Original Article

Severe hypoglycaemia-induced microglial inflammation damages microvascular endothelial cells, leading to retinal destruction

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Abstract

Human microglia (HMC) are stress-induced inflammatory cells of the retina. It is unknown whether severe hypoglycaemia causes inflammation in microglia, affects the permeability of human retinal microvascular endothelial cells (HRMECs), and causes retinal damage. This study aimed to explore the effects of severe hypoglycaemia on retinal microglial inflammation and endothelial cell permeability and evaluate the damage caused by hypoglycaemia to the retina. The CCK-8 assay was used to measure cell viability. Western blotting was used to detect IL-1β, IL-6, TNF- α, claudin-1, and occludin expression. ELISA was used to detect IL-1β, IL-6, and TNF- α. Transmission electron microscopy (TEM) and haematoxylin and eosin staining were used to observe the retinal structure. Immunohistochemistry and immunofluorescence staining assays were also used to detect IL-1β, IL-6, TNF- α, claudin-1, and occludin expression. Severe hypoglycaemia promoted inflammation in HMC3 cells. Inflammation caused by hypoglycaemia leads to the decreased expression of tight junction proteins. In vivo, severe hypoglycaemia induced structural damage to the retina, increased the expression of inflammatory factors, and decreased the expression of tight junction proteins. Our results suggest that severe hypoglycaemia leads to acute retinal inflammation, affecting the permeability of HRMECs and causing retinal damage.

Keywords

Severe hypoglycaemia, human microglia, human retinal microvascular endothelial cells, inflammation, retinal damage

Introduction

Maintaining normal blood glucose levels during diabetes treatment while avoiding hypoglycaemia is a major challenge for patients and physicians.[1] The effective management of diabetes can delay or prevent micro-and macrovascular complications. However, a single case of severe hypoglycaemia due to intensive glycaemic control may negate the benefits of maintaining blood glucose within the normal range throughout life.[2] The exact definition of hypoglycaemia is still being debated. The ADA Working Group has provided a comprehensive definition, defining "severe hypoglycaemia" as "a hypoglycaemic response that requires assistance with treatment."[3]

Hypoglycaemia may damage large and micro-vessels more severely than hyperglycaemia. The ACCORD and VADT trials showed that cardiovascular mortality increased in patients with diabetes, intensive glycaemic control increased the incidence of severe hypoglycaemia three-fold, and

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patients with diabetes with hypoglycaemia showed an increased risk of adverse cardiovascular events and mortality.[4,5] Several studies have shown that severe hypoglycaemia accelerates cognitive dysfunction and increases the risk of dementia in patients with diabetes and that severe hypoglycaemia is associated with increased mortality in people with dementia.[6] Current research on hypoglycaemic microvascular complications in patients with T2DM is limited. Patients with severe hypoglycaemia are at increased risk of microvascular complications and an increased risk of DR.[7]

Increased levels of inflammatory cytokines and leucocytosis after hypoglycaemia suggest a link between hypoglycaemia and inflammation, but the underlying mechanisms remain unclear.[8,9] Cytokines are key factors in the inflammatory response, and acute hypoglycaemia can elevate the expression of inflammatory factors such as TNF-α, IL-1β, IL-8, and IL-6.[10] Studies in Edinburgh have shown that severe hypoglycaemia in patients with T2DM increases the expression of plasma inflammatory markers IL-6 and TNF-α.[6] Schram et al. reported an independent and strong correlation between IL-6 and TNF-α levels and retinopathy in patients with T2DM and T1DM.[11]

As the main components of the iBRB, cells and intercellular junction proteins play a "gating" role in maintaining microvascular permeability and are important structures for maintaining homeostasis in the retinal microenvironment.[12] Occludin was the first protein identified in the TJs, and its high expression in iBRB ECs is associated with low EC permeability.[12] Claudins are the basic structure of EC TJs. Changes in claudin-1 expression are associated with increased vascular permeability.[12] The role of chronic inflammation in the pathogenesis of DR is well-established.[13] Growing evidence supports a strong relationship between the microglia and retinal inflammatory responses.[14,15] Microglia are the innate immune cells of the retina. Their overactivation promotes the formation of an inflammatory microenvironment in the retina and contributes to retinal EC damage.[16]

Although the effect of hypoglycaemia on DR has been confirmed, there is no evidence showing the direct effect of hypoglycaemia on retinal microglial inflammation or the resulting inflammation of ECs. Therefore, this study aimed to observe the effect of hypoglycaemia on microglial inflammation, the effects of hypoglycaemia-induced inflammation on endothelial cell permeability, and the effects of hypoglycaemia on the retinal structure and the expression of inflammatory factors and connexins in diabetic rats.

Methods

Cell culture and treatment

HMC3 human microglia were purchased from ProCell (Wuhan, China). HRMECs were purchased from the Bena Culture Collection (Beijing, China). All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. Cells in the logarithmic growth phase were used for the experiments. HMC3 cells were cultured with glucose at a concentration of 25 mmol/L. Subsequently, cells grown in 25 mmol/L glucose were used as the control group, while cells cultured in 1 or 2.5 mmol/L glucose were used as the hypoglycaemic group cells. Co-cultures of HRMECs and HMC3 cells with different concentrations of glucose (1 and 25 mmol/L) were also prepared.

CCK8 assay

A CCK-8 detection kit (Dojingdo Laboratories, CK04) was used to evaluate cell viability. Briefly, HMC3 cells were cultured with glucose (25 mmol/L) and then treated with different concentrations of glucose (1, 2.5, or 25 mmol/L). The cells were also treated with 1 mmol/L glucose for different durations (0, 3, 6, 12, or 24 h). Absorbance was measured at 450 nm using a monochromator microplate reader.

Western blotting

The membranes were incubated at 4°C overnight with rabbit polyclonal primary antibodies against TNF-α, IL-1β, IL-6, occludin, and claudin-1 (Wanleibio, China). GAPDH, actin, and β-tubulin were used as control and their expressions were detected using the corresponding rabbit polyclonal primary antibodies (Wanleibio). The membranes were then incubated with the corresponding fluorescent secondary antibodies (IRDye800 CW goat anti-rabbit, 926-32211 or IRDye680RD goat anti-mouse, 926-68070, 1:10000, LI-COR) at room temperature in the dark for 1 h. The protein bands were scanned using an Odyssey Infrared Laser Scan-imaging Instrument (LI-COR). ImageJ software was used for image analysis. All experiments were repeated thrice.

ELISA

The levels of TNF-α, IL-1β, and IL-6 were determined using ELISA kits (Elabscience Biotechnology, China) following the manufacturer's instructions. All experiments were repeated thrice.

Animal studies

Fifteen 6-week-old clean-grade male Sprague–Dawley rats were purchased from the Heilongjiang University of Traditional Chinese Medicine. After 7 days of adaptive feeding, the rats were randomly divided into the normal control group and the diabetes group. The normal control group (control) was fed regular feed, while the diabetes

group was fed a high-fat diet. After 4 weeks of feeding and then fasting for 12 h, the diabetes group was administered an intraperitoneal injection of streptozotocin (25 mg/kg) for three consecutive days, and the normal control group was injected with the same dose of normal saline. The criteria for successful modelling were fasting blood glucose ≥7.8 mmol/L or random blood glucose ≥16.7 mmol/L.

Animal model of hypoglycaemia. After successfully establishing a rat model of diabetes, rats in the diabetes group were randomized and grouped into the diabetes mellitus (DM group, $n = 5$) and diabetes mellitus hypoglycaemic groups (DMH group, $n = 5$). All rats were fasted for 12 h on the day before sampling and were not restricted regarding drinking water consumption. To measure blood glucose levels, blood was collected from the tail vein of the rats, and an intraperitoneal injection of rapid-acting insulin (8 U/kg; Novolin R, Novo Nordisk Pharmaceutical Co., Ltd) was used to induce hypoglycaemia in the DMH group. Equal doses of normal saline were administered to the DM and control groups. Blood was collected from the tail vein every 15 min, and blood glucose levels were monitored using a blood glucose meter. When the blood glucose level reached ≤1.5 mmol/L, a timer was started and maintained for 90 min; when the blood sugar tended to rise, insulin was administered in a timely manner.

TEM

The eyeballs of the rats were immediately separated and fixed in an ocular fixation solution. The left eye was used for electron microscopy and the right eye was used for immunohistochemistry and immunofluorescence assays. Cells and tissues were fixed with 1% OsO_4 and washed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer. The cells and tissues were dehydrated with graded alcohol and embedded in epoxy resin. Ultrathin sections were then prepared and stained with uranyl acetate and lead citrate, placed on copper grids, and imaged using a transmission electron microscope (JEOL, Tokyo, Japan).

H&E staining

The retinal tissue sections were embedded in paraffin, stained with H&E, dehydrated with ethanol and xylene, and sealed in neutral glue. Images were captured using a Nikon DS-F12 microscope.

IHC staining

Tissue sections were incubated with primary antibodies against TNF-α, IL-1β, IL-6 (Wanleibio) overnight at 4°C. The sections were then incubated with the corresponding secondary antibodies at room temperature for 1 h, followed by incubation with Vectastain Elite ABC reagent (Vector Laboratories) for 30 min. After a peroxidase reaction with diaminobenzidine (DAB Kit; Solarbio), the tissue sections were counterstained with haematoxylin (Solarbio). The mean optical density was analysed using Nikon DS-F12 software.

IF staining

The sections were incubated overnight with the prepared primary antibodies against occludin (Wanleibio) and Claudin-1 (Wanleibio) in a 4°C freezer. The residual primary antibodies were rinsed with PBS, and the prepared fluorescent secondary antibodies were added dropwise to the sections, incubating for 60 min at room temperature. After rinsing with PBS, DAPI (Aladdin, China) was added dropwise to the sections to counterstain the nuclei. Finally, an anti-fluorescence quenching agent was added dropwise to the sections (Solarbio) and observed using an OLYMPUS microscope.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM software. Data were presented as the means ± standard deviations. Unpaired t-tests were used to compare differences between two groups. Differences were considered statistically significant at $p < .05$.

Results

Hypoglycaemia reduced the viability and promoted inflammation in HMC3 cells cultured in a high-glucose environment

HMC3 cells were treated with different concentrations of glucose (1 or 2.5 mmol/L) for 6 h, and microglial viability was detected using the CCK-8 method. The viability of microglia gradually decreased as the glucose concentration decreased compared to that in the normal control group (25 mmol/L). As blood glucose concentrations decreased, microglial viability also decreased (Figure 1(A)). Next, the cells were divided into a normal control group (25 mmol/L) and a low-sugar group (1 mmol/L) and incubated for 0, 3, 6, 12, or 24 h to detect the optimal intervention time under low-sugar conditions. Microglial viability decreased progressively over time but decreased significantly after culture for 12 h (Figure 1(B)). Based on these results, we selected a concentration of 1 mmol/L and treatment for 6 h in subsequent experiments. Next, the cells were divided into the normal control (25 mmol/L) and hypoglycaemic

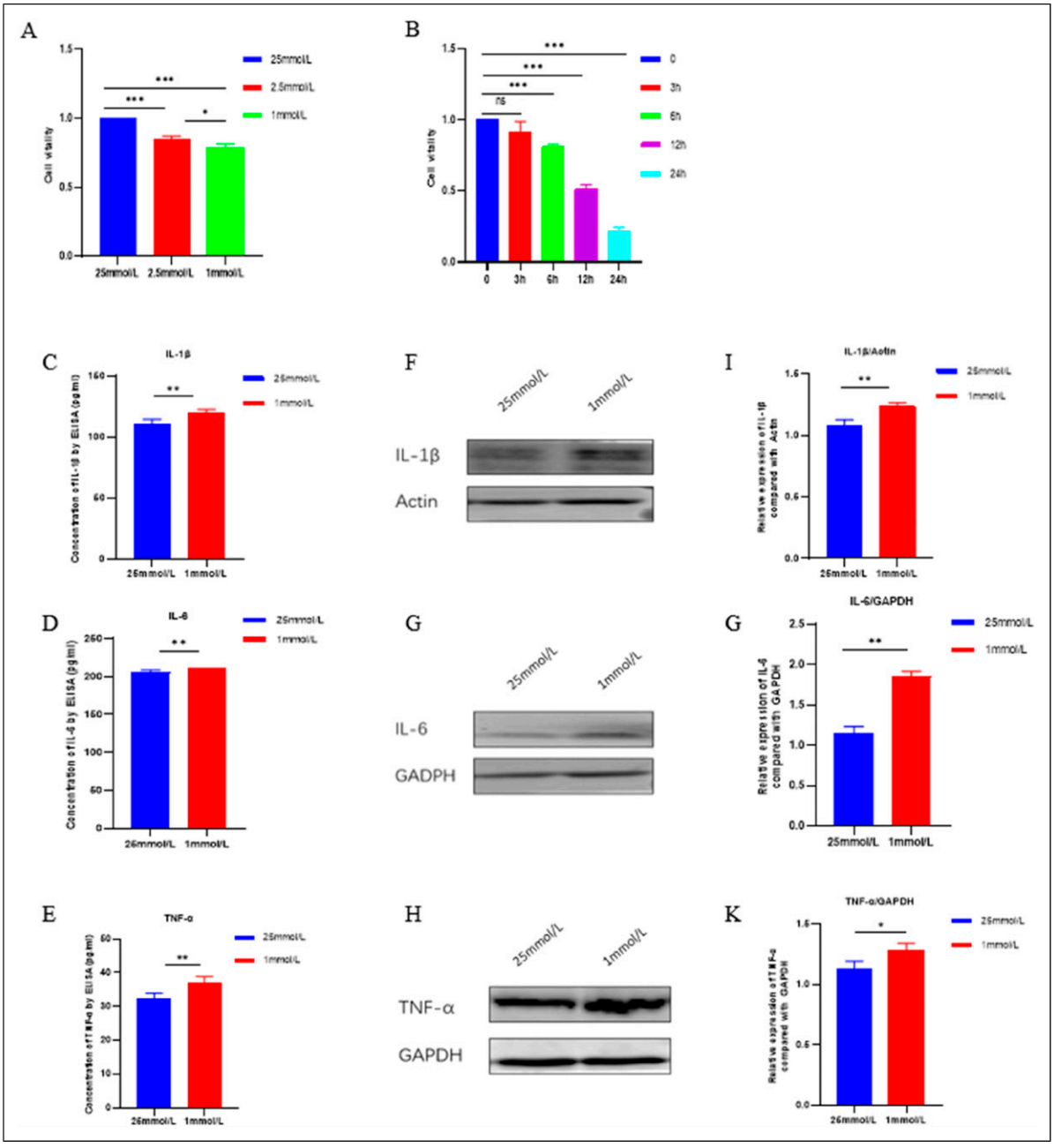

Figure 1. Variation of cell vitality in HMC3. (A) Effect of glucose concentration on HMC3viability for 6 h. (B) Effect of hypoglycaemic time on HMC3 viability at 1 mmol/L. The expression of inflammatory cytokines. (C–E) The expression levels of IL-1β, IL-6 and TNF-α in cell supernatant were determined by ELISA. (F–K) The intracellular expression levels of IL-1β, IL-6 and TNF-α were determined by WB. *$p < .05$, **$p < .01$, ***$p < .001$.

intervention (1 mmol/L) groups. The levels of inflammatory cytokines (TNF-α, IL-6, and IL-1β) in the cell supernatants were determined using ELISA. The results showed that compared with the normal control group, the levels of TNF-α, IL-6, and IL-1β in the cell supernatant of the hypoglycaemia group significantly increased (Figure 1(C)–(E)). The protein expression levels of intracellular inflammatory factors were also detected using western blotting (WB). Compared with the normal control group, the protein expression levels of intracellular inflammatory factors were significantly increased in the hypoglycaemia group (Figure 1(F)–(K)).

Hypoglycaemia-induced inflammation decreased the expression of endothelial permeability proteins

HMC3 cells and HRMECs were co-incubated to observe the effects of hypoglycaemia-induced inflammation on the permeability of HRMECs. The HRMECs were divided into a blank control group (NC, without any intervention), a control group (co-incubation with HMC3 cells treated with 25 mmol/L glucose), and a hypoglycaemia intervention group (co-incubation with HMC3 cells treated with 1 mmol/L glucose). The expression of connexin in the cell membrane of HRMECs was then observed using immunofluorescence staining. The results showed that, compared with the control and blank control groups, the expression of claudin-1 (Figure 2(A)) and occludin (Figure 2(B)) on HRMEC cell membranes decreased in the hypoglycaemia group. Meanwhile, compared with the hypoglycaemic intervention group and the control group, the expression of claudin-1 and occludin on the cell membrane decreased significantly in the blank control group. Intracellular connexin expression levels were then measured using WB. Compared with the blank control group, the intracellular claudin-1 and occludin expression levels in the control group decreased, while their expression levels in the hypoglycaemic intervention group decreased significantly compared with those in the control group (Figure 2(C) and (D)).

Changes in the body weight and blood glucose levels in rats

The results showed no difference in the initial body weight of rats among all groups before model establishment. Compared to the normal control group, the DM and pre-intervention groups had increased body weights (Figure 3(A)). Fasting blood glucose levels were also measured before and after the intervention. The results showed no differences in fasting blood glucose levels among the groups before modelling. However, after modelling, the fasting blood glucose levels of rats in the DM and pre-intervention groups were significantly increased compared to the normal group. After insulin administration, the fasting blood glucose levels of rats in the intervention group became significantly lower than that in the DM group, while the fasting blood glucose of rats in the intervention group also became significantly lower than that in the normal group (Figure 3(B)).

Hypoglycaemia caused the destruction of retinal structures in diabetic rats

H&E staining was used to assess the structural changes in the retina (Figure 4(A)). The retinal layers of rats in the normal control group were closely arranged and organized,

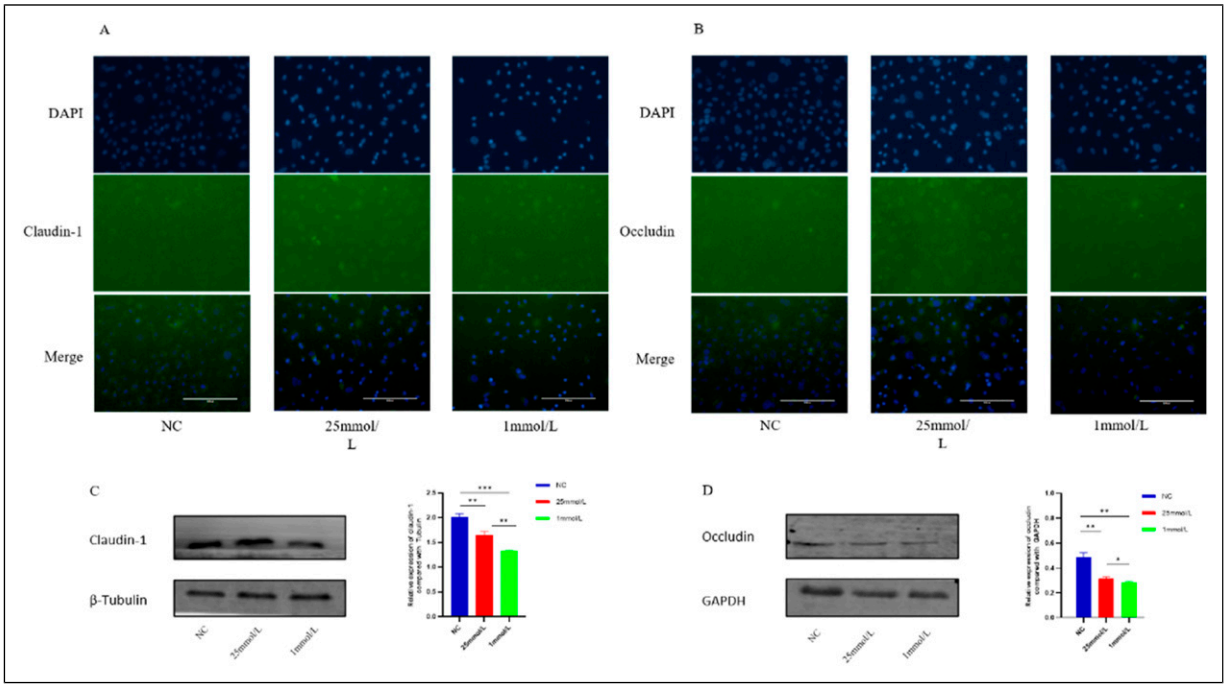

Figure 2. The expression of conjunctive protein. (A) The expression levels of claudin-1 were determined by IF. (B) The expression levels of occludin were determined by IF. (C–D) The expression levels of claudin-1 and occludin were determined by WB. *$p < .05$, **$p < .01$, ***$p < .001$.

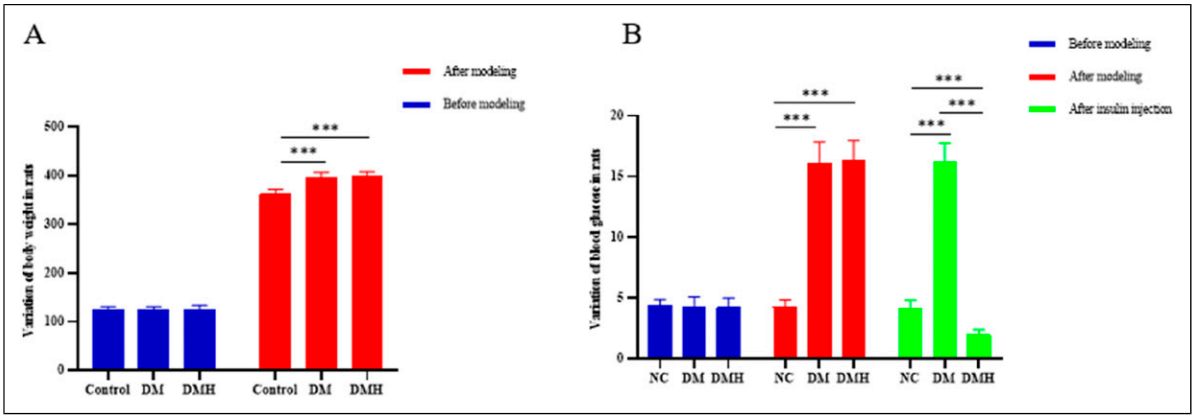

Figure 3. Variation of weight and blood glucose in rats. (A) Variation of weight in rats. (B) Variation of blood glucose in rats. *$p < .05$, **$p < .01$, ***$p < .001$.

and the structure was clearly hierarchical. The cells of various layers of the retina of the diabetic group were mildly disordered and irregular. In diabetic rats with persistent severe hypoglycaemia, the cell arrangement of all retina layers was obvious, and there was evident oedema and thickening of the NFL. In the GCL, the cells of the inner core layer were disorganized, and vacuole-like changes were obvious.

Electron microscopy was used to visualize the tiny cellular structures of the retina. Compared with the normal control group, the retinal cell arrangement and microstructure of the rats in the diabetes group were relatively disordered, while the retinas of the rats in the hypoglycaemia intervention group had more serious structural damage compared with the normal group (Figure 4(B)–(D)). In addition, compared to the normal control group, the retinas of the diabetic group were slightly thickened, and the ECs were slightly swollen. Moreover, compared to the normal control group, the retinal microvascular ECs of the rats in the hypoglycaemic intervention group were significantly swollen and mitochondrial damage was serious (Figure 4(E)–(G)).

Severe persistent hypoglycaemia led to an increase in the expression of inflammatory factors and a decrease in the expression of tight junction proteins in the retinas of diabetic rats

IHC was used to measure the expression of IL-1β, IL-6, and TNF-α in the rat retinal tissues. The results showed that the expression of IL-1β, IL-6, and TNF-α in the normal control group was mainly concentrated in the NFL and GCL. IL-1β, IL-6, and TNF-α were more highly expressed in the rat retina in the DM group compared with the normal control group. Compared with the normal control group, IL-1β, IL-6, and TNF-α expression in the retina of rats in the hypoglycaemic intervention group were significantly increased. In particular, IL-1β, IL-6, and TNF-α were more highly expressed in the retinal NFL and GCL in the hypoglycaemic intervention group compared to the diabetes group (Figure 5(A)–(C)).

An IF assay was also performed to detect the expression of connexins claudin-1 and occludin in the rat retinal tissues. The results showed that the expression of claudin-1 and occludin was reduced in diabetic rats compared to those in the normal control group. Similarly, compared to the normal control group, the expression of these proteins in the retina of rats in the hypoglycaemic intervention group was also reduced. Compared to rats in the DMH and rats in DM, the expression of claudin-1 and occludin was reduced in DMH (Figure 5(D) and (E)).

Discussion

Rapid and tight glycaemic control can worsen microvascular complications, particularly retinopathy. Studies have shown that the mechanism of hypoglycaemia on microvascular complications is attributed to ischemia due to decreased capillary blood flow. However, other studies have shown that hypoglycaemia-induced oxidative stress, inflammation, and endothelial cell dysfunction are also involved. Hypoglycaemia may exacerbate retinal neurodegeneration and microangiopathy through its presumptive effect on the local vasculature. Microglia activation in diabetic neuropathy may mediate diabetes-related complications.[17,18] However, whether microglia, as inflammatory cells, are involved in the damage to the diabetic retina during hypoglycaemia has not been studied. Therefore, in this study, we observed the expression of various inflammatory cytokines that stimulate the release of microglia, which further leads to retinal vascular damage, to provide new insights into the pathogenesis of hypoglycaemia in DR.

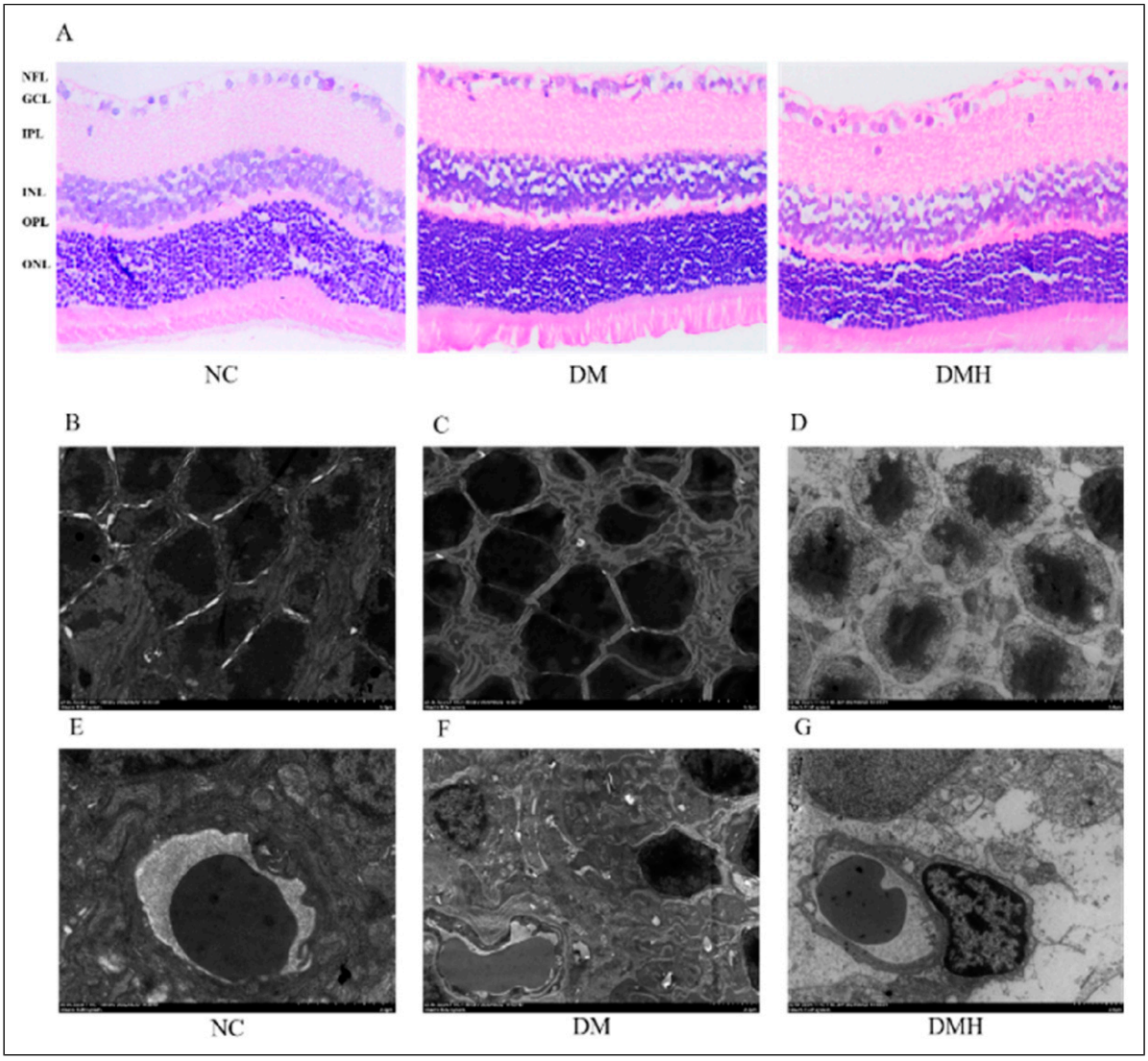

Figure 4. Destruction of retinal structures in diabetic rats. (A) H&E staining of retina. (B–G) TEM was used to visualize the cellular microstructures of the rat retina. (B–D) Cellular tiny structures. (E–G) Retinal microvascular and endothelial cells structures. NFL: nerve fibre layer; GCL: Ganglion cell layer; IPL: inner plexiform layer; INL: Inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear.

According to the 2022 ADA standards, hypoglycaemia is divided into three levels. Grade 1 is characterized by 3.0 mmol/L ≤ blood glucose <3.9 mmol/L, grade 2 is characterized by blood glucose <3.0 mmol/L, and grade 3 has no specific blood glucose value but is accompanied by changes in consciousness and/or physical conditions and requires the assistance of others to correct severe hypoglycaemia. Other studies have also reported that there is no exact blood glucose value to define severe hypoglycaemia, equivalent to grade 3 hypoglycaemia.[3] When the blood glucose falls within the range of 1.5–2.8 mmol/L, the body will begin to experience cognitive impairment, local neurological impairment, and neuronal necrosis or apoptosis. At blood glucose levels <1.5 mmol/L, the body is often accompanied by irreversible neuronal necrosis, consciousness impairment, convulsions, coma, and other serious consequences.[19,20] Therefore, in the in vivo experiments, we ensured that the blood glucose levels of rats in the hypoglycaemic intervention group were maintained below 1.5 mmol/L, even lower. Piarulli et al. used 2.5 mmol/L as the low-glucose condition to treat human monocytes.[21] Yin et al. incubated human tumour cells in 1 mmol/L glucose to simulate low-glucose conditions.[22] Ma et al. used 2.5 mmol/L the low-glucose condition to study the potential mechanism of apoptosis in human ovarian cancer cells.[23] Therefore, based on the above research methods, two glucose concentrations (1 and 2.5 mmol/L) were selected for our experiments. We then

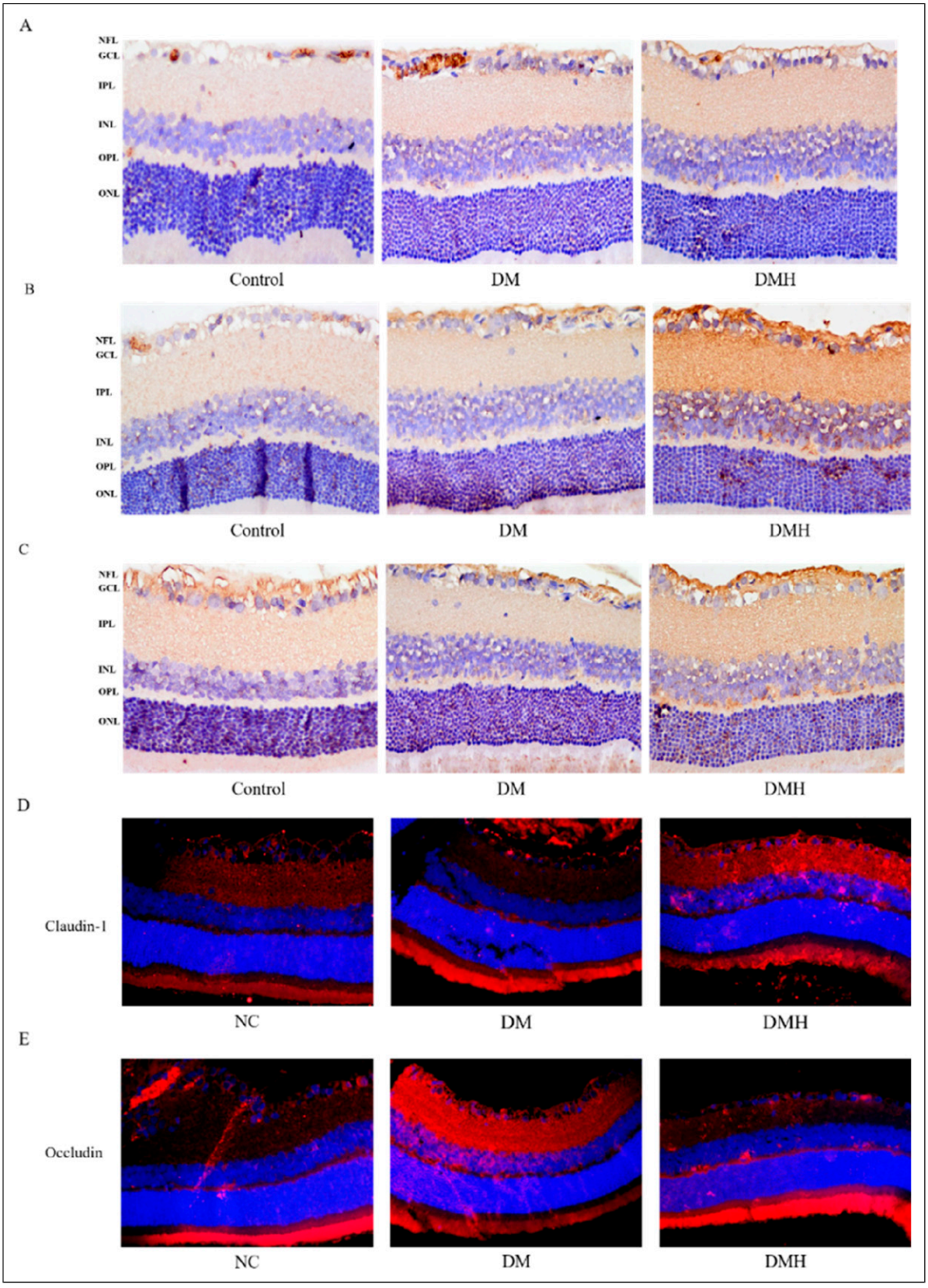

Figure 5. (A–C) The expression of inflammatory factors in rat retinal tissue by IHC. (A) The expression of IL-1β in rat retinal tissue by IHC. (B) The expression of IL-6 in rat retinal tissue by IHC. (C) The expression of TNF-α in rat retinal tissue by IHC cellular tiny structures. (D–E) The expression of connexins in rat retinal tissue by IF. (D) The expression of claudin-1 in rat retinal tissue by IF. (E) The expression of occludin in rat retinal tissue by IF.

further determined the optimal time for hypoglycaemia intervention. Microglia were cultured for 0, 3, 6, 12, and 24 h under hypoglycaemic (1 mmmol/L) conditions, and CCK-8 was used to determined cell viability. Prolonged hypoglycaemic intervention may have unpredictable effects on microglia cultured in vitro. Finally, we concluded that treating HMC3 microglia with 1 mmol/L glucose for 6 h effectively induced low-glucose conditions.

Overactivated microglia secrete a large number of pro-inflammatory mediators that cause chronic inflammation, leading to diabetic blood-retinal barrier damage and pathological deterioration in DR.[24] The results of our in vitro experiments showed that low-sugar conditions significantly induced the excessive activation of microglia cultured in high-sugar concentrations, promoting the expression and release of inflammatory factors TNF-α, IL-6, and IL-1β. This suggests that acute hypoglycaemia is a pro-inflammatory stimulus that brings microglia to a pre-activated or semi-activated state. The results of our experiments are also consistent with previous studies that reported low-glucose conditions promoted the expression of the pro-inflammatory cytokine IL-1β in monocytes[21] and that low glucose levels can induce an increase in pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6.[25] Our in vivo experimental results showed that the expression of inflammatory factors TNF-α, IL-6, and IL-1β in the retinal tissue of rats with severe hypoglycaemia was enhanced. These results further support the idea that hypoglycaemia can exacerbate inflammatory damage to the retina in patients with diabetes. Hypoglycaemia is not a short-lived pro-inflammatory stimulus; even if blood sugar levels return to normal, the effects of hypoglycaemia on the body can last for a long time. Hypoglycaemia has been shown to persist in the hematologic and inflammatory responses of patients with T2DM for several days.[26] Moreover, Kahal et al. showed that the induction of hypoglycaemia significantly increased the expression of inflammatory markers and markers of oxidative stress during hypoglycaemia, and that subjects with T2DM have increased inflammatory and oxidative stress 24 h after the onset of hypoglycaemia.[27] However, there are many unknown results regarding the long-term effects of acute or repeated hypoglycaemia on the body that need to be further studied.

As the first cells to sense changes in blood glucose, HRMECs are damaged early during blood glucose changes. Inflammation plays an important role in the development of diabetes. Therefore, we conducted in vitro and in vivo experiments to determine whether the inflammatory response induced by hypoglycaemia can damage the retinal microvascular endothelium, resulting in changes in microvascular permeability. The in vitro results showed a decrease in connexin expression in HRMECs after co-incubation with HMC3 cells. The in vivo results confirmed the results of the in vitro experiments. Recent studies have shown that TNF-α can induce leukocyte adhesion to retinal ECs and increase the permeability of the retinal vascular barrier.[28] Moreover, Valle et al. showed that IL-6 directly affects ECs, disrupting the human retinal microvascular barrier.[29] McVicar et al. observed microglial activation and a significant increase in the expression of the cytokines IL-6 and TNF-α in STZ-induced diabetic rat retinas, suggesting pro-inflammatory and neurotoxic effects.[30] Therefore, we believe that the chronic low-grade inflammatory response caused by hyperglycaemia damages ECs. In contrast, the acute inflammatory response caused by severe hypoglycaemia can cause damage to ECs within a short period of time, and is even greater than that caused by hyperglycaemia.

This study has some limitations. First, in our in vivo experiments, the control group was fed a regular diet, while the diabetic rats were fed a high-fat diet, consistent with the methods of previous studies. Weight gain caused by a high-fat diet may lead to insulin resistance and abnormal blood glucose levels, while feeding a regular diet ensured that the control group was unaffected by other factors. In addition, we built rat models of DM and DMH at the early stage of diabetes so that the weight of the rats in these groups could still increase. With the prolongation of diabetes, most rats in the diabetes group lost weight. Secondly, incubating cells with 1 mmol/L of glucose for 6 h to simulate hypoglycaemia is unlikely to be replicated in the real world and is, therefore, not clinically significant; it was an extreme hypoglycaemic challenge used to probe a cellular mechanism in vitro. However, in the in vivo experiments, we simulated severe hypoglycaemia that may occur in clinical practice by maintaining blood glucose levels below 1.5 mmol/L for 90 min. Third, the subjects of this study were human cells in vitro and rats in vivo, so the effects of their specific differences must also be acknowledged.

In summary, this study was the first to propose that severe hypoglycaemia can induce diabetic retinal microglia to release various inflammatory factors, which is an acute process. A mechanism underlying the acute inflammatory response caused by hypoglycaemia was proposed, which was shown to aggravate the damage to the retina by changing the permeability of ECs. Our results provide a theoretical basis for the body damage caused by hypoglycaemia in patients with clinical diabetes.

Conclusions

Severe hypoglycaemia leads to an acute inflammatory response in HMC3 cells and the retina of diabetic rats, which alters the permeability of HRMECs, further aggravating the damage caused by retinopathy.

Author contributions

YXH, ZL, and HXL conceived and designed the experiments. YXH, ZL, HXL, QX, XFM, WJL and CYX performed the experiments. YXH, MH and YHK analysed the data. YXH wrote the first draft. All authors contributed to the article and approved the submitted version.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical statement

Ethical approval

The research protocol was reviewed and approved by the Ethical Committee and Institutional Review Board of First Affiliated Hospital of Harbin Medical University.

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Data availability statement

All data generated or analysed during this study are included in this article. Further inquiries can be directed to the corresponding authors.

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Appendix

Abbreviations

ADA	American Diabetes Association
DM	Diabetes mellitus
DMH	Diabetes mellitus with hypoglycaemia
DR	Diabetic retinopathy
ECs	Endothelial cells
GCL	Ganglion cell layer
H&E	Haematoxylin and eosin
HRMECs	Human retinal microvascular endothelial cells
iBRB	Internal blood-retinal barrier
IL	Interleukin
NFL	Nerve fibre layer
TEM	Transmission electron microscopy
TJs	Tight junctions
TNF	Tumour necrosis factor
WB	Western blotting