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Original article

Morin attenuates STZ-induced diabetic retinopathy in experimental animals

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ABSTRACT

Diabetic retinopathy (DR) occurs in untreated diabetic patients due to the strong influence of oxidative stress. Bioflavonoids are well known for their antioxidant property. Morin, a bioflavonoid, has been demonstrated for its antioxidant as well as antidiabetic activity. Thus, this research work intended to determine the ameliorative impact of morin in DR rats using STZ-induced type 1 diabetic model. To induce type 1 diabetic in rats STZ (60 mg/kg) was administered intraperitoneally. Grouping of animals was done as described below (n = 6), where, group I – normal control, group II – diabetic control, group III – morin (25 mg/kg), group IV – morin (50 mg/kg), and group V – metformin (350 mg/kg) were used. All the animals underwent treatment for 60 days as given above. It was observed that supplementation of morin (25 and 50 mg/kg) showed a noteworthy decline in elevated serum glucose level. Moreover, decrease in the level of LPO and improved activity of endogenous antioxidants (GPx, CAT, and SOD) was observed in morin treated groups. It also notably drops the concentration of TNF-α, IL-1β, and VEGF in the tissue homogenate of the retina. Furthermore, it increased the retinal thickness and cell count in the ganglion cell layer of the retina in diabetic animals. Hence, we can conclude that morin encumbers the progression of DR in diabetic animals, which may be via antioxidant property and suppression of TNF-α, IL-1β, and VEGF.

1. Introduction

Diabetic retinopathy (DR) is a prolonged hurdle occurring in diabetic patients due to an uncontrolled increase in blood glucose level and, if untreated, leads to visual impairment (Klein, 2007). Several mechanisms are involved in DR viz., microvascular damage, pro-inflammatory cytokines upregulation, generation of ROS, as well as increased VEGF expression are engaged in development of DR (Joussen et al., 2004; Guzman et al., 2016). Besides, high production of polyol (sugar alcohol) in addition to AGEs is the main culprits in the pathogenesis of DR (Brownlee, 2005). On the other hand, PKC is activated by DAG which is involved in various cellular functions as a microchip in cell signalling (Xia et al., 1994). Beta isoform of this kinase protein is known as PKC β aid in the development of DR. Activation of PKC β by DAG has been demonstrated in the retina of hyperglycaemic animals, results in microvascular damage in the retina (Koya and King, 1998) which may be due to high generation of ROS via PKC pathway.

Further, activation of certain enzymes (NADPH oxidase and xanthine oxidase) decreases antioxidant guard and increases ROS oppression (Al-Shabrawey et al., 2008) which worsen the state of retina in DR. Henceforth, the utilization of antioxidants can be beneficial for the treatment of DR.

Morin (2′,3′,4′,5,7-pentahydroxyflavon) is a dietary bioflavonoid, initially obtained from the individuals of Moraceae family plants (Sreedharan et al., 2009). Much evidence has confirmed that Morin has beneficial effects on several diseases. It has

Abbreviations: AGEs, Advanced glycated end products; BGL, Blood glucose level; BRB, Blood retinal barrier; CAT, Catalase; DAG, Diacylglycerol; GPx, Glutathione peroxidase; IL-1β, Interleukin 1 beta; LPO, Lipid peroxidase; PKC, Protein kinase C; ROS, Reactive oxygen species; SOD, Superoxide dismutase; STZ, Streptozotocin; TNF-α, Tumor necrosis factor alpha; VEGF, Vascular endothelial growth factor.

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demonstrated to be effective against a wide range of pathological conditions. Besides many biological effects, morin exerts potent antidiabetic (Abuohashish et al., 2013; Alkhamees, 2013; AlSharari et al., 2014) and antioxidants (Rice-Evans et al., 1996; Wu et al., 1994) activities. Thus, in this experimental work, an attempt was taken to examine the effect of morin in STZ persuade DR in rats.

2. Methodology

2.1. Drug and chemicals

Blood glucose, TNF-α, IL1-β, and VEGF kits were purchased from Sigma-Aldrich, USA. Every other chemical for this present evaluation was a commercial product of analytical reagent grade.

2.2. Animals

Normal healthy Albino Wistar rats (male), body weight ranges from 180 to 200 g, were selected for the study. They were kept in cages made up of polypropylene on a 12 h day and 12 h night cycle. The temperature was maintained at 22 ± 1 °C. Acclimatization of animals was done for 7 days prior to the commencement of experiment. Standard pellet diet was supplied with fresh drinking water ad libitum to the animals. Animal Ethics Committee of Jinshan Hospital of Fudan University (Ethics Number: FDJS201900769) approved this experiment.

2.3. Experimental design

A single dose of STZ (60 mg/kg; i.p.) was used to induce diabetes in the animals. STZ solution prepared by diluting with freshly prepared citrate buffer (0.1 M) with maintained pH 4.5. 48 h after STZ injection tail vein blood was collected to estimate BGL. Animals with blood glucose concentration 200 mg/dl or more was considered in this experiment.

Wistar rats were alienated into 5 groups (n = 6).

Group I – Normal control (saline solution- 0.5 ml/100 g).

Group II – Diabetic control (saline solution- 0.5 ml/100 g).

Group III – Morin (25 mg/kg).

Group IV – Morin (50 mg/kg)

Group V – Metformin (350 mg/kg)

All the animals underwent the treatment for 60 days as mentioned above. Blood glucose of fasting animals of group I-V were determine on day 0, 7th, 30th, and 60th (Abuohashish et al., 2013; Alkhamees, 2013; AlSharari et al., 2014).

2.4. Endogenous antioxidants, pro-inflammatory cytokines and VEGF content in retina

Retinal tissue homogenate (10%w/v) was prepared using trisphosphate buffer (50 mM), and the pH maintained at 7.4. Then centrifugation of the tissue homogenate was done for 10 min at 4 °C. The obtained supernatant was used to determine LPO (Ohkawa et al., 1979), GPx (Roturck et al., 1973), CAT (Sinha, 1972), and SOD (Marklund and Marklunf, 1974). TNF-α, IL-1β, and VEGF estimation were performed as per the instruction of the manufacturer.

2.5. Morphometric study

A morphometric computer-assisted image analysis technique was used for morphometric study of the retinal tissue section. For this purpose, Image J software was used as per the instructions of Jiang and co-researchers' published article (Jiang et al., 2010).

2.6. Statistical design/study

Obtained results were displayed as mean ± standard error from 6 animals in each group. Further a one-way analysis of variance was used followed by Tukey Multiple Comparison Test using the latest graph pad prism software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Blood glucose level

It is well known that STZ utilize GLUT2 and enter into the β-cells. After entering into the β-cells it alkylates the DNA causes damage or death of β-cells. This condition decreases the synthesis or production of insulin results increase in glucose level in the plasma. From Table 1, we can interpret that diabetic animals of 2nd group (group II) showed a noteworthy or significant elevation of blood glucose levels on 7, 30, and 60 days. Diabetic animals supplemented with morin (25 and 50 mg/kg) resulted in a notable decline of blood glucose level at 7, 30, and 60 days.

3.2. Endogenous antioxidants, pro-inflammatory cytokines and VEGF content in retina

We know that endogenous antioxidants are helpful to defend our organs against ROS or oxidative assaults. It protects our biological system from daily exposure of chemicals and heavy metals. During metabolic disorders imbalance between endogenous antioxidants and oxidants promote cellular damage. In the case of antioxidant enzyme activity of retinal tissue, a noteworthy or significant elevation in the LOP level was observed in group II animals. morin (25 and 50 mg/kg) resulted in a notable decline of LPO. Besides, endogenous antioxidant enzymes (GPx, CAT, and SOD) activity were also significantly improved by morin (25 and 50 mg/kg) in a dose-reliant way when compare to group II animals (Table 2).

It is well known that increase in cellular markers like inflammatory cytokines and VEGF are responsible for DR. Hence, in the retinal tissue homogenate estimation of those parameters is very essential. From Table 3, we can interpret that diabetic animals of

Table 1
Notable decline of blood glucose level by morin.

Groups	Treatment	Days			
		0	7	30	60
I	Normal Control	94.72 ± 2.87	97.36 ± 0.74	94.49 ± 1.02	95.63 ± 1.93
II	Diabetic Control	95.20 ± 2.51	208.59 ± 3.18$^{###}$	299.26 ± 4.30$^{###}$	365.74 ± 4.84$^{###}$
III	Diabetic + Morin (25 mg/kg)	96.04 ± 1.86	170.28 ± 2.70**	210.79 ± 3.17**	190.68 ± 2.06***
IV	Diabetic + Morin (50 mg/kg)	95.48 ± 1.80	161.38 ± 2.10***	195.41 ± 2.31***	168.53 ± 2.19***
V	Diabetic + Metformin (350 mg/kg)	94.02 ± 1.21	156.25 ± 2.96***	179.73 ± 3.05***	160. 02 ± 2.98***

$^{###}$ $p < 0.001$ – Group I vs Group II.

** $p < 0.01$ and $^{***}p < 0.001$ – Group III-V vs Group II.

Table 2
Noteworthy improvement of endogenous antioxidants in retinal tissue by morin.

Groups	Treatment	LPO (ηm of MDA per mg of protein)	GPx (μMole of the oxidized GSH/min/mg of protein)	CAT (μm H_2O_2/mg of protein)	SOD (U/mg of protein)
I	Normal Control	2.18 ± 0.32	7.91 ± 0.71	10.08 ± 0.85	8.84 ± 0.94
II	Diabetic Control	10.81 ± 0.96###	2.18 ± 0.26###	3.83 ± 0.98###	1.17 ± 0.29###
III	Diabetic + Morin (25 mg/kg)	6.13 ± 0.64**	4.08 ± 0.82**	5.88 ± 0.45**	4.58 ± 0.90**
IV	Diabetic + Morin (50 mg/kg)	2.99 ± 0.61***	7.03 ± 0.75***	8.66 ± 0.90***	7.02 ± 0.81***
V	Diabetic + Metformin (350 mg/kg)	3.09 ± 0.22***	6.81 ± 0.59***	6.96 ± 0.84***	6.80 ± 0.29***

p < 0.001 – Group I vs Group II.
** p < 0.01 and *** p < 0.001 – Group III-V vs Group II.

Table 3
Estimation of inflammatory cytokines and VEGF in retinal tissue homogenate.

Groups	Treatment	TNFα (pg/ml)	IL1β (pg/ml)	VEGF (pg/mg)
I	Normal Control	7.99 ± 0.89	12.10 ± 0.97	3.04 ± 0.20
II	Diabetic Control	57.91 ± 1.63###	66.02 ± 1.69###	7.91 ± 0.78###
III	Diabetic + Morin (25 mg/kg)	34.21 ± 1.02***	37.72 ± 1.80***	4.13 ± 0.61***
IV	Diabetic + Morin (50 mg/kg)	19.09 ± 1.28***	25.42 ± 1.92***	5.18 ± 0.27***
V	Diabetic + Metformin (350 mg/kg)	26.15 ± 1.12***	30.04 ± 1.07***	4.87 ± 0.42***

p < 0.001 – Group I vs Group II.
*** p < 0.001 – Group III-V vs Group II.

2nd group (group II) showed a noteworthy or significant elevation of VEGF and inflammatory cytokines (TNF-α and IL-1β). Inflammatory cytokines and VEGF activity was also notable declined by Morin (25 and 50 mg/kg) in a dose-reliant way when compare to group II animals as shown in Table 3.

3.3. Morphometric analysis

In the morphometric analysis, there was a notable decrease in thickness of the retina. Besides, a noteworthy decrease in quantity of cells in ganglion layer of the animals in group II. A noteworthy improvement was observed in the morin treated groups in a dose-reliant way (Table 4).

4. Discussion

STZ is used to induce diabetic conditions in experimental animals. β-cells in pancreas contain GLUT2. With help of GLUT2, STZ enter into the β-cells, where it alkylates the DNA causes damage or death of β-cells (Szkudelski, 2001). This study showed a potent antihyperglycemic effect of Morin in STZ-induced type 1 diabetes in a dose-dependent fashion in male Wistar rats, which supports the previously reported antidiabetic activity of morin.

DR is one of the most widely recognized obstacles secondary to diabetes mellitus. An increase in ROS generation induced by high blood glucose levels in diabetic condition act as a pivotal factor in the progression of DR. In this condition, retinal cells are highly at risk to oxidative assaults as it contains a towering amount of fatty acids which are polyunsaturated in nature. A high level of glucose oxidation in retinal cells also aid in the pathogenesis of DR (Anderson et al., 1984). In this view, we have observed a notable increment in the LPO level and a noteworthy decrease in endogenous antioxidants (GPx, CAT, and SOD) level. Supplementation with morin significantly reduced LPO and restored the bustle of endogenous antioxidants (GPx, CAT, and SOD). In DR pathogenesis, ROS is mostly engaged (Wu et al., 2014; Pacher et al., 2005), which appears to be appropriate for the therapeutic interventions, for example, the use of antioxidants from natural sources. Morin is a dietary bioflavonoid that has demonstrated to show potent antioxidant properties in different diabetic models (Abuohashish et al., 2013; Alkhamees, 2013; AlSharari et al., 2014).

Inflammatory cytokines acts as a pivotal function in progress of both early and late stages of DR. A crucial factor, NFκβ is also engaged in upregulation of TNF-α and IL-1β in turn aid in the advancement of DR (Yang et al., 2006). Several metabolic factors in diabetes, including high production of AGEs and polyols and increased PKC activation activates NFκβ. Moreover, it also demonstrated that increased free radicals formation causes oxidative stress and, thus, the activation of NFκβ. A study reported that TNF-α and IL-1 accumulate in vitreous fluid of the DR patients (Sato et al., 2009). It is well known that TNF-α is one of the essential inflammatory cytokines which is another crucial factor in proliferation, differentiation, as well as cellular apoptosis (Gao et al., 2007). On the other hand, the presence of IL-1α in vitreous fluid suggests that it might have a role in DR. Further, upregulation of TNF-α can induce adhesion of leukocytes in the retina and BRB collapse (Ben-Mahmud et al., 2004; Huang et al., 2011). The influence

Table 4
Morin improved retinal morphology in diabetic rats.

Groups	Treatment	Retinal Thickness (μm)	Number of cells in ganglion cell layer/100 μm
I	Normal Control	649.82 ± 50.82	8.45 ± 0.63
II	Diabetic Control	351.16 ± 41.38###	4.10 ± 0.18###
III	Diabetic + Morin (25 mg/kg)	444.69 ± 46.51***	6.15 ± 0.30***
IV	Diabetic + Morin (50 mg/kg)	496.90 ± 51.76***	7.19 ± 0.26***
V	Diabetic + Metformin (350 mg/kg)	478.35 ± 31.62***	6.68 ± 0.86***

p < 0.001 – Group I vs Group II.
*** p < 0.001 – Group III-V vs Group II.

of this cytokine experimentally demonstrated in TNF-α knockout diabetic mouse where suppression of BRB breakdown suggests the crucial influence of TNF-α BRB breakdown (Huang et al., 2011).

VEGF helps in the progression of the early stage of DR. In diabetes high glucose level increases AGEs which regulates VEGF expression (Treins et al., 2001). Phospholipase C and PKC and Ca^{2+} also promote VEGF induced vascular permeability and neovascularization (Wu et al., 1999). It was discovered that intraocular administration of VEGF in non-diabetic animals elicited abnormalities in retina resemble DR (Aiello et al., 1997), which suggests its role in DR. In this study, we found that supplement of Morin remarkably reduced the bustle of TNF-α, IL-1β, and VEGF.

However, in morphometric study, animals of the diabetic control group showed a reduction in the thickness of the retina. Moreover, the decline in the number of cells in the ganglion cell layers diabetic control animals. Treatment with Morin improved both parameters indicates its defensive role against STZ-induced DR in animals.

5. Conclusion

From the above outcome of the experiment we can come to a conclusion that Morin ameliorates DR may be via suppression of cellular markers TNF-α, IL-1β, and VEGF. Besides, Morin also enhanced the thickness of the retina, and the number of cells in the ganglion cell layers. Hence, Morin showed its defensive role against STZ-induced DR in animals.

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