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ORIGINAL ARTICLE

Effect of taxifolin on development of retinopathy in alloxan-induced diabetic rats

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ABSTRACT

Purpose: Diabetic retinopathy (DR) is one of the leading causes of blindness. In DR patients, antioxidant defence is disrupted, and production of reactive oxygen species and pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumour necrosis factor alpha (TNF-α) increases. Taxifolin has been reported to suppress reactive oxygen species, IL-1β and TNF-α production. The aim of this study is to biochemically and histopathologically examine the protective effect of taxifolin against DR damage induced by alloxan.

Materials and methods: Alloxan received rats with a blood glucose level of ≥250 mg/dL were divided into taxifolin-treated (TAX) ($n = 6$), diabetic control (DC) ($n = 6$) groups. There were rats received only saline in non-diabetic control (NC) group ($n = 6$). Taxifolin (50 mg/kg) was orally administered to the TAX group rats. DC and NC rats received the same volume of saline as a solvent. This procedure was repeated once a day for 3 months. At the end of this period, animals were killed by high dose thiopental sodium anaesthesia. Histopathological examinations were then performed on excised rat eyes. Malondialdehyde (MDA), total glutathione (tGSH), IL-1β and TNF-α levels were measured in obtained blood samples.

Results: MDA, IL-1β and TNF-α levels were significantly increased in blood samples of DC group rats with hyperglycemia induced by alloxan compared with NC group ($p < 0.0001$), and decreased in the TAX group compared with the DC group ($p < 0.0001$). The levels of tGSH were significantly decreased in blood samples of DC group rats compared with NC group ($p < 0.0001$), and increased in the TAX group compared with the DC group ($p < 0.0001$). Histopathologically, retinal ganglion cells of the TAX group had a slightly dilated and congested blood vessel, and severe damage was inflicted to the retinal ganglion cell layer of the DC group.

Conclusions: Experimental results suggest that taxifolin may be beneficial in the treatment of DR.

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Alloxan; diabetic retinopathy; retina toxicity; taxifolin; rat

Introduction

Diabetes mellitus (DM) is an endocrine disease that develops by absolute or relative insufficiency of endogenous insulin and is characterized by chronic hyperglycemia[1]. Type 2 DM accounts for around 90% of all cases of diabetes[2]. In DM, microvascular damage is seen in many organs[3]. However, diabetic retinopathy (DR) is the most important microvascular injury associated with DM[4] DR is one of the leading causes of blindness that has recently become an issue in industrial countries[5]. It accounts for approximately 12% of blindness in USA[6]. It is shown to be the most common cause of adult blindness[7]. Although there are many scientific studies on the pathogenesis of DR, its pathogenesis is still not fully understood. There are various studies suggesting that overproduction of free oxygen radicals (FORs) is responsible for the development of DR[8]. In patients with DR, antioxidant defence is impaired, formation of free radicals increases and more cytotoxic aldehydes such as malondialdehyde (MDA) – a product of lipid peroxidation – are formed[9]. Furthermore, the risk of severe DR has been found to be associated with the presence of oxidative stress markers in diabetic patients[10]. Hyperglycemia has several mechanisms associated with each other that can explain the cause of elevated plasma free radical concentration[11]. Cinici et al.[12] also showed that hyperglycemia leads to retinopathy based on biochemical and histopathological results. In addition, the role of pro-inflammatory cytokines such as interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) in DR has also been reported[13,14]. Taxifolin (3,3′, 4′, 5,7-pentahydroxyflavone), of which we will investigate the protective effects against DR damage, is a flavanone found in the contents of onion, milk thistle, French maritime and Douglas fir bark[15]. Antioxidant activity of taxifolin has been proved[16]. It has been reported in another study that taxifolin suppressed the production of

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FORs, IL-1β and TNF-α[17]. All this information suggests that taxifolin may be beneficial in the treatment of hyperglycemia-induced retinopathy. No information or studies have been found in the literature on taxifolin's effect on DR. Therefore, in this study, we aimed to examine the effect of taxifolin on development of retinopathy in alloxan-induced diabetic rats via biochemical and histopathological methods.

Materials and methods

Experimental animals

Albino wistar male rats weighing 220–295 g were used in the experiments. All of the rats were obtained from Ataturk University Medical Experimental Application and Research Centre. Animals were housed and fed for 1 week at normal room temperature (22 °C) in the pharmacology laboratory before experimentation so that the rats could adapt to the environment. Animal experiments were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and were approved by the local animal ethics committee (2018–2/43)

Chemicals

Thiopental sodium used in the experiments was procured from Ulagay (Turkey), alloxan was supplied from SIGMA (USA), and taxifolin was obtained from Evalar (Russia).

Experimental groups

Rats were divided into taxifolin treated (TAX), diabetic control (DC) and non-diabetic control (NC) groups ($n = 6$ for each group).

Induction of diabetes

In order to create a Type 2 diabetes model, alloxan dissolved in distilled water was intraperitoneally injected into the rats for three consecutive days at a dose of 120 mg/kg. Three days after alloxan administration, fasting blood glucose was measured in blood samples taken from the tail veins of the rats. A blood glucose metre available on the market was used to measure blood glucose levels. Animals with a blood glucose level of 250 mg/dL or more were included in the TAX and DC groups. As is known, rats with a blood glucose level above 250 mg/dL are considered diabetic[18]. The rats of the NC group were received only saline.

Experimental procedure

Taxifolin (50 mg/kg) was orally administered to TAX group of diabetic rats. DC and NC rats were given the same volume of saline as a solvent. This procedure was repeated once a day for 3 months. At the end of this period, all rats were killed by high dose thiopental sodium anaesthesia and the eyeball was removed under sterile conditions. Histopathological examinations were then performed on the excised eyes. In addition, MDA, total glutathione (tGSH), IL-1β and TNF-α levels were measured in blood samples taken from the tail veins before the animals were killed. Biochemical and histopathological results obtained from TAX and NC groups were compared with the DC group.

Biochemical analyses

From the blood samples obtained from animals, serum MDA, tGSH, IL-1β and TNF-α levels were investigated.

MDA analysis in serum

MDA measurements were based on the method used by Ohkawa et al.[19] involving spectrophotometrical measurement of absorbance of the pink-coloured complex formed by thiobarbituric acid and MDA. The serum sample (0.1 mL) was added to a solution containing 0.2 mL of 80 g/L sodium dodesil sulphate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate, and 0.3-mL distilled water. The mixture was incubated at 95 °C for 1 h. Upon cooling, 5 mL of n-butanol:-pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane.

tGSH analysis in serum

The analysis was performed according to the method defined by Sedlak and Lindsay[20]. DTNB (5,5′-dithiobis [2-nitrobenzoic acid]) disulfite is chromogenic in the medium, and DTNB is reduced easily by sulfhydryl groups. The yellow colour produced during the reduction is measured by spectrophotometry at 412 nm. For measurement, a cocktail solution (5.85 mL 100 mM Na-phosphate buffer, 2.8 mL 1 mM DTNB 3.75 mL 1 mM Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) and 80 µL 625 U/L Glutathione reductase) was prepared. Before measurement, 0.1 mL meta-phosphoric acid was added to 0.1 mL serum and centrifuged for 2 min at 2000 rpm for deproteinization. The 0.15 mL cocktail solution was added to 50 µL of supernatant. The standard curve was obtained by using GSSG.

IL-1β and TNF-α analysis in serum

Serum IL-1β and TNF-α concentrations were measured using rat-specific sandwich enzyme-linked immunosorbent assay Rat IL-1β ELISA Kit (Cat no: YHB0616Ra, Shanghai LZ) and Rat TNFα ELISA kits (Cat no: YHB1098Ra, Shanghai LZ). Analyses were performed according to the manufacturers' instructions. Briefly, monoclonal antibody specific for rat IL-1β and TNF-α were coated onto the wells of the micro plates. The serum samples, standards and biotinylated monoclonal antibody specific and streptavidin-HRP were pipetted into these wells and then incubated at 37 °C for 60 min. After washing, chromogen reagent A and chromogen reagent B were added which is acted upon by the bound enzyme to produce a colour. It was incubated at 37 °C for 10 min than stop solution was added. The intensity of this coloured product is directly

proportional to the concentration of rat IL-1β and TNF-α present in the original specimen. At the end of the course, the well plates were read at 450 nm via an microplate reader (Bio-Tek, USA). The absorbance of the samples was estimated with formulas that used standard graphics.

Statistical analysis

The results are expressed as "mean ± standard error of mean" (x ± SEM). Significance of difference between the groups was determined using one-way analysis of variance (ANOVA) test followed by post-hoc Tukey test. All statistical analyses were performed with "SPSS statistical software" (Version 18) and p values < 0.05 were considered significant.

Results

Biochemical findings

Fasting blood glucose test

Fasting blood glucose levels of DC and TAX group rats were significantly higher than the NC group ($p < 0.0001$). The difference in fasting blood glucose levels between DC and TAX groups was not statistically significant ($p > 0.05$) (Figure 1).

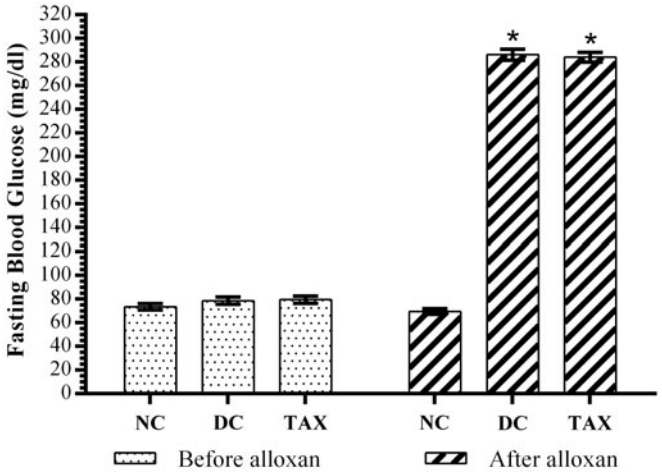

Figure 1. Fasting blood glucose levels of NC, DC and TAX groups Fasting blood glucose levels of study groups before and after alloxan administration. *$p < 0.0001$, according to NC group.

MDA and tGSH analysis results

The amount of MDA in the blood serum of the DC group was significantly higher than that of the NC group ($p < 0.0001$). Levels of MDA in the TAX group were lower than that of the DC group ($p < 0.0001$). Fasting blood glucose level in the TAX group was measured at approximately the same level as the NC group ($p > 0.05$). The levels of tGSH were significantly decreased in blood samples of DC group rats compared with NC group ($p < 0.0001$), and increased in the TAX group compared with the DC group ($p < 0.0001$) (Figure 2).

IL-1β and TNF-α analysis results

IL-1β and TNF-α levels were higher in the serum of DC group rats compared with NC group ($p < 0.0001$), and decreased in the TAX group compared with the DC group ($p < 0.0001$). The levels of IL-1β and TNF-α were very close to each other in the NC and TAX groups ($p > 0.05$) (Figure 3).

Histopathological findings

Figure 4a shows the normal histology of the ganglion cell layer (GCL), inner plexiform layer, inner nuclear layer (INL), outer plexiform layer and outer nuclear layer in the retina tissue of the NC group. Dilated and congested blood vessels (flat arrow) were seen in the retinal GCL of hyperglycemia-induced DC group, and destruction (double arrow) and polymorphonuclear leukocytes infiltration (striped arrow) were observed throughout all other layers (Figure 4b). In addition, severe GCL damage was detected in retinal tissue of DC group (Figure 4c). Dilated and congested (flat arrow) and papillary structure formation (round arrow) were also observed in the GCL of the retina (Figure 4d). Furthermore, oedema (striped arrow), dilated and congested blood vessels (straight arrow), and full-layer destruction involving outer and INL (double-sided arrow) was detected in retinal GCL in the DC group (Figure 4e). Retinal tissue of the TAX groups was

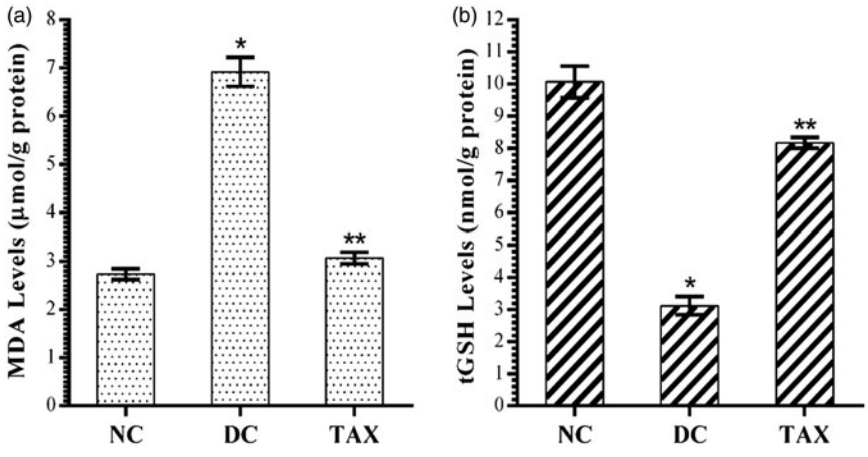

Figure 2. MDA and tGSH levels of NC, DC and TAX groups. *$p < 0.0001$, according to NC group. **$p < 0.0001$, according to DC group.

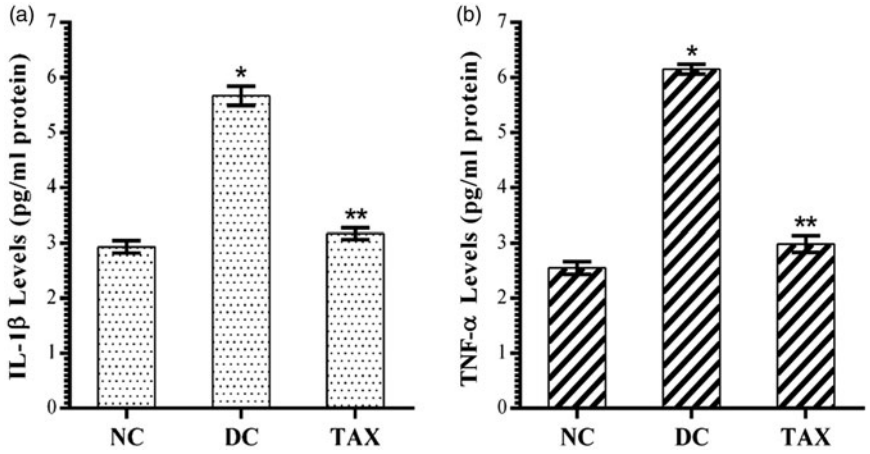

Figure 3. IL-1β and TNF-α levels of NC, DC and TAX groups. $^{*}p < 0.0001$, according to NC group. $^{**}p < 0.0001$, according to DC group.

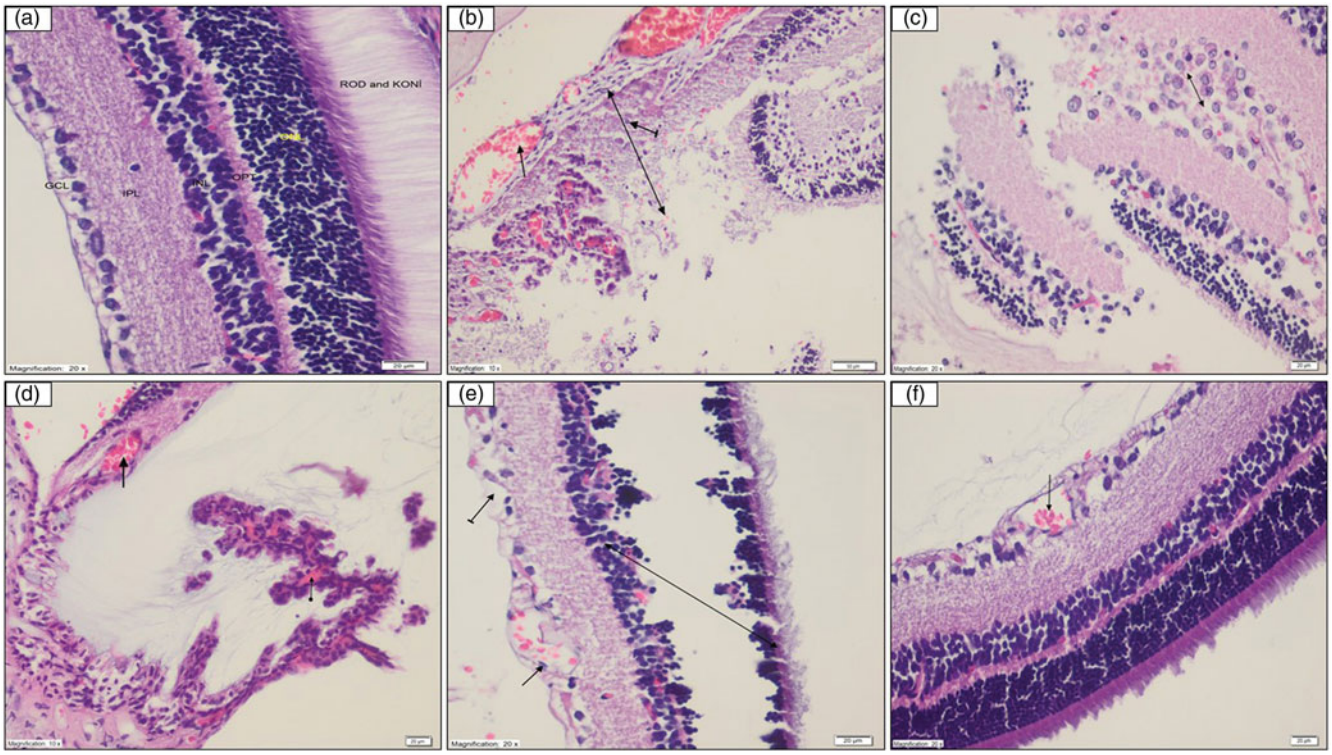

Figure 4. Histopathological appearance of the retina tissue in study groups. (a) Retina of the NC group. GCL: ganglion cell layer, IPL: Inner plexiform layer, INL: inner nuclear layer, OPT: outer plexiform layer, ONL: outer nuclear layer, ROD and KONI (H&E ×400) (b) Dilated and congested blood vessels in the GCL of damaged retina (flat arrows), and destruction along all other layers (double arrow), and PMNL (striped arrow) were seen in retina of the DC group rats (H&E ×200) (c) Severe GCL damage in retina of the DC group rats (H&E ×400) (d) Dilated and congested blood vessels (flat arrow) and papillary structure (round arrow) formation in the GCL in retina of the DC group rats (H&E ×400) (e) Oedema (striped arrow), dilated and congested (flat arrow), full-layer destruction involving outer and INL in the GCL in retina of the DC group rats (H&E ×400) (f) Near-normal appearance of GCL in retina tissue of TAX group, except continuing dilated and congested blood vessels (H&E ×400).

observed to be normal except for the dilated and congested blood vessels through the GCL (Figure 4f).

Discussion

In this study, the effect of taxifolin on retinopathy in rats with alloxan-induced hyperglycemia was investigated biochemically and histopathologically. Biochemical results showed that type II diabetes developed in animals treated with alloxan. In our study, hyperglycemia was taken as a base criterion for experimental diabetes in rats. As noted above, animals with a blood glucose level of 250 mg/dL or more are considered diabetic[18]. Previous studies have also reported that oxidative stress develops in the retinal tissue of animals treated with alloxan with a blood glucose level of 250 mg/dL or more[12]. There are studies arguing that hyperglycemia creates DR[21,22]. Information in the literature suggests that oxidative stress is not ignored in the development of DR[8]. As our experimental results indicate, MDA level increased significantly in the DC group and tGSH level decreased. As is known, MDA is a final product of lipid

peroxidation triggered by increased FORs produced as a result of oxidative stress[23]. MDA causes cross-linking of cell membrane components, inactivating membrane receptors and enzymes and leads to cell damage[24]. In experimental studies of Cinici et al.[12], histopathological damage has been reported in retinal tissue of hyperglycemic animals with high MDA levels.

Oxidative stress has been clearly documented in literature as a fundamental mechanism for the development of DR[25]. As is known, oxidative stress is explained by increased reactive oxygen species production and decreased antioxidant defence systems. Therefore, antioxidant administration is thought to inhibit retinal damage in diabetic animal models[26]. In our study, which is fully consistent with the literature, it was found that hyperglycemia caused a reduction in tGSH. Reduced GSH levels in diabetic rats were also shown by Al-Dosari et al.[27] and these findings confirm the validity of our results. Tugcu et al.[28] also reported that oxidative stress develops in the retinal tissue of diabetic rats with reduced GSH levels.

However, the role of pro-inflammatory cytokines such as IL-1β and TNF-α has also been reported in DR[13,14]. In this study which was conducted based on this literature information, IL-1β and TNF-α levels were found to be high in blood serum of hyperglycemic rats in which histopathological damage was detected. Pro-inflammatory IL-1β and TNF-α levels are also used as important parameters in different retinal damage models[29,30].

In our study, biochemical results were found to be consistent with histopathological findings. Dilated and congested blood vessels, oedema, destructive damage and formation of papillary structures were observed in the retinal GCL in the DC group. However, the TAX group was found to have low MDA, IL-1β and TNF-α levels and high tGSH levels showed a normal appearance of GCL in the retina except dilated and congested blood vessels. In the present study, neovascularization – known as an important sign of alloxan-induced DR – was not observed. Absence of neovascularization in alloxan-induced DR was also reported in the studies of Olurishe et al.[31]. In the study of Cinici et al.[12], it was emphasized that in addition to histopathological findings such as oedema and Ganglion cell loss, neovascularization was also observed in retinal tissue of rats with alloxan-induced hyperglycemia. These specified lesions are evaluated in literature as indicative of hyperglycemic damage[31]. Especially dilated and congested blood vessels are important indicators of DR[32]. It is known that oedema occurs at different rates in both non-proliferative retinopathies and in proliferative cases[33]. In many studies, diabetes has been experimentally shown to cause ganglion cell loss in retina[12,34]. There is no information that taxifolin prevents retinal damage induced by hyperlipidemia due to alloxan. However, taxifolin has been proven to be a potent antioxidant[16]. The beneficial effects of antioxidant activity in preventing retinal injury have been demonstrated in the literature[29]. Furthermore, doses of taxifolin that inhibited retinal injury also inhibited the elevation of IL-1β and TNF-a. Previous studies have reported that retinal tissue damage is associated with an increase in oxidants, IL-1β and TNF-α, and a decrease in tGSH[35]. In conclusion, blood glucose levels of rats treated with alloxan were elevated. Significant histopathological findings were observed in retinal tissue of rats with high MDA, IL-1β and TNF-α levels and low tGSH levels in the DC group. However, blood serum MDA, IL-1β, TNF-α and tGSH levels of TAX group were close to the NC group, and histopathological findings in the TAX group were nearly normal (except for dilated and congested vessels). These results suggest that taxifolin may be beneficial in the treatment of diabetic retinal damage.

Disclosure statement

No potential conflict of interest was reported by the authors.

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