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Original Article

Resveratrol ameliorates the detrimental effects of high glucose on Wharton's jelly-stem cells by the differential regulation of cytokines, chemokines, and growth factors

Samar Sultan[1], Ahmed Alzahrani[1], Abdulwahab Noorwali[2,3], Ghadeer AlRefaei[4], Farid Ahmed[5], Peter Natesan Pushparaj[5], Gauthaman Kalamegam[5,6]

[1]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia; [2]Stem Cell Unit, King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia; [3]Department of Clinical Biochemistry, College of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia; [4]Biology Department, Faculty of Sciences, University of Jeddah, Jeddah, Saudi Arabia; [5]Center of Excellence in Genomic Medicine Research (CEGMR), King Abdulaziz University, Jeddah, Saudi Arabia; [6]Faculty of Medicine, AIMST University, Semeling, Bedong, Kedah, Malaysia

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Abstract: Hyperglycemia can induce tissue damage, by increasing the oxidative stress and pro-inflammatory mediators, whereas the secretome of stem cells helps in the restoration of tissue homeostasis. Resveratrol (RSV), a polyphenolic compound, is reported to exert both anti-inflammatory and antioxidant effects on both *in vitro* and *in vivo* models of health and disease. In this study, we evaluated the effects of high glucose (HG), at either 28 mM, and 41 mM, RSV (17 μM), or in combination, on the cell morphology, proliferation, and regulation of cytokines in human Wharton's jelly-derived stem cells (hWJSCs) *in vitro*. Treatment with HG reduced the hWJSCs proliferation compared with the control but did not affect the cell morphology. In addition, exposure to RSV alone or in combination with HG significantly enhanced cell proliferation. The cytokine analysis of the supernatants of the hWJSCs by Multiple Analyte Profiling (xMAP) based on magnetic bead-based technology using MAGPIX instrument showed that the expression of pro-inflammatory cytokine interleukin-6 (IL-6) was significantly reduced in all the treatment groups, while the interleukin-8 (IL-8) or chemokine (C-X-C motif) ligand 8 (CXCL8) was reduced upon treatment with RSV or RSV with HG at 72 h. Meanwhile, the granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion, was differentially regulated in the treatment groups when compared with control. We, therefore, concluded that HG inhibited the proliferation and caused cellular damage in hWJSCs. However, the RSV treatment helped to reverse these detrimental effects possibly by the differential regulation of IL-6, IL-8, and GM-CSF in hWJSCs.

Keywords: Wharton's Jelly stem cells, cell proliferation, high glucose, resveratrol, cytokines, multiple analyte profiling

Introduction

Hyperglycemia induces tissue damage through increased oxidative stress, which is accompanied by the augmentation of pro-inflammatory pathways [1]. Stem cells, with their ability to secrete multiple cytokines and growth factors, helps to maintain the micro-environment and also contribute to tissue repair [2, 3]. At the cellular level, hyperglycemia can potentially reduce stem cell proliferation, impair angiogenesis, reduce the secretion of growth factors, increase senescence, and apoptosis [4]. Elevated glucose levels activate protein kinase C (PKC), which in turn stimulate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase resulting in increased reactive oxygen species (ROS) production in bone marrow mesenchymal stem cells (BM-MSCs) [5]. The cell-damaging effects of HG concentration may also be mediated by ROS, and advanced glycation end products (AGE). It has been reported that the differentiation capacity of MSCs is reduced by AGE and the neutralizing antibody anti-RAGE improves their differentiation into adipose, bone, and cartilage tissues [6].

MSCs can self-renew and differentiate into multiple mesodermal tissue types and are therefore useful in regenerative medicine. Among the different types of MSCs, the human Wharton's jelly stem cells (hWJSCs) appear to be beneficial for use in cell-based therapy because they are embryonic in origin. Also, unlike bone marrow aspiration the hWJSCs can be easily and non-invasively harvested from umbilical cords that are usually discarded as medical waste. Additionally, hWJSCs have no ethical issues, as opposed to embryonic stem cells, and they are also non-carcinogenic [7]. The hWJSCs secrete several important cytokines and growth factors under basal conditions, including pro-inflammatory IL-6, IL-8, and IL-1A as well as the hematopoietic growth factors G-CSF and GM-CSF [8-10]. Interestingly, these secretory factors support *ex vivo* expansion of hematopoietic stem cells (HSCs), reduce apoptosis, and protect the neural cells in cerebral ischemia [9]. These cytokines are also implicated in the recruitment and migration of stem cells during inflammation and are associated with secretion of adhesion molecules and chemokines to initiate immunosuppressive activities required for tissue repair [11, 12]. However, the effect of HG concentrations on the expression levels of these cytokines in hWJSCs yet remains to be clearly understood.

Resveratrol (RSV) is a natural polyphenol product derived from grapes [13] and is reported to have anti-oxidative and anti-inflammatory effects [14]. RSV is cytoprotective under inflammatory conditions; it inhibits the expression of IL-6 and IL-8 inflammatory cytokines through the suppression of tumor necrosis factor-α (TNF-α) in human stem cells [15-17]. The effects of RSV on the cytokines secreted by hWJSCs under hyperglycemic conditions remain to be investigated. In the present study, we aimed to test the effects of HG and RSV either alone or in combination on the hWJSCs morphology, proliferation, and cytokines secretion *in vitro*.

Materials and methods

All materials were purchased from UFC biotechnology (Riyadh, KSA) unless otherwise indicated.

hWJSC culture

hWJSCs isolated from the umbilical cord were obtained from ATCC (PCS-500-010, Manassas, USA). The cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/100 mg/ml), fungizone (2.5 µg/ml), and L-glutamine (2 mM). The cultured cells were maintained at 37°C under 95% air and 5% CO_2 humidified conditions and the medium was changed every 2-3 days until confluence.

Characterization of hWJSCs by flow-cytometry

The hWJSCs were screened for their expression of MSCs related CD makers using FACS analysis. Briefly, the hWJSCs were trypsinized using 0.25% TrypsinEDTA and centrifuged at 1000 rpm for 5 min to pellet the cells. The cell pellet was then resuspended in 5 ml of PBS containing 3% FBS to prevent nonspecific binding and aliquots (1×10^5 cells/15 ml tube) were stained with CD maker antibodies. A cocktail of CD marker antibodies for hWJSC surface markers (BD Pharmingen, California, USA) was prepared by diluting them 1:10 in phosphate buffer saline (PBS) containing 3% FBS (FACS buffer). They are as follows: MSC isotype cocktail (negative control); MSC positive cocktail 1 (labeled as Mix 1, containing CD45-APC, CD105-FITC, and CD73-PERCP) and MSC positive cocktail 2 (labeled as Mix 2, containing CD34-PE, CD44-PE-CY, and CD90-FITC). Respective CD marker cocktails were added to the different groups and incubated in the dark at 4°C for 30 min. The cells were washed twice with FACS buffer and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 500 µl of FACS buffer before analysis using FACS Aria III instrument (BD Biosciences, San Jose, USA) with the 488 nm (blue) and 561 nm (yellow-green) lasers for uncoupled excitation, detection of FITC, and PE fluorochromes.

WST-1 cell proliferation assay

The hWJSCs were seeded on 96-well plates at a density of (5×10^4 cells/well) and allowed to attach overnight (16-18 h) under standard incubation conditions. The hWJSCs were divided into six groups as follows: Group 1: Control (5 mM; physiological glucose level); Group 2: HG (28 mM); Group 3: HG (41 mM); Group 4: RSV (17 µM); Group 5: HG (28 mM) + RSV (17 µM); and Group 6: HG (41 mM) + RSV (17 µM). The cells in both control and treated groups were cultured and cell proliferation was assessed at 24 h, 48 h, and 72 h. Briefly, 10 µl of WST-1

reagent (Abcam, Cambridge, USA) was added to 100 µl of culture medium and the cells were incubated for 30 min at 37°C and 5% CO_2. Following the incubation period, the absorbance was quantified using a spectrophotometer at a wavelength of 450 nm.

In a separate experiment, the hWJSCs were evaluated for their cell proliferative effects in the presence of sucralose to exclude any osmolality effects on cell proliferation. The hWJSCs were seeded as above and exposed to sucralose (23 mM and 36 mM) was added to the medium containing 5 mM Glu to obtain the same concentrations as seen with the HG conditions. The cell proliferation was assessed after 24 h, 48 h, and 72 h.

Morphology of hWJSCs

The hWJSCs cultured under various conditions as mentioned above, for different time points (24 h, 48 h, and 72 h) were analyzed for changes in cell morphology if any, and imaged using an inverted phase-contrast microscope.

Multiplex bead-based immunoassays

The multiplex cytokine assay was performed on the hWJSCs cell culture supernatant and the cell lysate collected at 72 h following treatment from the different experimental groups (Groups 1-6). Cell lysis was performed on the hWJSCs using ice-cold NP40 cell lysis buffer that contained 50 mM Tris, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40 (NP40), 0.02% NaN_3, 1 mM PMSF and 1X protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, USA). Multiplex bead-based analysis for human cytokines 5-plex panel namely IL-1A, IL-6, IL-8, G-CSF, and GM-CSF was carried out using ProcartaPlex kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). Briefly, the antibody-coated polystyrene magnetic beads solution was vortexed for 30 s and 50 µl of this solution was added to each well. The plate was washed twice with 1X wash buffer and then the prepared standards (1:3 serial dilution) and samples (supernatant and lysed cells) were added to the plate and incubated at room temperature (RT) on an orbital shaker at 500 RPM for 2 h. Then, 25 µl of the biotinylated detection antibody mix (1X) was added to the plate and incubated for 30 min at RT, followed by another incubation at RT for 30 min after the addition 50 µl of the streptavidin-PE antibodies to each well. Washing with buffer (2-3 times) between each step was accomplished using a handheld magnetic bottom to keep the magnetic antibody beads adhered to the plate. Finally, the beads were resuspended in buffer (120 µl/well) and analyzed using the Luminex MAGPIX® instrument (Luminex Corporation, Austin, USA). The raw data were acquired and analyzed using the Luminex xPONENT® multiplex assay analysis software (v.4.2.1324.0, Luminex Corporation).

Statistical analysis

Comparison studies between control and experimental groups were analyzed using Student's *t*-tests for paired and unpaired data, as appropriate. The statistical analysis was performed using GraphPad Prism 8.0. All results are expressed as mean ± SEM from three replicates. Differences among means were considered statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

Results

CD marker in hWJSCs

The stemness of hWJSCs was assessed using flow-cytometry analysis for MSC-related CD markers expression. The hWJSCs showed high expression of MSC-positive CD markers, CD44, CD105, CD90, and CD73, relative to their respective isotype-matched controls (**Figure 1A** and **1B**). The hWJSCs were negative for the hematopoietic stem cell-related CD markers, CD34, and CD45 (**Figure 1C**).

Cell morphology of hWJSCs

The morphology of the hWJSCs in the control (Group 1) appeared as long spindle-shaped fibroblast-like cells and the same morphological pattern was demonstrated upon exposure to high glucose (Group 2, Group 3), resveratrol alone (Group 4), or combinations of both high glucose and resveratrol (Group 5 and Group 6) at 72 h (**Figure 2**).

The proliferation of hWJSCs

Cell growth rates were monitored using the WST-1 assay. Group 1 showed a time-depen-

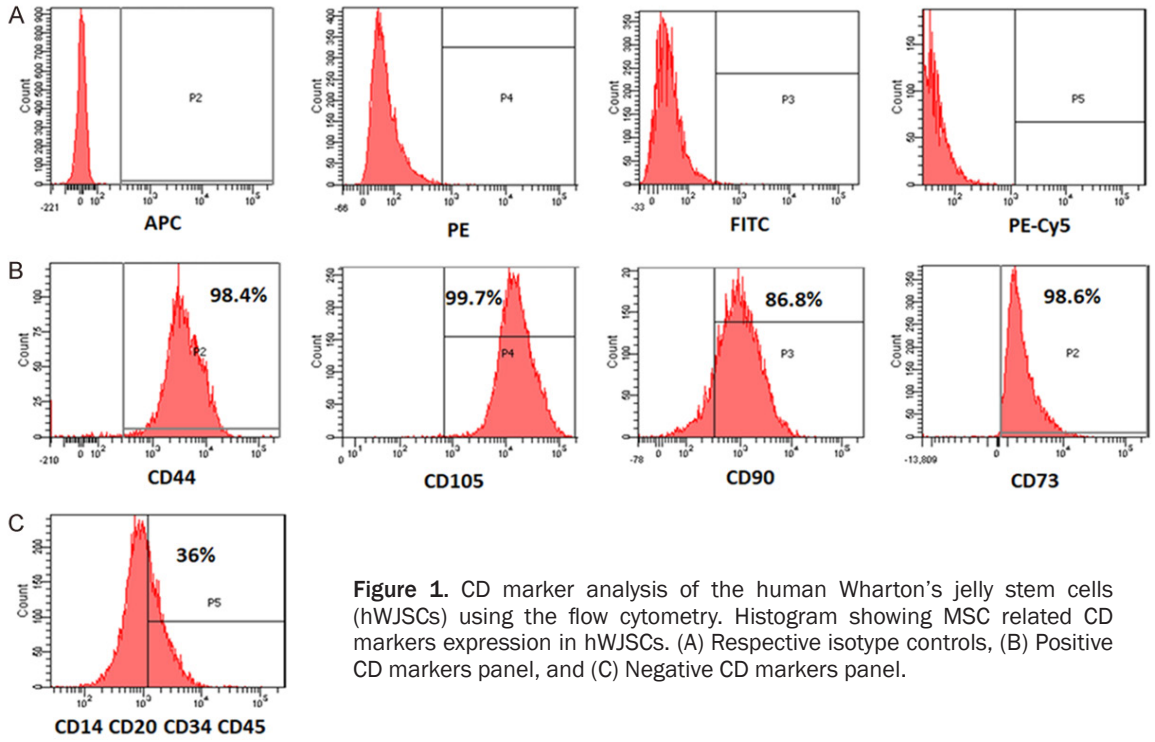

Figure 1. CD marker analysis of the human Wharton's jelly stem cells (hWJSCs) using the flow cytometry. Histogram showing MSC related CD markers expression in hWJSCs. (A) Respective isotype controls, (B) Positive CD markers panel, and (C) Negative CD markers panel.

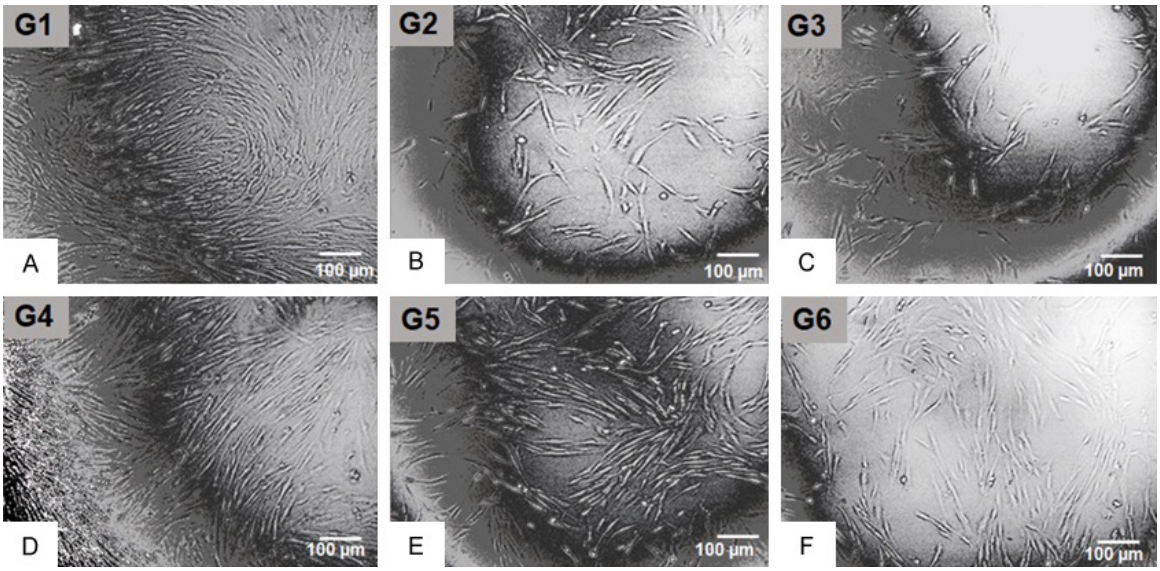

Figure 2. Representative inverted phase-contrast images of hWJSCs showing the morphology following culture for 72 h under the following stated conditions; A: G1 Control; B: G2 (HG 28 mM); C: G3 (HG 41 mM); D: G4 (RSV 17 µM); E: G5 (HG 28 mM + RSV 17 µM); and F: G6: (HG 41 mM + RSV 17 µM). Scale bar of images represents 100 µm.

dent increase in cell proliferation (**Figure 3A**). However, the hWJSCs in the treated groups (Group 2-6) showed either an increase or a decrease in proliferation (**Figure 3A**). Compared with the control, the treatments with HG in Group 2 and Group 3 demonstrated significant decreases in cell proliferation at 24 h ($P < 0.05$). Treatment with RSV alone (Group 4) resulted in a statistically significant increase in cell growth after 24 h ($P < 0.01$), 48 h ($P < 0.01$), and 72 h ($P < 0.05$) compared with the control. Group 5 and Group 6 that were treated with a combination of HG and RSV demonstrated a significant increase in proliferation (only at

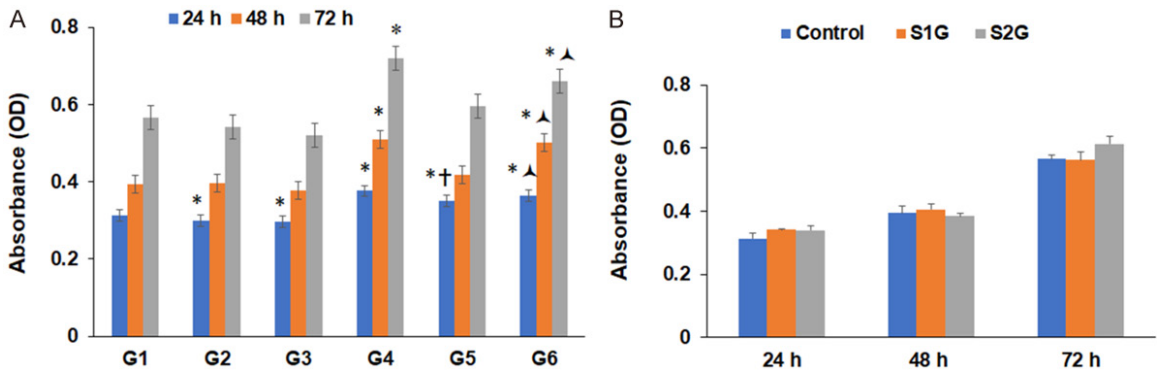

Figure 3. WST-1 proliferation assays. (A) hWJSCs proliferation following the stated treatment at 24 h, 48 h, and 72 h. (B) The osmotic effect on the hWJSCs proliferation was analyzed following treatment with S1G (23 mM sucralose (Sucr) + 5 mM glucose) and S2G (36 mM Sucr + 5 M glucose) for 24 h, 48 h, and 72 h. The values are expressed as mean ± SEM (n=3). * indicates $P < 0.05$ as compared with control; † indicates $P < 0.05$ as compared with G2; ⅄ indicates $P < 0.05$ as compared with G3.

24 h in Group 5) and at 24 h ($P < 0.05$), 48 h ($P < 0.01$), and 72 h ($P < 0.05$) in Group 6, compared with that of the control (**Figure 3A**).

Interestingly, compared with Group 2, the proliferation of Group 5 was enhanced significantly after 24 h ($P < 0.01$, **Figure 3A**). Similar results were obtained for Group 6 after 24 h ($P < 0.05$), 48 h ($P < 0.01$), and 72 h ($P < 0.01$) compared with Group 3. The hWJSCs exposed to sucralose (23 mM and 36 mM) (an osmotic control) in addition to the basal glucose level (5 mM) in the medium had increased cell proliferation, thereby excluding any osmotic related cell damage (**Figure 3B**).

Cytokine production in hWJSCs

Expression levels of cytokines in the hWJSCs cell lysate and the hWJSC cell supernatant were investigated using the multiplex cytokine assay. The IL-6, IL-8, G-CSF, GM-CSF, and IL-1A cytokines were analyzed from both the control (Group 1) and the experimental arms (Groups 2-6) at 72 h.

The level of IL-6 was significantly decreased in hWJSC cell lysate, in the experimental arms (Group 2-6) (**Figure 4A**). The mean values of IL-6 expression in the experimental groups 2-6 were 2016, 2278, 2727, 2104, and 2269 pg/ml, respectively, compared with that of the control (3957 pg/ml). Similarly, the IL-8 protein was significantly decreased in the RSV treated Group 4 and Group 5 ($P < 0.05$, **Figure 4B**). The mean values of IL-8 expression in Group 4 and 5 were 8.2 and 11.3 pg/ml, respectively, compared with that of the control (26.3 pg/ml). Notably, the GM-CSF, G-CSF, and IL-1A protein levels had similar patterns to IL-8; however, they were not statistically significant ($P > 0.05$, **Figure 4C-E**).

In the hWJSCs cell supernatant, the secreted level of GM-CSF was significantly low in HG Group 3 ($P < 0.05$, **Figure 5C**). The mean value of GM-CSF expression in Group 3 was 78.8 pg/ml compared with that of the control (194 pg/ml). Notably, there were no significant changes in the protein levels of IL-6, IL-8, G-CSF, or IL-1A (**Figure 5A**, **5B**, **5D**, **5E**).

Discussion

The chronic hyperglycemic state is detrimental to both cellular structure and function [18]. The present study demonstrated that HG inhibited hWJSCs cell proliferation and altered the basal expression levels of pro-inflammatory factors such as IL-6, IL-8, and GM-CSF. Treatment with RSV helped to overcome the adverse effects of HG on cell proliferation by reducing the expression of IL-6 and IL-8. The elevated glucose concentrations (28 mM and 41 mM) were used in the present study to mimic the hyperglycemic state based on the earlier study by Lou et al. in 2014 [19]. The hWJSCs cultured under HG levels for 24 h showed reduced proliferation. The decrease in cell proliferation with HG conditions is similar to earlier studies, which showed that HG (25 mM) decreased the cell proliferation while the reduction of the glucose concentration increased the growth rates in rat MSCs

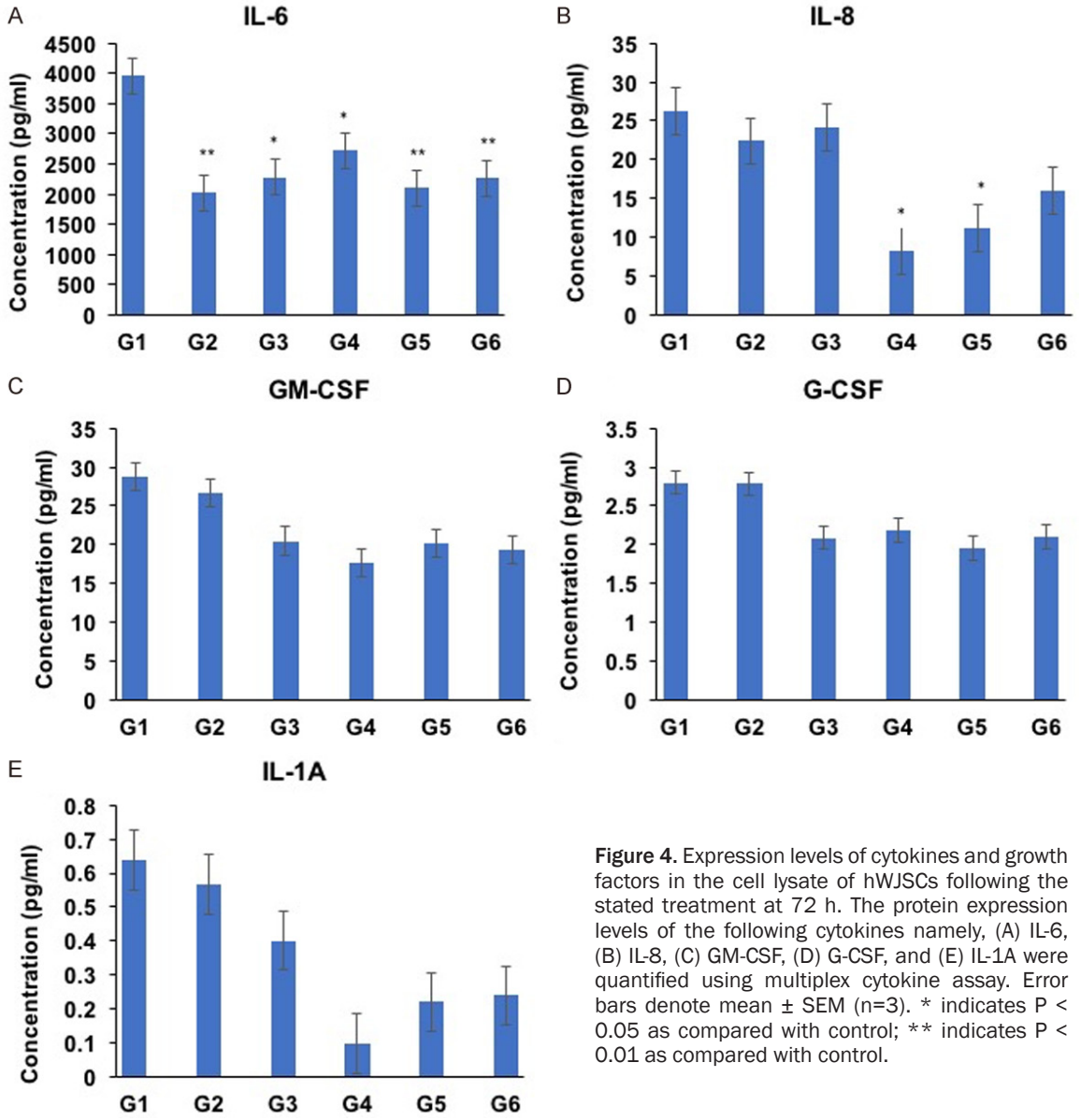

Figure 4. Expression levels of cytokines and growth factors in the cell lysate of hWJSCs following the stated treatment at 72 h. The protein expression levels of the following cytokines namely, (A) IL-6, (B) IL-8, (C) GM-CSF, (D) G-CSF, and (E) IL-1A were quantified using multiplex cytokine assay. Error bars denote mean ± SEM (n=3). * indicates $P < 0.05$ as compared with control; ** indicates $P < 0.01$ as compared with control.

[20] and BM-MSCs [21]. Although the cell numbers decreased under HG (28 mM and 41 mM), the hWJSCs morphology was not affected by HG concentrations (**Figure 2**). In line with our findings, an earlier study reported that HG (24 mM) did not change the morphology of the periodontal ligament stem cells cultured for 1, 3, 5, and 7 days [22]. In contrast, another study reported that the morphology of BM-MSCs became flat and rounded when cultured under HG (25 mM) for 28 days [5]. The changes in morphology could, therefore, be attributed to the differences of individual and other variables such as culture time, and conditions. The observed reduction in proliferation or cell damage in the hyperglycemic state is not primarily due to hyper-osmolality because the cells thrived well and demonstrated growth with high concentrations of sucralose (**Figure 3B**). Although glucose is the energy source for MSCs grown *in vitro,* elevated glucose concentrations may damage cell metabolic pathways and, hence, their growth rate. A few mechanisms have been reported by which the hyperglycemic state inhibits the MSCs growth rate. The epidermal growth factor receptor signaling required for cell proliferation is inhibited in HG conditions because of the formation of advanced glycation end products (AGEs) [23]. Additionally, oxidative stress induced by HG impairs cell growth

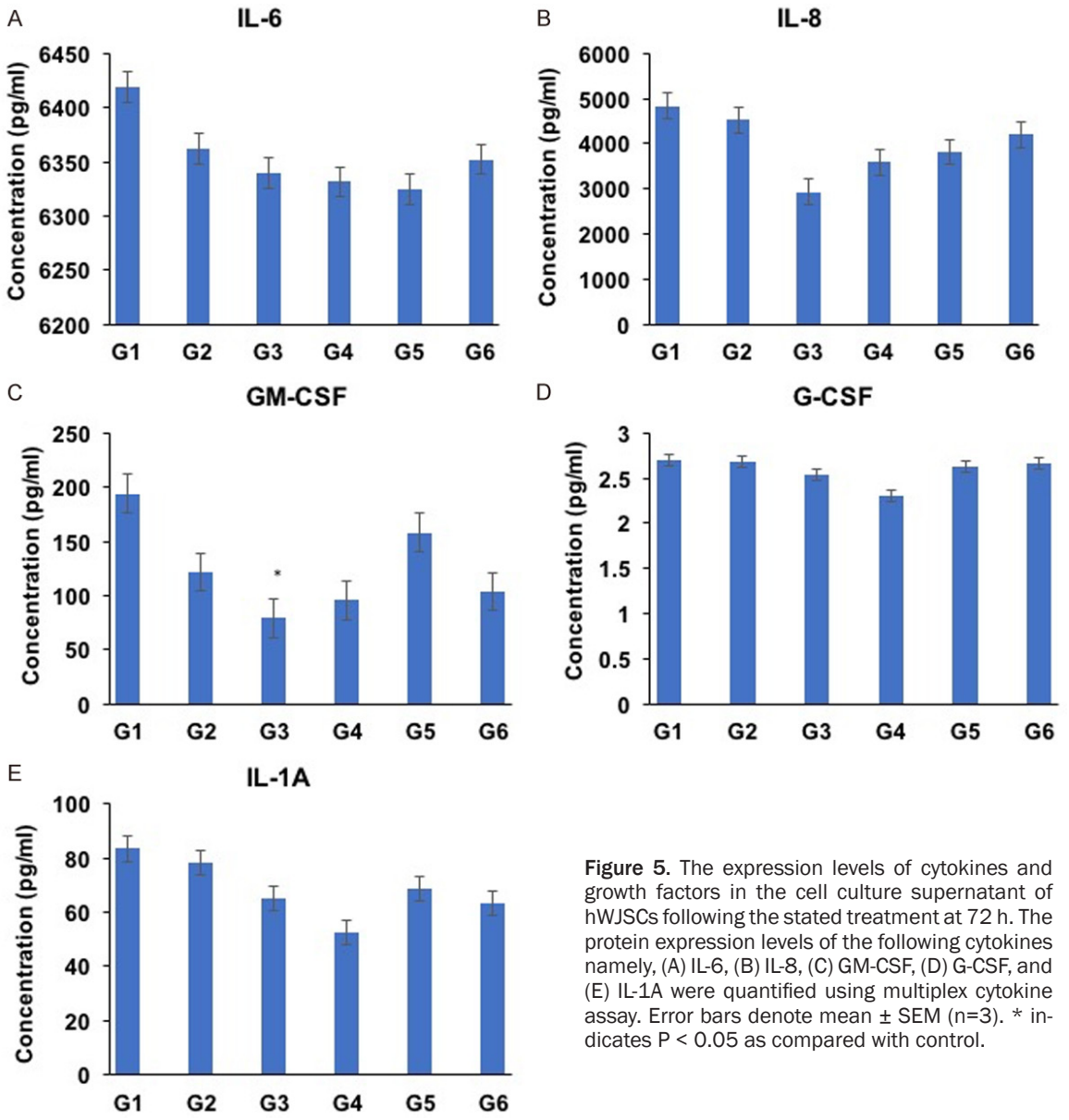

Figure 5. The expression levels of cytokines and growth factors in the cell culture supernatant of hWJSCs following the stated treatment at 72 h. The protein expression levels of the following cytokines namely, (A) IL-6, (B) IL-8, (C) GM-CSF, (D) G-CSF, and (E) IL-1A were quantified using multiplex cytokine assay. Error bars denote mean ± SEM (n=3). * indicates $P < 0.05$ as compared with control.

through the activation of PKC and the increase of NADPH oxidase [24].

Interestingly, the treatment with RSV (17 μM) either alone or even in the presence of HG (28 mM and 41 mM) demonstrated an increase in proliferation, which explains the role of RSV as a cytoprotective and survival factor. Earlier studies on MSCs have reported that RSV improved cell viability and proliferation [25] and reduced the oxidative stress and inflammatory response under diabetic conditions [26]. Following the initial optimization, we used 17 μM RSV in our experiments, and this concentration was within the pharmacological range reported by others [27-29]. RSV helps in scavenging a variety of oxidants [30]; therefore, the increased proliferation rates observed in the presence of RSV might be related to the prevention of ROS production or its active clearance. RSV supplementation could, therefore, serve to improve the hWJSCs replicative capacity under HG conditions.

Inflammatory cytokines are implicated in the pathogenesis of diabetes associated with hyperglycemia, and hence we analyzed their expression in the present study under mimicked HG conditions. Unlike HSCs, BM-MSCs, or AD-MSCs, the hWJSCs secrete higher con-

centrations of key cytokines, including IL-1A, IL-6, IL-8, GM-CSF, and G-CSF under basal conditions [8, 31]. Some of these cytokines enhance the *ex vivo* expansion of HSCs that may benefit therapeutic purposes [32, 33].

IL-6 levels were significantly lower in the cell lysate and not in the culture supernatant in the HG treated group at 72 h. Our findings are in line with a study, which showed that elevated glucose induced transforming growth factor-β1 (TGF-β1) production, which in turn suppressed the immune response and reduces IL-6 in peripheral blood mononuclear cells (PBMC) [34]. Moreover, prolonged HG conditions for up to 5 days reduced IL-6 secretion and compromised cellular functions including glucose metabolism, cell replication, and differentiation potentials of AD-MSCs [35]. In the current study, treatment with RSV alone or its combination with HG also led to reduced IL-6 compared to the untreated control, suggesting its anti-inflammatory function. RSV treatment attenuates IL-6 expression in diabetic rats through the [36].

IL-8 belongs to the CXC chemokine family and participates in neutrophil recruitment and increases angiogenesis through increased endothelial cell proliferation and matrix metalloprotease production [37]. The decreased amount of IL-8 in the hWJSC lysate was observed following exposure to RSV or co-treatment of RSV and HG conditions relative to the control. Our findings are in line with studies in other models that showed that RSV inhibits the expression of both IL-6 and IL-8 by preventing ROS production following conjugated linoleic acid-mediated inflammation in human adipocytes [38]. Interestingly, Li et al. reported that the low concentrations of IL-6 and IL-8 pro-inflammatory cytokines enhanced human placental stem cell proliferation, whereas their increased concentrations suppressed cell proliferation [39]. Based on this study, the enhanced cell proliferation upon the addition of RSV with or without HG may in part be due to its effect on the reduced IL-6 and IL-8 expression.

The hWJSCs have been reported to secrete more GM-CSF and G-CSF hematopoietic growth factors [40]. Overexpression of the GM-CSF protein promotes the survival and proliferation of *in vitro* cultured neural stem cells under cytotoxic conditions [41]. In this study, the level of the GM-CSF was significantly low under the HG condition in a concentration-dependent manner (**Figure 5C**). It is, therefore, possible that the observed decrease in cell growth upon treatment with HG (41 mM) may be partially due to the low expression of GM-CSF. The other two cytokines analyzed in the present study, IL-1A and G-CSF, did not show any significant changes in a cell lysate or supernatant.

In conclusion, HG induces hWJSC dysfunction, leading to the inhibition of proliferation and differential cytokine, chemokine, and growth factor expression. RSV treatment can rescue the hWJSCs proliferation, inhibit pro-inflammatory cytokines IL-6 and IL-8, and enhance GM-CSF expressions.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Samar Sultan, Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia. Tel: 00966126400000; E-mail: sasultan@kau.edu.sa

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