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Fisetin Prevented Amyloid Formation of Insulin and Attenuated Fibril-induced Cytotoxicity

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Authors' contributions

This work was carried out in collaboration between all authors. Author CMZ designed the study, wrote the protocol and supervised the work. Authors YJZ and NC carried out all laboratories work and performed the data analysis. Authors YJZ and NC contributed equally to this work. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Natural polyphenols are major constituents of plant foods and herbs. Numerous reports demonstrated that these compounds inhibited amyloid formation and destabilized the preformed amyloid fibrils. The present study, utilizing bovine insulin as a model peptide, examined the anti-amyloidogenic effects of fisetin (3,7,3',4'-tetrahydroxyflavone). Fluorescent probes, transmission electron microscopy and hemolytic assay were utilized to determine the roles of fisetin on amyloidogenesis of insulin. The results demonstrated that fisetin dose-dependently inhibited amyloid formation of insulin. Moreover, fisetin disaggregated the preformed insulin fibrils and transformed the fibrils into non-amyloid structures. Hemolysis was observed when erythrocytes were co-incubated with insulin fibrils. Fisetin inhibited fibril-induced hemolysis in a dose-dependent manner. Hydrophobic interaction is suggested to be a key driving force in the anti-amyloidogenic and fibril-disaggregating effects of fisetin. The results of the present work suggest that fisetin is an effective anti-amyloidogenic compound and may serve as a lead structure for the design of novel drugs for the treatment of amyloid diseases.

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Keywords: Fisetin; insulin; amyloid fibrillation; hemolysis.

ABBREVIATIONS

ANS: Anilinonaphthalene-8-sulfonic Acid; TEM: Transmission Electron Microscopy; ThT; Thioflavin T.

1. INTRODUCTION

Amyloid fibrils of proteins or peptide fragments have been found to be associated with more than 20 human diseases, such as Alzheimer's disease, type 2 diabetes, Parkinson's disease, hemodialysis-related amyloid deposition and a number of systemic amyloidoses [1,2]. These proteins, despite their unrelated amino acid sequences and tertiary structures, can unfold and assemble into fibrils with similar ultrastructures and identical biochemical properties, including long and unbranched fibrils with enriched β-sheet structure, increased surface hydrophobicity, fluorescence upon binding to thioflavin T (ThT) and the ability to disrupt cellular membranes [2].

Bovine insulin is a polypeptide consisting of a 21-residue A chain and a 30-residue B chain which are linked together by two interchain disulfide bonds. Numerous studies have shown that under denaturing conditions, including acidic pH and high temperature, monomeric insulin molecules are able to assemble into amyloid fibrils [3,4]. Since these fibrils have been well characterized by various methods, insulin serves as an ideal model system for studying amyloid aggregation. There is no evidence to show that endogenous insulin forms a pathogenic fibrillar assembly *in vivo*. However, the fibrillation of non-native insulin causes a variety of problems in the production, storage, and delivery of soluble insulin in the form of pharmaceutical formulations. A subsequent *in vivo* problem known as injection-localized amyloidosis can occur and an unwanted immune response may be triggered [5].

The therapeutic strategies proposed for the treatment of amyloid-related diseases are mainly based on inhibition of the amyloid formation and disruption of the fibrillar structures. Enzymatic inhibitors, hormones, antagonists, antibodies, peptide fragments, synthetic ligands and natural antioxidants have appeared in the list of screened candidates. Natural polyphenolic compounds from foods and traditional herbal medicines, having broad pharmacological activities and exhibiting inhibition of amyloid formation, have been extensively investigated in disruption of amyloid fibrils and reduction of the toxicity of fibrils to living cells [6,7].

Fisetin (3,7,3',4'-tetrahydroxyflavone; Fig. 1), one of the most important natural polyphenols, is a member of the flavone family and is found in many foods and herbs. Due to its potent antioxidant and metal ion-chelating capacity, fisetin possesses various biological effects including anti-inflammatory, anti-neoplastic and cardioprotective activities [8-10]. Recent investigations indicated that fisetin exhibited an inhibitory effect on the amyloid formation of β-amyloid peptides [11-13]. In the present study, the anti-amyloidogenic effects of fisetin were investigated *in vitro* using bovine insulin as a model protein. Fluorescent probes, transmission electron microscopy (TEM) and hemolytic assay were utilized to determine the roles of fisetin on amyloidogenesis of insulin.

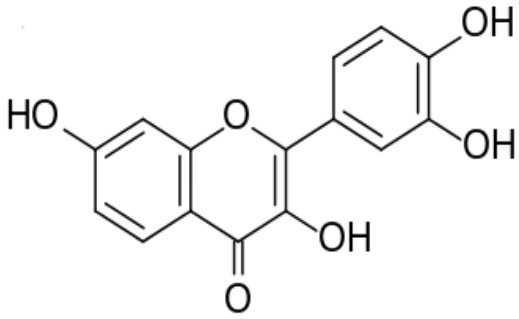

Fig. 1. The molecular structure of fisetin

2. MATERIALS AND METHODS

2.1 Chemicals and Blood Collection

Bovine pancreatic insulin (MW 5734 Da), anilinonaphthalene-8-sulfonic acid (ANS), Thioflavin T (ThT) and fisetin (MW 286 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were of analytical grade. Fresh blood was drawn from healthy volunteers using sodium citrate as an anticoagulant. The donors were informed by the ethical committee of the hospital on the need for the blood samples and they consented.

2.2 Preparation of Insulin Fibrils

A stock solution of fisetin (4 mg/mL) was prepared by dissolving the compound in DMSO. Insulin fibrils were prepared according to previous report with minor modification [14]. Briefly, bovine insulin was dissolved in 10 mM HCl solution (pH 2.0) and then diluted with the same HCl solution to a final concentration of 1 mg/mL with or without an aliquot of fisetin solution. Incubation was carried out at 50°C in sealed tubes to avoid solvent evaporation. Unless otherwise stated, all the control samples contained same volume of DMSO but devoid of fisetin. We confirmed that 1% DMSO had no obvious effect on the fibrillar structure of insulin, ThT and ANS fluorescence, and membrane permeability of human erythrocytes.

2.3 ThT Fluorescence Measurements

A stock solution of ThT (1 mM) was prepared by dissolving ThT in water which was stored at 4°C. Aliquots of insulin fibril solution were diluted with Tris-HCl buffer (50 mM, pH 8.0), followed by the addition of an aliquot of ThT solution. ThT fluorescence was measured using a Perkin Elmer LS 55 fluorescence spectrometer with excitation at 450 nm and emission at 485 nm. To study the fibril-disaggregating effects, fisetin was mixed with the preformed insulin fibrils (300 μg/mL) in 50 mM Tris-HCl buffer (pH 8.0) and incubated at 37°C. The mixtures were then diluted with same buffer prior to ThT assay.

2.4 ANS Binding Assay

The emission spectra of ANS fluorescence in the presence of insulin fibrils were recorded using an excitation wavelength of 350 nm. Aliquots of the incubated mixture were diluted in phosphate buffer (50 mM, pH 7.0) containing 10 μM ANS. The ANS fluorescence was then scanned immediately and the intensity at 470 nm was measured [15]. We also confirmed that, in the absence of insulin monomers or assemblies, fisetin (10-40 μg/mL) had no obvious effect on ThT and ANS fluorescence.

2.5 Transmission Electron Microscopy

An aliquot of insulin fibrils was diluted with water and dropped onto copper-mesh grids. Samples were negatively stained with 2% (w/v) uranyl acetate and air-dried at room temperature. Observations were carried out using a JEOL JEM-1400 electron microscope with an accelerating voltage of 80 kV.

2.6 Hemolytic Assay

Fresh blood was centrifuged at 1000 g for 10 min, and erythrocytes were separated from plasma and buffy coat and washed three times with isotonic phosphate buffered saline (pH 7.4). For the hemolytic assay, suspensions of erythrocytes of 1% hematocrit in isotonic phosphate buffered saline (pH 7.4) were incubated with insulin fibrils in the presence or absence of fisetin at 37°C for 1 h in a shaking water bath. Incubation was terminated by cooling the tubes at 4°C. The reaction mixtures were centrifuged at 1000 g for 10 min and the absorbance of the supernatant was measured at 540 nm using a Shimadzu UV-240 spectrophotometer. The hemolytic rate was calculated in relation to the hemolysis of erythrocytes in 10 mM phosphate buffer (pH 7.4) which was taken as 100%.

2.7 Statistical Analysis

Unless otherwise indicated, the experiments were performed in triplicate and the values are presented as means ± S.D. Student's t-test was utilized when two samples were compared.

3. RESULTS AND DISCUSSION

Incubation of insulin under conditions of low pH and elevated temperature resulted in formation of amyloid fibrils. Increased temperature, ionic strength or insulin concentration significantly shortened the lag time of fibril growth [3,4]. In the present study, we incubated 1 mg/mL insulin in 10 mM HCl solution (pH 2.0) at 50°C in the presence or absence of fisetin. The growth of amyloid fibrils was monitored and characterized by ThT fluorescence and transmission electron microscopy.

ThT is a fluorescent probe for monitoring the formation and growth of amyloid fibrils. The fluorescence intensity of ThT increased significantly when the compound specifically binding to the highly ordered β-sheet structure of amyloid fibrils [16]. As depicted in Fig. 2, a significant increase in ThT fluorescence emission was observed after incubating the control sample for 3 days. The intensity of ThT fluorescence increased gradually as the amyloid formation progressed until reached to a plateau after 7 days of incubation. Fisetin dose-dependently

inhibited the growth of insulin fibrils, resulting in a significant decrease in the final intensity of ThT fluorescence. No sigmoid curve was observed when insulin was incubated with 30 μg/mL and 40 μg/mL fisetin, indicating that fibril formation was inhibited.

Fig. 3 shows TEM images of the insulin assemblies prepared in the presence and absence of fisetin. In the absence of fisetin, the mature insulin fibrils showed a typical amyloid morphology, characterized by long, straight and partly bundled fibrils with diameters ranging between 5 and 40 nm (Fig. 3A). In the presence of fisetin, the insulin assemblies showed different morphologies, depending on the concentration of fisetin applied. Only a few small short fibrils were observed in the sample containing 40 μg/mL fisetin (Fig. 3B), indicating that amyloid fibrillation of insulin was inhibited, consistent with the ThT data.

ANS is a specific fluorescent dye for probing changes in surface hydrophobicity of protein molecules. Upon binding to a hydrophobic region of protein, the intensity of ANS fluorescence is significantly enhanced with a blue-shift of the maximum emission wavelength [15]. Incubation of insulin resulted in a significant increase in the ANS fluorescence (Fig. 4, curve a) at 470 nm, reflecting an increase in the solvent-exposed hydrophobic interior of the protein during fibril growth. In the presence of fisetin, only slight increases in ANS fluorescence (Fig. 4, curves b and c) were recorded, suggesting that fisetin inhibited exposure of the hydrophobic interior of the protein, causing an interruption of fibril assembly.

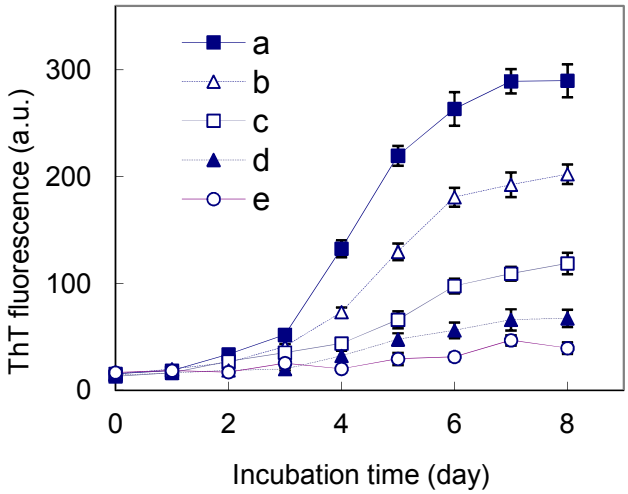

Fig. 2. ThT growth curves of insulin fibrils in the absence (a) and presence of fisetin 10 (b), 20 (c), 30 (d) and 40 μg/mL (e)

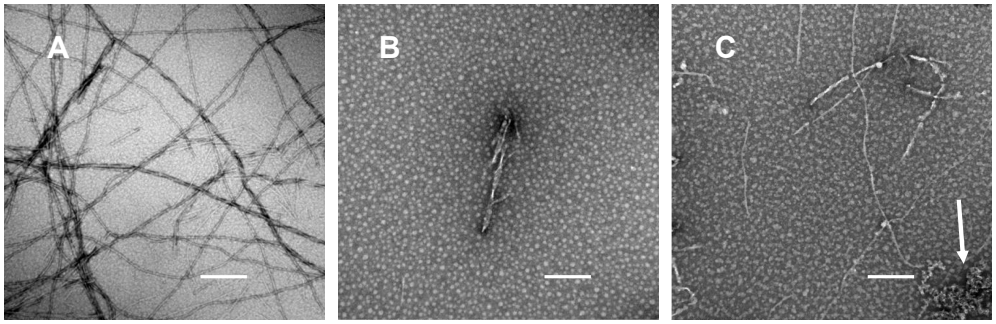

Fig. 3. TEM images of insulin assemblies. Samples were prepared by incubating insulin (1 mg/mL) at 50°C for 8 d in the absence (A) and presence of fisetin 40 μg/mL (B). (C) Fibril disaggregation induced by treating mature insulin fibrils (300 μg/mL) with fisetin (40 μg/mL) at 37°C for 6 h. Scale bars represent 200 nm

Treatment of preformed mature insulin fibrils with fisetin resulted in the disaggregation of the fibrils. As shown in Fig. 5. co-incubation of mature insulin fibrils with fisetin resulted in a decrease in the intensity of ThT fluorescence, suggesting that the fibrillar structure was transformed into non-amyloid structures. After incubating the fibrils with 40 μg/mL fisetin at 37°C for 6 h, a reduction in ThT fluorescence 78.5% was observed. The fibril-disaggregating effect of fisetin was both time- and dose-dependent. The decrease in affinity of insulin fibrils for ThT was accompanied with a change in fibrillar morphology. As shown in Fig. 3C, only a few short, thin and flexible fibrils were observed under TEM after treating insulin fibrils with 40 μg/mL fisetin at 37°C for 6 h. In addition, amorphous aggregates were also formed, as indicated by the arrow in Fig. 3C.

Our previous study [15] demonstrated that amyloid fibrils caused hemolysis and aggregation of human erythrocytes in an aging-dependent manner. The membrane disruption by amyloid fibrils involved intermolecular cross-linking of cytoskeletons, suggesting that the fibrils were able to interact with the inner part of cell membranes. In this study, hemolytic assays were performed to evaluate the cell-damaging capacity of insulin assemblies prepared in the absence or presence of fisetin. As shown in Fig. 6A, insulin fibrils induced hemolysis of erythrocytes under an isotonic environment in a dose-dependent manner. The hemolytic rates induced by 60 and 80 μg/mL insulin fibrils were 10.4% and 13.9%, respectively. Fisetin dose-dependently inhibited insulin fibril-induced hemolysis (Fig. 6B). A 55% reduction in hemolysis was achieved when 40 μg/mL fisetin was applied in the mixture of erythrocytes and 80 μg/mL insulin fibrils.

Amyloid fibrillation of a protein generally consists of a cascade of events including association/dissociation of the protein oligomer, nucleation, elongation and maturation of the fibrillar species [17], corresponding to different stages of the growth curve. Insulin is one of the proteins capable of forming amyloid fibrils with a variety of morphologies [3,4]. Under the experimental conditions in the present study, insulin transformed into mature amyloid fibrils in less than 8 days with a lag time of approximately 3 days, according to the ThT curves shown in Fig. 2. Fisetin inhibited amyloid fibrillation of insulin and altered the morphology of insulin assemblies. In the presence of fisetin, the lag time for insulin nucleation was longer than the control sample, suggesting that the early assembly of insulin monomers/oligomers was interrupted by fisetin. During nucleation, insulin molecules unfold and expose their hydrophobic regions to the solvent, as demonstrated by the ANS assay (Fig. 4). This process is thermodynamically unfavorable due to the increased surface area of insulin and the interactions between the hydrophobic region of the protein and water. The positive shift in free energy is compensated by the formation of aggregates which can serve as nuclei for amyloid assembly. The hydrophobic moiety of fisetin can bind with the exposed hydrophobic core of insulin and therefore the exposure of hydrophobic domains of insulin was suppressed.

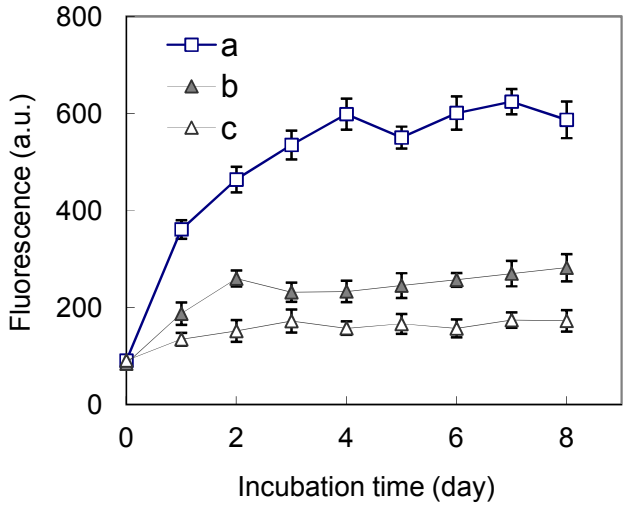

Fig. 4. ANS fluorescence of insulin fibril growth in the absence (a) and presence of fisetin 30 (b) and 40 μg/mL (c)

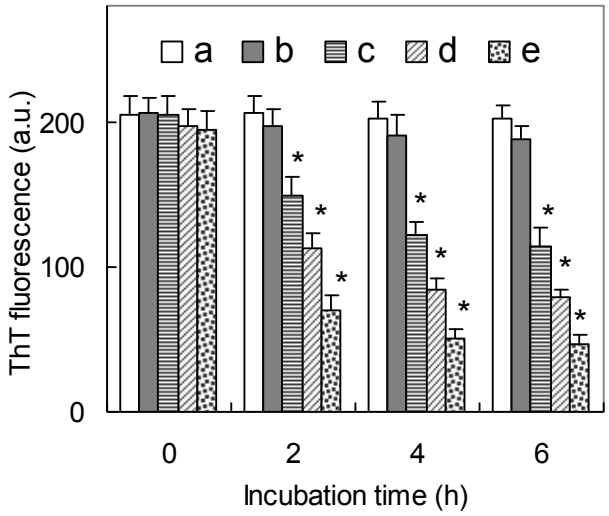

Fig. 5. Fibril disaggregation induced by fisetin. Mature insulin fibrils (300 μg/mL) were treated with 0 (a), 10 (b), 20 (c), 30 (d) and 40 μg/mL (e) fisetin at 37°C prior to ThT assay. Asterisks represent *P*<0.01 *vs* the values of insulin fibrils treated without fisetin (data a)

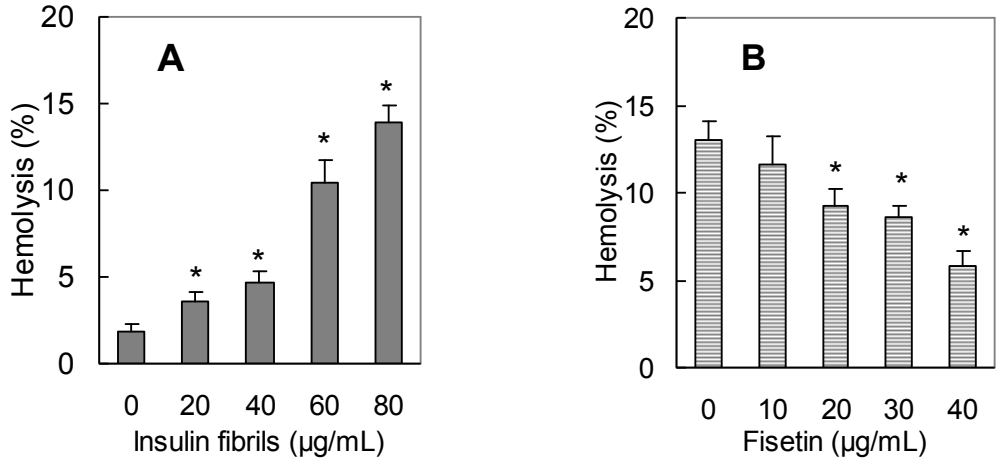

Fig. 6. Fisetin attenuated insulin fibril-induced cytotoxicity. (A) Dose-dependence of insulin fibril-induced hemolysis. Asterisks represent *P*<0.01 *vs* the value of control sample (without fibrils). (B) Inhibitory effects of fisetin on insulin fibril-induced hemolysis. Hemolysis of erythrocytes was induced by incubating the cell suspensions with insulin fibrils (80 μg/mL) in the absence or presence of fisetin. Native insulin (80 μg/mL) or fisetin (40 μg/mL) showed no hemolytic effect (data not shown). Asterisks represent *P*<0.01 *vs* the value of insulin fibrils in the absence of fisetin

Polyphenols, including flavonoids, are capable of binding to proteins to form soluble or insoluble complexes. The binding strength is positively correlated with hydrophobicity of the polyphenol [18]. Heating a protein can increase the binding affinity and binding capacity of polyphenols [19] due to increased exposure of the interior domains of the protein. According to the ANS data in Fig. 4, amyloid species have higher surface hydrophobicity than the native form of insulin. Accordingly, amyloid fibrillation of insulin enhanced the interaction between the protein and fisetin, resulting in the disruption of fibrillar structure. Previous reports have demonstrated that natural polyphenols are able to induce the transformation of amyloid fibrils into amorphous

aggregates [20,21]. The fibril-disrupting potency of a polyphenol is positively correlated to its hydrophobicity [21].

In addition to hydrophobic interactions, other forces may also be involved in the disruptive effect of fisetin on the fibrillar species of insulin. Recent investigations [22-25] have indicated that the aromatic stacking and hydrogen bonding capacity of a polyphenol play important roles in disaggregating amyloid fibrils. The transformation of a protein into amyloid fibrils may increase the opportunity for a polyphenol to bind specifically to amino acid residues such as tyrosine, phenylalanine and tryptophan. Myricetin, a flavonoid compound with a similar structure to fisetin, was found to bind specifically and reversibly to mature β-amyloid fibrils rather than to the monomeric peptide [26]. Hydrogen bonds and the interactions between the aromatic moieties were suggested to participate in the specific bindings. It has been reported that the 3', 4'-dihydroxyl group of the B-ring is essential for the anti-amyloidogenic effect of a flavonoid [11]. Inhibition of fibril growth and disaggregation of mature fibrils of β-amyloid peptide by baicalein, myricetin and quercetin were also observed [6,11,26].

The increased surface hydrophobicity of a protein upon amyloid formation enabled the fibrillar species to interact with the lipid bilayer of cell membranes, resulting in toxicity to living cells. The cytotoxicity of amyloid fibrils includes inducing hemolysis, damaging a cell to produce debris and triggering intermolecular cross-linking of cytoskeletal proteins [15,21]. In the present work, insulin fibrils induced hemolysis of erythrocytes in a dose-dependent manner. We attribute the disruption of cell membranes by insulin fibrils to the interactions between the hydrophobic domains of the peptide and lipid bilayers. Fisetin disaggregated the fibrillar structure and therefore reduced the hemolytic role of insulin fibrils.

Numerous reports have also suggested that the anti-oxidant property of a polyphenol is involved in its anti-amyloid activity [27-29]. Previous investigations demonstrated that the fibril-disaggregating activity of a polyphenol is positively correlated to its anti-oxidant capacity [21-23]. In this study, we determined whether oxidation of fisetin occurred under the conditions of the present study by using UV spectrometry. The results showed that the UV absorbance of fisetin at 366 nm decreased 35% in 8 days when incubated with 10 mM HCl solution (pH 2.0) at 50°C. When incubation was carried out in 50 mM Tris-HCl buffer (pH 8.0) at 37°C, the absorbance at 366 nm decreased 18% in 6 h. These data suggested that fisetin was unstable and possibly oxidized during the inhibition of insulin fibrillation and disaggregation of preformed fibrils. Recent report have demonstrated that the formation of reactive quinone though auto-oxidation and subsequently binding to peptide chains are prerequisites for the inhibition of amyloid growth by polyphenolic compounds [30]. The modification of peptides by quinones altered the interacting forces within intra- and inter-peptide chains, causing an interruption of amyloid fibrillation. Whether the redox property of fisetin is involved in its anti-amyloidogenic effects is still not clear and further research is required.

4. CONCLUSION

The present study demonstrated that fisetin had an inhibitory role on insulin fibrillation and a disaggregating effect on the preformed mature fibrils. The kinetics of fibril growth, the surface hydrophobicity of insulin assemblies, and disaggregation of preformed mature fibrils were affected by fisetin in a dose-dependent manner. Hydrophobic interaction is suggested to be the key driving force in the anti-amyloidogenic and fibril-destabilizing effects of fisetin. By virtue of its fibril-disaggregating capacity, fisetin attenuated the disruptive effects of insulin fibrils on erythrocyte membranes, resulting in an inhibition of hemolysis induced by insulin fibrils. The results of the present work suggest that fisetin is an effective anti-amyloidogenic compound and may serve as a lead structure for the design of novel drugs for the treatment of amyloid diseases.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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