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Evaluation of Aluminium, Manganese, Copper and Selenium Effects on Human Islets Amyloid Polypeptide Hormone Aggregation

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Abstract: Islet amyloid formation causes destruction of insulin-producing β-cells of the pancreas. The subsequent lack of insulin leads to increased blood and urine glucose. In this research, the fluorimetric assay was used to examine the effects of aluminium and some nutritionally essential trace elements including, manganese, copper and selenium on amyloid formation of human peptide of amylin under near-physiological circumstances. Results obtained from *in vitro* study showed that after 120 h incubation by shaker incubator in 37°C, copper and selenium at 8 μM inhibited amylin 8 μM from amyloid fibril formation by 22.1 and 11.3%, respectively ($p<0.05$) while the similar values of either aluminium and manganese promoted the formation of β-pleated sheet structure by 19.3 and 13.2% respectively ($p<0.05$). If islet amyloid is cytotoxic to β-cells then copper and selenium may be able to protect these cells against degeneration in diabetic patients especially in type 2 diabetes mellitus.

Key words: Diabetes mellitus, amylin, thioflavin T fluorescence, trace elements, toxic element

INTRODUCTION

The major aspect of diabetes is chronic hyperglycemia that leads to the disorders in metabolism of carbohydrates, fats and proteins. Although diabetes is classified as a single disease, but secondary complications such as cardiac abnormality, diabetic retinopathy, nephropathy and atherosclerosis may occur (Naqshbandi *et al*., 2008). The etiology of diabetes and its complications is still not clear; however several factors such as aging, obesity and oxidative stress have been implicated (Houstis *et al*., 2006; Roberts and Sindhu, 2009). Owing to the increasing prevalence of diabetes, multidisciplinary study aimed at preventing and treating is one of the world-wide research priorities. Aluminium (Al) is an element with extensive use in almost all modern industries as well as daily household life. It is present in medicines and is added to drinking water for purification (Ochmanski and Barabas, 2000). Impairments of glucose utilization, free radical-mediated cytotoxicity, lipid peroxidation, impact on gene expression, altered protein phosphorylation and changes in kinetic behaviors of the alkaline phopsphatase isoenzymes has been reported following Al toxicity (Strong *et al*., 1996; Mirhashemi *et al*., 2010). Manganese is the second common naturally occurring element in the environment. It is necessary for maintaining the proper function of many enzymes such as pyruvate carboxylase, arginase, superoxide dismutase and glycosyltransferase (Erikson *et al*., 2007; Crossgrove and Zheng, 2004; Gerber *et al*., 2002) and so its toxicity on hepatobiliary system was studied (Mirhashemi *et al*., 2009). Copper and selenium, are involved in many biochemical processes supporting life. The most important of these processes are cellular respiration, cellular utilization of oxygen, DNA and RNA reproduction, maintenance of cell membrane integrity and sequestration of free radicals. Copper, manganese and selenium are involved in destruction of free radicals through cascading enzyme systems. Superoxide radicals are reduced to hydrogen peroxide by superoxide dismutases in the presence of copper and manganese cofactors. Hydrogen peroxide is then reduced to water by the selenium-glutathione peroxidase couple. Efficient removal of these free radicals maintains the integrity of membranes, reduces the risk of pathological process (Chan *et al*., 1998). On the other hand, Native human islet amyloid polypeptide (hIAPP) or amylin is the primary component of the amyloid deposits found in the pancreas of the majority of patients with type 2 diabetes mellitus (type 2 DM). Amylin is a 37-amino-acid peptide hormone that is normally produced in the β-cells of the islets of Langerhans in the pancreas. This peptide is co-secreted from these cells with insulin and functions to control hyperglycemia by restraining the rate at which

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dietary glucose enters the bloodstream. Though the causal relationship between the development of hIAPP amyloid and the appearance of type 2 DM is unresolved, it has been documented that sites of hIAPP amyloid deposition in the pancreas are surrounded by areas of β-cell degeneration and hIAPP fibrils have been shown to be toxic to both human islet β-cells in culture (Rhoades *et al*., 2000).

For this reason, the major aim of this study was to investigate the possible effects of aluminium, manganese, copper, selenium and their combinations on human amylin hormone aggregation *in vitro*.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Company. This research project was conducted from March, 2010 to January, 2011 in Kashan University of Medical Sciences, Kashan, Iran.

Sample preparation: Synthesized human amylin (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) was purchased from Sigma-Aldrich. Its purity was 97% and the lyophilized salt included 70% peptide by weight. Peptide stock solution was prepared by adding 1.0 mL Dimethylsulfoxide (DMSO) to dry purified peptide, sonicating at room temperature for 15 min.

Peptide aggregation: Stock solution (1mg mL^{-1}) was diluted to a final concentration of 8 μM with PBS, pH: 7.4. Diluted solution was divided into eight groups so that each group had six samples. Aluminum, manganese, copper and selenium with concentration of 8 μM were prepared in amylin containing solution separately. The chosen concentration of total elements is physiologically relevant for manganese, copper, selenium and pathophysiologically significant for aluminium. In order to study the possible combined effects of several elements on human islet amyloid poly peptide amyloidogenesis, in addition to the five groups mentioned above, three additional groups were designed as follow: the 6th group included: amylin, aluminium and copper, the 7th group involved: amylin, manganese and copper and finally, the 8th group contained amylin, manganese, copper and selenium. The samples without any elements were selected as control group. All studied groups were incubated at 37°C for 168 h with shaking by a shaker incubator (GFL 3031, Germany).

Thioflavin T assay: To identify the formation of beta-pleated sheets of amyloid, Thioflavin T (ThT) assay was performed by adding 40 μL of incubated solution to 700 μL of 10 μM ThT solution (Sigma, USA) in PBS, pH 7.4. 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 (slit width = 10 nm) when excited at 440 nm (slit width = 5 nm) (Khan *et al*., 2005).

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 (Ward *et al*., 2008).

Light scattering assay: For determination of fibril growth endpoints, light scattering was performed at selective incubation times point 120 and 168 h. Light scattering was measured in a Perkin-Elmer LS55 fluorescence spectrophotometer at room temperature using a 1 cm path length quartz cell. Both the excitation and emission wavelengths were set to 405 nm with a spectral bandwidth of 1 nm.

Statistical analysis: Descriptive statistics was accomplished to obtain means and standard deviations. Between groups comparisons were performed with t-test. Statistic significance level was established at $p<0.05$. Analysis of data was performed using SPSS statistical software package.

RESULTS

As mentioned above, amylin samples without any the elements were selected as control group. In this group amylin itself readily aggregated and formed a ThT-Positive material, at zero time reading gave a value of 20.22 a.u. (arbitrary unit) which at 120 h had increased to mean value of 28.24 a.u. Added Al and Mn with concentration of 8 μM significantly ($p<0.05$) promoted amylin aggregation so that ThT fluorescence of amylin was increased by 19.3 and 13.2%, respectively after 120 h incubation in 37°C while Cu and Se significantly ($p<0.05$) inhibited amylin deposition and reduced ThT fluorescence by 22.1 and 11.3% respect to control group at the same time (Fig. 1). We more analyzed the total participation of amylin with the different elements at the incubation time point of 120 h, at which we believe that the interactions between amylin and the elements were at a plateau according to ThT results (Fig. 2). The data from

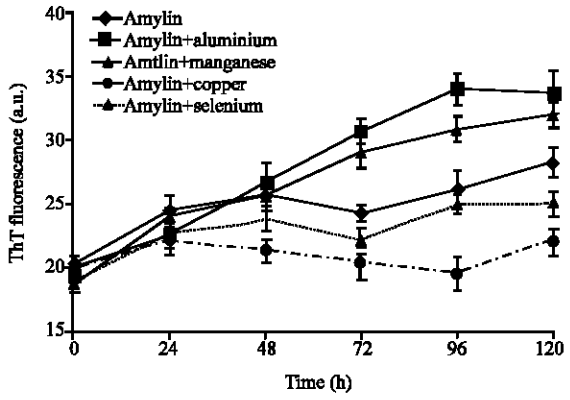

Fig. 1: The influence of incubation time on ThT fluorescence of studied groups. Amylin aggregation was monitored by ThT fluorescence in the absence and presence of aluminium, manganese, copper and selenium for 120 h at 37°C. There were statistically significant differences of amylin aggregation between treated groups and the controls ($p<0.05$). Data have been shown as Mean±SD, n = 6

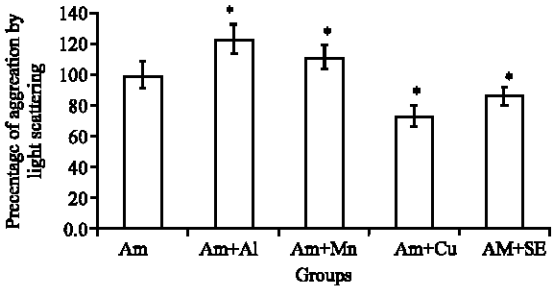

Fig. 2: Light scattering assay of amylin aggregation of studied groups. The data from light scattering show almost the same results from ThT assay. It confirmed that Al and Mn enhanced amylin beta pleated sheet formation, while Cu and Se had inverse effect. Data have been shown as Mean±SD, n = 6. *Indicates statistically significance at $p<0.05$ relative to control

light scattering show almost the same results from ThT assay. It confirmed that Al and Mn enhanced amylin beta pleated sheet formation, while Cu and Se had inverse effect (Fig. 2). In order to study the possible combined effects of several elements on human islet amyloid poly peptide amyloidogenesis, the 6, 7 and 8th groups were designed, as mentioned in the method section. ThT fluorescence assay showed that copper significantly reduced the toxic effect of aluminium and manganese on

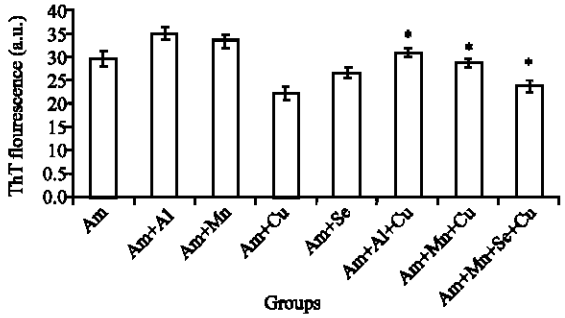

Fig. 3: Thioflavin T fluorescence assay of protective effects of copper and selenium on amylin deposition. All eight groups were incubated at 37°C for 168 h with shaking by a shaker incubator. * Indicates statistically significant protective roles of copper and selenium. Data have been shown as Mean±SD, n = 6

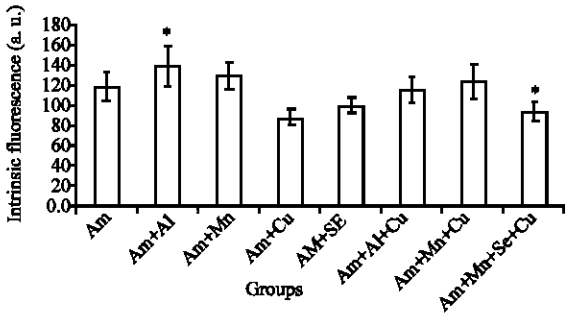

Fig. 4: Intrinsic fluorescence of the studied groups. Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the elements was measured after 168 h incubation in 37°C. Data have been shown as Mean±SD, n = 6. *Indicates statistically significance at $p<0.05$ relative to control

amylin aggregation by 11.1 and 13.7%, respectively ($p<0.05$) (Fig. 3). ThT fluorescence value was decreased by 28.4% ($p<0.05$) in 8th group which contained amylin, manganese, selenium and copper (Fig. 3) that implicated the protective role of combined selenium and copper. Figure 4 indicates that the addition of copper and selenium significantly ($p<0.05$) reduced the intrinsic Fluorescence (IF) of amylin while added aluminium showed a statistically significant ($p<0.05$) increase in intrinsic fluorescence relative to the no added metal treatment. Added manganese also showed a small increase in intrinsic fluorescence relative to single amylin (Fig. 4).

DISCUSSION

It is now well recognized that diabetes is an epidemic disease in most countries that are undergoing socio-economic transitions. Worldwide, an estimated 150 million people are affected by diabetes and this number is likely to reach 300 million by the year 2025 if successful strategies are not implemented for its prevention and control (King *et al*., 1998). The physiological role of amylin is unknown (Robrtson and Harmone, 2007), however, its aggregation *in vivo* as beta-pleated sheets and subsequent deposition in the islets of Langerhans is likely to be an abnormal process and has been greatly implicated in the deterioration of beta-cells in T2DM (Konarkowska *et al*., 2006; Lorenzo *et al*., 1994). Since there isn't any data in the literature concerning effects of manganese and selenium on amylin amyloidogenesis and so some evidences regarding correlation between copper and amylin depositions are controversial, thus the present study was designed. The main purpose of this project was to gain a further insight on the role of the mentioned elements on amylin aggregation. These findings showed that aluminium and manganese promote abnormal folding while copper and selenium inhibit beta-sheet formation of human islet amyloid polypeptide significantly ($p<0.05$), (Fig. 1-3). In concert with many other amyloidogenic peptides, the concentrations of amylin in the blood of normal persons is 1-20 pM rising to about 50 pM in subjects with insulin resistance (McCracken *et al*., 1989). This statement showed that the amylin value in the patients is less able to induce its self-aggregation *in vivo* and there maybe other factors which promote the precipitation and deposition of amylin *in vivo* (Lomas *et al*., 1992). There is evidence that Al influence the aggregation of amylin (Westermark *et al*., 1990) just as our results showed. The role of aluminium and manganese in induction of amyloidogenesis may be result from stimulation of ROS (Reactive Oxygen Species) production by these elements. ROS may impact disulfide bond formation (Cumming *et al*., 2004) and subsequently influence the development of IAPP (Islet Amyloid PolyPeptide) misfolding. Disulfide bonds formed in newly synthesized proteins in the ER (Endoplasmic Reticulum) of cells are important for proper protein folding, protein structure, biological activity and stability of many secreted and membrane proteins (Kopito and Ron, 2000; Anelli *et al*., 2002; Fassio and Sitia, 2002). In the case of copper there is conflicting information. Unlike our study, Masad *et al*. (2007) expressed that copper stimulated amyloidogenesis via stimulation of production of H_2O_2 (Masad *et al*., 2007) but Ward and *et al*. (2008) showed that copper inhibited the amylin amyloidogenesis, *in vitro*. Our findings were consistent with these results. The mechanism whereby copper and/or selenium inhibited amylin from forming beta-sheets of amyloid might involve its destabilisation of the intramolecular disulphide bridge, the presence of which in the peptide might be a prerequisite to amyloid formation as it is in another amyloidogenic peptide, ABri (Khan *et al*., 2004).

In summary, results obtained from this study revealed that in the absence of added elements, the 8 μM solution of amylin formed aggregates of peptide within the incubation time under physiological-like conditions, in vitro. We found that both aluminium and manganese stimulated amylin aggregation but copper and selenium inhibited amylin amyloidogenesis. We further found that copper and selenium could reduce the aluminium and manganese effects (Fig. 4) *in vitro*. It may be completed that if amyloidogenesis of amylin is involved in the etiology of diabetes mellitus then our results suggest that copper and selenium might protect against the toxicity of amylin.

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