Methylglyoxal and methylglyoxal-modified collagen as inducers of cellular injury in gingival connective tissue cells

I. N. Retamal[1], R. Hernández[1],
C. González-Rivas[1], M. Cáceres[2],
R. Arancibia[1], A. Romero[2],
C. Martínez[1], N. Tobar[3],
J. Martínez[3], P. C. Smith[1]

[1]Dentistry Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile, [2]Molecular and Cell Biology Program, Facultad de Medicina, Universidad de Chile, Santiago, Chile and [3]Institute of Nutrition and Food Technology, Laboratory of Cell Biology, University of Chile, Santiago, Chile

Retamal IN, Hernández R, González-Rivas C, Cáceres M, Arancibia R, Romero A, Martínez C, Tobar N, Martínez J, Smith PC. Methylglyoxal and methylglyoxal-modified collagen as inducers of cellular injury in gingival connective tissue cells. J Periodont Res 2016; 51: 812–821. © 2016 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objectives: Methylglyoxal is a toxic product derived from glucose metabolism that plays a role in inflammation, diabetes and aging. In addition, the periodontal pathogen *Tannerella forsythensis* may also generate this compound. However, the effects of methylglyoxal on gingival cells are still poorly understood. In the present study, we have explored whether methylglyoxal or methylglyoxal-treated collagen may modulate cell viability, death and proliferation in gingival connective tissue cells. In addition, we have searched for inflammatory mediators secreted by cells upon exposure to these conditions.

Material and Methods: Primary cultures of human gingival fibroblasts were stimulated with soluble methylglyoxal or cultured over a collagen matrix glycated by this agent. Cell viability was evaluated through the MTS assay. Cell death was assessed through DAPI nuclear staining, annexin V and propidium iodide assays. Cell proliferation was evaluated through double immunofluorescence for DAPI and Ki67. Protein levels of matrix metalloproteinases and cytokines were assessed through antibody arrays, enzyme-linked immunosorbent assay, real-time reverse transcription polymerase chain reaction and immunofluorescence. Statistical analysis was performed using the Kruskall–Wallis and Mann–Whitney tests.

Results: Soluble methylglyoxal, but not culture of gingival fibroblasts over a methylglyoxal-modified collagen matrix, induced a reduction on cell viability. Moreover, soluble methylglyoxal induced apoptotic cell death as indicated by DAPI nuclear staining, annexin V and propidium iodide assays. Neither soluble methylglyoxal, nor methylglyoxal-modified collagen modified cell proliferation. Using an antibody array, enzyme-linked immunosorbent assay and immunofluorescence assays, we determined that both, soluble methylglyoxal and methylglyoxal-modified collagen stimulated an increase in tissue inhibitor of metalloproteinase (TIMP)-1 protein levels.

Conclusions: Soluble methylglyoxal is a highly cytotoxic compound that induces cell death through apoptosis in gingival fibroblasts. TIMP-1 is induced in these cells upon direct exposure to methylglyoxal or after culture of gingival

Patricio C. Smith, DDS, PhD, Dentistry, Faculty of Medicine, Pontificia Universidad Católica de Chile, Marcoleta 391, Santiago, Chile
Tel: +56 2 2354 3408
Fax: +56 2 2633 1457
e-mail: psmithf@uc.cl

fibroblasts over methylglyoxal-treated collagen. As TIMP-1 has been implicated in cell survival and matrix remodeling, we propose that increased TIMP-1 protein levels may be part of a protective response of gingival connective tissue cells upon exposure to methylglyoxal or after the interaction with the collagen matrix that has been modified by this agent.

Methylglyoxal (MGO) is a toxic product derived from glucose metabolism (1). It corresponds to an alpha-oxoaldehyde generated from the triose phosphate intermediates of glycolysis, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (2). In the context of periodontal tissues, MGO may derive from several sources that include the periodontal pathogen *Tannerella forsythensis* (3) and eukaryotic glucose cell metabolism (2). MGO has been identified in the gingival crevicular fluid of healthy and periodontitis-affected sites and its concentrations have been correlated with the level of periodontal inflammation (4). In diabetes, increase in glucose levels trigger enhanced production of MGO that modifies proteins and other molecules to generate the so-called advanced glycation end products (AGEs) (2). As diabetes is one of the most prevalent diseases in Western countries and has a major impact on periodontal health (5), it is important to advance in the understanding of the cellular effects of MGO on gingival connective tissue cells. Given its electrophilic character, MGO may react with cysteine, arginine and lysine, inducing important post-translational changes in several proteins, including collagen (6). It has been demonstrated that MGO-modified collagen alters cell behavior in connective tissue cells (7,8). In gingival fibroblasts, MGO-treated collagen disrupts cell migration and adhesion (9). Moreover, the intracellular pathway of collagen degradation is also impaired in these cells due to an altered interaction between glycated collagen and β1 integrins (10).

It is important to consider that besides the pathological modification of extracellular matrix proteins, MGO may directly affect cell viability and function in several cell types (11–13).

Nevertheless, the potential cytotoxic effects of MGO on periodontal cells remain largely undefined. Fibroblasts represent the most abundant cell population in gingival tissues, and play a key role in the regulation of tissue structure and homeostasis through the secretion, organization and remodeling of the extracellular matrix and the regulation of inflammation (14). Matrix metalloproteinases (MMPs) represent a family of endopeptidases involved in the degradation of a wide array of extracellular matrix molecules, growth factors, cytokines and receptors. Although initially considered as strict mediators of tissue destruction, MMPs are now conceived as critical regulators of the biological activity of distinct molecules involved in tissue remodeling, inflammation and wound healing (15). Moreover, connective tissue homeostasis is controlled by fibroblasts that synthesize and degrade the collagen matrix in response to changes in the tissue microenvironment, including inflammatory cytokines and modifications in the composition and structure of the extracellular matrix. As fibroblasts may receive signaling from soluble molecules present in the periodontal tissues and from the extracellular matrix and, in particular, from collagen, the most abundant protein in periodontal tissues (14), it is important to consider whether the function of these cells is altered by MGO. Therefore, it is possible to consider at least two main mechanisms of action of MGO, one derived from its direct effects on cells as a soluble mediator, or as an agent that disrupts cell function in adherent cells modifying the extracellular matrix proteins. In the present study, we have studied whether MGO or MGO-modified collagen may alter cell viability/death

and the production of inflammatory mediators involved in tissue homeostasis and remodeling as part of the general response to these stimuli.

Material and methods

Cell culture

Explants were obtained from healthy gingiva surrounding third molars or during crown lengthening surgery of periodontally healthy individuals. Periodontal examination demonstrated sites with probing depth < 4 mm, no loss of attachment and no bleeding on probing. Tissues were obtained from five donors (18–25 years; two male and three female) with the informed consent of the patients. The Ethical Committee, Pontificia Universidad Católica de Chile approved the protocol for tissue obtainment. Patients did not report pre-existing medical or drug histories in the last 6 mo and no smokers were included. Cells were obtained through the explant method and cultured in α-minimal essential medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel), and penicillin streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells expanded between passages 4 and 10. Each experiment was performed and validated with at least two different cell cultures obtained from different donors.

Collagen treatment with methylglyoxal

MGO was used to glycate collagen as previously described (7,10). Culture plates were treated with 2 mL of a type I collagen solution (50 μg/mL)

and neutralized with 200 μL 1 N NaOH (Merck Millipore, Darmstadt, Germany) for 2 h at 37°C. This procedure facilitates the formation of collagen fibrils (10). MGO concentrations used in this study were selected from previous reports that identified MGO in gingival crevicular fluid of patients with periodontitis (4). Cell culture plates were then washed with phosphate-buffered saline twice and used for cell culture experiments.

Analysis of methylglyoxal-modified collagen

Cell culture plates were treated as described above and collagen was lysed in RIPA buffer supplemented with 10% sodium dodecyl sulfate at 70°C. Samples were fractioned using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8%) under reducing conditions followed by Coomassie blue staining.

Cell viability assay ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

Cells were cultured at semiconfluent conditions over collagen or MGO-treated collagen using 96-well plates (5000 cells per well). In addition, cells were exposed to soluble MGO. Cell viability was evaluated after 48 h through the MTS assay (Celltiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA). The product of the assay was finally read using a microplate reader (Biotek, Winooski, VT, USA) at 450 nm.

Annexin V and propidium iodide cell death assay

Cells were cultured at semiconfluent conditions on cell culture dishes previously treated with collagen as described above (400,000 cells per 60 mm culture dishes). In addition, serum-starved human gingival fibroblasts were exposed to soluble MGO (Sigma, St. Louis, MO, USA) or staurosporine (STA; Sigma) (1 μM), as a positive control of cell death. After

90 min, cells were released from cell culture plates using a trypsin–ethylenediaminetetraacetic acid solution (Life Technologies, Grand Island, NY, USA), and subsequently stained for annexin V-fluorescein isothiocyanate and propidium iodide (PI) using the fluorescein isothiocyanate Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA). Stained cells were examined using a flow cytometer (FACSCanto II; BD Biosciences, Becton Dickinson, San Jose, CA, USA) and data were analyzed using the BD FACSDIVA Software version 6.1.3 (BD Biosciences). To identify changes in fragmentation and/or condensation of the cell nucleus, cells were stained with DAPI (Invitrogen Molecular Probes Eugene, OR, USA) reagent as previously described (16).

Immunofluorescence

Cells were cultured at semiconfluent conditions over coverslips (25,000 cells per coverslip), previously treated with collagen and 1 mM MGO as described above. In addition, cells

were also stimulated with soluble 50 nM MGO. After 48 h of stimulation, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 for 4 min, blocked with bovine serum albumin (Merck) and incubated with the following antibodies: MMP-3 (Merck Millipore), tissue inhibitor of metalloproteinase (TIMP)-1 (Abcam, Cambridge, MA, USA), TIMP-2 (Abcam), Ki67 (Abcam) and interleukin (IL)-6 (Abcam). The antigen–antibody complex was washed and incubated with a fluorescein 5-isothiocynate secondary conjugated antibody (Invitrogen Molecular Probes). Nuclei were stained with DAPI (Invitrogen Molecular Probes). Cells were observed and photographed using a Zeiss Microscope (Zeiss Axioplan, Göttingen, Germany) equipped with a Micropublisher Camera model RTV (Qimaging, Surrey, BC, Canada).

Quantitative polymerase chain reaction

Human gingival fibroblasts were cultured on 60 mm cell culture dishes
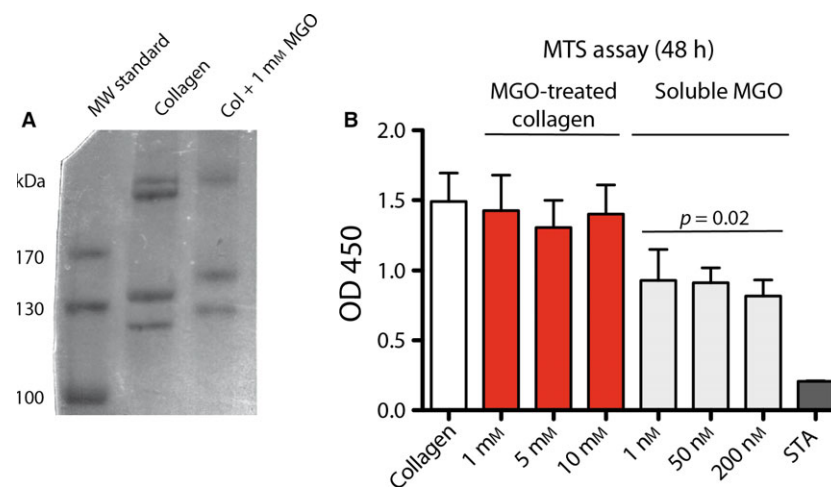
Fig. 1. Effect of MGO on collagen and cell viability. (A) Cell culture dishes were treated with a type I collagen solution for 2 h at 37°C. The collagen matrix was then treated with or without 1 mM MGO for 48 h at 37°C. Collagen samples were lysed, fractioned through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue. (B) Human gingival fibroblasts were seeded over a type I collagen matrix, treated with or without MGO (1–10 mM). Cells were cultured for 48 h in the presence, or not, of soluble MGO (1–200 nM). STA was used as a positive control of cell death. Cell viability was determined after 48 h through the MTS assay. Graph represents the average and standard error of MTS readings of three independent experiments. *p* value indicates significant difference between soluble MGO-stimulated cells and control. MGO, methylglyoxal; STA, staurosporine; MW standard, molecular weight standard.

(400,000 cells per dish) previously treated with collagen or MGO-treated collagen (1 mM). In addition, a soluble stimuli consisting of MGO (50 μM) or transforming growth factor-β1 (positive control), was added. After 24 h, total mRNA was isolated with Trizol (Ambion Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Messenger RNA levels were assessed through real-time quantitative polymerase chain reaction (qPCR) using LightCycler equipment (Roche Diagnostics, Mannheim, Germany). The reaction was performed using 100 ng of cDNA and LightCycler®FastStart DNA Master SYBR Green I kit (Roche) in a final volume of 20 μL. All reactions were performed in duplicate. The primers used were: TIMP-1, forward: 5′-ACTTCCACAGGTCCCACAAC-3′, reverse: 5′-GCATTCCTCACAGC-CAACAG-3′, PCR product size: 167 pb. Glyceraldehyde-3-phosphate dehydrogenase, forward 5′-TGCAC-CACCAACTGCTTAG-3′ 5′-GTTCAGCTCAGGGATGACC-3′. In every case, mRNA expression was normalized against glyceraldehyde-3-phosphate dehydrogenase as the loading control.

Antibody arrays

The secreted protein levels of inflammatory cytokines and MMPs were evaluated using two commercially available arrays (Ray Biotech, Norcross, GA, USA) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay

Protein levels for IL-6, TIMP-1, TIMP-2 and MMP-3 were determined in the cell culture media using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abcam) and a microplate reader (Biotek).

Statistical analysis

Data were analyzed using Kruskall–Wallis test and differences were determined using a Mann–Whitney test using the GRAPHPAD Software, Inc. (La Jolla, CA, USA). $p < 0.05$ was considered significant.

Results

Effects of methylglyoxal on collagen glycation and cell viability

We initially evaluated the effects of MGO on collagen glycation following a previously described protocol (17). To this end, collagen aliquots were treated with MGO and the electrophoretic migration of collagen molecules was evaluated through electrophoresis. As shown in Fig. 1A, MGO induced a minor change in the electrophoretic migration of this molecule when compared to non-treated collagen. This change is similar to previous studies reporting the effect of MGO on collagen (10). Afterwards we characterized the effects of MGO-treated collagen or soluble MGO on cell viability using primary cultures of human gingival fibroblasts. Cells were initially stimulated with a wide range of soluble MGO concentrations. We found that MGO induced a reduction
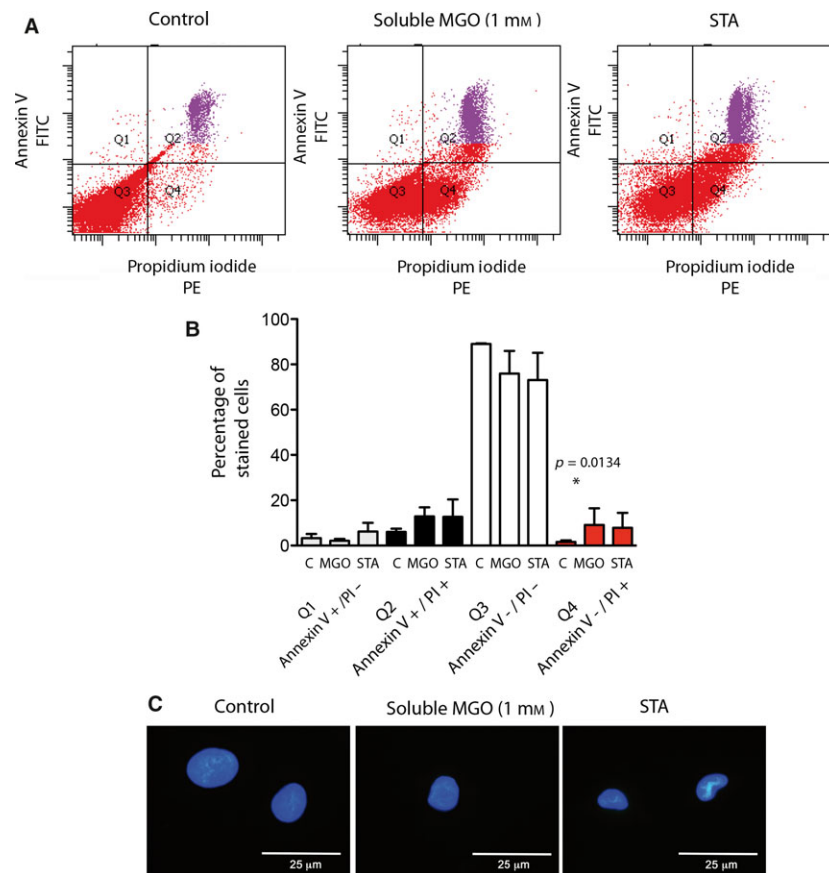

Fig. 2. Induction of cell death by soluble MGO. (A) Serum-starved human gingival fibroblasts were stimulated with soluble MGO or STA. After 90 min cells were released from cell culture plates, stained and analyzed through flow cytometry for annexin V and PI. Results are representative of three independent experiments. (B) Graph shows the average and standard error of cells stained for annexin V and PI identified in each quadrant defined in the flow cytometry experiment. Data were obtained from three independent experiments. (C) Serum-starved human gingival fibroblasts were stimulated with soluble MGO (1 mM) or STA. After 12 h cells were fixed and stained for DAPI to identify nuclear changes associated with cell death. Magnification bar equals 25 μm. FITC, fluorescein isothiocyanate; MGO, methylgyoxal; PE, phycoerythrin; PI, propidium iodide; STA, staurosporine.

on cell viability at the micromolar range (data not shown). Therefore, we selected lower concentrations of MGO (in the nanomolar range), to evaluate the effects of soluble MGO on cells. As shown in Fig. 1B, we used the MTS assay that evaluates the metabolic mitochondrial activity, to identify changes on cell viability when serum starved gingival fibroblasts were cultured over a collagen matrix previously treated with MGO (1, 5 or 10 mM), or exposed to soluble MGO (1, 50 or 200 nM). STA (1 μM), a potent inhibitor of cyclin-dependent kinases and protein kinase C (18), was used as a positive control of cell death. Figure 1B shows that culture of cells on MGO-treated collagen did not affect cell viability. However, soluble MGO, even at 1 nM was associated with a significant reduction on cell viability.

Effects of methylglyoxal on cell death

To identify the effects of soluble MGO on cell death, human gingival fibroblasts were stimulated with a higher concentration of MGO that would show the cytotoxic effects of this agent. We also reduced the amount of time of stimulation with MGO to identify early changes associated with cell injury. To this end, serum-starved human gingival fibroblasts were stimulated with 1 mM MGO as a soluble agent or 1 μM STA. After 90 min of stimulation, cells were released from cell culture plates, stained for annexin V and PI and analyzed through flow cytometry. As shown in Fig. 2A and B, stimulation of cells with MGO and STA was associated with an increase in annexin V-positive cells (early apoptotic) (Q1 in Fig. 2A), double positive for annexin V and PI (apoptotic) (Q2), and positive for PI (secondary necrotic) (Q4). The percentage of double negative cells for annexin V and PI (viable cells) (Q3) was reduced by MGO or STA when compared to vehicle-stimulated cells. Figure 2B shows a graph that represents the percentage of cells stained for annexin V and PI. Statistical analysis of these data showed a

significant increase in the number of double-positive cells in MGO-stimulated cells when compared to vehicle-treated cells. Additional experiments were performed to identify the effects of culturing cells over MGO-glycated collagen. In accordance to the MTS assays, culturing cells over a collagen matrix previously glycated with 1 mm MGO did not stimulate annexin V- or PI-positive cells (data not shown). To identify further the effects of MGO on cell death, serum-starved human gingival fibroblasts we stimulated 1 mM MGO (soluble) or 1 μM STA. After 12 h, cells were stained with DAPI to identify changes in the organization of the cell nucleus. As shown in Fig. 2C, MGO- and STA-stimulated cells presented signs of condensed nuclei, changes compatible with apoptotic cell death.

Effects of methylglyoxal on cell proliferation

We searched for the potential detrimental effects of MGO on cell prolif-eration. To this end, human gingival fibroblasts were seeded over collagen, treated with or without MGO (1 mM), or stimulated with soluble MGO (50 nM). As the proliferative stimuli, cells were exposed to 10% fetal bovine serum. After 48 h, cell proliferation was evaluated through double staining for Ki67 and DAPI. Figure 3A shows that a similar amount of Ki67-positive nuclei were observed in the three conditions under study. No significant changes were observed between these experimental conditions (Fig. 3B).

Effects of methylglyoxal on the release of inflammatory mediators, matrix metalloproteinases and tissue inhibitor of metalloproteinases

A proteomic approach was selected to identify inflammatory and tissue-remodeling mediators potentially involved in the response of cells to MGO. To this end, human gingival fibroblasts were seeded on collagen,
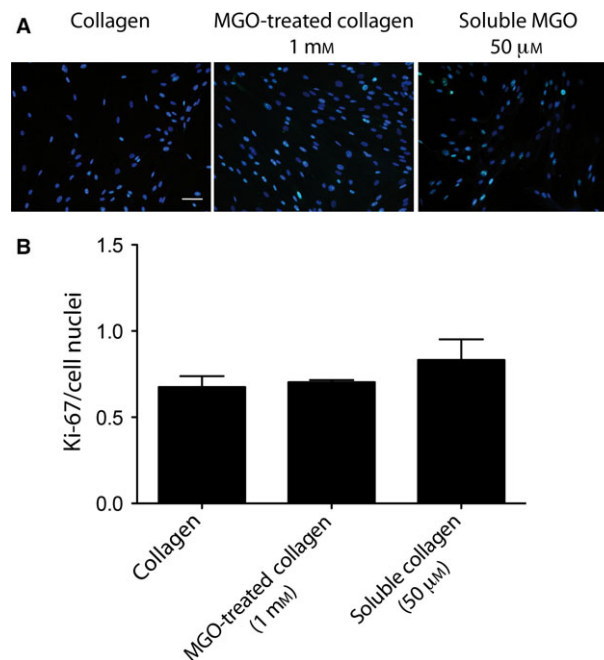
Fig. 3. Effects of MGO-treated collagen or soluble MGO on cell proliferation. Human gingival fibroblasts were seeded on collagen or MGO-treated collagen (1 mM) coverslips and treated with soluble MGO (50 μM). Cell proliferation was stimulated with 10% fetal bovine serum. After 48 h, cells were fixed and stained for Ki67 and DAPI. Magnification bar equals 10 μm (A). Graph represents the average and standard error of double-stained cells for Ki67 and DAPI (B). MGO, methylgyoxal.

and treated with or without 1 mM MGO. After 48 h of cell culture in the absence of serum, cell culture media was collected and analyzed using two antibody arrays. This initial analysis identified MMP3, TIMP-1, TIMP-2 and IL-6 as potential molecular mediators regulated by MGO-treated collagen in human gingival fibroblasts (Fig. 4A and B). As the antibody arrays used in this study are semiquantitative, we confirmed these observations using immunofluorescence and ELISA assays. In addition, cells were also stimulated with soluble MGO at relatively low concentrations (1, 50 and 200 nM) to ensure that the cytotoxic effects were minimal. Using immunofluorescence we evaluated the distribution of MMP-3, TIMP-1, TIMP-2 and IL-6 in gingival fibroblasts cultured over MGO-treated collagen (1 mM) or stimulated with soluble MGO (50 nM). As shown in Fig. 5, only TIMP-1 showed an increased immunofluorescence signal when cells were cultured over MGO-treated collagen (1 mM) or stimulated with soluble MGO (50 nM) (Fig. 5). To further quantify the protein levels of IL-6, MMP-3, TIMP-1 and TIMP-2, ELISA assays were performed from the conditioned media of cell cultures. To warrant that cell viability did not affect these results, protein levels under study were normalized against an MTS assay performed in control and treated cells in parallel to the ELISA assay. As a positive control of MMPs and cytokine production, cells were stimulated with 1 ng/mL IL-1β. As shown in Fig. 6, MGO-treated collagen and soluble MGO-stimulated TIMP-1 levels. However, MMP-3, TIMP-2 and IL-6 did not show significant changes in their protein levels neither when seeded on MGO-treated collagen nor when exposed to the soluble form of this agent. To confirm TIMP-1 regulation by MGO, gingival fibroblasts were cultured over MGO-treated collagen or stimulated with soluble MGO. After 24 h, quantitative evaluation of mRNA levels showed that only soluble MGO stimulated an increase in TIMP-1 mRNA. Meanwhile, culture of cells on MGO-treated

A

Collagen   Collagen + 1 mM MGO

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
2	POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
3	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	NEG	POS
4	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	NEG	POS

B

Collagen   Collagen + 1 mM MGO

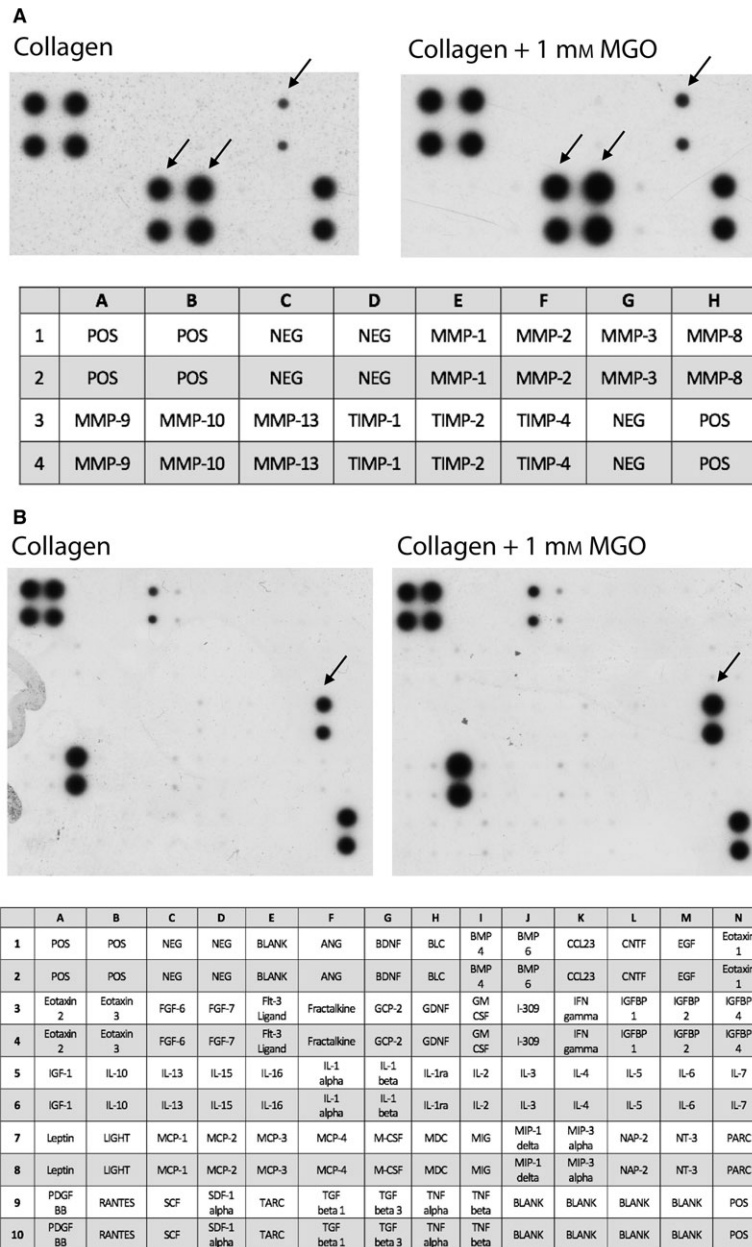|	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	BLANK	ANG	BDNF	BLC	BMP 4	BMP 6	CCL23	CNTF	EGF	Eotaxin 1
2	POS	POS	NEG	NEG	BLANK	ANG	BDNF	BLC	BMP 4	BMP 6	CCL23	CNTF	EGF	Eotaxin 1
3	Eotaxin 2	Eotaxin 3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM CSF	I-309	IFN gamma	IGFBP 1	IGFBP 2	IGFBP 4
4	Eotaxin 2	Eotaxin 3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM CSF	I-309	IFN gamma	IGFBP 1	IGFBP 2	IGFBP 4
5	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
9	PDGF BB	RANTES	SCF	SDF-1 alpha	TARC	TGF beta 1	TGF beta 3	TNF alpha	TNF beta	BLANK	BLANK	BLANK	BLANK	POS
10	PDGF BB	RANTES	SCF	SDF-1 alpha	TARC	TGF beta 1	TGF beta 3	TNF alpha	TNF beta	BLANK	BLANK	BLANK	BLANK	POS

Fig. 4. Proteomic screening of MMPs, TIMPs and cytokines. Human gingival fibroblasts were seeded over collagen or MGO-treated collagen (1 mM). After 48 h of cell culture in the absence of fetal bovine serum, cell culture media was analyzed using two antibody arrays for MMPs–TIMPs (A) and inflammatory cytokines (B). Arrows indicate dots with at least a 1.5-fold increase over control detected in three independent experiments. MGO, methylglyoxal; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases.

collagen induced a modest increase in TIMP-1 mRNA levels (Fig. 7).

Discussion

The main findings of the present study are that human gingival fibroblasts are highly sensitive to the cytotoxic effects of MGO. This observation derives from experiments showing that gingival connective tissue cells demonstrated an important reduction in mitochondrial cell metabolism that may reflect a decline on cell viability upon exposure to nanomolar concentrations of soluble
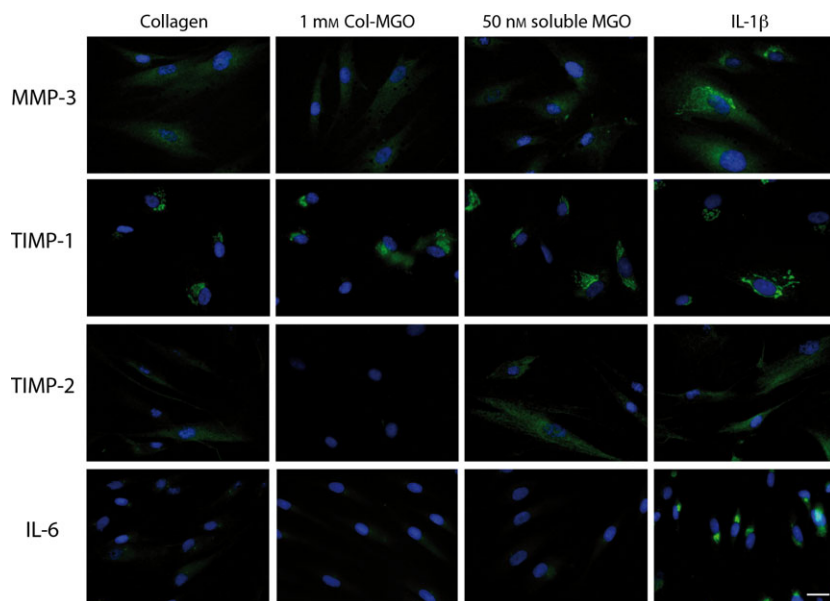
Fig. 5. Immunofluorescent detection of MMP-3, TIMP-1, TIMP-2 and IL-6. Human gingival fibroblasts were seeded over collagen or MGO-treated collagen. In addition, a soluble stimulus consisting of MGO (50 nM) or IL-1β (positive control) was used. After 48 h cells were fixed and processed for immunofluorescence to detect MMP-3, TIMP-1, TIMP-2 and IL-6. Nuclei were stained with DAPI. Magnification bar equals 10 μm. Col-MGO, methylgyoxal-treated collagen; IL, interleukin; MGO, methylgyoxal; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases.

MGO. In parallel, cell death was induced in gingival fibroblasts as demonstrated by an increase in annexin V- and PI-positive cells and nuclear condensation. On the other hand, MGO-treated collagen did not induce a reduction on cell viability or an increase in cell death. Moreover, our study also showed that among a wide range of MMPs, TIMPs and inflammatory cytokines evaluated, TIMP-1 was significantly increased when cells were exposed to both soluble MGO or when cultured over a glycated collagen matrix. Given that TIMP-1 may regulate the activity of MMPs and control cell survival pathways, these findings show new insights into the cell response of connective tissue cells against MGO or MGO-glycated collagen.

It is important to consider which MGO levels represent clinically relevant concentrations of this agent that may affect cell function. A previous study has evaluated MGO concentrations in gingival crevicular fluid of non-diabetic periodontitis patients and found MGO concentrations as

high as 23 mM (4). Our study used nano- and millimolar concentrations of MGO reaching up to 10 mM. Therefore, we believe our findings represent clinically relevant effects of MGO on cells. As MGO may derive from glucose metabolism and from periodontal pathogens such as *T. forsythensis* (3), MGO may contribute to tissue damage in inflammatory conditions such as periodontitis and this response might be augmented by diabetes. In addition, aging has also been explained by the cumulative action of MGO on cells and tissues (19). As an example, MGO-derived AGEs have been identified in the human lens and have been linked with the development of cataract (20). However, the specific role of MGO on periodontal aging has not been studied. Further studies are needed to clarify the precise contribution of MGO to diseases of the periodontium under these conditions.

MGO may induce apoptosis in several cell types, including human blood mononuclear cells, human endothelial cells, blastocysts and hep-

atic Hepg2 cells (11–13,21). Moreover, reactive oxygen species have been identified in the induction of cell death by MGO (12,13,21). However, to our knowledge this is the first study that evaluates the effects of MGO and MGO-treated collagen on cell death and viability in human gingival fibroblasts. Our findings reveal an important cytotoxic effect for soluble MGO on this cell type. On the other hand, glycated collagen by MGO did not affect cell viability or death. We believe this is an important finding as *in vivo* studies have identified that apoptotic cell death is increased during gingival wound healing of diabetic rats and mice (22). Our observations reveal a highly cytotoxic effect for soluble MGO on gingival connective tissue cells that may help to explain the reduction of available cells for wound healing in diabetic tissues.

Diabetes constitutes an important disease with a strong impact on periodontal health (5). Several studies have documented the role of diabetes as an important risk factor for the initiation and progression of periodontitis (23) and abnormal wound healing in periodontal tissues (22). Diabetes corresponds to a metabolic disease characterized by the maintenance of abnormally elevated glucose levels that influence the response of cells through several mechanisms (5). These include the direct action of glucose on cell metabolism and the formation of AGEs that alter cell and tissue function (5). Sustained hyperglycemia results in the generation of AGEs in proportion to blood glucose levels (5). The main pathogenic effects of diabetes have been attributed to the effects of AGEs on cells and tissues (23). MGO is derived from increases in blood glucose levels and therefore represents an important mechanism of action of diabetes on tissues. The present study has explored two potential mechanisms of action of MGO on gingival connective tissue cells. To this end, fibroblasts were directly stimulated with soluble MGO or cultured over a collagen matrix, previously exposed to this agent. This experimental
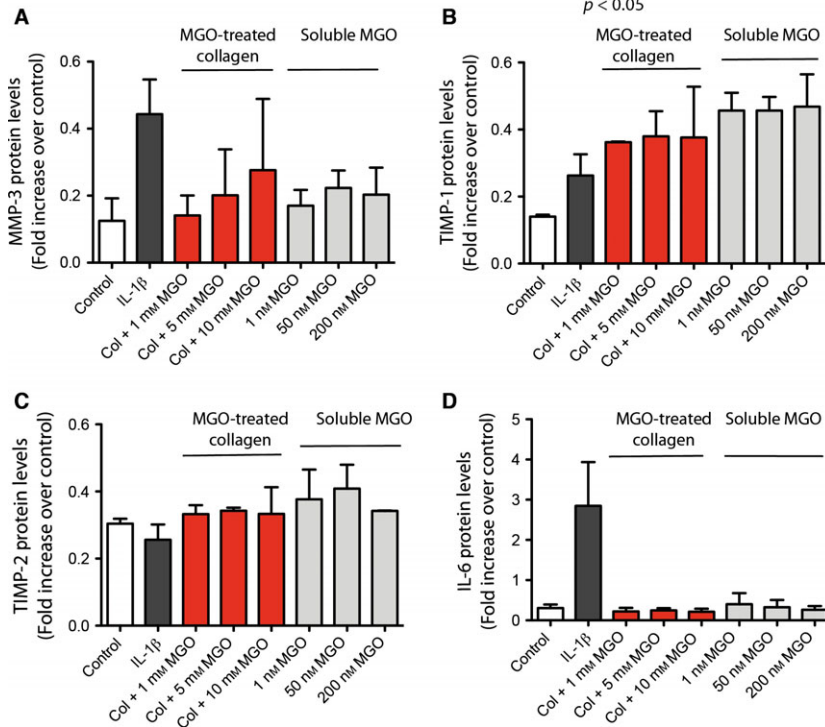
Fig. 7. TIMP-1 mRNA levels. Human gingival fibroblasts were seeded over collagen or MGO-treated collagen (1 mM) and stimulated with soluble MGO (50 nM). Total mRNA was extracted and mRNA levels were determined through quantitative polymerase chain reaction. TGF-β1 was used as a positive control of TIMP-1 expression. Graph represents the average and standard error of TIMP-1 mRNA levels normalized against GADPH mRNA. These data were obtained from three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MGO, methylgyoxal; MMPs, matrix metalloproteinases; TGF, transforming growth factor; TIMPs, tissue inhibitor of metalloproteinases

Fig. 6. MMP-3, TIMP-1, TIMP-2 and IL-6 protein levels. Human gingival fibroblasts were seeded over collagen or MGO-treated collagen (1–10 mM) or stimulated with soluble MGO (1–200 nM). After 48 h of cell culture in the absence of fetal bovine serum, cell culture media was collected, centrifuged and analyzed through ELISA. Protein levels for MMP-3 (A), TIMP-1 (B), TIMP-2 (C) and IL-6 (D) were normalized against cell viability measurements assessed through the MTS assay. Col, collagen; IL-1beta, interleukin 1beta; MGO, methylgyoxal; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases.

approach represented an acute stimulus on cells that probably occurs during sudden rises in glycaemia (soluble MGO). The second experiment probably corresponds to a chronic effect derived from the long-lasting exposure of connective tissues to increments in blood glucose levels (MGO-treated collagen). Only soluble MGO induced a reduction on cell and death. However, it is interesting to note that both experiments demonstrated increases in TIMP-1 protein levels among a long list of mediators evaluated. In this sense, TIMP-1 induction may represent an early response of cells against cell damage induced by MGO. Moreover, abnormal extracellular remodeling has been associated with diabetes (24). From our results, we can suggest that induction of TIMP-1 can counteract the degradation of the extracellular matrix to prevent tissue destruction.

To induce collagen glycation we followed a method in which type I collagen is incubated overnight with MGO (1–10 mM) (10). A detailed characterization of the effects of MGO on collagen have identified that arginine residues present in this molecule are specifically affected by this agent, decreasing the solubility, hydroxyproline content and the electrophoretic migration of this molecule (10). Using this method we obtained a similar migration pattern to the one observed by Chong *et al.* (10). Therefore, we believe that the protocol used in this study induced collagen modifications compatible with the glycation of this molecule.

Soluble MGO may have affected different pathways that include activation of the cell surface receptor for AGEs (RAGE) (2,25) and the modification of intracellular regulators of gene transcription (26). RAGE has
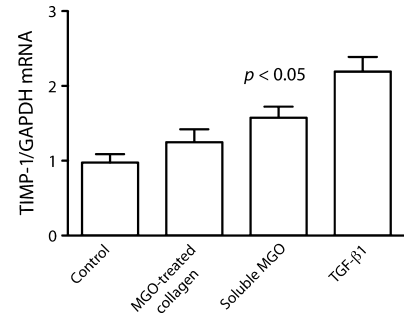
been associated with the activation of inflammatory mediators that may include TIMP-1 (25). On the other hand, treatment of collagen with MGO involves the glycation of this matrix, changing cell signaling in collagen binding cells such as fibroblasts (17,27). This response involves modifications in integrin signaling derived from the interaction between cells and the glycated collagen matrix (27). To our knowledge, this is the first study that links MGO or MGO-treated collagen with the regulation of TIMP-1. Further studies are needed to characterize this effect.

To analyze the effects of soluble MGO or MGO-treated collagen on inflammatory mediators, MMPs and TIMPs, several experimental approaches were used, including antibody arrays, immunofluorescence, ELISA and qPCR. Antibody arrays suggested that MMP-3, TIMP-1, TIMP-2 and IL-6 were stimulated when cells were cultured over MGO-treated collagen. As antibody arrays are semiquantitative (28), these results were confirmed

using ELISA assays. This new approach showed that only TIMP-1 protein levels were regulated by MGO-treated collagen or soluble MGO. In addition, we performed qPCR experiments to identify whether TIMP-1 mRNA levels were regulated by these two conditions. Surprisingly, only soluble MGO stimulated TIMP-1 mRNA levels. Meanwhile, MGO-treated collagen induced only a modest increase in this response. As mRNA levels were only measured at one time point (24 h), differences in the signaling pathways and transcriptional regulation initiated by both stimuli (MGO-treated collagen or soluble MGO) might account for this inconsistency. TIMP-1 has been identified as a target of microRNA suggesting a complex regulation for this gene (29). Further studies are needed to clarify this response.

TIMP-1 has been shown to control the activity of MMPs by forming 1 : 1 stoichiometric non-covalent complexes with these enzymes consequently regulating extracellular matrix remodeling in different physiological processes (30). In addition to the regulation of the extracellular matrix, TIMP-1 may regulate the inflammatory response through a still-undefined mechanism (31). TIMP-1 plays important roles in the protection of fibroblasts against cell death (32–34). Recent studies in keloid fibroblasts have also shown that silencing the TIMP-1 gene increases apoptosis in this cell type (35). Our study identified that soluble MGO or culture of cells over MGO-treated collagen was associated with an increase in TIMP-1 protein levels. It has been also identified that CD-63 and β1-integrin may act as cell surface receptors for TIMP-1 that, through activation of focal adhesion kinase, phosphatidylinositol 3-kinase and extracellular signal-regulated kinase, exert an anti-apoptotic effect in human breast cancer cells (36). We believe that this response might be involved in the protection of cells against cell injury induced by MGO.

The present findings suggest that soluble MGO, representing the acute exposure of cells to this agent, may reduce the amount of connective tissue cells necessary to preserve tissue homeostasis. TIMP-1 may represent a protective response of cells exposed to MGO-modified collagen or soluble MGO that may be important in tissue remodeling and/or protection against cell death induced by MGO. These responses might be relevant to inflammation and diabetes in gingival tissues.

Acknowledgements

Conflict of interest and sources of funding statement

The authors declare that they have no conflicts of interest to disclose.

References

1. Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 1999;**48**:1–9.
2. Ramasamy R, Yan SF, Schmidt AM. Methylglyoxal comes of AGE. *Cell* 2006;**124**:258–260.
3. Maiden MF, Pham C, Kashket S. Glucose toxicity effect and accumulation of methylglyoxal by the periodontal anaerobe *Bacteroides forsythus*. *Anaerobe* 2004;**10**:27–32.
4. Kashket S, Maiden MF, Haffajee AD, Kashket ER. Accumulation of methylglyoxal in the gingival crevicular fluid of chronic periodontitis patients. *J Clin Periodontol* 2003;**30**:364–367.
5. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. *Nat Rev Endocrinol* 2011;**7**:738–748.
6. Degenhardt TP, Thorpe SR, Baynes JW. Chemical modification of proteins by methylglyoxal. *Cell Mol Biol* 1998;**44**:1139–1145.
7. Dobler D, Ahmed N, Song L, Eboigbodin KE, Thornalley PJ. Increased dicarbonyl metabolism in endothelial cells in hyperglycemia impairs anoikis and impairs angiogenesis by RGD and GFOGER motif modification. *Diabetes* 2006;**55**:1961–1969.
8. Pedchenko VK, Chetyrkin SV, Chuang P et al. Mechanism of perturbation of integrin-mediated cell-matrix interactions by reactive carbonyl compounds and its implication for pathogenesis of diabetic nephropathy. *Diabetes* 2005;**54**:2952–2960.
9. Murillo J, Wang Y, Xu X et al. Advanced glycation of type I collagen and fibronectin modifies periodontal cell behavior. *J Periodontol* 2008;**79**:2190–2199.
10. Chong SA, Lee W, Arora PD et al. Methylglyoxal inhibits the binding step of collagen phagocytosis. *J Biol Chem* 2007;**282**:8510–8520.
11. Hsieh MS, Chan WH. Impact of methylglyoxal and high glucose co-treatment on human mononuclear cells. *Int J Mol Sci* 2009;**10**:1445–1464.
12. Figarola JL, Singhal J, Rahbar S, Awasthi S, Singhal SS. LR-90 prevents methylglyoxal-induced oxidative stress and apoptosis in human endothelial cells. *Apoptosis* 2014;**19**:776–788.
13. Huang FJ, Chin TY, Chan WH. Resveratrol protects against methylglyoxal-induced apoptosis and disruption of embryonic development in mouse blastocysts. *Environ Toxicol* 2013;**28**:431–441.
14. Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 2006;**40**:29–49.
15. Apte SS, Parks WC. Metalloproteinases: a parade of functions in matrix biology and an outlook for the future. *Matrix Biol* 2015; **44-46C**: 1–6.
16. Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem J* 1998;**333**:291–300.
17. Yuen A, Laschinger C, Talior I et al. Methylglyoxal-modified collagen promotes myofibroblast differentiation. *Matrix Biol* 2010;**29**:537–548.
18. Senderowicz AM. Inhibitors of cyclin-dependent kinase modulators for cancer therapy. *Prog Drug Res* 2005;**63**:183–206.
19. Rabbani N, Thornalley PJ. Dicarbonyl stress in cell and tissue dysfunction contributing to ageing and disease. *Biochem Biophys Res Commun* 2015;**458**:221–226.
20. Ahmed N, Thornalley PJ, Dawczynski J et al. Methylglyoxal-derived hydroimidazolone advanced glycation end-products of human lens proteins. *Invest Ophthalmol Vis Sci* 2003;**44**:5287–5292.
21. Seo K, Ki SH, Shin SM. Methylglyoxal induces mitochondrial dysfunction and cell death in liver. *Toxicol Res* 2014;**30**:193–198.
22. Desta T, Li J, Chino T, Graves DT. Altered fibroblast proliferation and apoptosis in diabetic gingival wounds. *J Dent Res* 2010;**89**:609–614.

23. Taylor JJ, Preshaw PM, Lalla E. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. *J Periodontol* 2013;**84**: S113–S134.

24. Wu YY, Xiao E, Graves DT. Diabetes mellitus related bone metabolism and periodontal disease. *Int J Oral Sci* 2015;**26**:63–72.

25. Sorci G, Riuzzi F, Giambanco I, Donato R. RAGE in tissue homeostasis, repair and regeneration. *Biochim Biophys Acta* 2013;**1833**:101–109.

26. Yao D, Taguchi T, Matsumura T *et al.* Methylglyoxal modification of mSin3A links glycolysis to angiopoietin-2 transcription. *Cell* 2006;**124**:275–286.

27. Talior-Volodarsky I, Connelly KA, Arora PD, Gullberg D, McCulloch CA. α11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. *Cardiovasc Res* 2012;**96**:265–275.

28. Copeland S, Siddiqui J, Remick D. Direct comparison of traditional ELISAs and membrane protein arrays for detection and quantification of human cytokines. *J Immunol Methods* 2004;**284**:99–106.

29. Li SH, Guo J, Wu J *et al.* miR-17 targets tissue inhibitor of metalloproteinase 1 and 2 to modulate cardiac matrix remodeling. *FASEB J* 2013;**27**:4254–4265.

30. Arpino V, Brock M, Gill SE. The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol* 2015; **44-46C**: 247–254.

31. Kim KH, Burkhart K, Chen P *et al.* Tissue inhibitor of metalloproteinase-1 deficiency amplifies acute lung injury in bleomycin-exposed mice. *Am J Respir Cell Mol Biol* 2005;**33**:271–279.

32. Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992;**298**:29–32.

33. Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci* 1994;**107**:2373–2379.

34. Murphy FR, Issa R, Zhou X *et al.* Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. *J Biol Chem* 2002;**277**:11069–11076.

35. Aoki M, Miyake K, Ogawa R *et al.* siRNA knockdown of tissue inhibitor of metalloproteinase-1 in keloid fibroblasts leads to degradation of collagen type I. *J Invest Dermatol* 2014;**134**:818–826.

36. Jung KK, Liu XW, Chirco R, Fridman R, Kim HR. Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. *EMBO J* 2006;**25**:3934–3942.