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Comments
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PURPOSE. The metabolic alterations due to chronic hyperglycemia are well-known to cause diabetes-associated complications. Short-term hyperglycemia has also been shown to cause many acute changes, including hemodynamic alterations and osmotic, oxidative, and inflammatory stress. The present study was designed to investigate whether diabetes-associated hyperglycemia can cause rapid-onset detrimental effects on the tear film, goblet cells, and glycocalyx and can lead to activation of an inflammatory cascade or cellular stress response in the cornea.

METHODS. Mouse models of type 1 and type 2 diabetes were used. Tear film volume, goblet cell number, and corneal glycocalyx area were measured on days 7, 14, and 28 after the onset of hyperglycemia. Transcriptome analysis was performed to quantify changes in 248 transcripts of genes involved in inflammatory, apoptotic, and stress response pathways.

RESULTS. Our data demonstrate that type 1 and type 2 diabetes-associated hyperglycemia caused a significant decrease in the tear film volume, goblet cell number, and corneal glycocalyx area. The decrease in tear film and goblet cell number was noted as early as 7 days after onset of hyperglycemia. The severity of ocular surface injury was significantly more in type 1 compared to type 2 diabetes. Diabetes mellitus also caused an increase in transcripts of genes involved in the inflammatory, apoptotic, and cellular stress response pathways.

CONCLUSIONS. The results of the present study demonstrate that diabetes-associated hyperglycemia causes rapid-onset damage to the ocular surface. Thus, short-term hyperglycemia in patients with diabetes mellitus may also play an important role in causing ocular surface injury and dry eye.

Keywords: cornea, ocular surface, diabetes, hyperglycemia, tear film

The global epidemic of diabetes mellitus has resulted in an alarming increase in the incidence of diabetes-associated complications.1 Diabetes-associated complications are categorized as microvascular, which include retinopathy, nephropathy, and neuropathy, or macrovascular which cause cardiovascular or cerebrovascular events such as myocardial infarction and stroke. Chronic hyperglycemia is an important etiological factor in causing diabetes-associated complications by initiating a variety of insidious metabolic changes. Such metabolic changes, including glycation leading to the formation of advanced glycation end products, excessive activation of hexosamine biosynthesis, polyol pathway, and PKC activation, increased growth factor/cytokines levels, and altered synthesis of the extracellular matrix are well-known mechanisms contributing to chronic hyperglycemia-associated metabolic changes.5,6

Besides chronic effects, hyperglycemia has also been shown to cause many rapid-onset biological effects. These rapid-onset effects of high blood glucose can be especially relevant in patients with poor glycemic control experiencing short-duration of fasting or post-prandial hyperglycemia. Hyperglycemia has been shown to rapidly cause hypovolemia, increased blood osmolality, and diuresis as early as 7 days in the mouse model of type 1 diabetes.4 Furthermore, hyperglycemia or glucose clamp infusion cause rapid cardiovascular changes in animal models of diabetes, in patients with diabetes, or in healthy non-diabetic human volunteers.5,6 Multiple studies in mice models of diabetes, in healthy volunteers with glucose clamp, in patients with diabetes, and using cell culture have demonstrated that hyperglycemia or exposure to high glucose promptly causes oxidative stress, leads to endothelial dysfunction, activates coagulation factors, and increases the expression of adhesion molecules and cytokine levels.6,10 Additionally, high glucose can also rapidly trigger inflammatory pathways because the exposure of peripheral blood mononuclear cells to high glucose has been shown to increase the activation of nuclear factor-κB and other pro-inflammatory transcription factors leading to increased expression of matrix metalloproteinases, tissue factor, plasminogen activator inhibitor-1, and pro-inflammatory cytokines.15–20
The tear film is a thin layer of fluid covering the ocular surface and plays a vital role in maintaining ocular surface health. Both preclinical and clinical studies have shown that diabetes mellitus affects tear film, causes dry eye, and damages the corneal epithelium. A decrease in tear film has also been reported. Damage to the lacrimal gland in the mouse model of type 1 hyperglycemia of several weeks has been shown to cause damage to the lacrimal gland in the mouse model of type 1 diabetes. A decrease in tear film has also been reported in rodent models of type 1 and type 2 diabetes at 8 weeks and 14 weeks after the onset of hyperglycemia. The chronic hyperglycemia-associated metabolic damage to the lacrimal gland and the associated decrease in the tear film likely contribute to dry eye disease in patients with diabetes mellitus. However, the tear film is extremely sensitive to changes in plasma volume, such as those caused by reduced hydration or drugs like diuretics. Additionally, changes in osmolarity and inflammatory stimuli can also have a major harmful effect on the tear film and ocular surface epithelial cells. Because hyperglycemia, even for a short duration, can cause rapid changes in blood volume, osmolarity, and oxidative and inflammatory stress levels, and given the fact that tear film is sensitive to all these noxious stimuli, it is important to investigate whether hyperglycemia can cause any rapid-onset detrimental effects to the tear film and ocular surface. Therefore, the present study was designed to assess whether diabetes-associated hyperglycemia can have rapid-onset detrimental effects on the tear film, goblet cells, and glyocalyx. We further investigated whether these deleterious effects are dependent on the severity of hyperglycemia by comparing these effects in mouse models of type 1 and type 2 diabetes that have differential levels of hyperglycemia. We also tested the effect of restoration of euglycemia using insulin implants. These novel insulin implants provide prolonged and consistent euglycemia by sustained release of insulin. Finally, using transcriptome analysis, we also examined whether short-duration (28 days) or less) hyperglycemia can cause the activation of an inflammatory cascade or cellular stress response in the cornea. Our data raise the interesting possibility that poorly controlled blood glucose even for a short duration may play an important role in causing ocular surface injury and dry eye.

METHODS

Mouse Models of Type 1 and Type 2 Diabetes Mellitus

The animal protocol was approved by the Institutional Animal Care and Use Committee of Chapman University. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The 4 week old mice were used for the type 1 and type 2 diabetes models and the non-diabetic control group, thus the animals were age-matched. For the induction of type 1 diabetes, 4-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were administered a single intraperitoneal injection of 200 mg/kg streptozotocin dissolved in citrate buffer, pH 4.5. Age-matched (4-week-old) male C57BL/6 mice received an intraperitoneal injection of citrate buffer only and served as non-diabetic controls. For type 2 diabetes, a 4-week-old male db/db strain (The Jackson Laboratory, Bar Harbor, ME, USA) of mice was used. These mice carry a genetic mutation for the leptin receptor and develop obesity, insulin resistance, and hyperglycemia at around 4 weeks of age. Blood glucose levels after streptozotocin injection or db/db mice were confirmed by measuring blood glucose levels using the OneTouch Verio glucometer. The mice were not subjected to any fasting before streptozotocin injection or blood glucose measurement and a postprandial blood glucose level of >250 mg/dL was considered as diabetic. The duration-dependent effects of type 1 and type 2 diabetes-associated hyperglycemia on the tear film, goblet cells, glyocalyx, and mucins were evaluated on days 7, 14, and 28 after the onset of diabetes.

Restoration of Euglycemia by Insulin Treatment

Type 1 diabetic mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) and subcutaneously implanted with one sustained-release insulin implant (LinShin, Toronto, ON, Canada). Briefly, hair on the mid-dorsal skin of mice was clipped and a 16-gauge disposable hypodermic needle was used to prick the skin. The pellet was implanted at this site using a delivery device.

Tear Film Quantification

Tear volume was quantified using commercially available phenol red thread (Zone Quick FCI Ophthalmics, Pembroke, MA, USA), which is pH sensitive and changes from yellow to red upon wetting by tears. The thread was placed in the temporal end of the lower eyelid of the mouse for 30 seconds. The mice were gently restrained in a cloth while performing the test but were not anesthetized. After 30 seconds, the thread was removed and the length of color change was measured in millimeters. The measurement was done for both the eyes of each animal to obtain an average reading. A standard curve was used to convert the phenol red thread measurement in mm to nanoliter. This standard curve was generated by placing the phenol red thread in a known volume of tears and measuring the length of the wetted thread.

Tissue Harvesting for Goblet Cell Staining, Glycocalyx Staining, and mRNA Isolation

The mice were euthanized to harvest the cornea from the right eye. The harvested cornea was immediately placed in liquid nitrogen for mRNA extraction to quantify gene expression of mucins (Muc) 1, 4, and 16 and for nanoring multiplex gene expression analysis. The left set of eyes along with eyelids were harvested and fixed in 4% paraformaldehyde overnight, followed by sequential dehydration in 15% sucrose and 30% sucrose. The tissues were then snap frozen in optimal cutting temperature (OCT) compound using 2-methyl butane cooled over liquid nitrogen. The frozen tissues were cut into sagittal halves to perform goblet cell and glyocalyx staining.

Goblet Cell Staining and Quantification

The 8-μm thin sagittal sections were obtained from the medial part of the frozen eye tissue using a cryostat (Leica, Wetzlar, HE, Germany) and stained for goblet cells with Periodic Acid Schiff’s (PAS) using a commercially available...
The stained sections were imaged at 100 × magnification using a bright-field microscope (Keyence Corporation of America, Itasca, IL, USA). The goblet cells were counted in a blind fashion in the fornix of 20 of these PAS-stained tissue section images obtained from the medial part of the eye for each mouse. Because 6 mice per group (type 1 diabetic, type 2 diabetic, and age-matched non-diabetic groups) and per time point (days 7, 14, and 28) were used, therefore, a total of 120 images (6 mice × 20 images/mice = 120) were counted to obtain goblet cells number for each time point/group.

**Glycocalyx Staining and Quantification**

For glycocalyx staining, corneas were blocked in 5% bovine serum albumin (BSA) solution in 1X PBS for 20 minutes. The corneas were incubated in a 1.5 μg/mL solution of Alexa 488-conjugated wheat germ agglutinin lectin (Thermo Fisher Scientific, Hanover Park, IL, USA) for 20 minutes at room temperature. The corneas were then washed with 1 × PBS 3 times for 10 minutes each. The stained corneas were mounted on microscope slides using DAPI mounting media. The slides were imaged at 200 × magnification using a confocal microscope (Nikon, Melville, NY, USA) to obtain a Z-stack of the stained glycocalyx. The glycocalyx-stained area was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) in a blinded manner. The quantitative data was obtained from 6 mice for each time point for type 1 diabetic, type 2 diabetic, and age-matched non-diabetic groups. For each cornea, 3 areas were imaged, thus, the quantitative data for each time point is obtained from 18 images.

**Gene Expression Quantification of Mucin 1, 4, and 16**

For gene expression quantification, RNA was isolated from the corneas using a commercially available kit (RNaseasy Mini Kit; Qiagen, Valencia, CA, USA). The mRNA was immediately reverse-transcribed into cDNA (SuperScript III First-Strand; Invitrogen, Carlsbad, CA, USA), which was used for quantifying Mucin 1, 4, and 16 gene expression using real-time PCR. β-actin was used as the housekeeping gene. Briefly, 20 μL reaction mixtures consisting of 2 μL of cDNA, 10 μL of SYBR Master Mix, 2 μL of forward primer, 2 μL of reverse primer, and 4 μL of DEPC water were run at a universal cycle (95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 55°C for 60 seconds) using a real-time thermocycler (Biorad, Hercules, CA, USA). Results were normalized to β-actin to calculate ΔΔCt and fold change in gene expression using the ΔΔCt method.

**Transcriptome Analysis for Inflammatory Cascade and Cellular Stress Response**

RNA isolated from corneas was also used to perform multiplex gene expression analysis using NanoString technology. Mouse Inflammation V2 assay panel and the nCounter Pro Analysis System (NanoString, Seattle, WA, USA) were used to quantify changes in gene expression of 248 genes. The data were analyzed using nSolver and ROSALIND software. Heatmaps were generated from z-scores obtained from software analyses and plotted using GraphPad Prism.

**Statistical Analysis**

The data are presented as mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism software (GraphPad Prism, version 9; GraphPad, San Diego, CA, USA). Two-way ANOVA followed by Bonferroni post hoc test was used to analyze the data. Statistical significance was determined as a P value < 0.05.

**RESULTS**

All the mice become diabetic (postprandial hyperglycemia > 250 mg/dL) within 24 hours of streptozotocin injection. The subsequent postprandial blood glucose levels were 501 ± 52 mg/dL, 580 ± 15 mg/dL, and >600 mg/dL at 7 days, 14 days, and 28 days after streptozotocin injection (Fig. 1A). The db/db mice included in this study had hyperglycemia...
of >250 mg/dL at 4 weeks of age. The subsequent mean postprandial blood glucose levels were 375 ± 51 mg/dL, 384 ± 21 mg/dL, and 531 ± 41 mg/dL at 7 days, 14 days, and 28 days, respectively, after the onset of hyperglycemia (see Fig. 1A). As expected, db/db mice had remarkably higher body weight compared to both the age-matched non-diabetic mice (*P < 0.05) and the streptozotocin-injected mice (ψ P < 0.05; Fig. 1B).

Effect of Type 1 and Type 2 Diabetes Mellitus on Tear Film Volume

To assess the effect of type 1 and type 2 diabetes mellitus on the tear film, we measured the tear film volume in mice at 7 days, 14 days, and 28 days after the onset of hyperglycemia. Our results demonstrate that compared to age-matched non-diabetic mice, mice with type 1 and type 2 diabetes mellitus showed a significant (*P < 0.05) decrease in tear volume (Fig. 2). The decrease in tear volume was noted as early as 7 days after the onset of hyperglycemia and it remained significantly (*P < 0.05) reduced at the tested time points of 14 and 28 days compared to age-matched non-diabetic mice, although a trend toward a slight increase was observed at days 14 and 28 compared to day 7. Furthermore, it is interesting to note that although the severity of hyperglycemia in mice with type 2 diabetes mellitus was significantly less compared to mice with type 1 diabetes mellitus (see Fig. 1A), the decrease in tear film volume noted in mice with type 2 diabetes was similar to that observed in mice with type 1 diabetes (see Fig. 2).

Effect of Type 1 and Type 2 Diabetes Mellitus on Goblet Cells

Goblet cells are specialized cells located in the conjunctival epithelium that, in mice, secrete Muc5AC and Muc5B onto the ocular surface. To test the effect of type 1 and type 2 diabetes on goblet cells, PAS staining was performed. Figure 3 shows representative images of PAS-stained goblet cells in the fornix of ocular tissue sections obtained from mice with type 1 and type 2 diabetes and age-matched non-diabetic mice. Compared to age-matched non-diabetic mice, tissue sections of mice with type 1 diabetes showed a significant (*P < 0.05) decrease in goblet cells at 7, 14, and 28 days after the onset of hyperglycemia. Further-
Effect of Type 1 and Type 2 Diabetes Mellitus on Corneal Glycocalyx

The corneal glycocalyx is important for the barrier, lubrication, and hydration functions of the ocular surface. Glycocalyx is a network formed by membrane-tethered mucins, their glycosylated and silated side chains, and galectin-3. Wheat germ agglutinin is a lectin that specifically binds to N-acetylglucosamine and sialic acid residues of mucin glycosyl side chains and has been used to stain ocular surface glycocalyx. Therefore, to test the effect of type 1 and type 2 diabetes on glycocalyx, we stained the corneas with wheat germ agglutinin. Figure 4 shows the representative immunostained Z-stack confocal images of mouse corneas obtained from age-matched non-diabetic mice and mice with type 1 and type 2 diabetes. As is evident from the immunostaining, non-diabetic corneas showed uniform and intact glycocalyx staining distributed throughout the cornea. Corneas obtained from mice with type 1 diabetes showed a decrease in glycocalyx and this loss of glycocalyx was punctuated at 7 days after the onset of hyperglycemia but became more evident with larger areas showing loss of glycocalyx on days 14 and 28 after the onset of hyperglycemia. Quantification of the images using ImageJ revealed that the type 1 diabetic corneas had an average of 48 ± 0.3%, 30 ± 0.3%, and 27 ± 0.2% glycocalyx stained area at days 7, 14, and 28, respectively, after the onset of hyperglycemia compared to 84 ± 0.3%, 90 ± 0.3%, 92 ± 0.2%, respectively, in the age-matched non-diabetic corneas.

Type 2 diabetes also caused a decrease in glycocalyx area but the decrease was significant only at days 14 (46 ± 0.5% stained area) and 28 (38 ± 0.5% stained area) after the onset of hyperglycemia. Furthermore, the decrease in glycocalyx area was significantly (ψ P < 0.05) less severe compared to mice with type 1 diabetes mellitus.

Effect of Type 1 and Type 2 Diabetes Mellitus on Gene Expression of Membrane-Tethered Mucins

Next, we tested whether the corneas of mice with type 1 and type 2 diabetes show any changes in corneal epithelial cell membrane-tethered mucins 1, 4, and 16 gene expression. Our data show that the onset of hyperglycemia due to type 1 and type 2 diabetes did not cause any significant change in the gene expression of Muc1 and Muc16 at any of the tested time points. However, a significant increase in Muc4 was observed at 14 days after the onset of hyperglycemia only in mice with type 1 diabetes (Fig. 5).

Restoration of Euglycemia and Attenuation of Diabetes Mellitus-Mediated Ocular Surface Injury

Next, we tested whether restoration of euglycemia by insulin will prevent the detrimental effects of hyperglycemia in mice...
Figure 5. Mucin (Muc) 1, 4, and 16 gene expression in the corneas of type 1 and type 2 diabetic mice. Mucin gene expression was quantified using real-time PCR in the corneas obtained at 7, 14, and 28 days after the onset of hyperglycemia. The data represent mean ± SEM. *P < 0.05 compared to age-matched non-diabetic mice.

Figure 6. Effect of insulin-mediated restoration of euglycemia (A), tear film volume (B), goblet cells (C), and glycocalyx (D) in type 1 diabetic mice at 7, 14, and 28 days after insulin pellet implantation. The data represent mean ± SEM. *P < 0.05 compared to age-matched non-diabetic mice and Φ P < 0.05 compared to type 1 diabetic mice.
with type 1 diabetes. We evaluated the effects of insulin in only the mice model of type 1 diabetes because type 2 db/db mice are already hyperinsulinemic and have insulin resistance, thus, they do not respond to insulin treatment. Our data show that an insulin implant caused effective restoration of euglycemia (Fig. 6A) and blood glucose levels in insulin-implanted animals were comparable to age-matched non-diabetic mice at day 7 and day 14. However, blood glucose levels started to rise again on day 28 likely due to the dissolution of the insulin implant resulting in somewhat diminished levels of insulin. Furthermore, restoration of euglycemia by insulin completely prevented the damaging effect of type 1 diabetes-associated hyperglycemia on goblet cells (Fig. 6C) and glycocalyx (Fig. 6D) because no decrease in goblet cell number or glycocalyx was noted. Furthermore, the goblet cell number and corneal glycocalyx area in insulin-treated mice were comparable to the age-matched non-diabetic mice. Restoration of euglycemia by insulin treatment also prevented the detrimental effect of diabetes-associated hyperglycemia on the tear film and the tear film volume in insulin-implanted mice was comparable to age-matched non-diabetic mice at days 14 and 28 after the insulin implant (Fig. 6B). However, it is interesting to note that there appears to be an apparent lag in both the onset and wearing off of the protective effect of insulin-mediated euglycemia on tear film because the tear film volume was still significantly lower at 7 days after the implant, although the mice were euglycemic at this time and the protective effect of the insulin implant was very significant on tear film at 28 days although the blood glucose levels started to rise by this time (see Fig. 6B).
Effect of Type 1 and Type 2 Diabetes Mellitus on Inflammatory Genes Transcriptome of Cornea

Diabetes mellitus-mediated pathological changes, including a decrease in tear film volume, a drop in goblet cell number, and a decline in glycoalyx area, can significantly compromise ocular surface health and may potentially lead to an inflammatory response. Thus, we tested whether the corneas of mice with type 1 and type 2 diabetes mellitus show differential changes in genes involved in inflammatory pathways. To test this hypothesis, we used NanoString technology nCounter mouse inflammation V2 panel to test gene transcriptome changes in 248 inflammatory gene transcripts. We used a threshold of 1.2-fold increase or decrease at a $P$ value of 0.08 to identify genes that were differentially expressed in the corneas obtained from mice with type 1 and type 2 diabetes, compared to the corneas obtained from age-matched non-diabetic mice. Figure 7 shows the volcano plots of the differential changes in the tested genes in the corneas of mice with type 1 and type 2 diabetes at 14 and 28 days after the onset of hyperglycemia. At 14 days after the onset of hyperglycemia, only 14 genes in mouse corneas with type 1 diabetes and 23 genes in mouse corneas with type 2 diabetes showed a notable change in expression. Furthermore, these genes were mostly downregulated, 13 in mice with type 1 diabetes and 21 in mice with type 2 diabetes. On the other hand, at 28 days after the onset of hyperglycemia, 48 genes in the corneas of
mice with type 1 diabetes and 42 genes in the corneas of mice with type 2 diabetes showed a notable change in expression. In contrast to 14 days data, most of these genes were upregulated, 30 in type 1 diabetes and 29 in type 2 diabetes.

Next, we performed pathway analysis to identify changes in groups of genes in the context of their biological relevance in inflammation. To accomplish this, genes were clustered according to pathways, and corresponding z-scores were plotted on heat maps (Fig. 8). It is worthwhile to note that similar changes in gene expression involved in the inflammatory pathways were identified in both models of type 1 as well as type 2 diabetes at 28 days after the onset of hyperglycemia. Upregulation of genes involved in TLRs and their ligands (TLR1, TLR2, and TLR4), IL-1, IL-6, and IFNγ family and their signaling (IL-18, STAT1, STAT2, STAT3, and MAPK3), and eicosanoid receptors family (LTB4R1 and LTB4R2) were identified. Furthermore, several genes (7 out of the 17 genes) involved in the complement cascade pathway were downregulated. Last, there were no notable changes in genes involved in the C-type lectin receptors signaling, chemokines and their receptors, and the NF-κB pathway. Genes involved in IL-2, IL-5, IL-9, IL-12, IL-17, and IL-22 signaling cascades were not detected in the corneas.

Finally, heat maps of the transcriptome data also show that several genes involved in the apoptosis pathway and cellular stress response were upregulated in the corneas obtained from mice with type 1 as well as type 2 diabetes at 28 days after the onset of hyperglycemia (Fig. 9). Furthermore, we validated the transcriptome analysis data by running real-time PCR for some of the genes that showed the most notable increase, which was detected at 28 days time point in mice with type 1 diabetes mellitus. The real-time PCR data showed that in agreement with the transcriptome data, there was a 1.2 ± 0.4 fold increase in DAXX, 2.8 ± 0.5 fold increase in MAPKAPK2, 3.1 ± 1.7 fold increase in TLR1, 1.3 ± 0.18 fold increase in TLR2, 2.2 ± 1.1 fold increase in TLR4, 1.78 ± 0.6 fold increase in STAT1, 3.3 ± 1.9 fold increase in STAT2, 1.9 ± 0.5 increase in LTB1R, and 1.2 ± 0.4 fold increase in LTB2R gene expression in corneas of mice at 28 days after the onset of hyperglycemia in mice with type 1 diabetes compared to age-matched non-diabetic control mice. Thus, the real-time PCR data validates the gene expression changes detected by transcriptome analysis.

**DISCUSSION**

Poor glycemic control is a well-known cause of long-term complications of diabetes mellitus. The biochemical and metabolic changes due to chronic hyperglycemia leading to microvascular damage are the major mechanism responsible for diabetes-associated neuropathy, nephropathy, and retinopathy. The resultant changes in cytokine milieu and extracellular matrix deposition cause structural alterations and tissue damage leading to compromised function of these organs.3,5 Multiple studies using animal models have shown that diabetes causes injury to the tear film and lacrimal gland and this damage is apparent after prolonged hyperglycemia of 5 to 10 weeks duration.39,25 Interestingly, the results of the present study demonstrate that diabetes-associated hyperglycemia of shorter duration can cause detrimental damage to the ocular surface by initiating a rapid-onset decrease in the tear film and an early loss of goblet cells which further leads to a decrease in glycosylglyx and triggers an inflammatory and cellular stress response. Our data demonstrate that diabetes-associated hyperglycemia causes a prompt decrease in tear volume and a loss of goblet cells that is apparent as early as 7 days after the onset of hyperglycemia. Furthermore, after this initial decline in tear volume and goblet cell loss, no
additional deterioration is noted during the tested short-duration (28 days) hyperglycemia in this study. The tear film is sensitive to hemodynamic and osmotic changes\(^{30,32,34}\) and because hyperglycemia has been shown to cause acute hemodynamic and osmotic changes,\(^{1-4}\) our data raises an intriguing possibility that the noted rapid-onset decrease in tear volume may be due to hyperglycemia-mediated hemodynamic and osmotic changes. Chronic hyperglycemia-associated metabolic changes deteriorate with the progression of time. But no time-dependent decline in tear film volume or goblet cell loss was noted with short-term hyperglycemia in the present study, it further supports the contention that the noted effect of short-term hyperglycemia on the tear film and goblet cells may be due to non-metabolic effects, such as hemodynamic or osmotic changes. However, further studies are needed to directly prove this mechanism.

The effect of hyperglycemia on goblet cell loss was severity rather than duration-dependent because it was significantly greater in mice with type 1 diabetes compared to mice with type 2 diabetes, but did not deteriorate further within the tested duration of 28 days after the initial cell loss. Hyperglycemia-mediated goblet cell death alone or in combination with reduced tear volume could potentially account for this noted loss of goblet cells. Similar effects of hyperglycemia-mediated cell death have been reported in lactotrophs and photoreceptors in mice model of type 1 diabetes.\(^{40,41}\) Future studies are warranted to further investigate the mechanism of hyperglycemia-induced ocular surface goblet cell death. Further, our data also show that hyperglycemia caused a decrease in the corneal glycocalyx. The damage to glycocalyx was both severity and duration-dependent because the damage was significantly more in mice with type 1 diabetes compared to type 2 diabetes and it progressed in a time-dependent manner in both the models. The decrease in tear film and goblet cells preceded the damage to the glycocalyx, which may partially be responsible for glycocalyx injury given the vital role of tear film and goblet cells in ocular surface health. The reduction in epithelial cells either due to increased apoptosis or reduced proliferation could be another potential mechanism contributing to this noted decrease in the corneal glycocalyx because our laboratory and other laboratories have reported that high glucose can cause corneal epithelial cell apoptosis and decrease the expression of corneal epithelial cell cycle and proliferation proteins.\(^{42,43}\) Interestingly, an increase in the corneal gene expression of membrane-tethered Muc4 was noted in the mice with type 1 diabetes at day 14 after the onset of hyperglycemia. Muc4 is the most abundant corneal mucin in mice and is a major component of glycocalyx. The noted increase in Muc4 gene expression could potentially be a compensatory mechanism to partially circumvent the glycocalyx damage. Finally, our data also demonstrate that restoration of euglycemia by insulin in type 1 diabetes attenuates the determinantal effects of diabetes-associated hyperglycemia on the tear film, goblet cells, and glycocalyx. We used a novel insulin implant to restore euglycemia. These implants provide a sustained release of insulin, thus achieving steady plasma glucose levels compared to the twice-daily injection of long-acting insulin which could be stressful for the mice. Furthermore, we did not evaluate the effect of insulin in type 2 db/db mice because they are already hyperinsulinemic and have insulin resistance, thus do not respond to insulin treatment.

Healthy corneal epithelium and intact glycocalyx play an important barrier function to keep the cornea protected from environmental pathogens.\(^{44-47}\) Therefore, we further hypothesized that the noted decrease in tear volume, glycocalyx, and goblet cells due to this short-term hyperglycemia may predispose the cornea to environmental pathogens and allergens triggering an inflammatory response. This hypothesis is supported by the results of our transcriptome analysis, which demonstrate the upregulation of several genes involved in inflammatory pathways in the corneas of mice with type 1 and type 2 diabetes. Furthermore, the noted upregulation in the pro-inflammatory genes was observed only 28 days after the onset of hyperglycemia. Because hyperglycemia-mediated damage to the tear film, goblet cells, and glycocalyx preceded the upregulation of inflammatory genes, it further supports our hypothesis that these detrimental events are the likely triggers to initiate the subsequent inflammatory cascade. The upregulated genes included toll-like receptors, leukotriene receptors, and pro-inflammatory cytokines signaling. The upregulation of pro-inflammatory genes was also accompanied by a concomitant increase in the upregulation of genes involved in apoptosis and cellular stress.

In conclusion, the results of the present study demonstrate that diabetes-associated hyperglycemia causes rapid-onset damage to the ocular surface including a decrease in the tear film, loss of goblet cells, and decrease in corneal glycocalyx. This damage may in turn trigger an inflammatory and cellular stress cascade in the cornea. Therefore, besides long-term glycemic control, management of short-duration hyperglycemia may also be important for preventing ocular surface injury and dry eye in patients with diabetes mellitus.

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