Retinal Mitochondrial DNA Mismatch Repair in the Development of Diabetic Retinopathy, and Its Continued Progression After Termination of Hyperglycemia

Manish Mishra and Renu A. Kowluru

Kresge Eye Institute, Wayne State University, Detroit, Michigan, United States

Correspondence: Renu A. Kowluru, Kresge Eye Institute, Wayne State University, 4717 St. Antoine, Detroit, MI 48201, USA; rkowluru@med.wayne.edu.

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Purpose. Mitochondrial DNA (mtDNA) is damaged in the retina in diabetes, and mitochondria copy numbers are decreased. The displacement-loop (D-loop) of the mtDNA, the region with transcription/replication elements, experiences more damage than other regions of mtDNA. Our aim was to examine the role of DNA mismatch repair (MMR) in mitochondria homeostasis in diabetic retinopathy, and in its continued progression after cessation of hyperglycemia.

Methods. Effect of hyperglycemia on sequence variants in the D-loop region was investigated in retinal endothelial cells and in the retina from streptozotocin-induced diabetic rats using mismatch-specific surveyor nuclease. The role of MMR machinery in mtDNA damage and mitochondrial respiration was investigated in retinal endothelial cells overexpressing *Mlh1*, an MMR enzyme mainly associated with mtDNA polymerase gamma, or *Msh2* (associated with nuclear polymerase beta).

Results. Hyperglycemia increased sequence variants in the D-loop region. While overexpression of *Mlh1* in endothelial cells ameliorated glucose-induced increase in D-loop sequence variants, decrease in respiration rate and increase in apoptosis, overexpression of *Msh2* did not protect the mitochondria damage. Termination of hyperglycemia failed to reverse decrease in MMR enzymes and increase in *D-loop* sequence variants.

Conclusions. Due to a compromised MMR system, the sequence variants in the D-loop region were not repaired, and that resulted in impaired mtDNA transcription. Mitochondria become dysfunctional, and they continued to be dysfunctional even after hyperglycemia was terminated, contributing to the development, and progression of diabetic retinopathy. Thus, strategies targeting mitochondrial MMR machinery could help maintain mitochondria homeostasis, and inhibit the development of diabetic retinopathy and its continued progression.

Keywords: diabetic retinopathy, DNA repair, mitochondria damage, mtDNA mismatch, metabolic memory

Diabetic retinopathy remains a complex disease with multiple interrelated pathways contributing to its pathogenesis. Retinal mitochondria are dysfunctional, superoxide levels are elevated and the mitochondrial enzyme responsible for scavenging superoxide radicals in mitochondria is compromised.[1,2] Superoxide radicals are considered to act as causal links between elevated glucose and the major abnormalities associated with the vascular complications of diabetes, including retinopathy.[3]

Mammalian mitochondria have a small circular (mitochondria) DNA (mtDNA), and mtDNA is prone to oxidative damage as it is packed as nucleoid-like structures without any histones, and is in close proximity to the reactive oxygen species (ROS)-generating electron transport chain.[4] We have shown that in diabetes, retinal mtDNA is damaged and its biogenesis is impaired, and the copy numbers are decreased.[5,6] Mitochondria DNA has a large, noncoding sequence, the displacement-loop (D-loop), which contains essential transcription and replication elements,[7] and our studies have shown that this region experiences more damage than other regions of the mtDNA.[8]

Mitochondria are also equipped with efficient repair machinery to repair damages in their DNA. In diabetes, although the gene transcripts of the base excision repair (BER) system[9] are increased in the retina, these enzymes fail to reach to the mitochondria to repair the damaged mtDNA.[5,10] In addition to the BER system, mitochondria also possess another repair mechanism, the mismatch repair (MMR) system. This MMR system repairs uncomplimentary base pairs incorporated into the DNA sequences and the insertion and/or deletion loops that are formed during DNA replication.[11] The system has a number of proteins that assist in detecting the mismatch and directing repair machinery to it (e.g., Msh2 of MutS family recognizes mismatches and Mlh1 of MutL family cuts the mismatch).[11,12] Recent studies have shown that, while Msh2 is largely associated with nuclear DNA polymerase beta, Mlh1 is associated with mtDNA polymerase gamma (POLG).[13] We have shown that POLG, the rate-limiting enzyme for mtDNA replication, is subnormal in the retina and its capillary cells in diabetes, and the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) are increased in the mitochondria.[5,14] How diabetes affects the mitochondrial MMR system in the retina is unclear.

Hyperglycemia has long lasting detrimental consequences on diabetic retinopathy, and retinopathy continues to progress for a considerable period even after hyperglycemia is corrected, suggesting a metabolic memory phenomenon.[15–20] We have shown that in the retina of diabetic rats, the mitochondria continue to be dysfunctional with D-loop region damaged and POLG subnormal. In addition, the BER system and mtDNA transcription remain subnormal even after normal glycemia is reinstituted.[14,21] However, the role of MMR system in the continued progression of diabetic retinopathy remains elusive.

The objective of this study was to examine the role of MMR system in the mitochondria homeostasis in the development of diabetic retinopathy and its resistance to arrest after removal of hyperglycemic insult. Using retinal endothelial cells, the site of histopathology of diabetic retinopathy, and the rat model of diabetic retinopathy, we have investigated the effect of hyperglycemia on mitochondrial MMR system. The specific role of Mlh1 and Msh2 in the regulation of mitochondria homeostasis was investigated by using retinal endothelial cells in which *Mlh1* or *Msh2* was overexpressed.

Methods

Retinal Endothelial Cells

Bovine retinal endothelial cells from the fifth to eighth passage were incubated in Dulbecco's modified Eagle's medium containing 2% fetal calf serum (heat inactivated), 10% replacement serum (Nu-serum; Bioscience, San Jose, CA, USA), heparin (50 μg/mL), endothelial growth supplement (10 μg/mL), and antibiotic/antimycotic in an environment of 95% O_2 and 5% CO_2 in the presence or absence of 20 mM glucose. For reversal of hyperglycemia experiments, the cells were incubated in 20 mM glucose for 4 days, followed by 5 mM glucose for 4 additional days (20-5 group). The cells incubated in continuous 5 mM glucose or 20 mM glucose media served as controls. For osmotic control, the cells were incubated in 20 mM mannitol instead of 20 mM glucose.[6,14,22] To investigate the specific role of mismatch in mtDNA damage, the cells were transfected with 5 μg of *Mlh1* or *Msh2* expression plasmid (OriGene, Rockville, MD, USA). After transfection, the cells were incubated in 5 or 20 mM glucose for 4 days.[8,23] Parallel incubations were carried out using the transfection reagent alone.

Rats

Male Wistar (Harlan Labs, South Easton, MA, USA) rats were made diabetic by streptozotocin injections, and soon after the induction of diabetes they were divided into three groups. Rats in group 1 were maintained in poor glycemic control (PC, glycated hemoglobin GHb ~12%) for 6 months. Group 2 had the rats that were maintained in poor glycemic control (PC group) for 3 months followed by good glycemic control (GHb ~6%) for another 3 months (Rev group), and group 3 rats remained in good glycemic control for the entire 6 months (GC group). To maintain good glycemic control, insulin was administered two times a day (total of 5–7 IU/d). These methods are routinely being used in our laboratory.[20,24,25] These animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and are approved by the Wayne State University's Institutional Animal Care and Use Committee (Detroit, MI, USA).

Gene Expression

Total RNA was extracted from the retina or BRECs with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green assay in ABI 7500 Cycler detection system (Applied Biosystems, Foster City, CA, USA). The threshold cycle (Ct) was determined, and the relative gene expression subsequently was calculated following ddCt method.[5,26,27] A list of specific primers used in this study is included in the Table.

Protein Expression

Protein (retina or endothelial cells, 50–60 μg) was separated on a 10% SDS polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with 5% nonfat milk for 1 hour. The membranes were incubated with antibodies against the Mlh1 (Abcam, Cambridge, MA, USA) or Msh2 (Pierce, Thermo, Rockford, IL, USA) for overnight at 4°C, and the expression was detected using ECL Western blot detection system (Thermo, Rockford, IL). β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as the loading control.[5,23]

Sequence Variants in mtDNA

Variations in mtDNA sequences were analyzed using Surveyor Mutation Detection kit from Transgenomics (Omaha, NE, USA). DNA pellet, isolated by the phenol/chloroform method,[14,25] was suspended in the elution buffer, and DNA concentration was quantified by using Quant-iT DNA detection kit (Invitrogen). The D-loop and Cox regions of the mtDNA were amplified using 50 ng DNA, region-specific primers and a high fidelity Elongase enzyme mix (Invitrogen). To rule out that the contaminating DNA is acting as a template, 'no template' negative control was run simultaneously. Mismatch in the D-loop region was investigated by digesting the amplified mtDNA products with a surveyor nuclease, a mismatch-specific endonuclease with high specificity for the sites of base-substitution mismatch. In a 60-μL incubation medium, 6 μL of the surveyor nuclease reaction buffer, 1 μL each of the surveyor enhancer and surveyor nuclease, and 15 μL of each of the PCR-amplified product were incubated at 42°C for 20 minutes, and the reaction was stopped by adding 6 μL of the stop solution. The digested products were finally electrophoresed on a 2% agarose gel and analyzed for DNA fragmentation by visualizing under a UV transilluminator.[28,29] Band intensity of an amplicon after surveyor nuclease digestion was normalized against undigested products from the same sample, and undigested amplicon was used as an internal control.

Mitochondrial Respiratory Rate

The cellular respiration, reflecting the aerobic metabolic activity, of the retinal endothelial cells was recorded by high resolution respirometry (OROBOROS Instruments, Innsbruck, Austria). After calibrating the oxygraph with PBS, cells (1×10^6/2 mL) were transferred into a respiratory chamber, and the samples were continuously stirred at 37°C. The measurements were initiated soon after closing of the chamber. Reoxygenation was performed by opening the chamber and exposing the stirred medium to air until the return to the preanoxia level of O_2 concentration. Subsequently, the respiration chamber was closed again, and the routine respiration rate monitored. The slopes of O_2 consumption, representing the routine respiration of the cells, were calculated using the OROBOROS oxygraph software (DatLab 4.0).[30]

Cell Apoptosis

Endothelial cell death was determined using Cell Death Detection ELISAPLUS kit from Roche Diagnostics (Indianapolis,

TABLE. Primer Sequences

Gene	Sequence	Product Length (bp)
Bovine		
Mlh1	5′-CCTATGGCTTTCGGGGTGAG-3′ 5′-CTGCACACGGTTTAGGAGGT-3′	144 (371–514)
Msh2	5′-GCTTCCATTGGTGTTGTGGG-3′ 5′-GGGAACTCACACAGTCCCAG-3′	110 (483–592)
Msh6	5′-ATACCGGGCTCCCAGTTTTG-3′ 5′-ACCAAGAGCAGAGAGGGCTA-3′	192 (1861–2052)
Yb1	5′-TGATGGAGGGTGCTGACAAC-3′ 5′-TTCATTGCCGTCCTCTCTGG-3′	134 (790–923)
Pms2	5′-TCAATCGGAGGCCTTGTGAC-3′ 5′-CTGCACACGGTTTAGGAGGT-3′	126 (875–1000)
D-loop	5′-AACACGCCCATACACAGACC-3′ 5′-AGTGCGTCGGCTATTGTAGG-3′	1302 (15916–829)
Cox region (*Cox 1* & *Cox 2*)	5′-GTTGTAACCGCACACGCATT-3′ 5′-TAAGCCTGGACGGGACGATA-3′	2092 (5855–7946)
β-actin	5′-CGCCATGGATGATGATATTGC-3′ 5′-AAGCCGCCTTGCACAT-3′	66 (89–154)
Rat		
Mlh1	5′-CGCCATGCTGGCCTTAGATA-3′ 5′-AGTCTGCAAGCATCTTGGCT-3′	119 (1761–1879)
Msh2	5′-GCAACGAACATTGGTGCCTT-3′ 5′-AGCGAGTTCAGCCACATGAA-3′	192 (2311–2502)
Msh6	5′-TGATCGCCACTGTTCCAGAC-3′ 5′-CCTGAAGGCAAGATGACCAGT-3′	131 (1824–1954)
Yb1	5′-AGACAAAAGCAGCCGATCCA-3′ 5′-TGTTGGATGACCAAACCGGA-3′	117 (1022–1138)
Pms2	5′-TAATCAGCTCGGACAGGGGA-3′ 5′-AGTCTGCAAGCATCTTGGCT-3′	185 (699–883)
D-loop	5′-ATGAATCGGAGGCCAACCAG-3′ 5′-AATTTGAGGAGGGTGACGGG-3′	2099 (15143–928)
Cox1	5′-TGGCTTCAATCTACTTCTCCCG-3′ 5′-TGGCGTCTTGTAAGCCAAGT-3′	1903 (5140–7042)
β-actin	5′-CCTCTATGCCAACACAGTGC-3′ 5′-CATCGTACTCCTGCTTGCTG-3′	215 (957–1171)

IN, USA). This method determines the relative amounts of oligonucleosomes generated from the apoptotic cells by using monoclonal antibodies directed against DNA and histones, respectively. The cell homogenate was incubated with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled anti-histone in a streptavidin-coated plate. The plate was washed thoroughly, incubated with 2,2′-azino-di-[3-ethylbenz-thiazoline sulfonate] diammonium salt (Roche Diagnostics), and the absorbance was measured at 405 nm.[1,31]

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was carried out using SigmaStat statistical software (Systat Software, Inc., Chicago, IL, USA). For multiple group comparison, one-way ANOVA followed by Student-Newman-Keuls test was used for data with normal distribution, while Kruskal-Wallis one-way analysis followed by Dunn's test was performed for data that did not present normal distribution. A P value less than 0.05 was considered as statistically significant.

RESULTS

Retinal Endothelial Cells

Mismatch Repair Proteins. Gene expression of *Mlh1,* a DNA MMR protein which removes sections of DNA with mismatch pairs, was decreased significantly in the cells exposed to high glucose compared with the cells in normal glucose (Fig. 1A). Expression of *Msh2*, another MMR protein, which, in association with Msh3 and Msh6, identifies mismatch locations, was also significantly decreased in retinal endothelial cells exposed to high glucose compared with the cells exposed to normal glucose (Fig. 1B). Incubation of cells with 20 mM mannitol, instead of 20 mM glucose, had no effect on the expression of *Mlh1* or *Msh2*, suggesting that the detrimental effects of glucose were not due to increased osmolarity. However, high glucose had no significant effect on some of the other MMR proteins investigated, including *Msh6*, *Yb1*, and *Pms2*; their expressions remained similar in the cells exposed to normal or high glucose (Fig. 1C).

Sequence Variants/Mismatch in the *D-Loop* Region. Decrease in *Mlh1* and *Msh2* by high glucose was accompanied by increased sequence variants in the D-loop region (Fig. 2A). To further confirm glucose-induced sequence variants in the D-loop region, amplicon intensity was quantified, and as shown in Figure 2B, the intensity of this band was significantly decreased in the cells incubated in high glucose compared with the cells in normal glucose ($P < 0.05$). However, contrary to the D-loop region, glucose had no effect on the sequence variation in the Cox region (*Cox1* and *Cox2*) of the mtDNA (Fig. 2C). "No template" negative control had no amplification, and the D-loop region template, without surveyor digestion, did not present any clear fragmentation (Fig. 2D). Please note

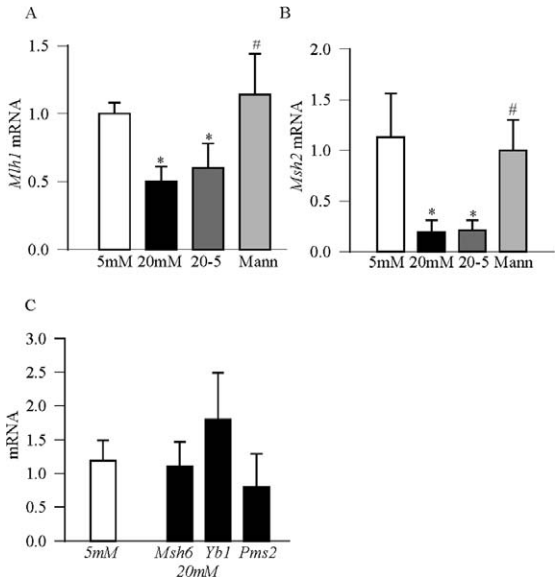

Figure 1. Effect of high glucose on MMR machinery. Retinal endothelial cells were analyzed for mRNA expressions of MMR genes by qRT-PCR using β-actin as a housekeeping gene. Each measurement was made in duplicate using cells from three to four different cell preparations, and the values are represented as mean ± SD. (**A**) Mlh1, (**B**) *Msh2*, and (**C**) *Msh6*, *Yb1*, and *Pms2*. $^*P < 0.05$ and $\#P < 0.05$ compared with 5 mM glucose and 20 mM glucose, respectively. 5 mM, 5 mM glucose; 20 mM, 20 mM glucose; 20-5, 4 days of 20 mM followed by 4 days of 5mM glucose; Mann, 20 mM mannitol.

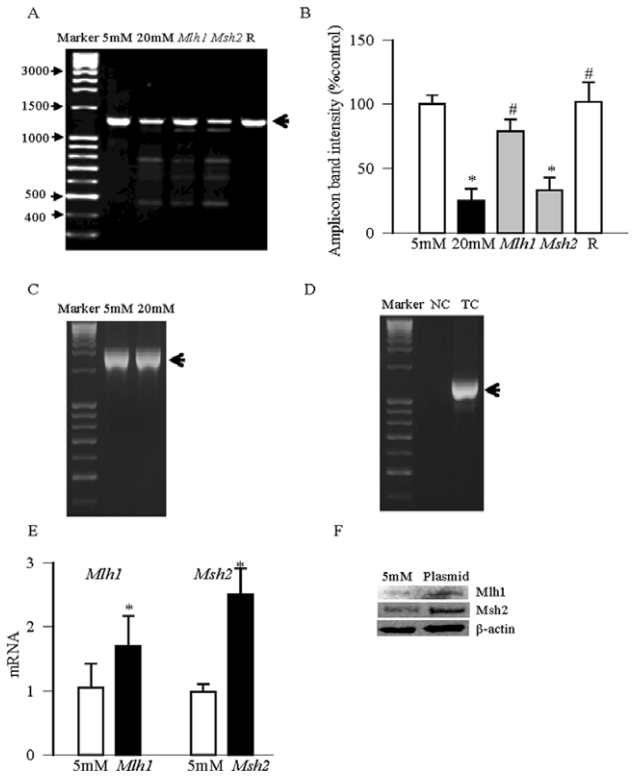

Figure 2. Sequence variants in the D-loop region. (**A**) DNA was amplified using semiquantitative PCR for D-loop regions of mtDNA, and after digesting the samples with mismatch-specific surveyor nuclease, they were analyzed on an agarose gel. The *arrow* indicates the amplicon product length. (**B**) The amplicon intensity of the D-loop region was quantified, and the intensity of the band in 5 mM glucose was considered as 100%. (**C**) For comparison, amplification for the Cox region (*Cox1* and *Cox2*) of the mtDNA was analyzed after mismatch-specific surveyor nuclease digestion. (**D**) 'No template' (NC), D-loop template control without surveyor nuclease digestion (TC). (**E**) Gene transcripts and (**F**) protein expression were quantified in cells transfected with *Mlh1* or *Msh2* by q-PCR and Western blot techniques, respectively. Values are from three to four different experiments. $^*P < 0.05$ compared with 5 mM glucose and $\#P < 0.05$ compared with 20 mM glucose. 5 mM and 20 mM, 5 mM and 20 mM glucose, respectively; *Mlh1* and *Msh2*, cells after transfecting with *Mlh1* or *Msh2*, incubated in 20 mM glucose for 4 days; R, cell incubated with transfection reagent alone during transfection, followed by incubation in 5 mM glucose for 4 days.

that the extra bands in Figure 2A represent the digested products of the fragmented DNA.

In cellular respiration, high glucose exposure resulted in a progressive slowdown of the routine respiration of the cells compared with the cells incubated in normal glucose. In the same cells, the respiration rate was decreased by approximately 3-fold in the cells incubated in high glucose compared with the cells in normal glucose (Fig. 3).

Upregulation of Mismatch Enzymes and Mitochondria Homeostasis. The specific role of Mlh1 and Msh2 in the mismatch in mtDNA sequence was determined in the cells overexpressing *Mlh1* or *Msh2*. The transfection efficiency of *Mlh1,* and that of *Msh2* in retinal endothelial cells was greater than 70%, as determined by quantifying the gene expressions (Fig. 2E), or by protein expressions (Fig. 2F).

Cells overexpressing *Mlh1* had decreased glucose-induced sequence variants in the D-loop region and increased amplicon intensity compared with the untransfected cells exposed to high glucose ($P < 0.05$; Figs. 2A, 2B). In the same cell preparations, glucose-induced decrease in the respiration rate (Fig. 3), and increase in capillary cell apoptosis (Fig. 4) were also ameliorated, the values obtained from the cells transfected with *Mlh1* were not different from those obtained from cells incubated in normal glucose. However, transfection of cells with the reagent alone had no detrimental effects on mtDNA mismatch fragmentation and cell apoptosis. In contrast to *Mlh1*, overexpression with *Msh2*, which is mainly associated with nuclear DNA polymerase beta, did not produce any significant protection in the glucose-induced fragmentation of mtDNA (Figs. 2A, 2B). Similarly, decrease in the routine respiration and increase in cell apoptosis, experienced by cells in high glucose conditions in *Msh2*-transfected cells, were also not different from those obtained from cells without any transfection (Figs. 3, 4).

Rat Retina

Mismatch Repair Enzymes. Gene and protein expressions of Mlh1 were decreased by over 50% in the retina from diabetic rats compared with that from age-matched normal rats (Figs. 5A, 5B). Consistent with Mlh1, Msh2 was also subnormal in diabetes, its gene expression was reduced by over 70%, and its protein expression by over 85% (Figs. 5C, 5D). In contrast to Mlh1 and Msh2, diabetes had no effect on the gene expression of some of the other MMR proteins, including *Yb1*, *Msh6*, and *Pms2* (Fig. 5E).

Sequence Variants/Mismatch. Consistent with the results from retinal endothelial cells in high glucose, diabetes resulted in sequence variants in the mtDNA representing the D-loop region, as evident by increase mismatch in the nuclease digest (Fig. 6A). Increased sequence variant in the D-loop region was accompanied by an approximate 50% decrease in the amplicon intensity (Fig. 6B). Contrary to the D-loop region, mtDNA representing the *Cox1* region did not show any sequence variants (Fig. 6C). As a control, there was no major

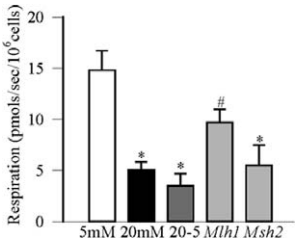

FIGURE 3. Effect of high glucose on mitochondrial respiration: the cellular respiration was recorded by high-resolution respirometry using 10^6 retinal endothelial cells. Respiration rate was calculated using the OROBOROS oxygraph software (DatLab 4.0). *$P < 0.05$ and #$P < 0.05$ compared with 5 mM glucose and 20 mM glucose, respectively.

amplification in the "no template" negative control, and no fragmentation in the D-loop template control without surveyor nuclease digestion (Fig. 6D).

Effect of Cessation of Hyperglycemia

Reinstitution of good glycemic control, which had followed a period of poor glycemic control, does not reverse retinal mtDNA damage, and the retinopathy continues to progress.[5,14,20,24,32] Here, our results show that despite removal of hyperglycemic insult, the gene and protein expressions of both Msh2 and Mlh1 continued to be subnormal, and the values were similar to those obtained from rats in continuous poor control (Fig. 5). Consistent with the expression of Msh2 and Mlh1, in the same animals, reinstitution of good glycemic control had no significant effect on the sequence variants, the number of mismatches and the intensity of the parent band in the D-loop region remained similar to that obtained from the rats in PC group (Fig. 6). However, when good glycemic control was instituted soon after induction of diabetes, expressions of mismatch proteins, Mlh1 and Msh2, were significantly higher compared with the values obtained from the rats in the reversal group, and the number of sequence variants was also similar to that obtained from the rats that remained normal throughout the experiment (Figs. 5, 6). These results suggest that the high dose of insulin, administered to maintain good glycemic control, did not influence mtDNA mismatch machinery.

In accordance with our results from the in vivo model, removal of high glucose insult in endothelial cells also had no favorable effect on *Msh2* and *Mlh1*; the expression of these enzymes in the cells incubated in 20 mM glucose for 4 days, followed by 5 mM glucose for 4 additional days (20-5 group) were not different from those obtained from the cells incubated in continuous 20 mM glucose, but were significantly

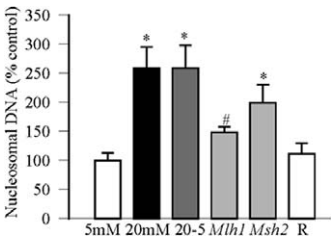

FIGURE 4. Overexpression of mismatch proteins and cell apoptosis: apoptosis was measured by performing ELISA for cytoplasmic histone-associated DNA fragments using an assay ELISA kit. Values are presented as mean ± SD of three different experiments, each performed in duplicate. The values obtained from cells in normal glucose were considered as 100%. *$P < 0.05$ and #$P < 0.05$ compared with 5 mM glucose and 20 mM glucose, respectively.

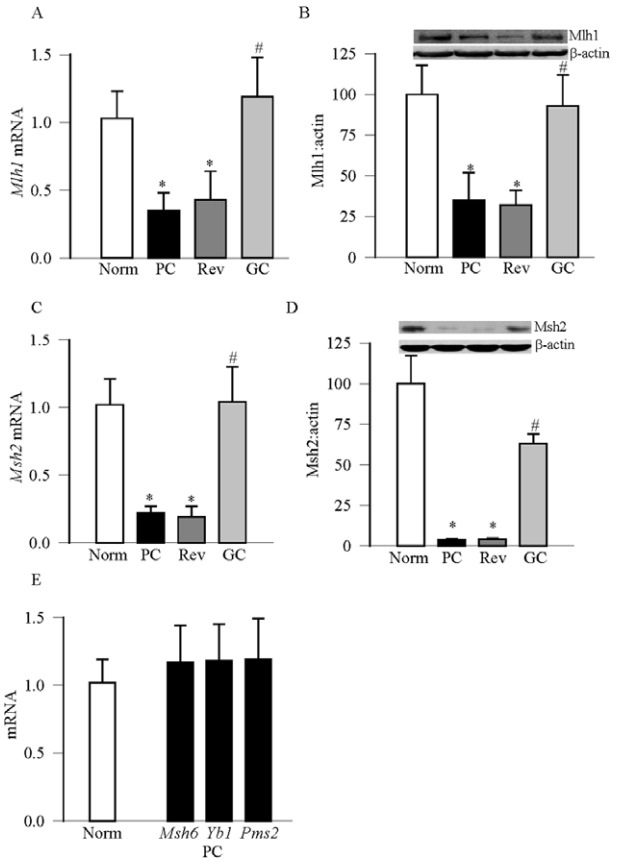

FIGURE 5. Effect of diabetes on MMR machinery. Gene expression of the mismatch genes were quantified in the rat retina by qRT-PCR, and the Ct value of each gene was normalized to β-actin in that sample. Protein expressions were quantified by Western blot technique using β-actin as the loading proteins. Each measurement was made in duplicate, and the values are represented as mean ± SD of four to six rats/group. (**A**, **B**) Gene and protein expressions of Mlh1, and (**C**, **D**) represent those of Msh2. (**E**) The gene expressions of *Msh6*, *Yb1*, and *Pms2*. *$P < 0.05$ compared with normal and #$P < 0.05$ compared with poor control. Norm, normal rats; PC and GC, rats in continuous poor control or good control, respectively, for 6 months. Rev, rats in poor control for 3 months followed by good control for 3 additional months.

lower ($P < 0.05$) than those from the cells in continuous 5 mM glucose (Figs. 1A, 1B). Furthermore, 4 days of normal glucose exposure that had followed 4 days of high glucose had no beneficial effect on the respiration rate (Fig. 3) and cell apoptosis (Fig. 4), and the values remained significantly different from those obtained from the cells in continuous normal glucose.

DISCUSSION

In diabetes, mitochondria become dysfunctional in the retina and its capillary cells initiating the apoptotic machinery, mtDNA is damaged and electron transport chain is compromised, and mtDNA biogenesis and the copy numbers are decreased.[1,5,14,24,33] Mitochondria are equipped with multiple pathways to repair the damaged mtDNA, including direct reversal, BER, single-strand break repair, and MMR systems.[11,34,35] We have shown that, despite increase in the mRNA levels of BER enzymes in the retina in diabetes, these enzymes fail to reach to the mitochondria.[5,10] Here, we show that the MMR system is compromised in the mitochondria; the levels of two of the major MMR proteins, Mlh1 and Msh2, are

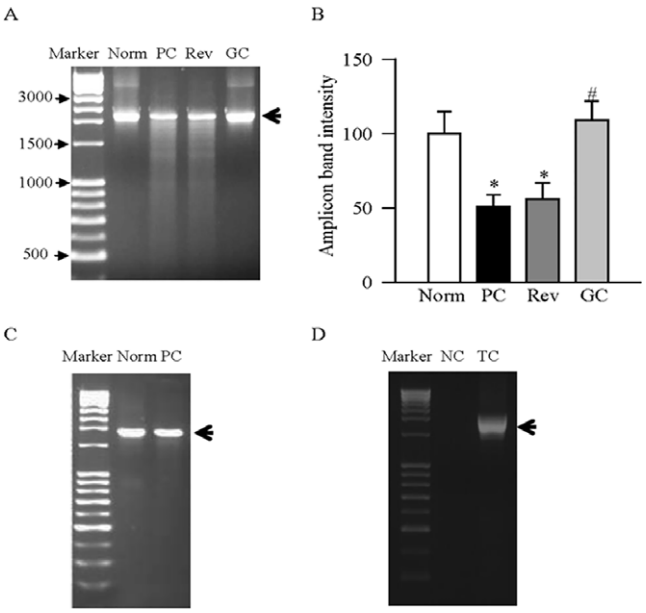

FIGURE 6. Effect of diabetes on retinal mtDNA sequence variants. (**A**) Retinal DNA was amplified for mtDNA region encoding D-loop using semiquantitative PCR, digested with mismatch specific surveyor nuclease, and separated on an agarose gel. The *arrow* indicates the D-loop amplicon product length. (**B**) The amplicon intensity of the D-loop region was quantified, and the intensity of the band obtained from normal rat retina was considered as 100%. (**C**) Amplification for the *Cox1* region of the mtDNA was analyzed after mismatch specific surveyor nuclease digestion. (**D**) "No template" (NC) and D-loop template control without surveyor nuclease digestion (TC) were analyzed by agarose gel electrophoresis. Values are from four to six rats in each group. $^{*}P < 0.05$ and $\#P < 0.05$ compared with normal and poor control, respectively. Norm, normal rats; PC and GC, rats in continuous poor control or good control respectively, for 6 months; Rev, 3 months of poor control followed by 3 months of good control.

decreased in the retina, and the number of sequence variants is significantly increased in the mtDNA. The number of sequence variants is significantly higher in the D-loop region of the mtDNA compared with the Cox region. While overexpression of *Mlh1* helps ameliorate mismatch in the D-loop region, protects mitochondria function (respiration rate), and prevents increase apoptosis that the capillary cells experience in high-glucose conditions, overexpression of *Msh2* does not provide such benefit and the cells continue to undergo apoptosis. Furthermore, we show that diabetes-induced increase in mtDNA sequence variants continue to progress even after removal of hyperglycemic insult. Together, these results strongly suggest that inefficient MMR system could be one of the mechanisms responsible for mtDNA damage in diabetes. Since termination of hyperglycemia does not benefit impaired MMR system, this suggests its role in the continued progression of diabetic retinopathy even when the hyperglycemic insult is terminated. In support, mitochondrial genome mismatch has been reported in other chronic ocular diseases including AMD and Leber hereditary optic neuropathy.[29,36]

The MMR pathway is responsible for correcting base substitution matches and insertion–deletion mismatches during DNA replication by capturing the errors in the newly synthesized DNA strands that are missed by the polymerase proof reading.[11,12] In the pathogenesis of diabetic retinopathy, retinal mtDNA replication machinery is compromised, and mtDNA replication enzyme POLG becomes subnormal and its activity is inhibited.[14] In addition to the subnormal BER enzymes in the retina in diabetes,[5,10] here we show that the MMR system is also inefficient, suggesting that any mismatch in the retinal mtDNA, caused by the diabetic environment, is not properly corrected.

The MMR system consists of a number of proteins; DNA mismatches are mainly recognized by the MSH2/MSH6 heterodimer allowing Mlh complex (consisting of Mlh1 and Pms2) to recruit, and stabilize the MutSα:DNA interaction.[12] During replication, cytosine or adenine bases incorporate into 8-OHdG resulting in guanine-cytosine → thymine-adenine mismatch, and 8-oxoguanine glycosylase (OGG1) of the BER system repairs 8-OHdG lesions.[37,38] While DNA polymerase beta is responsible for incorporating the correct base in the nuclear DNA, POLG is important for the mtDNA.[13,39,40] In the pathogenesis of diabetic retinopathy, 8-OHdG levels are elevated and OGG1 accumulation in the mitochondria is attenuated, mtDNA damage is more extensive at the D-loop region than other regions of mtDNA, and the binding of POLG at the D-loop region is compromised and mtDNA transcription is impaired.[5,8,10] Our results show that in diabetes, while the Cox region of the mtDNA does not present sequence variants, the D-loop region has a number of variants, further confirming the vulnerability of the D-loop region to greater oxidative damage than other regions of the mtDNA.[8] Consistent with this, the D-loop region, which contains essential transcription and replication elements, and is the starting point of the replication of the mtDNA, tends to accumulate mutations at a higher rate than other regions of mtDNA, including the region encoding Cox.[41,42] To limit the number of sequence variants and correct addition of adenosine opposite 8-OHdG, BER glycosylase enzyme MUTYH, removes that adenine.[43] However, MUTYH also becomes subnormal in the retinal mitochondria in diabetes,[5] further contributing to mtDNA sequence variants and damage.

Overexpression of *Mlh1* significantly ameliorates hyperglycemia-induced DNA sequence variants in the D-loop region; the overall number of variants is lower and the intensity of the amplicon band is significantly higher in *Mlh1*-tranfected cells compared with the untransfected cells exposed to high glucose. This decrease in number of sequence variants is accompanied by restoration of mitochondrial function, and decrease in capillary cell apoptosis, a phenomenon that precedes the development of vascular histopathology characteristic of diabetic retinopathy,[44,45] implying that Mlh1 has an important role in maintaining the integrity of the mtDNA and mitochondria homeostasis in diabetes.

Although Yb1, a multifunctional protein implicated in melting mismatch, has been shown to regulate mtDNA mismatch-binding/repair and cellular respiration,[46] our results show that *Yb1* and other MMR proteins *Msh6* and *Pms2*, are not affected by diabetes; the reason for such discrepancy is not clear. Furthermore, despite decrease in retinal Msh2 in diabetes, its overexpression failed to protect the mitochondria from the damage. These results suggest that these components of MMR system might not be playing a significant role in the repair of the damaged mtDNA, induced by the diabetic milieu.

Clinical and experimental studies have shown that diabetic retinopathy does not halt after termination of hyperglycemic insult, the retinal mtDNA remains damaged with impaired BER system and mtDNA biogenesis and replication.[5,6,8,18,47] The results presented here clearly show that the MMR system also remains compromised. Cessation of hyperglycemia does not provide any benefit to retinal Mlh1 and Msh2 levels, and the mtDNA continues to present sequence variants. As a result of poor efficiency of mtDNA repair mechanisms (MMR and BER systems), mtDNA damage, induced by prior poor glycemic control, does not benefit from the good control which follows it, mtDNA transcription remains deficient and the electron

transport machinery compromised, and the retinopathy continues to progress.

Mismatch repair pathway is implicated in both, the predisposition to cancer and in the response to therapy, and its role in this chronic disease is being investigated by many laboratories.[48–51] Our study, using molecular, biochemical, and functional approaches, clearly demonstrates that retinal MMR system is impaired in diabetes, suggesting that this system plays a significant role in the damage of mtDNA and its transcription. Regulation of *Mlh1*, which is associated with mitochondrial replication enzyme POLG,[13] protects mitochondria homeostasis and accelerated apoptosis, a phenomenon which precedes the development of histopathology associated with diabetic retinopathy, strongly implying that the strategies targeting mitochondrial MMR system could have potential to protect the retina from undergoing oxidative damage in the diabetic milieu, and inhibit the progression of the debilitating blinding disease in diabetic patients.

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