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Morin hydrate prevents diabetic nephropathy by suppressing oxidative stress and 8-hydroxydeoxyguanosine in kidney tissues

Mohammad Alhilal[1], Esra Dereli[2], Hüseyin Eren Yıldırım[2], Metin Kılıçlıoğlu[2], Suzan Alhilal[3], Serkan Yıldırım[2,4]

[1]*Department of Nursing, Faculty of Health Sciences, Mardin Artuklu University, Mardin, Turkiye*
[2]*Department of Pathology, Faculty of Veterinary Medicine, Ataturk University, Erzurum, Turkiye*
[3]*Department of Medical Services and Techniques, Vocational School of Health Services, Mardin Artuklu University, Mardin, Turkiye*
[4]*Department of Pathology, Faculty of Veterinary Medicine, Kyrgyzstan-Turkey Manas University, Bishkek, Kyrgyzstan*

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ABSTRACT

Aims: The aim of this study was to investigate the protective effect of morin hydrate either individually or in combination with metformin against diabetic nephropathy by targeting oxidative stress and 8-hydroxydeoxyguanosine (8-OHdG) in the kidney tissue of rats with diabetic nephropathy.

Methods: In this experimental study, diabetic nephropathy was induced in rats by injection of streptozotocin (STZ). The ability of morin hydrate to inhibit diabetic nephropathy was tested by screening lipid peroxidation (LPO), glutathione, glutathione peroxidase, superoxide dismutase, and catalase as parameters of oxidative stress; 8-OHdG as a marker of DNA damage and kidney injury molecule-1 (KIM-1) and aquaporin as indicators of kidney injury in renal tissues; and serum creatinine and blood urea nitrogen as markers of renal function using biochemical, immunohistochemical, and immunofluorescence methods.

Results: Significant increases ($p<0.0001$) in LPO, 8-OHdG, KIM-1, and aquaporin levels and significant decreases ($p<0.0001$) in glutathione, glutathione peroxidase, superoxide dismutase, and catalase levels were observed after STZ administration, indicating the development and progression of diabetic nephropathy. Treatment with morin hydrate, especially in combination with metformin, suppressed the oxidant levels and improved the antioxidant system and the histopathological integrity of the kidney, which was positively reflected in the levels of KIM-1, aquaporin, and kidney function parameters.

Conclusion: Morin hydrate prevents diabetic nephropathy resulting from diabetes mellitus by suppressing oxidative stress and 8-OHdG levels in kidney tissues. Therefore, this bioflavonoid represents a promising candidate for patients with diabetic nephropathy. Moreover, the combination therapy of morin hydrate and metformin achieved better effectiveness than the single treatment, which emphasizes an important synergistic role of morin hydrate and metformin in managing patients with diabetic nephropathy.

Keywords: Morin hydrate, oxidative stress, 8-OHdG, diabetic nephropathy, KIM-1

INTRODUCTION

Diabetes mellitus is one of the most widespread diseases, with an estimated more than half a billion people affected worldwide, and this number is expected to reach 629 million by 2045.[1] Among the various complications of diabetes, diabetic nephropathy is the second most frequent and prevalent complication.[2] Diabetic nephropathy is an important cause of chronic kidney failure by damaging the glomerulus due to the excretion of large amounts of albumin in the urine.[3] This complication develops in approximately 35%– 40% of patients with diabetes[4] and is a major cause of end-stage renal disease, especially in developed countries where it is responsible for up to 50% of all end-stage renal disease cases. Diabetic nephropathy is a cause of 40% of dialysis and kidney transplant cases in the United States. The negative impacts of diabetic nephropathy are not limited to the kidney alone but extend to other organs. Patients with diabetic nephropathy have more than twice the probability of developing cardiovascular disease than those without diabetic nephropathy.[5] Furthermore, diabetic nephropathy increases the mortality rate in diabetic patients[6] and is the major reason for hospitalizations related to the chronic complications of diabetes.[7] These data indicate that diabetic nephropathy is a global health, social, and economic burden, which requires the development of new therapeutic strategies. In this regard, we believe that the development of treatments for diabetic nephropathy depends primarily on a deep

Corresponding Author: Mohammad Alhilal, dr.mkh.1981@gmail.com

understanding of the pathophysiology of this complication. Increasing evidence indicates a critical role of oxidative stress in the pathophysiology of diabetic nephropathy.[2,8,9] Oxidative stress is defined as an excess of reactive oxygen species (ROS) coupled with a weakened cellular antioxidant system. Oxidative stress contributes to diabetes and its complications by exacerbating ROS production, impairing insulin production, increasing insulin resistance, and stimulating inflammatory processes and cell death.[8] Furthermore, the persistent oxidative stress in diabetic patients causes damage to the genetic material of mitochondria and increased levels of 8-hydroxydeoxyguanosine (8-OHdG), which stimulates apoptosis in various tissues.[2] On the basis of these data, antioxidant agents are hypothesized to mitigate diabetic nephropathy. In recent years, there has been an increasing use of natural products, especially flavonoids isolated from plants, in numerous studies due to their therapeutic properties without side effects. In this context, morin hydrate, a bioflavonoid extracted primarily from various parts of plants belonging to the Moraceae family, has emerged as a promising candidate against various chronic and life-threatening degenerative diseases owing to its antioxidant, anti-inflammatory, and antiapoptotic properties.[10] Studies have shown that morin hydrate exerts hepatoprotective effects in rats with type 2 diabetes,[11] inhibits the adverse effects of diesel exhaust particles on the pancreas of rats with type 2 diabetes,[12] and decreases diabetes-induced diabetic encephalopathy[13] by suppressing ROS production and improving the antioxidant defense system. Lawal et al.[14] hypothesized that morin hydrate protected the kidney in rats with type 2 diabetes exposed to diesel exhaust particles by inhibiting the Notch1/Snail signaling pathway, although they did not screen for oxidative stress and DNA damage parameters in renal tissues. Based on these promising results, it is hypothesized that morin hydrate exerts protective effects against diabetic nephropathy associated with diabetes. Accordingly, we conducted this study to investigate the therapeutic effects of morin hydrate either individually or in combination with metformin in a rat model of streptozotocin (STZ)-induced diabetic nephropathy by targeting oxidative stress and 8-OHdG. For this purpose, we used several biochemical, histopathological, immunohistochemical, and immunofluorescence techniques.

METHODS

Ethics

Ethical approval for this study was obtained from the Ethics Committee of Animal Experiments at Atatürk University (Date: 22.12.2022, Decision No: 292). The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Experimental Protocol and Study Groups

A total of 60 adult female Sprague Dawley rats weighing 220–250 g were provided by the Medical Experimental Application and Research Center at Atatürk University. The sample size is appropriate to provide statistically good results. With 10 rats per group, the sample size provides robust results and enhances the generalizability of the conclusions. We confirmed this issue using the G*power program (Version 3.1.9.7, Düsseldorf, Germany), which yielded the following parameters: Total sample size=60, Effect size f=10.8537124, α err prob=0.05, power (1-β err prob)=1.0000000, Number of groups=6. The rats were randomly distributed into six groups. The experimental groups and treatments are described in **Table 1**.

Table 1. Details of the experimental groups and treatments

Group name	Number of rats	Description
Control	10	Physiological serum 50 mg/kg
Diabetes	10	STZ 50 mg/kg
Dia+Met	10	STZ 50 mg/kg+metformin 100 mg/kg
Dia+Met+MH100	10	STZ 50 mg/kg+metformin 100 mg/kg+ morin hydrate 100 mg/kg
Dia+MH100	10	STZ 50 mg/kg+morin hydrate 100 mg/kg
MH100	10	Morin hydrate 100 mg/kg

Dia: Diabetes, Met: Metformin, MH: Morin hydrate, STZ: Streptozotocin

Diabetes mellitus was induced by an intraperitoneal administration of a single dose (0.5 ml) of 50 mg/kg STZ (BioVisionCat No: 1930-1000) dissolved in cold citrate buffer (0.1 M, pH 4.5). After 1 week of STZ administration (on the 8th day), fasting glucose (12-h) levels were measured using a glucometer, and rats with fasting blood glucose levels of >250 mg/dl were considered diabetic. The experiment lasted for 4 weeks [15], and freshly prepared morin hydrate (100 mg/kg) was diluted with physiological serum and administered daily via gastric gavage. 100 mg/kg of metformin was diluted with physiological serum and administered daily via gastric gavage. The rats of the control and MH100 groups were intraperitoneally injected with a single dose of 50 mg/kg citrate buffer without STZ to make the experience conditions equal in all groups.

The doses of STZ and morin hydrate were determined based on previous studies.[15-17] Morin hydrate, metformin, and STZ were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

On the 28th day after STZ administration, blood samples were collected under general anesthesia for biochemical assessment, and renal tissues were collected after euthanization by cervical dislocation for histopathological, immunohistochemical, and immunofluorescence examinations. Blood samples were centrifuged at 3500–4000 rpm for 10 min, and the obtained sera were stored at −80°C until biochemical analysis. Renal tissues were immediately placed in 10% formalin solution.

Biochemical Examination of Renal Tissue and Serum

Standard commercial kits acquired from SunRed Biological Technology Company (Shanghai) were used for measuring the biochemical parameters in renal tissues and serum according to the manufacturer's instructions. Renal tissues were homogenized in PBS and centrifuged at 4°C for 10 min at 3000 rpm. The resulting supernatant was used to determine the oxidative stress parameters according to the kit's manufacturer's instructions.

Histopathological Examination of Kidney Tissue

Renal tissues were subjected to a consecutive routine series of alcohol and xylol washes. Then, 4-µm-thick sections were

prepared from the tissue samples and embedded in paraffin. After staining with hematoxylin–eosin (H&E), the sections were examined under a light microscope (Olympus BX 51, Japan). Sections were evaluated as no (–), mild (+), moderate (++), and severe (+++) according to the severity of lesions.[18]

Immunohistochemical Evaluation of the Kidney Tissue

Kidney injury molecule-1 (KIM-1) expression was evaluated according to the renal immunohistochemistry protocol procedures of the previous study.[18] KIM-1 Cat no (ab184787; diluted 1:100) was used as the primary antibody. The stained sections were examined under a light microscope (Zeiss AXIO, Germany).

Double Immunofluorescence Evaluation of the Kidney Tissue

For evaluating aquaporin expression, aquaporin Cat no ab110418 and FITC Cat no: ab6785; diluted 1:1000 were used as the primary antibody and secondary immunofluorescence antibody, respectively. 8-OHdG expression was evaluated using 8-OHdG Cat no: sc66036 and Texas Red Cat no: ab6719; diluted 1:1000 as the second primary antibody and secondary immunofluorescence antibody, respectively. All procedures were performed as described previously.[18] Finally, the stained sections were examined under a fluorescence microscope (Zeiss AXIO, Germany).

Statistical Analysis

The data analysis was performed using the GraphPad prism (version 8.0.2) software. One-way ANOVA followed by Tukey's test was used to determine significant differences between groups ($p<0.05$). To determine the intensity of positive staining from the images obtained through immunohistochemistry and immunofluorescence staining, five random areas were selected from each image, and their evaluations were conducted on the ZEISS Zen Imaging Software program. One-way ANOVA followed by Tukey's test was performed to compare positive immunoreactive cells and immunopositive stained areas with healthy controls.

RESULTS

Biochemical Examination

The effects of morin hydrate on lipid peroxidation (LPO), glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) in renal tissues are illustrated in **Figure 1**. LPO levels significantly increased in the diabetes group (125.6±3.74) compared with those in the control group (66.40±1.95) ($p<0.0001$). Administration of morin hydrate at a dose of 100 mg/kg prevented the increase in LPO levels. A significant decrease ($p<0.0001$) in LPO levels was observed in the Dia+MH100 group (91.01±0.98) compared with that in the diabetes group. The LPO levels also significantly decreased ($p=0.0019$) in the Dia+Met+MH100 group (77.61±2) compared with those in the Dia+MH100 group. The GSH levels and SOD, GPx, and CAT activities significantly decreased ($p<0.0001$) in the diabetes group (6.27±0.07, 13.64±0.33, 3.74±0.27, 7.32±0.31, respectively) compared with those in the control group (8.53±0.10, 26.55±0.39, 17.28±0.19, 19.76±0.35, respectively). This decrease was prevented by the administration of morin hydrate at a dose of 100 mg/kg. The Dia+MH100 group showed significant increases ($p<0.0001$) in GSH levels (7.48±0.11) and SOD (19.78±0.26), GPx (11.79±0.34), and CAT (14.45±0.62) activities compared with the diabetes group. The Dia+Met+MH100 group also showed significant increases in GSH levels (8.27±0.05) ($p=0.0078$) and SOD (23.65±0.34) ($p=0.0002$), GPx (15.68±0.33) ($p<0.0001$), and CAT (17.83±0.52) ($p=0.0090$) activities compared with the Dia+Met group (7.75±0.11, 21.27±0.31, 12.57±0.33, 15.45±0.50, respectively).

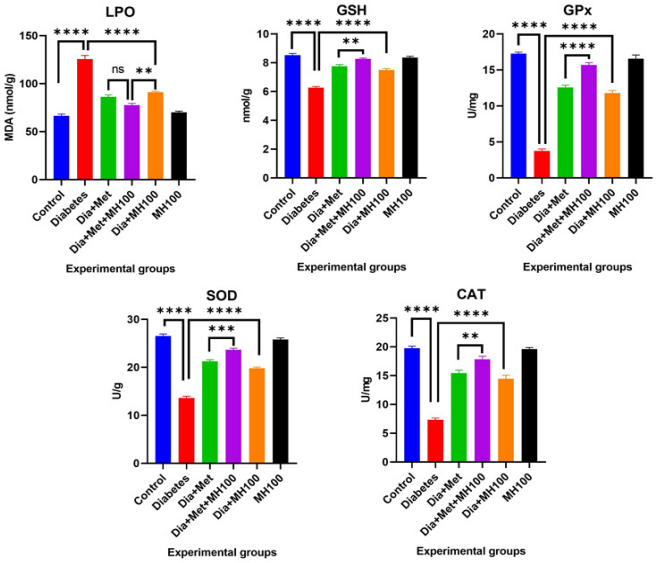

Figure 1. Effect of morin hydrate on oxidative stress parameters in renal tissues
Dia: Diabetes, Met: Metformin, MH: Morin hydrate. Findings are expressed mean±SE., (****: $p<0.0001$; ***: $p=0.0002$; **: For LPO $p=0.0019$, For GSH $p=0.0078$, For CAT $p=0.0090$; ns: non-significant $p>0.05$)

The effects of morin hydrate on the serum levels of creatinine and blood urea nitrogen (BUN) are depicted in **Figure 2**. Creatinine and BUN levels increased in the diabetes group (3.46±0.15 and 41.33±1.41, respectively) compared with those in the control group (0.98±0.12 and 18.14±0.39, respectively) ($p<0.0001$). In contrast, the Dia+MH100 group showed significantly decreased creatinine and BUN levels (1.61±0.12 and 22.68±1.03, respectively) compared with the diabetes group ($p<0.0001$).

Histopathological Examination

Blinded evaluation of tissue slides was conducted for the histopathological, immunohistochemical, and immunofluorescence outcomes and image analyses.

Control and MH100 groups: Normal histological structure was observed in these groups (**Table 2, Figure 3**).

Diabetes group: Severe degeneration and severe necrosis were detected in the renal tubular epithelial cells, and severe hyperemia was observed in the glomerular vessels (**Table 2, Figure 3**).

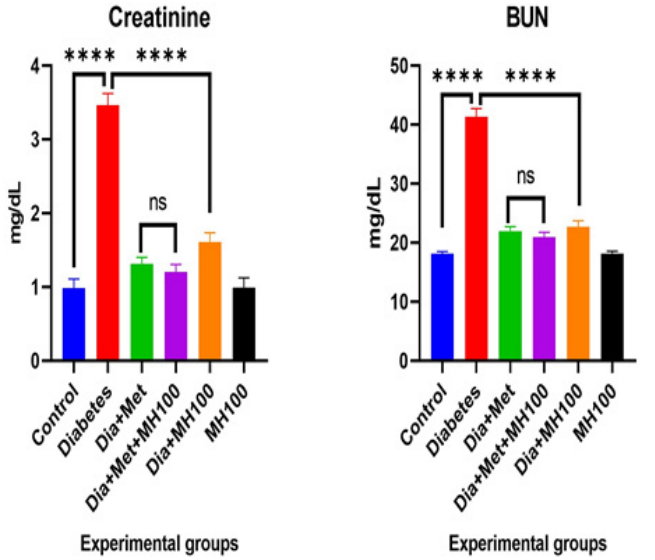

Figure 2. Effect of morin hydrate on renal function tests in serum
Dia: Diabetes, Met: Metformin, MH: Morin hydrate, BUN: Blood urea nitrogen. Findings are expressed mean±SE. (****: p<0.0001; ns: non-significant p>0.05).

Table 2. Scoring of histopathological findings observed in kidney tissues

Groups	Degeneration	Necrosis	Hyperemia
Control	-	-	-
Diabetes	+++	+++	+++
Dia+Met	++	+	+++
Dia+Met+MH100	+	-	++
Dia+MH100	++	+	+++
MH100	-	-	-

Sections were evaluated as no (-), mild (+), moderate (++) and severe (+++) according to the severity of lesions. Dia: Diabetes, Met: Metformin, MH: Morin hydrate

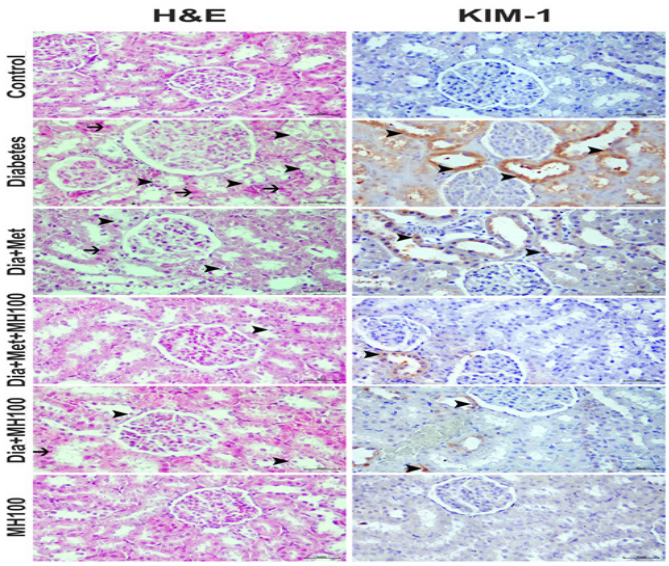

Figure 3. Photomicrography of histopathological and immunohistochemical assessment of renal tissues
Histopathological images: Arrowheads: degeneration; Arrows: necrosis. H&E, Bar: 40μm.
Immunohistochemical images: Arrowheads: KIM-1 expression in the renal tubular epithelial cells. IHC-P, Bar: 40μm. Dia: Diabetes, Met: Metformin, MH: Morin hydrate.

Dia+Met group: Moderate degeneration and mild necrosis were found in the renal tubular epithelial cells (Table 2, Figure 3).

Dia+Met+MH100 group: Mild degeneration was detected in the renal tubular epithelial cells (Table 2, Figure 3).

Dia+MH100 group: Moderate degeneration and mild necrosis were detected in the renal tubular epithelial cells (Table 2, Figure 3).

Immunohistochemical Evaluation

KIM-1 expression was negative in the control and MH100 groups (Figure 3, 4). However, the diabetes group showed significantly increased (p<0.01) KIM-1 expression in the renal tubular epithelial cells compared with other groups (Figure 3, 4). In contrast, treatment with morin hydrate significantly (p=0.0079) reduced the KIM-1 expression in the renal tubular epithelial cells of the Dia+MH100 group compared with that in the diabetes group (Figure 3, 4).

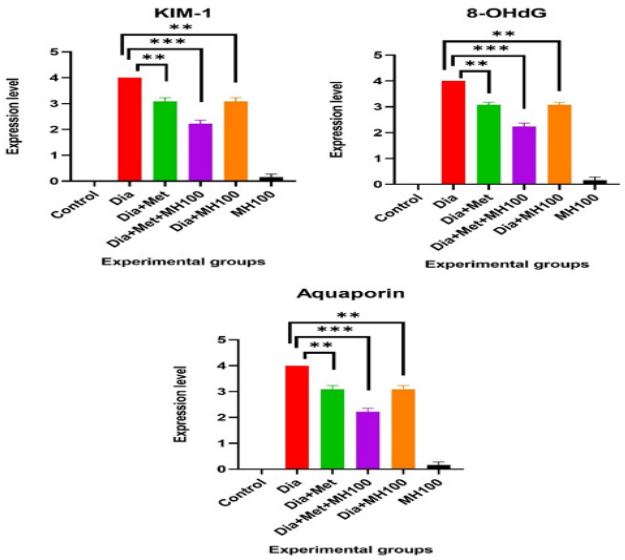

Figure 4. Statistical analysis results of KIM-1, 8-OHdG, and aquaporin expressions in renal tissues
KIM-1: kidney injury molecule-1, 8-OHdG: 8-hydroxydeoxyguanosine, Dia: Diabetes, Met: Metformin, MH: Morin hydrate. The results are presented as mean±SE. (***: p<0.001; **: p=0.0079)

Double Immunofluorescence Evaluation

8-OHdG and aquaporin expression levels were evaluated using the immunofluorescence technique to strengthen the results of this study. The control and MH100 groups showed no expressions of 8-OHdG and aquaporin (Figure 4, 5). In contrast, the diabetes group showed significant increases (p<0.01) in 8-OHdG and aquaporin expressions in the renal tubular epithelial cells compared with other groups (Figure 4, 5). These increases were prevented by morin hydrate administration at 100 mg/kg (Figure 4, 5).

DISCUSSION

This study has demonstrated the ability of the bioflavonoid morin hydrate either individually or in combination with metformin to inhibit diabetic nephropathy by targeting oxidative stress and 8-OHdG. We believe that this bioflavonoid and the targeted approach will contribute to providing new insights into the management of diabetic nephropathy. Several parameters were screened in blood and renal tissues

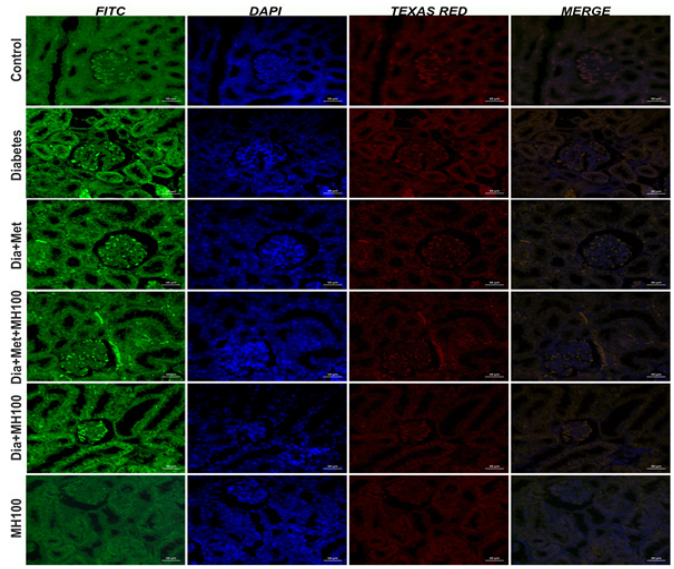

Figure 5. Double immunofluorescence findings of aquaporin (FITC) and 8-OHdG (TEXAS RED) in renal tissues
Dia: Diabetes, Met: Metformin, MH: Morin hydrate. IF, Bar: 50µm

using multiple techniques to accurately verify the potential pharmacological effects of morin hydrate.

Oxidative stress in diabetes develops from increased cellular reliance on the beta-oxidation of free fatty acids for energy as a compensatory mechanism for impaired glucose uptake and cellular metabolism, resulting in increased generation of ROS and insulin resistance.[8] This excessive production of ROS, especially in mitochondria, exceeds the ability of the cell to produce endogenous antioxidants, thereby promoting oxidative stress, which causes oxidative damage to the sensitive cellular macromolecules (viz., lipids, proteins, and nucleic acids).[2] Therefore, there exists a relationship between oxidative stress and podocyte damage, proteinuria, and tubulointerstitial fibrosis.[19] Measuring ROS levels to detect oxidative stress is difficult due to their short half-lives. Instead, the products of ROS action, including LPO, are measured to detect oxidative stress.[20] Thus, the excessive increase in LPO levels and breakdown of the antioxidant system in the diabetes group is an indicator of the development of oxidative stress in the kidneys of this group.

Elevated LPO levels and decreased SOD, CAT, and GPx activities have been detected in patients with diabetic nephropathy.[21] SOD, CAT, and GPx enzymes and tripeptide GSH scavenge ROS; hence, their decreased activities and levels in the present study not only strengthen oxidative stress but are also evidence of the exposure of renal glomeruli and tubular epithelial cells to free radical attack. The use of morin hydrate exerted promising therapeutic effects, including an obvious ability to inhibit LPO and strengthen the antioxidant system in kidney tissues. Previous studies have also demonstrated the ability of morin hydrate to suppress ROS production and improve the antioxidant defense system in models of various organ damage resulting from diabetes.[11-13] The positive effects of morin hydrate in our study can be attributed to its powerful antioxidant capacity that mitigates free radical accumulation and promotes the antioxidant system, thereby restoring cells to homeostasis. Morin hydrate chemically contains a double bond between C2 and C3, as well as a hydroxyl group that activates the double bond at the position C-3. This chemical structure also contains two hydroxyl groups in the B ring as depicted in **Figure 6**. This unique structure imparts morin hydrate the antioxidant potential, particularly its capacity to inhibit LPO.[10,22]

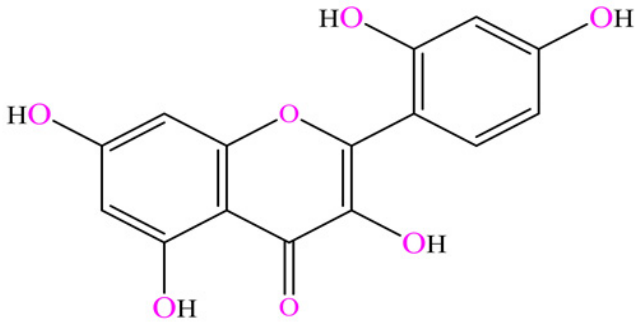

Figure 6. The chemical structure of morin hydrate

We also investigated the effect of the combination therapy of metformin and morin hydrate against STZ-induced diabetic nephropathy. This combination therapy achieved better effectiveness than the single treatment in terms of improving antioxidant enzyme and GSH levels and inhibiting DNA damage, thereby underscoring an important synergistic role of morin hydrate and metformin in managing patients with diabetic nephropathy. Similarly, Bahramzadeh et al.[23] reported that the combination of metformin with morin reduced insulin resistance, inflammation, and oxidative stress better than the single treatment in the skeletal muscle of mice fed on a high-fat diet.

The genetic material in the cell also does not survive the attack of ROS. Persistent oxidative stress resulting from diabetes causes DNA damage, which is greater and more persistent in mitochondrial DNA than in nuclear DNA. Mitochondrial DNA damage results in mitochondrial dysfunction, which exacerbates ROS production and complicates the oxidative damage.[24] 8-OHdG is a biomarker of DNA damage and is formed due to the attack of hydroxyl radicals on the nucleobase guanine (at the C8 position) in the genetic material.[25] In our study, elevated levels of 8-OHdG were detected in the diabetes group, indicating damage to the genetic material in the kidney tissue. Previous studies have also reported increased 8-OHdG levels in the serum and urine of patients with diabetic nephropathy compared with those in healthy individuals,[26,27] indicating the presence of an association between the progression of diabetic nephropathy and elevated levels of 8-OHDG. In addition to being an important marker of DNA damage and diagnostic indicator in diabetic nephropathy, 8-OHdG contributes to promoting apoptosis by activating the p53/Bax/cytochrome c/caspase-3 pathway[28] because of which it was targeted in this study. The decrease in 8-OHDG levels after morin hydrate administration indicates the capacity of this bioflavonoid to protect the genetic material. Moreover, the reduction in 8-OHdG suggests a potential decrease in DNA damage-related apoptotic signaling. We believe that morin hydrate exerts these effects through its ability to scavenge ROS and improve the antioxidant defense system.

Aquaporins are transmembrane channel proteins that facilitate the transport of water across biological membranes.[29] In diabetic nephropathy, aquaporins at the plasma membrane of epithelial tubular cells are dysregulated.[30] Increased expression of aquaporin was detected in the renal tubular epithelial cells of the diabetes group, which we believe is a response to increased ROS production, as aquaporins allow ROS permeation in oxidative stress.[31] Thus, morin hydrate exerted its protective effect on STZ-induced diabetic nephropathy by reducing the ROS-mediated activation of aquaporins.

Expression of KIM-1, a transmembrane protein, is increased in the apical surface of damaged proximal tubular epithelial cells.[32] This elevated expression is associated with a low glomerular filtration rate in diabetic nephropathy.[33] Therefore, KIM-1 is considered a biomarker for detecting kidney injury.[34] The elevated expression of KIM-1 in the renal tubular epithelial cells in our study may be a cellular response to increased damage in these cells, as increased expression of KIM-1 promote tissue repair and stimulate phagocytosis. The decrease in these expressions in the experimental groups treated with morin hydrate is evidence of the ability of this bioflavonoid to reduce renal tissue damage. A previous study also reported that morin decreased KIM-1 expression in a rat model of ifosfamide-induced nephrotoxicity.[35]

We also measured serum creatinine and BUN levels for evaluating the glomerular filtration rate. Creatinine is produced continuously in the muscle and is a final product of creatine and dietary protein metabolism. Elevated levels of serum creatinine are eliminated by kidneys.[36] BUN is the primary human protein metabolite used to evaluate kidney function, and its levels are elevated when the glomerular filtration rate is decreased.[37] High levels of creatinine and BUN are produced due to severe histopathological changes in the renal tissue, such as degeneration and necrosis. Morin hydrate successfully maintained the integrity of the renal tissue. This improvement in renal tissue was positively reflected in creatinine and BUN concentrations.

Limitations

Screening the proteins that regulate apoptosis, including B-cell lymphoma 2, Bax, Bad, and cytochrome c, and the levels of glucose and hemoglobin A1C (HbA1C) would certainly have strengthened the results of this study, which is a limitation of this study. Nevertheless, we inferred the therapeutic effects of morin hydrate against diabetic nephropathy using a wide range of biochemical parameters in the blood and renal tissues.

CONCLUSION

Based on our results, we summarize the conclusions as follows: 1) this study has confirmed the crucial role of oxidative stress in the pathophysiology of diabetic nephropathy, 2) morin hydrate exerts promising therapeutic effects against diabetic nephropathy without any toxic effects, 3) morin hydrate exhibited these pharmacological effects by suppressing oxidative stress and 8-OHdG levels in renal tissues, and 4) the combination therapy of morin hydrate and metformin achieved better effectiveness than the single treatment, emphasizing their synergistic role in the management of patients with diabetic nephropathy.

ETHICAL DECLARATIONS

Ethics Committee Approval

Ethical approval for this study was obtained from the Ethics Committee of Animal Experiments at Atatürk University (Date: 22.12.2022, Decision No: 292).

Informed Consent

Informed consent was not required because it was an animal study.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

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Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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