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Full Length Research Paper

To evaluate likely antiamyloidogenic property of ferulic acid and baicalein against human islet amyloid polypeptide aggregation, *in vitro* Study

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Aggregation of human islet amyloid polypeptide (hIAPP) as islet amyloid is associated with increased beta cell apoptosis and reduced beta cell mass in type 2 diabetes mellitus. Inhibition of the formation of β-amyloid fibrils, as well as the destabilization of preformed β-amyloid in the pancreas, would be attractive therapeutic targets for the treatment of diabetes mellitus. Using fluorescence spectroscopic analysis with Thioflavin T, we examined the effects of Ferulic acid and Baicalein on the formation, and destabilization of β-amyloid *in vitro*. The results showed that after 192 h incubation by shaker incubator in 37°C, Ferulic acid at 10 and 40 µM repressed amylin amyloid formation by 22.6 and 27.7% respectively ($p<0.05$) and the similar values of Baicalein inhibited the formation of β-sheet structure by 20.4 and 30.9% respectively ($p<0.05$). The obtained data also confirmed that amyloidal sheet opening was induced by Ferulic acid and Baicalein significantly ($p<0.05$). It may conclude that islet amyloid cytotoxicity to β-cells may be reduced by these two herbal extracts and these compounds should be key molecules for the development of the therapeutics for Diabetes mellitus.

Key words: Amylin, diabetes, ferulic acid, baicalein, amylin fibrils.

INTRODUCTION

Type 2 diabetes mellitus is a common endocrine disorder in which carbohydrates, fats and proteins metabolism have been interrupted and results in diabetic complications include cardiac abnormality, diabetic retinopathy, nephropathy and atherosclerosis (Naqshbandi et al., 2008). The etiology of diabetes and its complications is still not clear; but it has been implicated that human islet amyloid polypeptide (hIAPP) aggregates in type II diabetics to form oligomers that interfere with beta-cell function, eventually leading to the loss of insulin production (Reddy et al., 2011). Human Islet Amyloid Polypeptide (hIAPP, also known as amylin) is a 37 residue peptide hormone secreted from pan-creatic β-cells (Brender et al., 2008). In its normal physiological role, hIAPP is associated with appetite suppression and, in conjunction with insulin, in maintaining proper glycemic levels (Scherbaum, 1998). However, a change in the cellular environment in the early stages of type II diabetes, causes it to aggregate into dense, insoluble fibrillar deposits that accumulate in the pancreas and contributes to islet β-cell dysfunction in type 2 diabetes (Haataja et al., 2008; Soong et al., 2009). The disruption of the integrity of the cellular membrane may be one of the primary mechanisms by which hIAPP causes cellular death (Butterfield and Lashuel, 2010). Human islet amyloid polypeptide has been also shown to cause significant impairment of the integrity of the phospholipids membrane (Brender et al., 2007; Engel et al., 2008). Owing to the increasing prevalence of diabetes, multidisciplinary study aimed at preventing and treating is one of the world-wide research priorities. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (Figure 1) is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine. It occurs primarily in seeds and leaves both in its free form and covalently linked to lignin and other biopolymers. Due to its phenolic nucleus and an extended side chain

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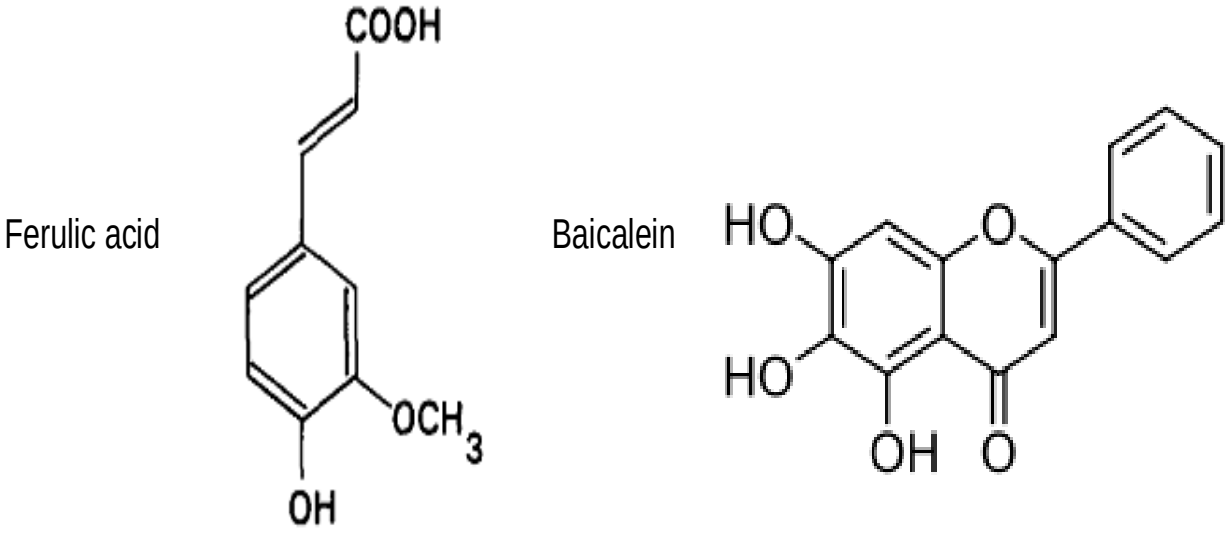

Figure 1. Structures of Ferulic acid and Baicalein.

conjugation, it readily forms a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential (GgAF, 1992 and Barone et al., 2009). Baicalein (5, 6, 7-Trihydroxy-2-phenyl-chromen-4-one) (Figure 1) is the predominant flavonoid isolated from the roots of Scutellaria lateriflora Georgi.

It has been reported that this compound exhibits many different pharmacological activities, including that of an antioxidant (Gao et al., 2001), anti-inflammatory (Shen et al., 2003) and as an anti-viral (Xu et al., 2010). Recently, the neuroprotective effect of baicalin against Aβ-induced neurotoxicity has been reported, and this effect is thought to be associated with reduction of oxidative stress (Yina et al., 2011)

Inhibition of the formation of β-amyloid fibrils, as well as the destabilization of preformed β-amyloid in the pancreas, would be attractive therapeutic targets for the treatment of diabetes mellitus, so the present study was designed to assay the potential roles of two flavonoids compounds named Ferulic acid and Baicalein against amyloidogenic property of hIAPP under near physiologic conditions.

MATERIALS AND METHODS

Human amylin peptide and other materials were prepared from Sigma-Aldrich Company.

Amylin stock solution

Human amylin used in this project had the following characteristics: (1-37)(Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH2, intra-molecular disulfide bridge: between Cys2 and Cys7). Its purity was 97% and the lyophilized salt included 70% peptide by weight. Amylin stock solution was prepared by adding 1.0 ml dimethylsulfoxide (DMSO) to dry purified peptide, sonicating at room temperature for 15 min.

Experiments were performed in the two different phases as follows.

The first series of experiment

In order to assay the effects of different concentrations of Ferulic acid and Baicalein on amylin aggregation and amyloidogenesis, control and treated groups were considered. The peptide stock solution was diluted by PBS 50 mM, pH: 7.5, to the final concentration of 10µM. Different concentrations of Ferulic acid (10, 40 µM) and Baicalein (10, 40 µM) were prepared in PBS buffer containing 10 µM amylin as treated groups, separately. The samples without Ferulic acid and Baicalein were selected as the control group. All studied groups were incubated at 37°C for 192 h with shaking by a shaker incubator (GFL 3031, Germany).

The second series of experiments

The second series of experiments were carried out to elucidate the destabilizing effect of the two herbal components on preformed amyloid sheet of amylin. For this purpose, prepared amyloid from the previous step was used. Amyloid was incubated with different concentrations of each of the agents for 6 hours in 37°C.

Amyloid formation and destabilization assay

To determining the level of amyloid beta-pleated sheets at the end of the two series of experiments, Thioflavin T (ThT) fluorescent assay was used. Thioflavin T assay was performed by adding 40 µl of each incubated solution to 700 µl of 10 µM ThT solution. Fluorescence measurements were recorded in a Perkin-Elmer LS55 fluorescence spectrometer (Perkin-Elmer LS55, USA) at room temperature using a 1 cm path length quartz cell. The ThT signal was quantified by averaging the fluorescence emission at 485 nm

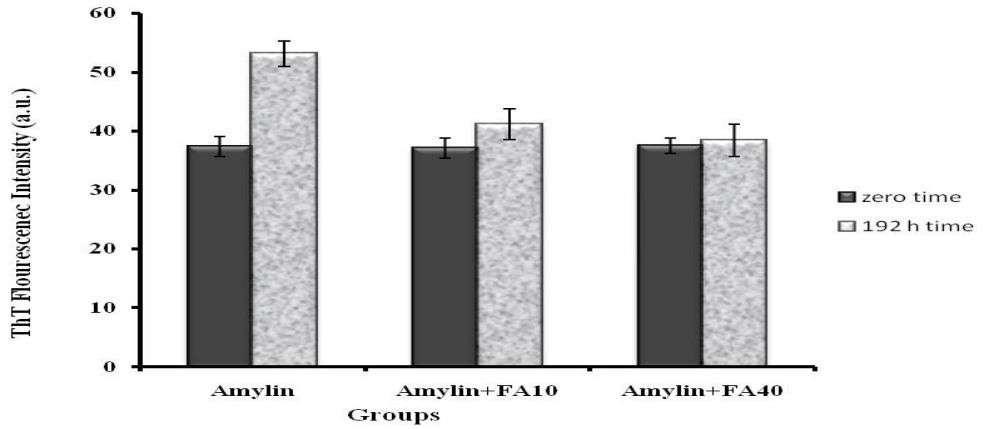

Figure 2. Thioflavin T fluorescence assay of protective effects of Ferulic acid on amylin Fibrils. All groups were incubated at 37°C for 192 h with shaking by a shaker incubator. At zero time (before incubation) there were no significant differences between three groups: amylin, amylin+FA 10 and amylin+FA40 ($p>0.05$). Ferulic acid (FA) inhibited amylin aggregation in a dose-dependent manner. Data have been shown as Mean±SEM, n=5.

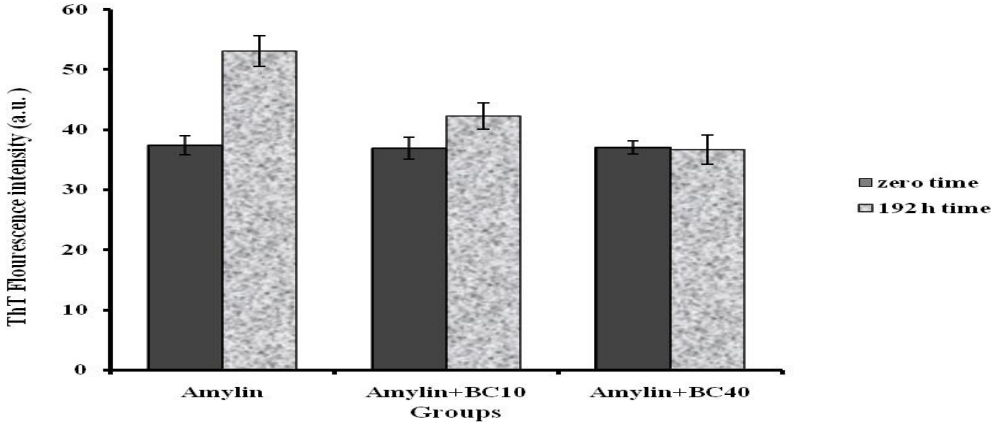

Figure 3. Thioflavin T fluorescence assay of protective effects of Baicalein on amylin aggregation. All groups were incubated at 37°C for 192 h with shaking by a shaker incubator. At zero time (before incubation) there were no significant differences between three groups: amylin, amylin+BC10 and amylin+BC40 ($p>0.05$). Baicalein (BC) inhibited amylin aggregation in a dose-dependent manner. Data have been shown as Mean±SEM, n=5.

(slit width = 10 nm) when excited at 440 nm (slit width = 5 nm).

IF assay

The intrinsic fluorescence of the peptide tyrosine residue was measured for the studied groups after 168 h by averaging the fluorescence emission at 304 nm when excited at 270 nm.

Statistical analysis

Descriptive statistics was accomplished to obtain means and standard deviations. Statistic significance level was established at $p<0.05$. Analysis of data was performed using SPSS statistical software package.

RESULTS AND DISCUSSION

The first run of experiments showed that amylin itself readily aggregated and formed a ThT-Positive material in control group. ThT-fluorescence mean value for control group at zero time, was 37.4 had increased to mean value of 53.1 at 192 h ($p<0.05$). Data indicated that ThT-fluorescence mean value for control group which. In Ferulic acid treated groups, ThT fluorescence assay indicated that 10 µm and 40 µM of Ferulic acid inhibited amyloid formation by 22.6 and 27.7% respectively after 192 h incubation at 37°C ($p<0.05$) Figure 2. Different concentrations effects of Baicalein on amylin aggregation were demonstrated in Figure 3. These data indicated that

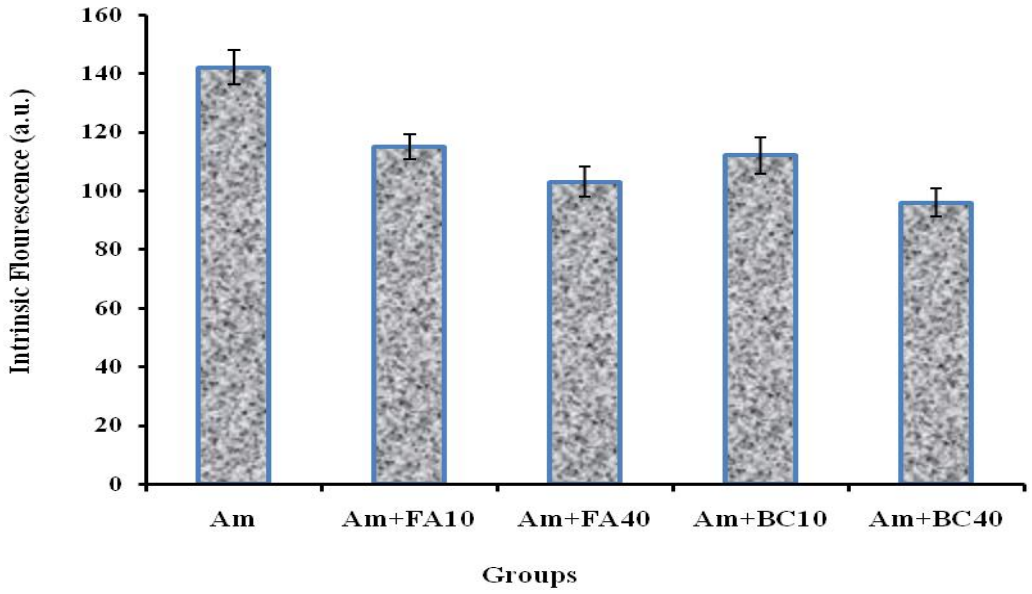

Figure 4. Intrinsic fluorescence of the control and treated groups. Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the agents was measured after 168 h incubation in 37°C. Data have been shown as Mean±SEM, n=5.

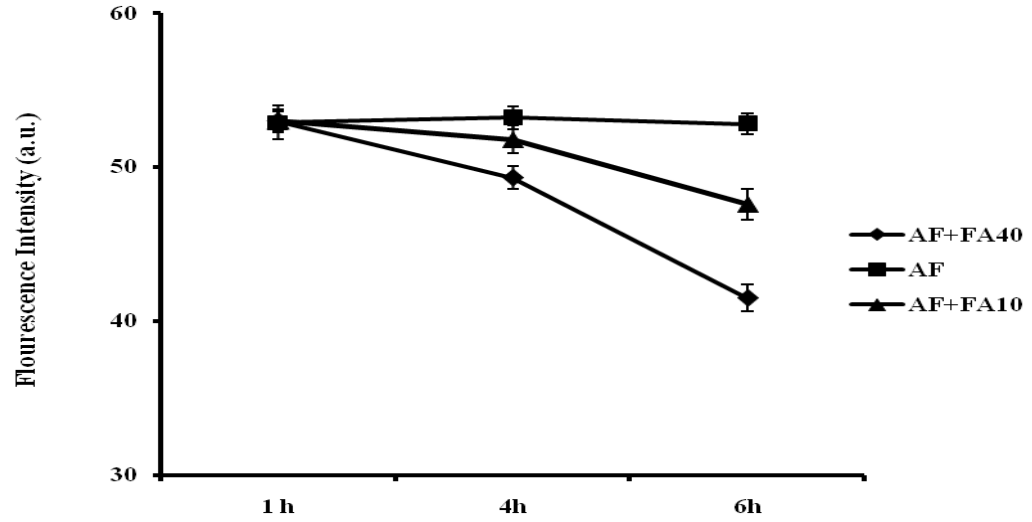

Figure 5. Ferulic acid effect on amylin fibril destabilization. Ferulic acid (FA) with two different concentrations destabilized amylin fibril (AF) significantly after 6 hours incubation.

compared to control group, ThT-fluorescence was increased significantly in the presence of 10 and 40 µM of Baicalein by 20.4 and 30.9% respectively ($p<0.05$). Figure 4 indicates that the addition of Ferulic acid and Baicalein significantly ($P<0.05$) reduced the intrinsic fluorescence (IF) of amylin relative to the control (Figure 4).

Amyloid destabilizing effects of these components were shown in Figures 5 and 6. The obtained data from the 2nd run of experiments confirmed that both compounds were able to open the amyloid sheet significantly ($p<0.05$). Diabetes is the most common endocrine disorder characterized by hyperglycemia. Diabetes mellitus persists to generate a significant burden on healthcare services worldwide because of its high prevalence, so multidisciplinary study aimed at preventing and treating is one of the world-wide research priorities. It is implicated that human amylin is a small fibrillogenic protein that is

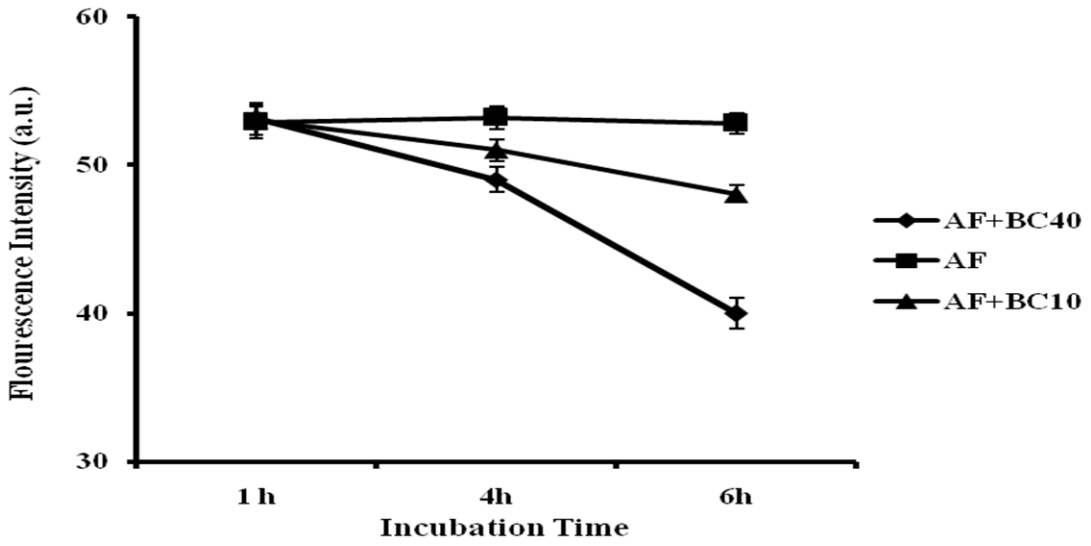

Figure 6. Baicalein effect on amylin fibril destabilization. Baicalein with two different concentrations destabilized amylin fibril (AF) significantly after 6 hours incubation in a dose dependent manner.

the major constituent of pancreatic islet amyloid, which occurs in most subjects with type 2 diabetes (Konarkowska et al., 2006; Zheng et al., 2010; Wang et al., 2011). Although the amyloid contribution to islet β-cell dysfunction is well clarified in the diabetic patients, but the factors affecting this process remain elusive. There are evidences that some essential and toxic elements influence the aggregation of amylin (Mirhashemi et al., 2011a, b and c; Ward et al., 2008) in vitro. This project showed significant inhibitory role for Ferulic acid and Baicalein concerning amylin amyloidogenesis and so demonstrated the β-sheet opening ability for these compounds. It was indicated that these compound induced their effects in a concentration-dependent manner. The formation of amyloid fibrils, via self-assembly of peptide, is assumed to be a crucial step in the pathogenesis of many amyloid diseases, including type-2diabetes mellitus (Tabner, 2001). Previous investigations have shown that fibrillization of several polypeptides such as amylin is accompanied by formation of free radicals. In turn, reactive oxygen species (ROS), mainly free radicals, accelerate fibril formation , possibly via oxidation reactions (Schoneich, 2005; Shoval et al., 2007). ROS may impact disulfide bond formation (Cumming et al., 2004) and subsequently influence the development of IAPP misfolding. Disulfide bonds are important for proper protein structure, biological activity, and stability of many secreted and membrane proteins (Kopito and Ron, 2000; Anelli et al., 2002; Fassio and Sitia, 2002). Although the exact mechanism by which Ferulic acid and Baicalein inhibit amyliod formation remains unclear, it may be suggested that the antiamyloidogenic power of these compounds may be due to their antioxidant properties. Several lines of evidence have shown that flavonoids and monophenolic compounds such as Ferulic acid and Baicalein act as a potent antioxidant in vitro, due to its ability to scavenge free radicals (Barone et al., 2009; Yina et al., 2011). Further study is required to elucidate the exact mechanism.

Conclusion

Mainstays of therapy for type 2 diabetes involve drugs that are insulin-centric that is, they are designed to increase insulin secretion and decrease insulin resistance. The mechanism for deterioration of β-cell function is related to chronic oxidative stress. This suggests that drug discovery should not exclusively focus on insulin-centric targets, but also include glucose-centric strategies, such as antioxidant protection of the β-cell. The antioxidant activity of Ferulic acid and Baicalein was examined on the formation, and destabilization of amylin amyloid fibril, in vitro. For the first time in the literature, we expressed that these two compounds inhibited amylin amyloid formation significantly. In addition, they destabilized preformed amylin fibrils. It may be concluded that these compounds should be key molecules for the development of the therapeutics for diabetic patients.

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