Egypt. J. Biophys. Biomed. Eng., **Vol., 25** No. 1 pp.25- 35 (2024)

"The Improvement of Retinal Protein by Glycine and the Possible Mitigation of Experimental Diabetic Retinopathy"

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THIS study aimed to explore the improving role of glycine against induced diabetic retinopathy (DR) in experimental animals. Eighty- four female Wister rats were involved in the study and were divided into four groups: (1) The control group (C, n=21); (2) The glycine-supplemented group (G) with 10 gm/L in their drinking water (n=21); (3) Diabetic retinopathy group (DR) was intraperitoneally injected with STZ (n=21); and (4) The treated group with glycine (DR+G) (n=21). The protein content, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and the damage to the DNA for retinal protein were evaluated after one week, two weeks, and four weeks, respectively, to assess the efficacy of glycine in improving the retinal protein. The elevation of protein concentration induced by DR was reduced by glycine and revealed a maximal decrease of 15.08% ($P<0.05$), 7.06% ($P>0.05$), and 2.05 %($P>0.05$) after 1, 2, and 4 weeks, respectively. The protein structure was improved by glycine and was indicated by the SDS-PAGE pattern. The damage in the DNA of retinal protein was enhanced by glycine after four weeks at the level of the number of tailed cells, tail length, the percent of DNA in the tail, and tail moment. Supplementing the amino acid glycine to an experimental model of rats with DR suggests therapeutic means for DR by improving the retinal protein.

Keywords: Glycine, diabetic retinopathy, protein concentration, SDS-PAGE, DNA damage

Introduction

Diabetic retinopathy (DR) is a substantial complication of Diabetes Mellitus (DM) on the microvascular level. It is the most abundant cause of blindness worldwide [1]. An analysis conducted in 2010 reported that DR is responsible for about 0.25% of blind people worldwide [2]. This percentage increased dramatically to about 1.1% by 2015 [3]. DR is characterized by the growth of new blood vessels in the retina (neovascularization) [1]. The pathogenesis of DR is considered a multifactorial condition. The structural and functional alteration in the retina related to DR mainly results from the hyperglycemic condition associated with DM. Elevated levels of circulating glucose promote protein glycation and the establishment of advanced glycation end products (AGEs), activation of polyol pathways, protein kinase cytokines (PKC), releasing of reactive oxygen species (ROS), and increased oxidative stress [4]. Additional attributes characterizing DR include inflammation, apoptosis, blood-retinal barrier (BRB) breakdown, and consequent permeability changes [1]. Although microvasculopathy is a characteristic of DR, apoptosis of retinal neurons and consequent neurodegeneration were indicated in many studies [4,5]. Recent work revealed that neurodegeneration occurs more rapidly than vascular degeneration in DR [6-8].

Numerous therapeutic techniques have been widely assessed and used for managing and controlling DR [3]. Laser photocoagulation was the most frequently used [1]. Many studies were interested in the anti-diabetic effect of some amino acids and the possible intake into treatment and protection trials against DR [1,9,10]. One such amino acid is glycine, which is the simplest and most important nonessential amino acid in the human body. It is now considered a new trend in treating DR due to its high antioxidant and anti-glycation effects [11]. Glycine is synthesized endogenously from serine, threonine, and choline [12]. However, some conditions such as diabetes,

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DOI : 10.21608/EJBBE.2024.267322.1071
Received 2/2/2024 , accepted 5/3/2024

primarily type 2, showed an acute shortage of glycine, thus requiring dietary supplementation [13]. This study aimed to investigate the protective role of glycine in rats with streptozotocin (STZ)-induced DR. Total protein concentration, retinal protein structure by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and DNA damage using comet assay were evaluated with or without treatment with glycine.

Materials and methods

Experimental animals

Eighty-four Wistar rats (weighing 200±20 gm) were obtained from the animal house of the Research Institute of Ophthalmology. This study was performed with the approval of the Institutional Animal Care and Use Committee at Cairo University for using laboratory animals in ophthalmic and vision research. The rats were kept in a standard cycle of 12 h. of light and 12 h. of dark with a balanced diet and free access to water at 25°C ± 2°C. Before the experiment, all rats' eyes were examined by a slit-lamp biomicroscope to indicate the absence of any signs of intraocular or periocular defects in all eyes. Four main groups of rats were divided as follows: (1) The control group (C) that had not received STZ or any treatment (n=21). (2) The glycine group (G) received 10 gm/L glycine in their free accessed drinking water for 1, 2, and 4 weeks (n=21) according to Gholami et al. 2019 **[11]**. (3) The diabetic retinopathy group (DR) in which diabetes was induced by a single intraperitoneal injection of 40 mg/kg STZ in 0.1 M (pH=4.4) freshly prepared sodium citrate buffer (n=21). (4) Another diabetic retinopathy group (n=21) was treated with glycine (DR+G). After the onset of diabetes, 6-8 weeks are required to establish DR according to previous works [31, 32]; the rats freely received glycine in drinking water for 1, 2, and 4 weeks, respectively. A glycine solution with a molarity of 130 mM and a concentration of 1% was prepared by dissolving 10 gm of glycine in one liter of distilled water. Further, all groups were sacrificed after the estimated periods.

Sample collection

Fasting blood glucose levels were monitored after 72 h of STZ injection. Animals with blood glucose levels >240 mg/dl were considered diabetic. Rats were sacrificed under anesthesia (by injection of high doses of xylazine and ketamine hydrochloride), and retinae were dissected from the eyeballs and collected carefully. Part of the retinae was used for DNA damage evaluation. The rest was weighed accurately and homogenized with RIPA (protein lysis buffer with protease inhibitor) (SigmaAldrich, Inc., St. Louis, MO, USA) (5 mg tissue/300 µL protein lysis buffer) using cell homogenizer (type Tübingen 7400, Germany). The homogenate was placed on ice for 45 min, sonicated for 20 s, and centrifuged for 15 min. at 10,000 rpm (Awel centrifuge MF 20, France). The supernatant was separated for total protein measurements and SDSPAGE analysis.

Total protein measurement

The total protein content of the retina was evaluated according to Lowry et al., 1951 [14]. The absorbance readings for all samples were taken at 750 nm using a spectrophotometer (Thermo Fisher Scientific, Madison WI 53711, USA, Evo 600). The total protein concentration in each sample was calculated using a known standard protein concentration of bovine serum albumin.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The retinal protein structure was analyzed according to Laemmli 1970 [15] using 3% stacking gel and 12% separating gel. The gel was scanned using a scanner model SG800 imaging densitometer (BioRad Laboratories Inc., USA).

Comet assay

The DNA damage was evaluated using comet assay, a simple and sensitive electrophoresis method, according to Olive and Banáth, 2006 [16]. DNA comets were visualized using a Nikon Optiphot-2 epifluorescence microscope and photographed with an attached camera (Sony CCD-IRIS, Minato, Tokyo, Japan). The system was connected to Pentium 1133 MHZ PC, which provided images on the comet assay II software (Perceptive Instruments, UK). The software analyzes duplicated comet images for the tailed cells, tail length (µm), percent of DNA in the tail, and tail moment.

Statistical evaluation

The results were presented as mean ± standard deviation, and the deviations between distinct groups were investigated using a commercially available software package (SPSS 11 for Windows; SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance and Student's t-test was used to compare the distinct groups. The result was considered significant at a $P < 0.05$.

Results

Total protein content

The total soluble protein contents of rats' retinas for different experimental groups after one, two, and four weeks are shown in Figure (1). The total protein content for the control group was 15.58 ± 0.35 mg/g tissue wet wt. The total protein contents of the rat's retina treated with glycine were 16.58 ± 0.28 mg/g tissue wet wt. ($P>0.05$), 17.78 ± 0.36 mg/g tissue wet wt. ($P<0.05$), 17.70 ± 0.54 mg/g tissue wet wt. ($P<0.05$) with percentage changes of 6.41%,14.12%, and 13.6% compared to the control group after 1, 2, and 4 weeks, respectively. Moreover, the total protein contents progressively increased in DR groups after 1, 2, and 4 weeks to 18.05 ± 0.14 mg/g tissue wet wt. ($P<0.05$), 20.67 ± 0.3 mg/g tissue wet wt. ($P<0.01$), 21.97 ± 0.32 ($P<0.001$) mg/g tissue wet wt. with percentage changes of 15.58%, 32.67%, and 41.01% compared to the control group. Furthermore, the DR group treated with glycine showed improved retinal total protein content with values of 17.93 ± 0.15 mg/g tissue wet wt. ($P<0.05$), 16.68 ± 0.62 mg/g tissue wet wt. ($P>0.05$), 15.9 ± 0.26 mg/g tissue wet wt. ($P>0.05$). It reduced the percentage change to 15.08%, 7.06%, and 2.05 % after 1, 2, and 4 weeks, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic pattern with different optical densities and molecular weights of the retinal protein after one week for the control (C), glycine (G), diabetic retinopathy (DR), and diabetic retinopathy treated with glycine (DR+G) are shown in Figure (2). The electrophoretic pattern of the control group was separated into nine fractions at molecular weights of 160 kDa,122 kDa, 99 kDa, 86 kDa, 70 kDa, 65 kDa, 58 kDa, 42 kDa, and 25 kDa, respectively. The group treated with glycine for one week (fig. 2) showed a slight shift in the 160 kDa peak to 165 kDa and 122 kDa to 126 kDa. In addition, a new peak appeared with a molecular weight of 36 kDa and an insignificant reduction in the intensity of the peaks at the molecular weights of 99 kDa and 25 kDa. After one week of DR, the separation pattern revealed a marked increase in the molecular weight of the peak at 160 kDa to 180 kDa with further increased intensity. Moreover, there was a slight shift of the peak at molecular weight of 122 kDa to 126 kDa and a disappearance of the peak at 99 kDa. Furthermore, the peak at 86 kDa was shifted to 81 kDa, accompanied by increased peak intensity. In addition, the pattern was characterized by a decrease in the intensity of the peaks at 65 kDa, 58 kDa, and 42 kDa; the disappearance of the 70 kDa; the formation of two new peaks at 48 kDa and 36 kDa; and a significant increase in the intensity of the peak at 25 kDa. Treatment of DR with glycine for one week partially improved the shift of 160 kDa and 122 kDa and reduced the intensity of the

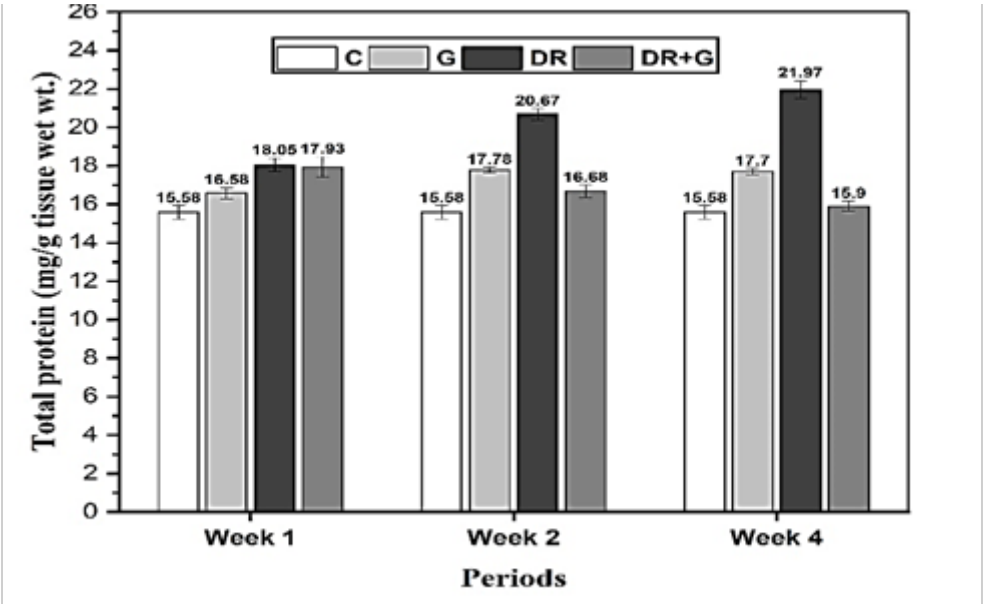

Fig. 1. Total soluble protein content of rats' retina for the control(C), glycine(G), diabetic retinopathy (DR), and diabetic retinopathy rats treated with glycine (DR+G) after 1, 2, and 4 weeks, respectively.

newly formed 81 kDa peak. Moreover, the new peaks at 48 and 36 kDa showed reduced intensity and improvement in the intensity of the peak at 25 kDa.

Glycine treatment for two weeks (fig. 3) produced a separation pattern similar to one week and a slight shift in the 99 kDa and 70 kDa toward high molecular weight. After two weeks of DR, the SDS-PAGE pattern revealed an increase in the molecular weight of the peaks at 160 kDa and 122 kDa. There was a slight shift in molecular weight and the intensities of the peaks at 65, 58, and 42 kDa. In addition, the pattern was characterized by a disappearance of 70 kDa, the formation of three new peaks at 110 kDa, 52 kDa, and 36 kDa, and a slight shift in the peak at 25 kDa to 22 kDa with a remarkable increase in its intensity. The DR group treated with glycine (DR+G) for two weeks was characterized by a noticeable improvement of the 160 kDa peak and in the intensities at 65 kDa, 58 kDa, 42 kDa, and 25 kDa. In addition, the pattern revealed that the two peaks that appeared at 52kDa and 36 kDa in the 2 W DR group disappeared in the 2 W DR+G group. Moreover, the 110 kDa peak showed reduced optical density with glycine. The group treated with glycine for four weeks (fig. 4) showed no deviation from the control except for the shifting of the fraction from 99 kDa to 105 kDa.With the progression of DR for four weeks, the change in separation pattern was more noticeable, especially in the low molecular weight region characterized by new peaks that appeared at 51 kDa, 45 kDa, and 41 kDa. Moreover, the peak shift at 160 and 122 kDa toward higher molecular weight was more than two weeks of DR. Impressively, treatment with glycine for four weeks significantly restored the SDS-PAGE pattern of retinal samples.

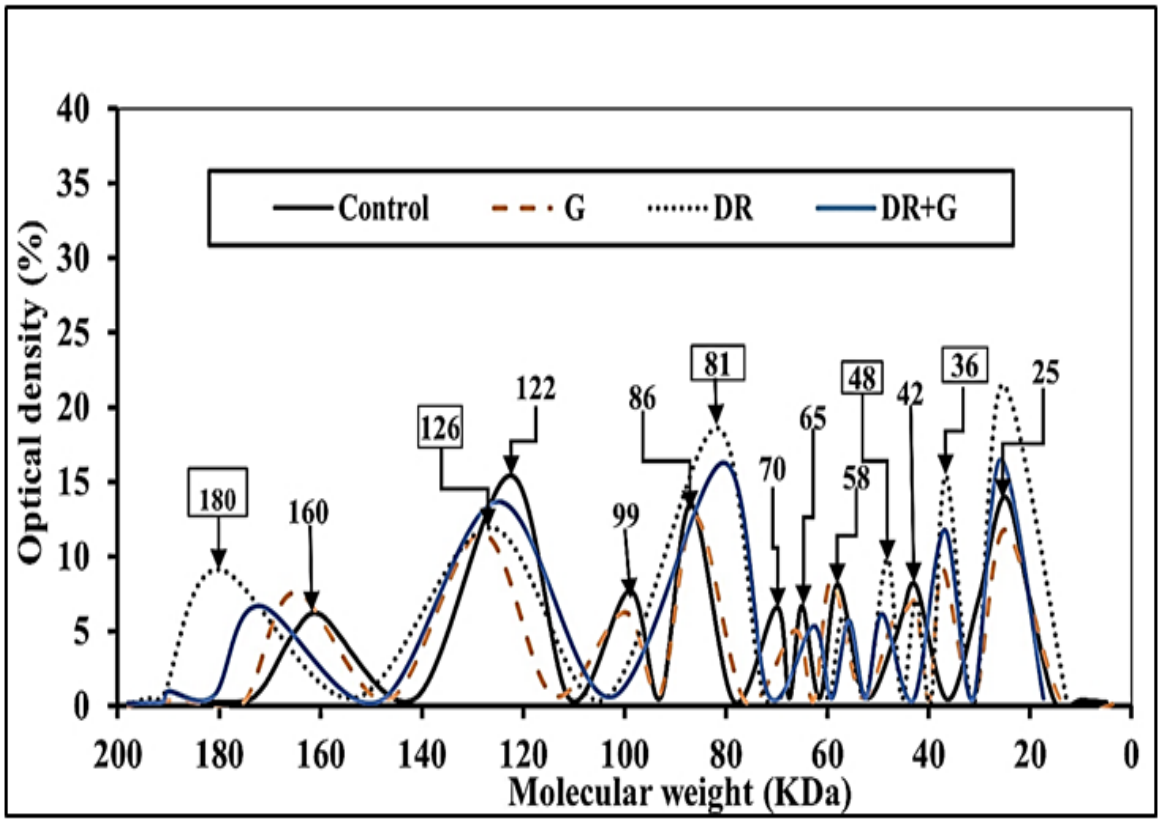

Fig. 2. The electrophoretic pattern for the control(C), glycine(G), diabetic retinopathy (DR), and diabetic retinopathy rats treated with glycine (DR+G) after one week.

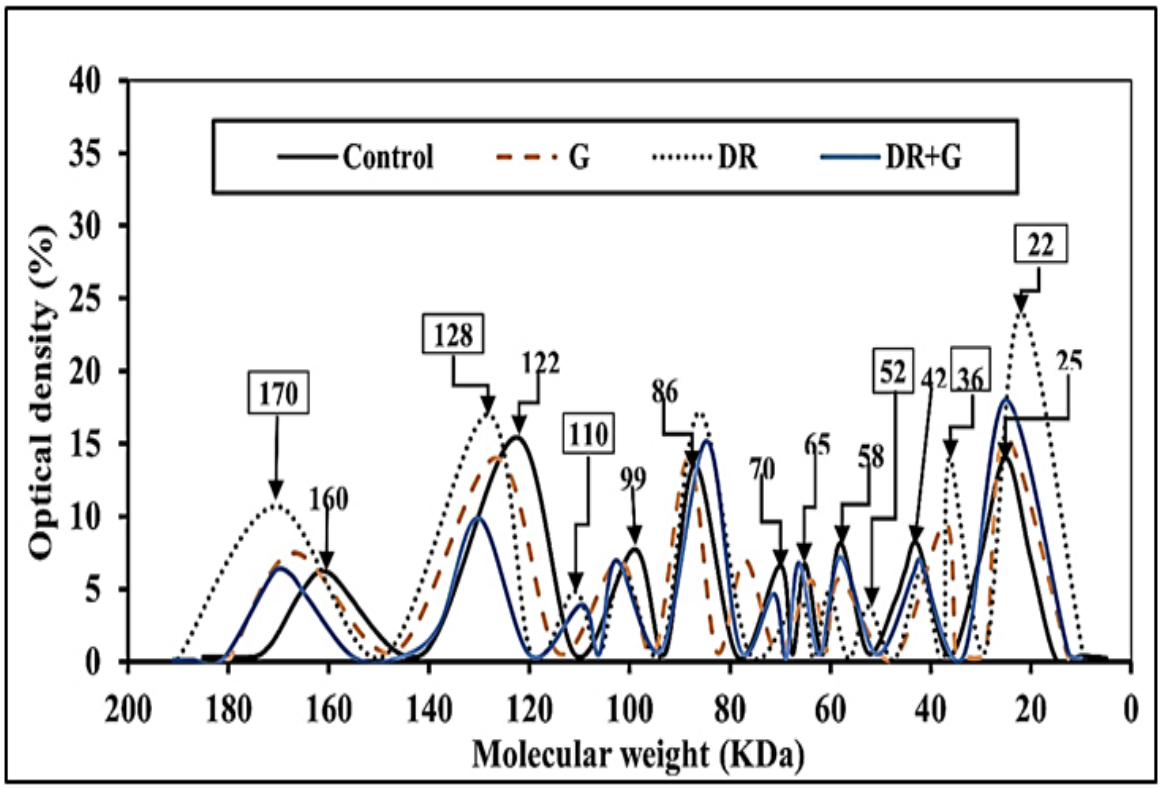

Fig. 3. The electrophoretic pattern for the control(C), glycine(G), diabetic retinopathy (DR), and diabetic retinopathy rats treated with glycine (DR+G) after two weeks.

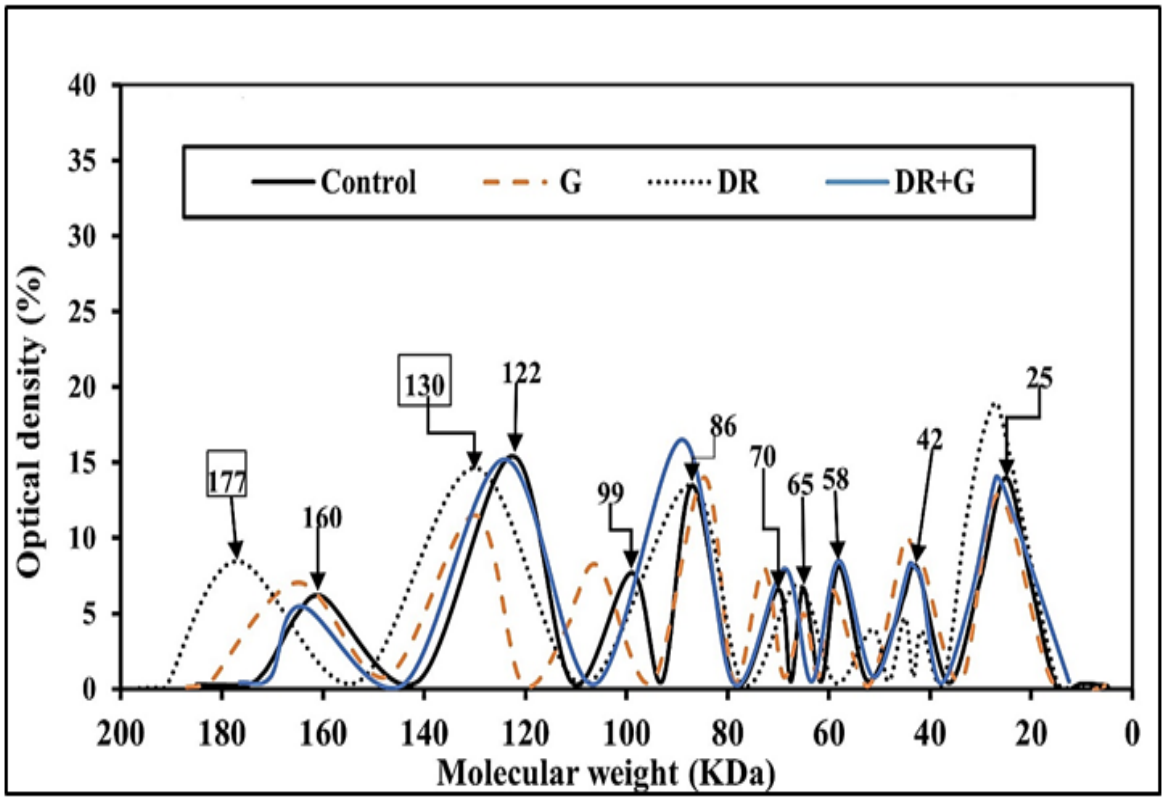

Fig. 4. The electrophoretic pattern for the control(C), glycine(G), diabetic retinopathy (DR), and diabetic retinopathy rats treated with glycine (DR+G) after four weeks.

DNA Damage

Comets' photos of control, glycine, diabetic retinopathy, and diabetic retinopathy groups that received glycine are shown in Figure 5. The small round spots represent the intact DNA without migration. The faint comet-shaped areas represent DNA migrating fragments. Moreover, the number of tailed cells, tail length, percent of DNA in the tail, and tail moment of all groups are presented in Table 1.

The results revealed that after one week, the percentage changes in tail lengths were 9.5%, 149.5%, and 112.4% for the G group, DR group, and DR+G group, respectively, compared to the control. After two weeks, the percentage change in tail lengths was 12.4%, 180.0%, and 120.0% for the G group, DR group, and DR+G group, respectively. Whereas after four weeks, the percentage change in tail lengths was 8.6%, 213.3%, and 134.3% respectively, for the same groups.

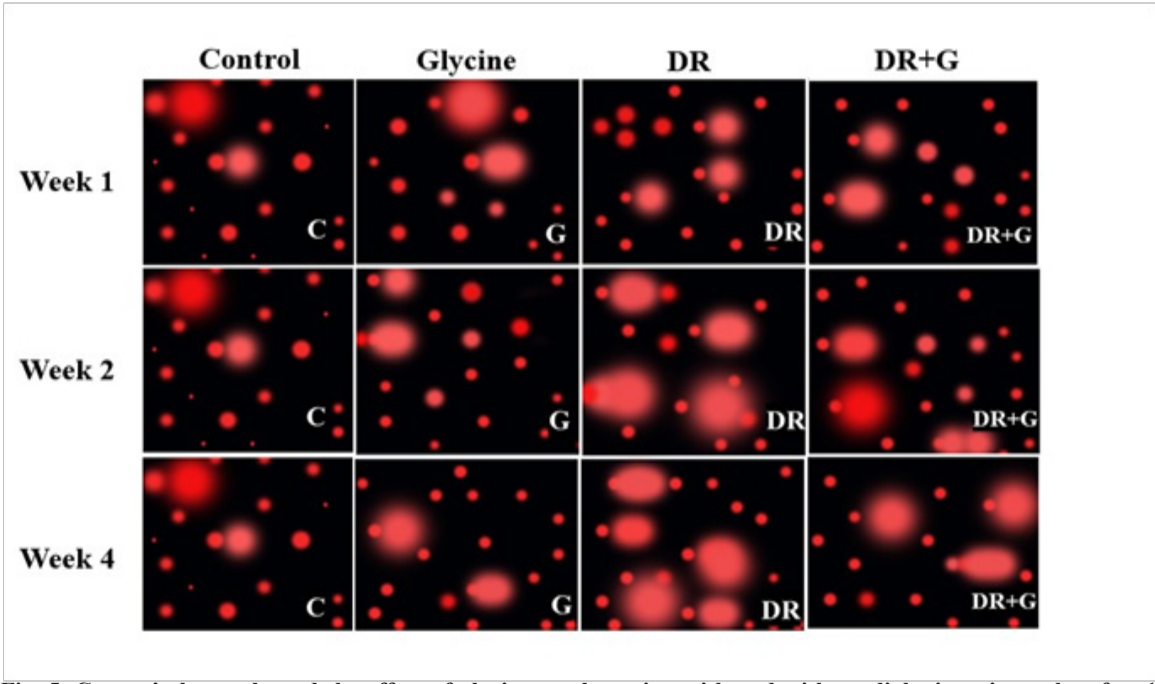

Fig. 5. Comets' photos showed the effect of glycine on the retina with and without diabetic retinopathy after 1 week, 2 weeks, and 4 weeks, respectively. The control (C), glycine (G), diabetic retinopathy (DR), and diabetic groups received glycine (DR+G).

TABLE 1. The percentage of tailed cells, tail length, percent of DNA in the tail, and the tail moment for the control group, Glycine (G), Diabetic retinopathy (DR), and Diabetic retinopathy with Glycine (DR+G).

Groups	Tailed cells (%)			Tail length (µm)			Tail DNA (%)			Tail moment (Units)		
Periods (week)	1	2	4	1	2	4	1	2	4	1	2	4
Control		5.0			1.05			1.15			1.21	
G	4.0	5.0	4.0	0.95	1.18	1.14	1.05	1.16	1.21	1.00	1.37	1.38
DR	14.0	17.0	23.0	2.62	2.94	3.29	2.69	2.77	3.05	7.0ᵒ	8.14	10.03
DR+G	9.0	12.0	16.0	2.23	2.31	2.46	2.16	2.23	2.4	4.81	5.15	5.9

Regarding the percentage changes in DNA percent in the tail, after one week, the G group, DR group, and DR+G group showed percentages of -8.7%, 133.9%, and 87.8%, respectively, compared to the control group. After two weeks, the percentage change in DNA percent in the tail was 0.8%, 140.9%, and 93.9% for the G group, DR group, and DR+G group, respectively, compared to the control group. After four weeks, the percentage change in DNA percent in the tail was 5.21%, 165.2%, and 108.7% for the G group, DR group, and DR+G group, respectively, compared to the control group.

Furthermore, concerning the tail moments, the percentage changes after one week were -17.3%, 482.6 %, and 297.5 for the G group, DR group, and DR+G group, respectively, concerning the control group. After two weeks, the percentage changes concerning the control group were 13.2%, 572.1 %, and 325.6% for the G group, DR group, and DR+G group, respectively. Finally, after four weeks, the percentage changes were 14.0%, 728.9%, and 387.6% for the G group, DR group, and DR+G group, respectively, concerning the control group.

Discussion

In hyperglycemic conditions, the interaction of proteins with glucose results in the production of advanced glycation end products (AGEs), which accumulate in the blood. These AGEs, in turn, bind to their receptors on the membrane of endothelial cells, causing elevation of the vascular endothelial growth factor (VEGF) [1, 17]. VEGF was reported to be associated with the breakdown of tight junctions and an increase in permeability [18, 19].

A wide range of factors contributing to BRB breakdown and enhancing permeability are associated with diabetes Mellitus. These factors include hypoxia-inducible factors, oxidative stress, and proinflammatory cytokines such as tumor necrosis factors and interleukin [17]. The reduction of fluid leakage achieved by glycine treatment seems to be the outcome of BRB function maintenance. Studies have shown that glycine could alleviate the levels of VEGF induced by AGEs in retinal cells and regulate the production of proinflammatory cytokines. Moreover, glycine reduced the elevated nitric oxide (NO) level in diabetes, thereby modulating the permeability [1, 12, 20, 21].

In this work, the nondiabetic group administrated with glycine showed a slight elevation in the retinal total protein ($P<0.05$) with percentage changes of 14.12% and 13.6% compared to the control group after two and four weeks, respectively. This elevation can be attributed to the fact that, in normal conditions, the endogenously synthesized glycine is adequate for its various functions conducted in the human body [21]. However, in diabetes Mellitus, there is an acute shortage of glycine and thus requires dietary glycine supplementation [13, 22]. In addition, the endogenous synthesis of glycine depends on glucose metabolism (glycolysis) [23]. Furthermore, glycine concentration in the retina is about five times greater than in plasma and is transported into the retina with concentration-dependent uptake values [24]. Therefore, increasing glycine in plasma by oral administration may result in glycine accumulation in the retina and elevated levels of retinal proteins. The elevation in retinal protein may be responsible for the protein structure change indicated by the SDS-PAGE. The electrophoretic patterns showed shifting in some protein fractions at molecular weights of 160 kDa, 122 kDa, 99 kDa, and 70 kDa toward higher molecular weights in addition to the appearance of a new peak with a molecular weight of 36 kDa. However, the group (4 W G) showed no deviation from the control except for the fraction at 99 kDa. Correspondingly, there was no significant DNA damage in the (G) group compared with the control group. These data indicated that glycine has no harmful effects on healthy rats' protein content, protein structure, and retinal DNA.

During the study, significant changes were detected in the retinal protein of the DR groups, whereas glycine supplementation significantly alleviated these changes. After four weeks, the treated group (DR+G) showed improved retinal total protein content ($P>0.05$) with a reduced percentage change to 2.05 % compared to the control group. Additionally, glycine treatment almost restored the SDS-PAGE pattern of retinal samples to its regular pattern.

Furthermore, the comet assay results for DR+G indicated a noticeable improvement in the DNA parameters. In the DR+G groups, the number of tailed cells was less than in the DR groups, and both groups were more than the control group. The result indicated that the DNA change of the retina with DR was more significant than in the control

(P<0.001) and four weeks treated group (P< 0.01) (Table 1). The tail moment, which specifies the amount and the spreading of DNA in the tail, became a common descriptor along with the tail length and percentage of DNA in the tail. The % change in the tail moment for the DR group was 482.6%, 572.7%, and 728.8% and was reduced for the DR+G group to 297.5%, 325.6%, and 387.6% compared to the control samples after one week, two weeks, and four weeks, respectively.

Thus, this result investigated that glycine could protect DNA from DR-related alterations. Moreover, the improvement may be attributed to the favorable effects of glycine exerted on the retinal protein through its antioxidant effect. This improvement is consistent with the previous work by Zhong et al., 2003 [25], who determined glycine as an antioxidant agent that works through glycine-gated chloride channels in leukocytes and macrophages, which modulates intracellular Ca^{2} levels, regulating the generation of superoxides, modulating the production of cytokines, thus, exerting anti-inflammatory response [25-27].

Another previous work suggested that oral dietary glycine supplementation in diabetic individuals restored the synthesis rates of GSH to the levels observed in nondiabetic individuals [28]. Glycine supplementation overcame the deficiency in GSH synthesis in patients with type 2 DM. Moreover, in vitro, improving glycine transport into diabetic β-cells has increased glutathione synthesis and protects against oxidative stress [28, 29]. Overcoming the deficiency of GSH is thought to be a protective mechanism against retinal disorders in which mitochondrial dysfunction is involved [30]. Thereby, it has a significant aspect in increasing the antioxidant capacity of retinal cells [27]. Additionally, research has shown that treatment with glycine had a favorable antidiabetic effect by increasing insulin secretion and improving glucose tolerance [22]. The improvement in protein content observed in the DR+G groups may be attributed to the decreased leakage of blood proteins through the blood-retinal barrier (BRB). Consequently, glycine can potentially mitigate the disruption of BRB integrity characteristic of diabetic retinopathy (DR).

Conclusion

In conclusion, the findings of this study suggest that glycine supplementation has beneficial effects in the context of diabetic retinopathy (DR). DR is associated with protein changes in content and structure, DNA alterations, and blood-retinal barrier (BRB) disruption. Glycine treatment appears to mitigate these effects by reducing the leakage of blood proteins across the BRB and alleviating the accumulation of advanced glycation end products (AGEs), which contribute to endothelial cell dysfunction and increased growth factor levels. The antioxidant properties of glycine and its ability to enhance glutathione synthesis and protect against oxidative stress are thought to play a role in its protective effects on retinal cells. Overall, these findings highlight the potential of glycine as a therapeutic intervention for DR, offering protection against protein and DNA alterations while preserving the integrity of the retinal tissue.

Conflicts of interests

There are no conflicts to declare.

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تحسين بروتين الشبكية بواسطة الجليسين والتخفيف المحتمل من اعتلال الشبكية السكري التجريبي

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تهدف هذه الدراسة إلى استكشاف الدور المحسن للجلسرين ضد اعتلال الشبكية السكري المستحث (DR) في حيوانات التجارب.

استخدمت في هذه الدراسة أربعة وثمانون من اناث فئران ويستر. وتم تقسيمها كالتالي (i) المجموعة الضابطة (العدد= ٢١)، (ii) المجموعة المعالجة بالجليسين (G) مع ١٠ جم/لتر في مياه الشرب (العدد = ٢١)، (iii) مجموعة اعتلال الشبكية السكري (DR) والتي تم حقنها بمادة الاستربتوزوتوسين (STZ) داخل الغشاء البريتوني (العدد = ٢١) و(iv) المجموعة المستحث بها اعتلال الشبكية السكري عن طريق الحقن بمادة الاستربتوزوتوسين (STZ) وتمت معالجتها بالجليسين (DR+G) (العدد = ٢١).

تم تقييم محتوى البروتين داخل نسيج الشبكية، اجراء الفصل الكهربي للبروتين باستعمال جيل البولي اكريلاميد الصوديوم (SDS-PAGE)، كما تم تقييم الأضرار التي لحقت الحمض النووي لبروتين الشبكية بعد أسبوع واحد، أسبوعين، وأربعة أسابيع، على التوالي. ومن النتائج وجد أنه تم تقليل ارتفاع تركيز البروتين داخل نسيج شبكية العين الناجم عن DR وذلك بواسطة الجليسين وتوصلنا لأقصى انخفاض قدره ١٥,٠٨٪ (P <٠,٠٥)، ٧,٠٦٪ (P < ٠,٠٥)، و٢,٠٥٪ (P < ٠,٠٥) بعد أسبوع واحد، أسبوعين، وأربعة أسابيع، على التوالي.

وأيضا تم تحسين بنية البروتين بشكل ملحوظ بواسطة الجليسين كما هو موضح في نتائج الفصل الكهربي SDS-PAGE. بالإضافة الي ذلك تم تخفيف الضرر في الحمض النووي لبروتين الشبكية بواسطة الجليسين بعد أربعة أسابيع على مستوى عدد خلايا الذيل وطول الذيل ونسبة الحمض النووي في الذيل وعزم الذيل. وعلي ذلك فإن إضافة الجليسين إلى نموذج تجريبي للفئران مع DR يدعم بشدة ويقترح استخدام الجليسين ضمن الوسائل العلاجية لاعتلال الشبكية السكري وذلك عن طريق النتائج المستفادة في تحسين بروتين الشبكية.