by 74%. However, a recovery in cell proliferation was noted, especially on cells exposed to 100 μM MGO. This data indicated a delayed proliferation of primary epidermal keratinocytes due to MGO. The proliferation of keratinocytes is an essential part of cutaneous wound healing and is described as more favorable than its differentiation. This delayed proliferation was previously reported to occur due to epithelial closure and was also reported to be influenced by the presence of neutrophils in the wound site (Florin et al., 2006).

The cellular toxicity of MGO on HEKa may come from its nature as a highly reactive dicarbonyl species which interacts with amino groups of biomolecules of the cells to form AGE (Elmhiri et al., 2014). According to reports, approximately 90% of these interactions result in the formation of Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) following the interaction of MGO with arginine. An AGE molecule in the form of Nε-(1-carboxyethyl)-lysine (CEL) was also generated as a product of the MGO reaction with lysine (Nickel et al., 2021; Wetzels et al., 2019). Reaction involving MGO with DNA molecule also causes the glycation of guanine, which ultimately leads to DNA strand breaks and replication error due to the formation of MGO-DNA adducts (Guo et al., 2021).

AGE molecules generated by the excessive presence of MGO inside the cells could bind to cell surface RAGE (Gkogkolou and Böhm, 2012). In addition to promoting the expression of NF-κB, the activation of RAGE also stimulates the expression of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX), which catalyzes the synthesis of free radicals. The AGE-RAGE interaction also reduced the intracellular antioxidant system by inhibiting the activity of superoxide dismutase (SOD), catalase (CAT), and the level of glutathione (GSH) and ascorbic acid. This situation causes redox imbalance, which eventually results in oxidative stress (Chen et al., 2022; Gkogkolou and Böhm, 2012).

The key role of MGO in inducing redox imbalance has been described as causing cellular toxicity, including in skin cells. The exposure of 400 μM MGO increased the intracellular ROS by 2.24-fold and led to reduced viability of HaCaT by 61,7% (Sheng et al., 2020). In another study, 400 μM MGO also elevated the ROS level by two-fold and resulted in almost 50% of HaCaT death in vitro (Cepas et al., 2021). In accordance with experiments in keratinocytes, treatment of MGO also significantly increased the level of ROS in primary human fibroblast (Nickel et al., 2021). Similar physiological mechanisms may potentially occur in this study since we observed reduced proliferation accompanied by a significant elevation of intracellular ROS concentration due to MGO.

Interestingly, lower concentrations of ROS have been reported to stimulate the proliferation of the HaCaT cell line. Small and physiologically accepted amounts of ROS may be involved in the signaling pathway of proliferation by increasing the number of mitotic cells, therefore elevating the proliferation of cells exposed to low concentrations of ROS (Blázquez-Castro and Stockert, 2015). A similar physiological response may have a role in the increased cell viability of HEKa exposed to low concentrations of MGO in our study, although more data is required.

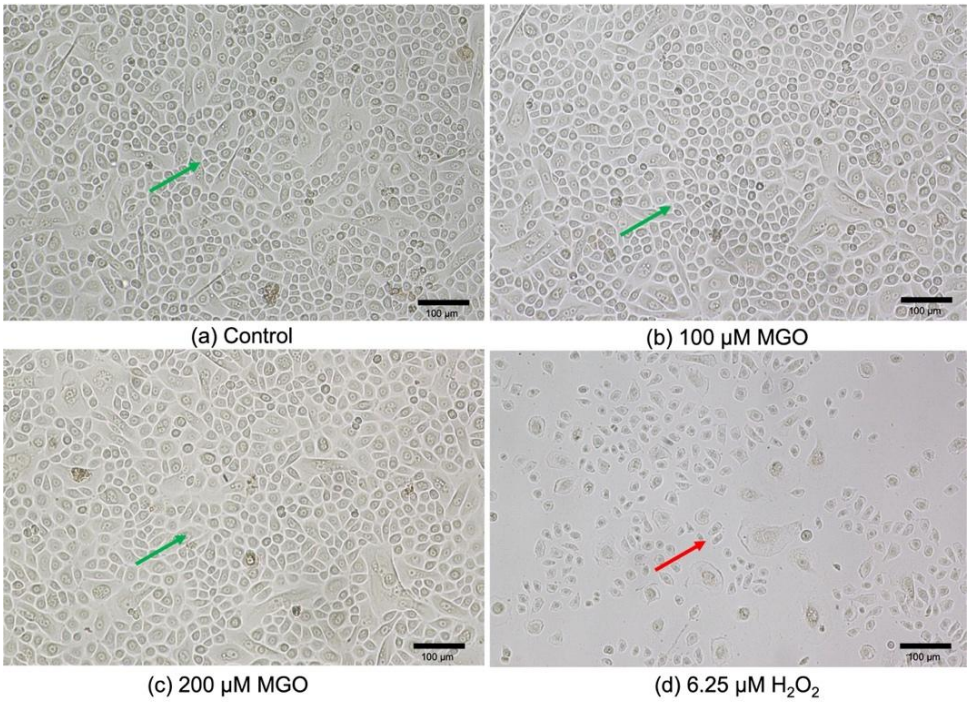

Fig. 4. Comparison of HEKa morphology after 24 hours. Note the absence of visual changes on cells exposed to MGO, while distinct morphology and the loss of cell-cell contact were visible on HEKa treated with 6.25 µM H_2O_2.

In regards to morphology, we did not observe noticeable indicators of cellular damage, such as the presence of blebs and irregular borders as a result of apoptosis (Blázquez-Castro and Stockert, 2015). Moreover, the common characteristics of pyroptosis, such as cell swelling and the rupture of the membrane, were also absent in our study (Blázquez-Castro and Stockert, 2015). Although more data is imminent, this study may indicate a different mechanism of regulated cell death (RCD) (Eckhart and Tschachler, 2018), including a possible terminal differentiation that results in the death of keratinocytes into corneocytes induced by MGO as a specific mechanism for primary cells.

4. Conclusion

Our study revealed the biphasic response of primary human epidermal keratinocytes exposed to different concentrations of MGO. Cells treated at 25 and 50 µM of MGO resulted in higher viability, while the exposure to higher levels of MGO (100, 200, and 300 µM) induced cellular toxicity after 24 hours of culture and was accompanied by increasing intracellular ROS concentrations. Reduced proliferation capacity of primary epidermal keratinocytes treated with 100 and 200 µM MGO were also noted after 24 and 48 hours. This study suggests a lower concentration of MGO was required to induce cellular toxicity in primary epidermal keratinocytes, indicating the responsiveness of primary cells compared to the cell line.

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Conflict of interest

The authors declare no conflict of interest in this research.

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