To estimate effective antiamyloidogenic property of melatonin and fisetin and their actions to destabilize amyloid fibrils

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Abstract: Aggregating of amylin as pancreatic deposition is connected with pancreas degeneration in type 2 diabetes mellitus. Suppression of the amylin accumulation and so instability of the pre-formed pancreatic β-amyloid, may be attractive curative goal for mediation of diabetes mellitus. Fluorimetric assay by Thioflavin-T was utilized for investigating the properties of melatonin and fisetin on the generation and instability of β-amyloid near to physiological conditions. The results showed that after 168 hours incubation by shaker incubator in 37°C, melatonin at 10μM and 40 μM repressed amylin amyloid formation by 20.1% and 27.5% respectively ($p<0.05$) and the similar values of fisetin inhibited the formation of β-sheet structure by 16.5% and 23.2% respectively ($p<0.05$).The obtained data also confirmed that amyloidal sheet opening was induced by melatonin and fisetin significantly ($p<0.05$). It may be concluded that islet amyloid cytotoxicity to β-cells may be reduced by melatonin and fisetin, and they should be important constituents of new drugs for diabetes mellitus treatment.

Keywords: Amylin, Diabetes Mellitus, Melatonin, Fisetin, Amylin fibrils.

INTRODUCTION

Diabetes mellitus considered to be a persistent illness and its complications have become causes of morbidity, disability and death in developed and developing countries (Bai *et al*., 2014). Causation of diabetes occurrence and its pathologic consequences is not well clear up to now; but it has been implicated that human islet amyloid polypeptide accumulates in type II diabetes to form β-amyloid that disturbs pancreatic task, finally leading to the failure of insulin creation (Reddy Nanga *et al*., 2011; Dupuis *et al*., 2011). Human amylin has 37 amino acids in extent, is released from pancreas. Amylin acts as a controller of gastric discharge, blood sugar homeostasis, and other metabolic behaviors (Cao *et al*., 2013). Amylin accumulation results in the development of diabetes and confers to the dysfunction of β-cells (Haataja *et al*., 2008 & Seeliger 2012).

Melatonin (N-acetyl 5-methoxytryptamine) (fig. 1), an indoleamine hormone, is released mainly from the pineal and so presents in herbs (Espino *et al*., 2011). The exclusive characteristics of melatonin such as scavenging of free radicals have been widely considered. Dissimilar to further antioxidants, for example vitamin C and dihydrolipoic acid, melatonin does not act as a pro-oxidant (Zhu *et al*., 2009).

Miller *et al*., 2014 and Abdolsamadi *et al*., 2014). Fisetin (3, 7, 3', 4'-tetrahydroxyflavone) (fig. 1), is a unique natural flavonol exists in many plants and fruits. It displays a broad type of pharmacological characteristics, counting antioxidant, anti-inflammatory as well as anti-carcinogenic property (Prasath 2011 & Ying *et al*., 2011). Due to the rising occurrence of diabetes, multiform study intended at aborting and remedy is one of the universal investigate priorities. A hallmark of diabetes mellitus is the presence of amyloid deposits in the islets of Langerhans. Suppression of the amylin accumulation, and so instability of the pre-formed pancreatic β-amyloid, may be attractive curative goal for mediation of diabetes mellitus. To the best of our facts, this is the first study to appraise possible antiamyloidogenic property of melatonin and fisetin and their actions to destabilize β-amyloid sheet.

MATERIALS AND METHODS

Synthetic peptide of human amylin and additional applied materials were bought from Sigma-Aldrich Corporation, Products of USA.

Amylin stock solution

Human amylin used in this project had the following characteristics: (1-37) (*kcntatcatqrlanflvhssnnfgailss-tnvgsnty*, disulfide bridge: between 2 and 7) (Mirhashemi *et al*., 2011). It was purified by 97% along with the lyophilized salt involved 70% peptide by mass. Amylin stock solution was ready by addition 1.0ml dimethylsulfoxide (DMSO) to lyophilized peptide, sonicating at room temperature for 15 min.

In vitro 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 melatonin and fisetin on amylin aggregation and

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amyloidogenesis, control and four treated groups were considered. The peptide stock solution was diluted by PBS (Phosphate Buffer Saline) 50mM, pH: 7.4, to the final concentration of 10µM. Different concentrations of melatonin (10 and 40µM) and fisetin (10, 40µM) were prepared in PBS buffer containing 10µM amylin as treated groups, separately. The samples without melatonin and fisetin were selected as the control group. All studied groups were incubated at 37°C for 168 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 melatonin and fisetin 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 9 hours in 37°C.

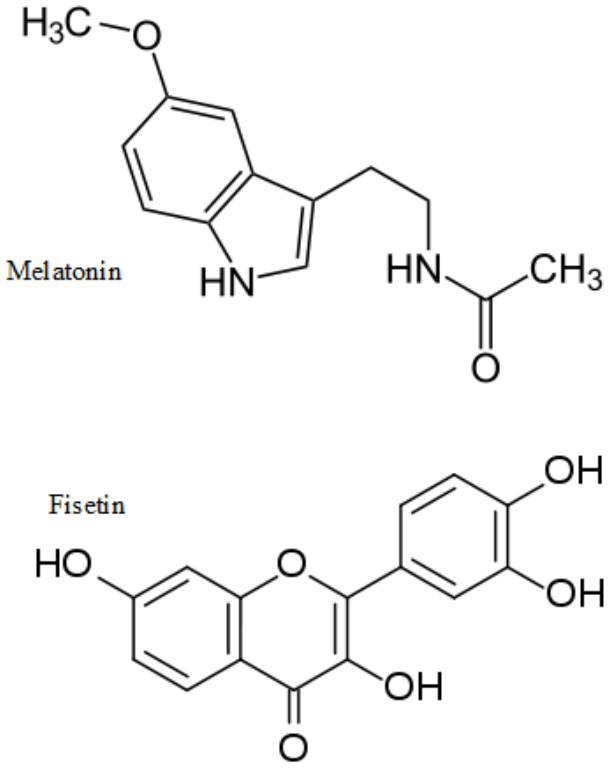


Fig. 1: Structures of melatonin and fisetin

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. The test was performed by addition 40µl of each incubated solution to 700µl of 10µM ThT solution. Fluorimetric evaluation was performed using spectrofluorimeter (Perkin-Elmer LS55, USA) at 25°C.

IF assay

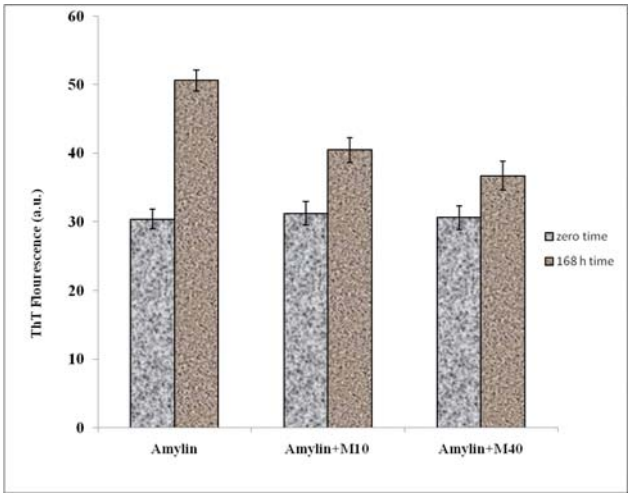
The intrinsic fluorescence of the peptide tyrosine residue was measured for the studied groups after 144h by emission at 304 nm when excited at 270nm.

STATISTICAL ANALYSIS

Descriptive statistics was accomplished to obtain means and standard deviations. Statistic significance level was established at $p<0.05$. Data were analyzed by SPSS software.

RESULTS

The first run of experiments indicated that amylin itself readily aggregated and formed a ThT-Positive material in control group. Data indicated that at zero time, ThT-fluorescence mean value for control group was 30.38 which at 168h had increased to mean value of 50.62 ($p<0.05$). In melatonin treated groups, ThT fluorescence assay indicated that 10µm and 40µM of melatonin inhibited amyloid formation by 20.1% and 27.5% respectively after 168 h incubation at 37°C ($p<0.05$) table 1 & fig. 2). Different concentrations effects of fisetin on amylin aggregation were demonstrated in table 1 and fig. 3. These data indicated that compared to control group, ThT-fluorescence was increased significantly in the presence of 10 and 40µM of fisetin by 16.5% and 23.2% respectively ($p<0.05$). The data indicated that the inhibitory effect of these components versus amyloid formation were dose-dependent significantly ($p<0.05$). fig. 4 indicates that the addition of melatonin and fisetin notably ($P<0.05$) reduced the IF of amylin relative to the control (fig. 4). Amyloid destabilizing effects of these components were shown in figs. 5 and 6. The obtained data from the 2nd run of experiments confirmed that both compounds were able to open the amyloid sheet significantly ($p<0.05$).

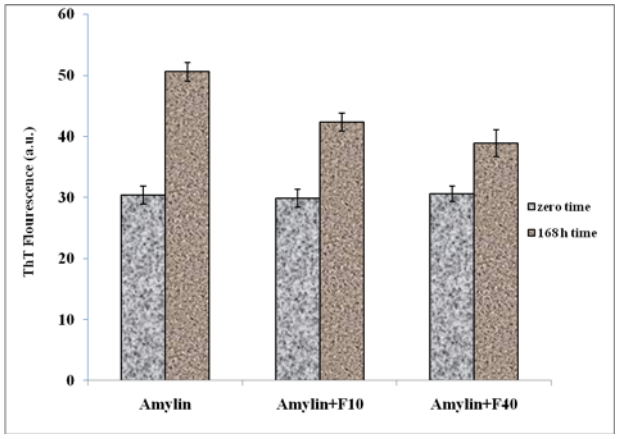


All groups were incubated at 37°C for 168 h with shaking by a shaker incubator. At zero time (before incubation) there were no significant differences between three groups: amylin, amylin+M10 and amylin+M40 ($p>0.05$). Melatonin (M) inhibited amylin aggregation in a dose-dependent manner ($p<0.05$). Data have been shown as Mean ±SEM, n=5.

Fig. 2: Thioflavin T fluorescence assay of protective effects of melatonin on amylin Fibrils.

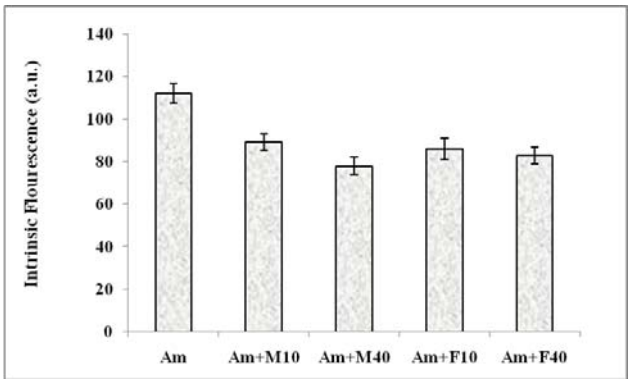
DISCUSSION

Amyloid sediments in the pancreas are the hallmark of diabetes mellitus. These deposits are formed by hIAPP (human Islet Amyloid Polypeptide). Normal solution form of hIAPP tolerates misfolding giving rise to beta-amyloid fibrils in the pancreas of diabetic individuals that impair the functionality and viability of beta-cells and may lead to apoptosis (Cao *et al*., 2013; Seeliger, 2012 and Fernandez, 2014). Accumulation of hIAPP into pancreatic sediments has been supposed to be one of the fundamental subscriber to pancreatic β-cell loss in about 95% of type 2 diabetes (Bahramikia, 2013 and Sinopoli *et al*., 2014).Thus, materials that avert the toxicity of aggregations, should be regarded as a new drug for medication of this illness.



All groups were incubated at 37°C for 168 h with shaking by a shaker incubator. At zero time (before incubation) there were no significant differences between three groups: amylin, amylin+F10 and amylin+F40 ($p>0.05$). Fisetin (F) inhibited amylin aggregation in a dose-dependent manner ($p<0.05$). Data have been shown as Mean ±SEM, n=5.

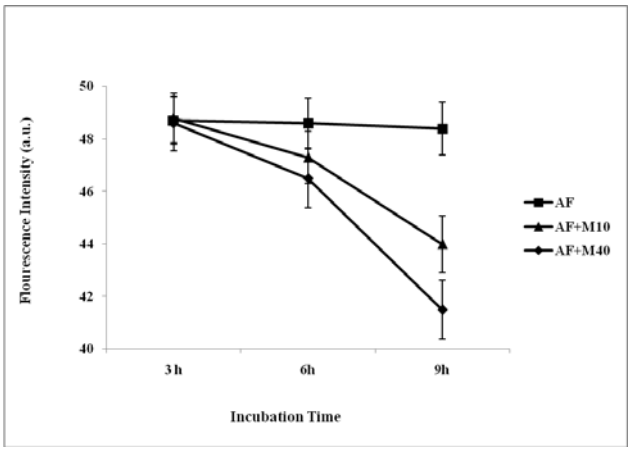
Fig. 3: Thioflavin T fluorescence assay of protective effects of fisetin on amylin aggregation



Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the agents was measured after 144 h incubation in 37°C. Data have been shown as Mean ±SEM, n=5.

Fig. 4: Intrinsic fluorescence of the control and treated groups.

In view of the fact that there isn't any data in the literature concerning the effect of melatonin and fisetin on amylin amyloidogenesis thus the present study was designed. This study showed that melatonin and fisetin inhibited amylin aggregation significantly ($p<0.05$) and so demonstrated the β-sheet destabilizing 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 the peptide, is considered to be a critical stage in the creation and development of numerous amyloid disorders, counting type-2diabetes mellitus (Pannuzzo *et al*., 2013 and Seeliger *et al*., 2013). Previous studies have revealed that fibrillization of several polypeptides such as amylin is occurred by creation and development of oxidative reactions (Schoneich, 2005 and Shoval *et al*., 2007). ROS (Reactive Oxygen Species) may impact disulfide bond formation and subsequently influence the development of hIAPP misfolding. Disulfide bonds have significant role in the native folding of secretory and membrane proteins (Okumura *et al*., 2014 and Hidaka 2014). Although the exact mechanism for preventive effects of melatonin and fisetin on amylin accumulation stays ambiguous, but it may be suggested that the inhibitory power of these compounds on amyloid fiber formation may be due to their free radical scavenging properties. Further study is required to elucidate the exact mechanism.



Melatonin (M) with two different concentrations destabilized amylin fibril (AF) significantly after 9 hours incubation.

Fig. 5: Melatonin effect on amylin fibril destabilization.

CONCLUSION

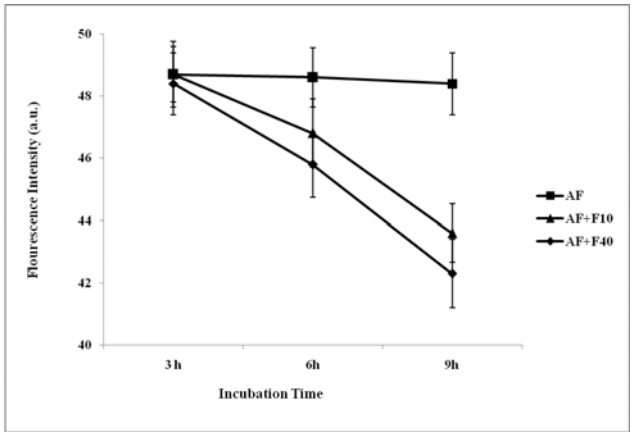
Backbones of the diabetes treatment engage medications that are insulin-based, i.e., these drugs get higher insulin discharge and diminish insulin resistance. In as much as, fall of β-cell capacity is associated to persistent oxidative stress, thus drug designing should not completely aim attention at insulin, but also consist of glucose-based approach, such as antioxidant safety of the pancreas. The antioxidant activity of melatonin and fisetin was examined on the formation, and destabilization of amylin

Table 1: Thioflavin T fluorescence assay of protective effects of melatonin and fisetin on amylin aggregation

Groups	ThT Fluorescence (a.u.) at zero time	ThT Fluorescence (a.u.) at 168 h time	Amylin aggregation (%)
Amylin	30.38±1.44	50.62±1.51	Increased 66.6
Amylin + Melatonin 10	31.22±1.73	40.46±1.82	Decreased 20.1
Amylin + Melatonin 40	30.58±1.69	36.7±2.08	Decreased 27.5
Amylin + Fisetin 10	29.84±1.51	42.28±1.49	Decreased 16.5
Amylin + Fisetin 40	30.58±1.27	38.88±2.17	Decreased 23.2

All groups were incubated at 37^{o}C for 168 h with shaking by a shaker incubator. At zero time (before incubation) there were no significant differences between five groups, ($p>0.05$). Melatonin and Fisetin inhibited amylin aggregation in a dose-dependent manner after 168h incubation ($p<0.05$). Data have been shown as Mean ±SEM, n=5.

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 melatonin and fisetin should be important molecules for the expansion of the therapeutic substances for diabetic patients.



Fisetin (F) with two different concentrations destabilized amylin fibril (AF) significantly after 9 hours incubation in a dose dependent manner.

Fig. 6: Fisetin effect on amylin fibril destabilization.

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