



Transient exposure to elevated glucose levels causes persistent changes in dermal microvascular endothelial cell responses to injury

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Background: The purpose of this study was to determine whether elevated glucose can induce a dermal microvascular endothelial cell metabolic memory, thus affecting angiogenesis in the repair process of mammalian cutaneous wound. We hypothesized that transient elevated glucose levels cause sustained alteration of endothelial cell responses to injury and persistent epigenetic changes in gene expression.

Methods: Human dermal microvascular endothelial cells were exposed to experimental conditions with or without 30 mM D-glucose. The control group was maintained at 5 mM D-glucose; while in the transient glucose group, after being exposed to 30 mM D-glucose for two days, then being put under the control conditions during the experiment. Besides, in the whole process of the experiment, the chronic glucose group was kept in the condition with 30 mM D-glucose. Proliferation, migration, tube formation, gene expression and histone methylation were assessed for individual conditions.

Results: Transient elevated glucose caused sustained effects on endothelial cell migration, tube formation and TIMP3 gene expression. The effects on TIMP3 expression were associated with persistent changes in histone modification at the 5' end of the TIMP3 gene, suggesting an epigenetic effect.

Conclusions: Hyperglycemia induced metabolic memory could promote the regulation of TIMP3, and it can be used as a possible innovative molecular target for therapeutic intervention in the treatment of chronic non-healing diabetic wounds.

Keywords: Diabetes; angiogenesis; hyperglycemia; metabolic memory; epigenetics

Submitted Nov 23, 2020. Accepted for publication Mar 10, 2021.

doi: 10.21037/atm-20-7617

View this article at: <http://dx.doi.org/10.21037/atm-20-7617>

Introduction

Chronic non-healing wounds are a common and debilitating complication of type 2 diabetes mellitus (1). It is estimated that up to 25% of the diabetic population develop a non-healing foot ulcer and an estimated 12% of individuals with diabetic foot ulcers require a surgical operation (2). On the basis of WHO's estimation, it was revealed that 347 million

people suffer diabetes mellitus around the world (WHO/ADA Fact sheet), and a global healthcare crisis is caused by the occurrence and prevalence of non-healing foot ulcers to some degree. Although non-healing diabetic ulcers have been strongly associated with underlying neuropathy (3), vascular insufficiency (4,5) and tissue hypoxia (6), the influence of hyperglycemia on the repair of the wound

seems to be little known and little understood.

Hyperglycemia contributes to the development of diabetic complications in part by causing endothelial dysfunction and subsequent vascular injury (4). Unfortunately, some detrimental effects of hyperglycemia persist even after glucose levels have been normalized. Many large-scale clinical trials and animal models give convincing evidence for the support this phenomenon, which was commonly called as metabolic memory (7). The prospective Diabetes Control and Complications Trial and the ensuing follow-up Epidemiology of Diabetic Interventions and Complications study demonstrated that vascular complications develop and progress despite intensive insulin therapy after periods of hyperglycemia (8). Similar results have been observed in diabetic dogs and rats, with tight glycemic control in both species failing to prevent (9).

Emerging data suggest that hyperglycemia-induced metabolic memory is mediated by epigenetic regulation of gene expression (10). Epigenetic regulation changes the chromatin structure by histone methylation or acetylation and/or DNA methylation. In a diabetic rat model of metabolic memory, hyperglycemia induced epigenetic modifications at both the *SOD₂* and gene promoters that were maintained after return to normoglycemia (11,12). In a zebrafish model of metabolic memory (13), hyperglycemia induced persistent changes in DNA methylation causing sustained effects on gene expression. Similarly, Transient exposure to hyperglycemia induced persistent epigenetic changes at the *p65* gene in cultured macrovascular endothelial cells (14,15). Collectively these studies implicate epigenetic regulation as an important mechanism for hyperglycemia-induced metabolic memory.

A literature search suggests that the only published study on contributions of hyperglycemia-induced metabolic memory on wound repair involved defects in wound closure and caudal fin regeneration in zebrafish which persisted after return to normoglycemia (16). Despite these promising results, the role of metabolic memory in mammalian wound repair has not been reported.

The study was aimed at finding out whether hyperglycemia can induce metabolic memory in human dermal microvascular endothelial cells and confirming the known participants in cutaneous angiogenesis during mammalian wound repair. We hypothesized that Transient hyperglycemia cause sustained effects on endothelial cell responses to injury and persistent epigenetic changes in gene expression.

The following article is presented in accordance with the

MDAR checklist (available at <http://dx.doi.org/10.21037/atm-20-7617>).

Methods

Cell culture

Primary human adult dermal microvascular endothelial cells (HMVECad) were gained in a commercial manner (Life Technologies, Carlsbad, CA, USA) and cultured on tissue culture plastic, which was coated with Attachment Factor Protein (Life Technologies, Carlsbad, CA, USA) and kept constantly at 37 °C and 5% CO₂ in Medium 131 supplemented with 5% Microvascular Growth Supplement, 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies, Carlsbad, CA, USA). Two biological replicates (females aged 48 and 26 year old) were used for these studies with excellent reproducibility.

For all experiments, on study day -2, dermal microvascular endothelial cells were seeded at 5×10^3 cells/cm² in medium 131, which contained 5 mM D-glucose. While on day 0, we exposed cells to experimental environment consisting of elevated levels of glucose (30 mM D-glucose; Life Technologies, Carlsbad, CA, USA) added to Medium 131; all experiments included three study groups of endothelial cells: Control, Transient glucose, and Chronic glucose. The control group was kept constantly at 5 mM D-glucose. While the Transient group was kept at a high-level glucose for two days, and then being put into Control Medium 131 conditions during experiment. The Chronic group was put into high glucose during the experiment. The another mannitol group was at 30 mM mannitol as control for hyperosmolarity. Endothelial cell passage numbers 6–9 were used for all experiments. Experiments were performed with 4–6 replicates, with the repeated test at least 3 times.

Cell proliferation assay

Cell morphology and proliferation were assessed on days 0, 2 and 4. For morphology, cells were photographed using a Nikon Digital Sight Camera (DS-Fi1) and the camera was mounted on a Nikon TE300 inverted light microscope which was equipped with phase contrast objectives. Then images were imported into Adobe Photoshop CS3[®] and a standard linear adjustment to brightness was applied to the entire image; no other adjustments to microscopic images were made. For cell proliferation, a Vicell Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA) was used to

count cells.

Apoptosis assay

The percentage of live, early apoptotic, late apoptotic and dead cells was confirmed by the use of the Muse™ Annexin V and Dead Cell Assay Kit (Millipore, Billerica, MA, USA) on days 0, 2 and 4. In brief, supernatant was collected and floating cells were pelleted and pooled with the pellet of trypsinized cells. The total cell pellet was resuspended in medium 131 and incubated with Annexin V reagent for 20 minutes at room temperature in the dark. And the cells were loaded onto the Muse™ Cell Analyzer to separate the live, early apoptotic, late apoptotic and dead cells.

Scratch wound closure assay

The influence of elevated glucose levels on endothelial cell migration was measured by the use of a scratch wound closure assay as previously described (17). In brief, cells were seeded at 5×10^4 cells/well in a 6-well plate and grown to confluence. The day of confluence was defined as day 0 for the Glucose treatment. On day 2, the scratch wound was created using sterile p200 pipette tip. Images were acquired with Nikon Eclipse TE300 along the scratch guidelines within the original three circles at multiple areas at the different timepoint (prior to scratch wound, immediately after scratch wound, and every 24h before the wound confluent). All the images saved as TIFF files and imported into Adobe Photoshop CS3 to measure the remaining defect in the cell layer. Image analysis plug-in Fovea Pro version 4.0 (Reindeer Graphics, Asheville, NC, USA) was used to measure the cell free space and the perimeter of each scratch wound. For each time point, they were normalized to the area of the original day 0 scratch defect. All data were shown as percent scratch wound closed. Images were acquired with Nikon Eclipse TE300 along the scratch guidelines within the original three circles at multiple areas at the different timepoint (prior to scratch wound, immediately after scratch wound, and every 24 h before the wound confluent). All the images saved as TIFF files and imported into Adobe Photoshop CS3 to measure the remaining defect in the cell layer. Image analysis plug-in Fovea Pro version 4.0 (Reindeer Graphics, Asheville, NC, USA) was used to measure the cell free space and the perimeter of each scratch wound. For each time point, they were normalized to the area of the original day 0 scratch defect. All data were shown as percent scratch wound

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Tube formation assay

Microvascular endothelial cells were seeded into Matrigel™ (BD Biosciences, Bedford, MA, USA) on day 4 in the process of tube formation assay. Phase contrast images (4x magnification) were taken in the center of each well of 6-well plate after 24 hours in the Matrigel™ using a Nikon Eclipse TE300 inverted microscope which is equipped with a QImaging QICAM Monochrome Fast 1394 c12-bit cooled camera. Digital images were generated as TIFF files and imported into Adobe Photoshop CS3 containing the plug-in FoveaPro version 4.0 (Reindeer Graphics, Asheville, NC, USA). Images were converted to a black and white binary image and skeletonized in order to determine total branch points and total line length, which are both accepted measures of tube formation (18).

RNA extraction, cDNA synthesis and real time PCR

RNA was extracted from the dermal microvascular endothelial cells by the use of Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and then the purification with the PureLink RNA Mini Kit (Life Technologies, Grand Island, NY, USA) was carried out, in which a DNase digestion step was included. By the use of an Omniscript RT kit (Qiagen, Valencia, CA, USA), cDNA was synthesized. Real time PCR using customized Primers (*Table 1*) was performed in a ViiA™7 instrument (Applied Biosystems, Foster City, CA, USA) using Quantitect SYBR green PCR kit (Qiagen, Valencia, CA). The comparative C_t method ($2^{-\Delta\Delta C_t}$) was selected for the qualification of gene expression levels, which were normalized to the housekeeping gene β -actin, ACTB; GFA had no notable influence on ACTB cycle threshold in the microvascular endothelial cells.

Matrix ChIP assay

Sample preparation and chromatin immuno-precipitation were done as previously described (19). Cells were fixed on day 4 with 1% formaldehyde for 10 minutes followed by quenching with 125 mM Glycine for 5 minutes at room temperature. Cells were washed with PBS, mechanically harvested, spun down at 1,600 xg for 5 minutes, washed with 1 mL PBS, spun down again and stored at -80°C as pellets. The pellet was

Table 1 Primers for ChIP and RT-PCR

Variable	Sequence
Human TIMP-3, Exon 1 (ChIP & RTPCR)	
Forward	CAGTCATGTCCAACCCAGAA
Reverse	GGACATCCATGAATGCACAG
Human TIMP-3, Exon 5 (ChIP & RTPCR)	
Forward	TCCTTTGGGCATCTCTTCTG
Reverse	GGGTTCGAGATCTCTTGTGG
Human ACTB, Ex4-Ex5 (ChIP)	
Forward	AGAGCTACGAGCTGCCTGAC
Reverse	AAGGTAGTTTCGTGGATGCC
Human ACTB (RTPCR)	
Forward	CTGGAACGGTGAAGGTGACA
Reverse	AAGGGACTTCCTGTAACAATGCA

resuspended in 100 μ L of immunoprecipitation (IP) buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM PMSE, pH 7.5). The chromatin was sheared using a Bioruptor sonicator (Diagenode, Philadelphia, PA, USA) with 30 s on-off cycles for 15 minutes at high intensity on ice. Lysates were cleared by centrifugation at 10,000 \times g at 4 $^{\circ}$ C for 10 minutes.

In the Matrix ChIP assay, we used UV-treated polypropylene 96-well plates coated with 0.5 μ g protein A in 100 μ L PBS per well. After a wash with 100 μ L PBS per well, the wells were blocked with 200 μ L blocking buffer (IP buffer supplemented with 5% BSA and 0.1 mg/mL salmon sperm DNA) for 30 minutes at room temperature. Blocking buffer was aspirated and the wells were incubated with 0.25 μ g antibody in 100 μ L of blocking buffer per well for 60 minutes at room temperature. The below components are included in primary antibodies: rabbit anti-human H3K4m3 (Thermo Fisher Scientific, Waltham, MA, USA), mouse anti-human H3K27m3 (ab6002, Abcam, Cambridge, MA, USA) and rabbit anti-human H3 (ab1791, Abcam, Cambridge, MA, USA). Wells were cleared and chromatin samples (equivalent of 50,000 cells in 100 μ L of blocking buffer) were added to wells (100 μ L/well) and plates were floated in ultrasonic water bath for 60 minutes, 4 $^{\circ}$ C. Wells were washed three times with 100 μ L ice-cold IP buffer and once with 100 μ L ice cold TE buffer (pH 7.5). Wells were incubated with 100 μ L elution buffer (20 mM Tris-base, 1 mM EDTA, 0.1 mg/mL proteinase K) for 15 minutes at

55 $^{\circ}$ C, and then it were stored for 10 minutes at 95 $^{\circ}$ C in a thermocycler. Input DNA was purified in parallel by adding an aliquot of chromatin sample to 100 μ L of elution buffer. DNA samples were stored at 4 $^{\circ}$ C. qPCR analysis of ChIP DNA samples was performed using primers listed in *Table 1*. The reaction mixture was made of 2.5 μ L 2 \times SYBR Green PCR master mix (SensiMixTM SYBR[®] Hi-ROX Kit, Bioline USA Inc, Taunton, MA, USA), 0.25 μ L TIMP-3 specific primers (10 μ M of each), 0.25 μ L H₂O and 2 μ L DNA sample in 5 μ L final volume in a 384-well optical reaction plate (Applied Biosystems, Foster City, CA, USA). Amplification (three steps, 40 cycles), data acquisition and analysis were carried out by the use of the 7900HT Real time PCR system (Applied Biosystems, Foster City, CA, USA). In each PCR run, dilutions of human genomic DNA were included that were used to generate standard curves for relative quantitation of qPCR. ChIP DNA data were normalized to input DNA and actin DNA, and were expressed as % of actin DNA [(TIMP3/inputs)/(actin/inputs)].

Statistical analyses

All values are shown as a mean \pm standard deviation. We used the Student's *t*-test or ANOVA to find out the obvious statistical differences among in these groups, with P values less than or equal to 0.05, which was considered as significant. A minimum of two biological replicates was analyzed for each end point. Each independent experiment

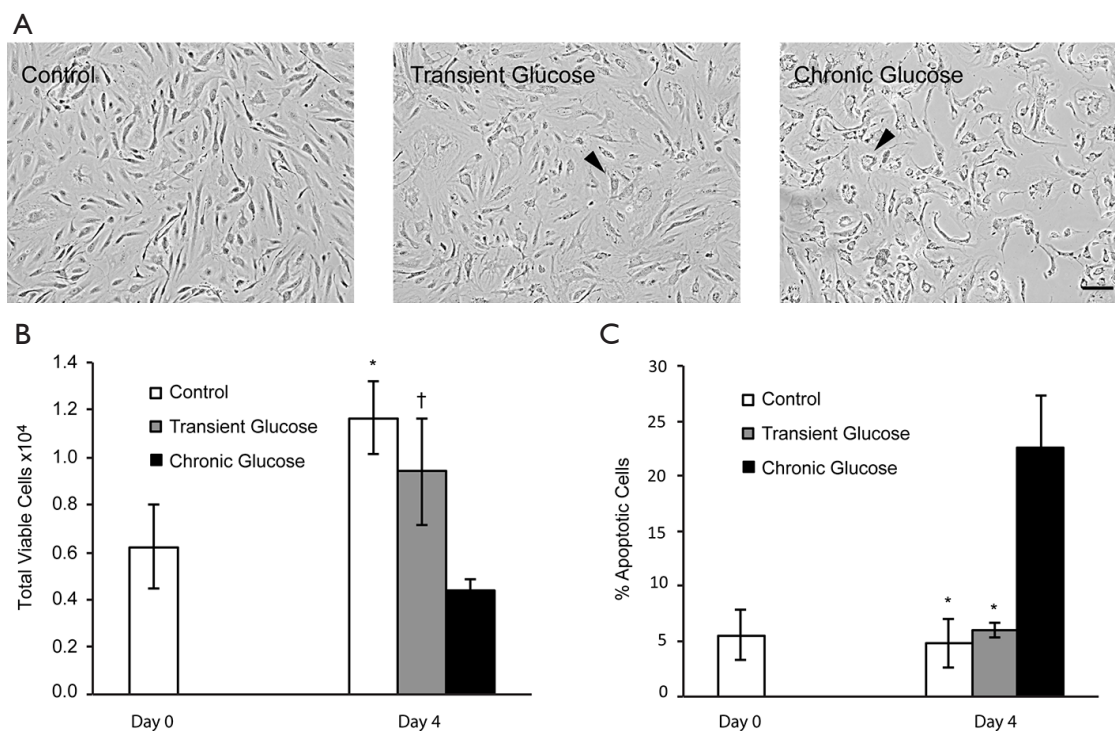


Figure 1 Transient glucose exposure showed sustained effects on morphology changes but not on cell viability of dermal microvascular endothelial cell. (A) Both cells treated in transient glucose and chronic glucose on day 4 shows lost spindle shape and appear in oval shape. The arrows show the fatty acid droplets accumulated in the nucleus (arrowhead). The magnification bar equals 50 μ m. (B) Effects of Glucose on proliferation profile in dermal microvascular endothelial cell culture over time. * $P < 0.01$ vs. chronic GFA, † $P < 0.05$ vs. chronic glucose. (C) Effect of glucose on apoptosis of dermal microvascular endothelial cell over time. * $P < 0.01$ vs. chronic glucose. Data displayed indicate mean \pm SD of a single representative experiment with $N = 6$ for individual condition.

was performed with six wells under individual condition, with the repeated tests at least 3 times.

Results

Effects of Glucose on dermal microvascular endothelial cell proliferation and viability

To model metabolic memory *in vitro*, primary dermal microvascular endothelial cells were exposed to elevated levels of glucose for two days and then returned to physiological levels of glucose for a minimum of two days. Control cells were cultured in physiological levels of glucose, and Chronic group cells were exposed to elevated levels of glucose for the duration of the experiment.

We first investigated the effect of high glucose exposure on endothelial cell viability by examining cell morphology, cell proliferation and apoptosis (Figure 1). Cells exposed to high glucose treatment exhibited considerable altered

morphology compared to controls (Figure 1A) and evidence of “fried egg” shaped cells with fatty acid droplets in the nucleus (Figure 1A, arrowhead). Whereas, these morphologic changes were most dramatic in the chronic group, they were also present in the Transient group. As a osmolarity control, cells exposed to mannitol treatment showed no changes in morphology compared to controls (Figure S1A). Despite this altered morphology, Transient Glucose had little to no effect on cell proliferation and total viable cell number compared to control after four days in culture (Figure 1B). There was also no significant increase in the percentage of apoptotic cells in the Transient Glucose group compared to the control (Figure 1C). In contrast, Chronic Glucose exposure induced endothelial cell apoptosis as evidenced by reduced viable cell number (Figure 1B) and a remarkable increase in the percentage of apoptotic cells (Figure 1C). Also, there were no significant changes of cell proliferation and apoptosis in mannitol treated group compare with the controls

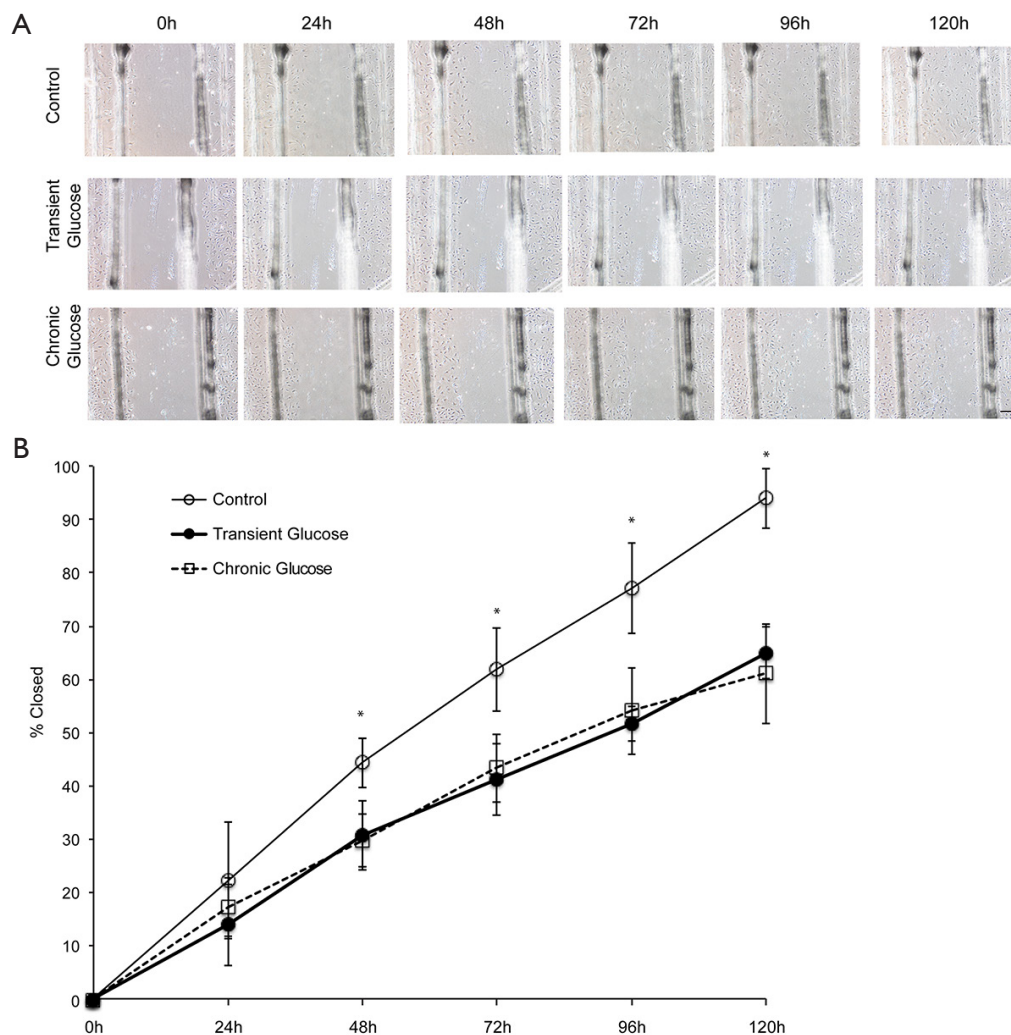


Figure 2 Transient glucose inhibit migration of dermal microvascular endothelial cells in a wound “scratch” assay. (A) Dermal microvascular endothelial cells were treated in M131 with or without elevated glucose and fatty acid for 2 days, and then scratched. The scratched cells were culture in conditional medium as follows: control group, continuous M131 without elevated glucose; transient glucose group, withdraw of elevated glucose and chronic glucose group, continuous M131 with elevated glucose. Images were captured immediately post scratch 0, 24, 48, 72, 96 and 120 h. (B) Transient elevated glucose and chronic glucose repressed notably the migration speed of human dermal microvascular endothelial cells at 48, 72, 96 and 120 h (all $*P < 0.05$ compared with transient glucose and chronic glucose group). Data displayed indicate mean \pm SD of a single representative experiment with $N=6$ for individual condition. And the value of magnification bar is 200 μm .

(Figure S1B,C). Collectively these results suggest that transient glucose exposure had no sustained effects on microvascular endothelial cell viability.

Effects of glucose exposure on dermal microvascular endothelial cell migration

We found out the influence of Transient Glucose exposure on endothelial cell migration in a scratch wound assay

(Figure 2). Images of scratch defects at 48, 72, 96 and 120 hours after scratching show that Chronic Glucose cells exhibited a significant delay in scratch wound closure when it was in comparison with the control (Figure 2A). Quantification of the percentage of scratch wound closure provided the evidence that this delay has an important statistical meaning (Figure 2B). When compared with the chronic glucose cells, it can be found that transient glucose exposure showed the similar tendency, which also delayed

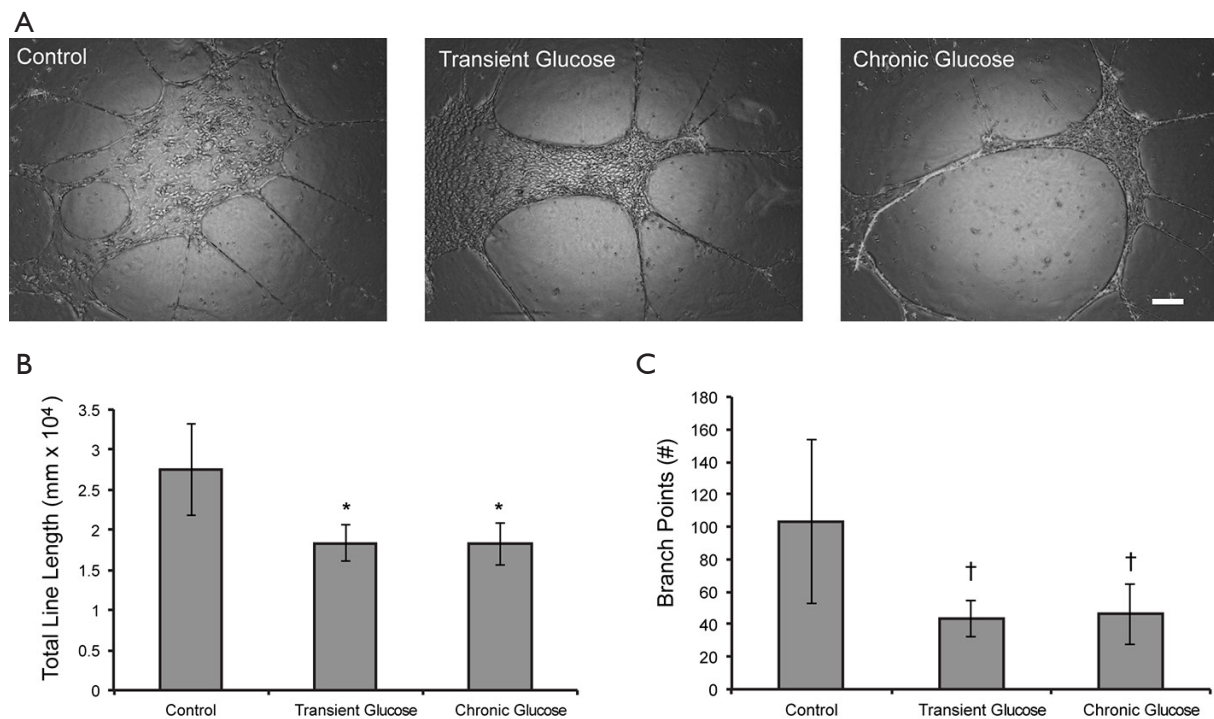


Figure 3 Transient glucose inhibit tube formation in dermal microvascular endothelial cells *in vitro*. (A) Tube formation assay was done as described in Methods. Cells were cultured for entire 24-h incubation period on matrigel. (B) Transient elevated glucose and (C) chronic glucose significantly reduced the total line length and branch point compared with control group. Error bars show SD. * $P < 0.01$, † $P < 0.05$ when compared with control. The magnification bar equals 50 μm .

endothelial cell migration into the scratch wound area, what's more, in the rate of endothelial migration between the transient and chronic glucose cells, no significant difference was found. The result also suggests that transient glucose exposure has a metabolic memory effect on endothelial cell migration. Also, there were no significant changes of cell migration in mannitol treated group compare with the controls (Figure S2).

Effects of glucose exposure on endothelial tube formation

Chronic glucose exposure decreased dermal microvascular endothelial cell tube formation when cultured on Matrigel™ (Figure 3A) as evidenced by significantly reduced tube length (Figure 3B) and total number of branch points (Figure 3C) compared to control conditions. Likewise, transient glucose significantly reduced both tube length and total number of branch points. Again, there was no significant difference between the chronic glucose and transient glucose cellular responses. As a validated *in vitro* model of angiogenesis, these results suggest that

transient exposure to elevated levels of glucose may impair angiogenesis during cutaneous wound repair.

Effects of glucose exposure on TIMP3 gene expression

We hypothesized that the sustained effects of transient glucose exposure on endothelial cell migration and tube formation were due in part to persistent changes in gene expression. We compared expression of 84 angiogenesis-related genes among the control, transient glucose and chronic glucose groups using a commercially available PCR array (SABioSciences/Qiagen, Valencia, CA, USA). Only the TIMP3 gene showed changes in expression that persisted after return to physiological levels of glucose (data not shown). This result was confirmed by RT qPCR analysis of TIMP3 expression (Figure 4) using customized primers (Table 1). Similar to endothelial cells exposed to chronic glucose treatment, TIMP3 gene expression in endothelial cells in the transient glucose group was down-regulated compared to control treatment; TIMP3 expression levels were not notably different between the

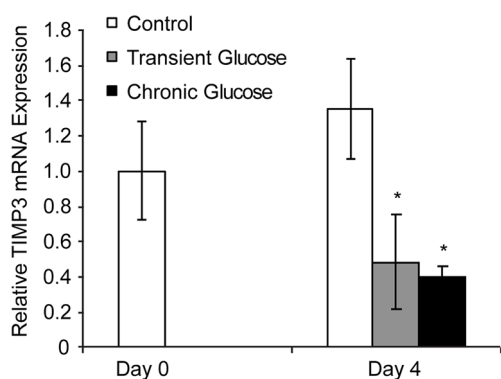


Figure 4 Transient elevated Glucose exposure induces a persistent change on *Timp3* gene expression by dermal microvascular endothelial cell. *Timp3* mRNA measured by RTPCR shows down-regulated in both transient glucose group and chronic group (* $P < 0.05$ compared with control group on day 4). There is no significant difference between control group on day 0 and day 4. The Data displayed indicated mean \pm SD of a single representative experiment with $N=6$ for individual condition.

transient and chronic glucose groups. Mannitol used as a control treatment to control for osmolarity had no notable influences on *TIMP3* gene expression (Figure S3). These results suggest that transient Glucose exposure induces a persistent change in *TIMP3* gene expression by dermal microvascular endothelial cells that may be explained by chromatin or DNA modification.

Glucose-induced epigenetic changes at the *TIMP3* gene

Using a chromatin immunoprecipitation assay (20), we examined glucose-induced changes in histone modifications at the *TIMP3* gene. Specifically, we measured the density of H3K27m3 a histone modification associated with silenced genes and H3K4m3, a histone modification associated with transcribed genes (20) (Figure 5). Compared to the control treatment, glucose exposure significantly increased the density of the repressive marker, H3K27m3 and significantly decreased the density of the activating marker, H3K4m3 at the 5' end of the *TIMP3* gene (exon 1). Glucose exposure did not change H3K27m3 and H3K4m3 levels at the 3' end of the gene (exon 5). There was no difference in density of histone modifications between the Transient and chronic glucose treatment groups. Glucose exposure had no significant effect on the density of either histone modification locus at the 5' end of the control housekeeping gene *ACTB* (exon 1). Collectively these results demonstrate

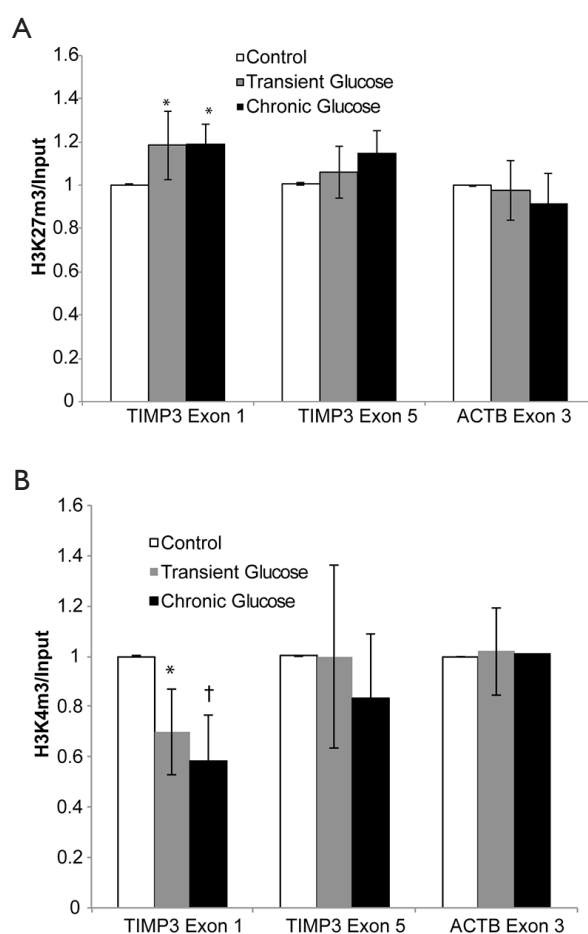


Figure 5 Transient exposure of elevated Glucose induces changes in histone modifications. ChIP analysis of histone H3 modification density at *TIMP3* Exon 1 and Exon 5. Matrix ChIP was proceeded with antibodies to (A) H3K4m3 and (B) H3K27m3 as per summarized in the section of Method. Through the gene-specific primers (*TIMP3* exon 1 and exon 5), the analysis of precipitated DNA completed by qPCR. After calculation, ChIP results are shown as fraction of input DNA. What's more, the results represent mean \pm SD. Signal ratios of PCR done in triplicate. *, † $P < 0.05$ compared with control.

that the sustained effects on *TIMP3* gene expression by transient exposure to elevated levels of glucose are related with alternations in histone modifications at the 5' end of the *TIMP3* gene, which indicating an epigenetic effect.

Discussion

In response to cutaneous injury, dermal microvascular

endothelial cells proliferate, migrate and differentiate to form new blood vessel sprouts from pre-existing vessels. To date, few publications have reported the effect of hyperglycemia on dermal microvascular endothelial cells (21) and wound angiogenesis (22). Moreover, in the available publications, exposure to high levels of glucose has been continuous. Data reported here are the first to demonstrate sustained effects of transient exposure to elevated levels of glucose on dermal microvascular endothelial cell migration, tube formation, TIMP3 gene expression and epigenetic changes at the 5' end of the TIMP3 gene. Collectively our findings are consistent with the hypothesis that transient exposure to hyperglycemia induces state of metabolic memory in dermal microvascular endothelial cells. Whereas previous studies have demonstrated that continuous exposure to high glucose levels inhibits dermal microvascular endothelial cell migration and tube formation (23), our data indicate that transiently elevated glucose induce persistent biological cellular responses after normalization of conditions, consistent with a metabolic memory.

Our observation that elevated levels of glucose reduce TIMP3 mRNA level in dermal microvascular endothelial cells is novel, as changes in TIMP3 expression in dermal microvascular endothelial cells have not been previously reported. In spite of strong evidence that TIMP3 regulates inflammation, angiogenesis and extracellular matrix remodeling (24), which are key responses to injury during cutaneous wound repair, there is scant information about TIMP3 expression in acute wounds (25). Previous published studies have focused on TIMP-1 and TIMP-2 expression in diabetic wounds (26,27). However, in one recent publication, Menghini and colleagues reported reduced TIMP3 mRNA levels in ischemic wounds compared to neuropathic diabetic foot ulcers (28); a limitation of their study is that they did not compare TIMP3 in diabetic wounds to non-diabetic wounds. Hence the temporal and spatial contributions of TIMP3 to normal or dysfunctional wound repair processes is not well defined.

Our observation that down-regulation of TIMP3 mRNA after transient Glucose exposure is associated with persistently increased levels of the silencing mark H3K27m3 and decreased levels of the activation mark H3K4m3, supports our hypothesis that TIMP3 gene expression in microvascular endothelial cells is regulated by epigenetic factors involving chromatin modifications. Our results corroborate previous reports that H3K27m3 is associated with reduced TIMP3 transcription and H3K4me3 is associated with increased TIMP3 expression in prostate (28) and ovarian cancer cell

lines (29). The relevance of our observations is also supported by reports that hyperglycemia alters the density of the H3K4m1, H3K9m2 and H3K9m3 on the p65 gene in aortic endothelial cells, increased. H4K20m3 density on the SOD2 promoter in mouse retina (30) and decreased H3K9me3 on the IL-6 gene in rat cardiomyocytes (31). All of these publications reported that the epigenetic change in histone methylation persisted after restoration of normoglycemic conditions.

The mechanism by which elevated glucose alter microvascular endothelial cellular biologic, genomic, and epigenetic responses warrants further analysis. One explanation that the observed changes result from hyperosmotic effect of glucose is unlikely given that an equal concentration of mannitol as a control for osmolarity did not alter the tested phenotypic responses. We have previously reported that elevated glucose causes oxidative responses including hydrogen peroxide production and that the responses could be attenuated with the antioxidants, alpha-tocopherol succinate and L-ascorbic acid (32). Studies have shown that hyperglycemia may induce apoptosis of vascular endothelial cells *in vitro*. In the study, transient glucose may also induce apoptosis of vascular endothelial cell, but more endothelial cells became apoptotic after persistent hyperglycemia. The impact of hyperglycemia on increasing the oxidative stress of endothelial cells has been confirmed. which might be ascribed to the long-term exposure to hyperglycemia used in this study. Therefore, it is possible that the epigenetic effects are caused by oxidative stress. Finally, TIMP3 has been previously reported to inhibit angiogenesis; as such, our results regarding changes in endothelial cell migration and tube formation as well as epigenetic effects on TIMP3 gene expression may be independent and may not represent an association between the endothelial cell biological responses and regulation of TIMP3 expression. Importantly, our results do indicate that a gene specific persistent alteration in epigenetic regulation is associated with transient exposure to hyperglycemia.

In summary, this novel report of metabolic memory in dermal microvascular endothelial cells indicated the effects of transiently elevated levels of glucose on angiogenesis, a critical stage of dermal wound repair. Transient exposure to elevated glucose levels had constant influence on endothelial cell migration, tube formation and TIMP3 expression. The Effects of glucose on TIMP3 gene expression were related with alternations in histone methylation that persisted after normalization of glucose levels. Overall, our results suggest that metabolic memory contributes to the impaired

neovascularization observed in chronic non-healing diabetic wounds and define potential molecular targets for therapeutic intervention.

Acknowledgments

Funding: This work was supported by the National Natural Science Fund of China (81272112). Thanks Dr. Nicole Gibran, Dr. Oleg Denisenko, Mrs Lara A Muffley and Dr. Anne M Hocking from University of Washington for research design and training.

Footnote

Reporting Checklist: The authors have completed the MDAR checklist. Available at <http://dx.doi.org/10.21037/atm-20-7617>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/atm-20-7617>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-20-7617>). Dr. LQ reports grants from National Natural Science Fund (81272112), during the conduct of the study. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This project received an ethical exemption from the Ethics Committee of Ruijin Hospital.

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Cite this article as: Wang Q, Song F, Dong J, Qiao L. Transient exposure to elevated glucose levels causes persistent changes in dermal microvascular endothelial cell responses to injury. *Ann Transl Med* 2021;9(9):758. doi: 10.21037/atm-20-7617