*Full Length Research Paper*

# To determine the possible roles of two essential trace elements and ascorbic acid concerning amyloidal beta-sheet formation in diabetes mellitus

**Seyyed Mehdi Mirhashemi\* and Mohammad-Hosein Aarabi**

Biochemistry Department, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, I. R. Iran.

Amylin is a peptide hormone that is made and co-secreted along with insulin. Human amylin is the main component of amyloid beta-sheet found in the pancreas of majority of diabetic patients. Amyloidogenesis causes destruction of pancreatic β-cells. The subsequent lack of insulin leads to increased blood and urine glucose. In this article, the fluorimetric assay was used to examine the role of ascorbic acid and two essential trace elements including zinc and iron on beta-amyloid formation of human peptide of amylin hormone under near-physiological circumstances. Results obtained from *in vitro* study showed that after 120 h incubation by shaker incubator at 37°C, zinc element at 10 μM inhibited amylin 10 μM from amyloid fibril formation by 9.1% ($p<0.05$) while the similar value of iron element promoted the formation of β-sheet structure by 13.1% ($p<0.05$). The obtained data also demonstrated that ascorbic acid with concentration of 150 μM had inhibitory effects on formation of beta-amyloid sheet significantly ($p<0.05$). It may be concluded that if islet amyloid is cytotoxic to β-cells then zinc and ascorbic acid should protect these cells against degeneration in diabetic patients.

**Key words:** Vitamin C**,** diabetes mellitus, amylin hormone, zinc, iron

## INTRODUCTION

Diabetes mellitus is a metabolic disease that is characterized by hyperglycemia that results in the disturbance of carbohydrates, fats and proteins metabolism. 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 et al., 2009). Owing to the increasing prevalence of diabetes, multidisciplinary study aimed at preventing and treating is one of the world-wide research priorities. Zinc and iron are two essential trace elements for life, playing a central role in many metabolic processes. More than 300 zinc metalloenzymes occur in all six categories of enzyme systems. Proteins can form domains able to bind tetrahedral zinc atoms by coordination with histidine and cysteine to form folded structures that have become known as zinc fingers. These biologically active molecules have important roles in gene expression and play a key role in developmental biology and also in the regulation of steroid, thyroid and other hormone synthesis (Falchuk, 1998; Berg et al., 1996). Zinc also have examined as insulin mimetic and used in diabetes treatment (Sakurai et al., 2005; Meyer et al., 2009). Numerous cellular enzymes and coenzymes require iron, either as an integral part of the molecule or as a cofactor. Nearly half of the enzymes of the Krebs cycle, hemoglobin, peroxidase and catalase enzymes, cytochromes have iron. 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

─────────────
\*Corresponding author. E-mail: mirhashemism@gmail.com. Tel: +98 361 5550021. Fax: +98 361 5551112.

(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 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 main objective of the present study was to investigate the possible roles of ascorbic acid and two essential trace elements including zinc and iron concerning amyloid beta- sheet formation of human amylin hormone, *in vitro*.

## MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Company.

### 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-$NH_2$, 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

Aggregation was induced by adding stock solution to modified Krebs-Hensleit buffer, pH: 7.4, to a final concentration of 10 µM. Diluted solution divided into eight groups so that each group had six samples. The samples without any agents were selected as the first or control group. The 2nd, 3rd and 4th groups were composed of zinc, iron and ascorbic acid, respectively. Zinc and iron with concentration of 10 µM and so L-ascorbic acid with concentration of 150 µM were prepared in amylin containing solution separately. In order to study the possible combined effects of the two elements and ascorbic acid on human islet, amyloid poly peptide amyloidogenesis, four additional groups were designed as follow: the 5th group included: amylin, zinc and ascorbic acid, the 6th group involved: amylin, iron and ascorbic acid, the 7th group involved: amylin, zinc and iron, and finally, the 8th group contained amylin, zinc, iron and ascorbic acid. 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).

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).

### Intrinsic fluorescence (IF) assay

The intrinsic fluorescence of the peptide tyrosine residue was measured for the studied groups after 72 h by averaging the fluorescence emission at 304 nm when excited at 270 nm.

### Light scattering assay

For determination of fibril growth endpoints, light scattering was performed at selective incubation time point 120 h. Light scattering was measured in the 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 independent sample t-tests. Statistic significance level was established at $p<0.05$. Analysis of data was performed using SPSS statistical software package.

## RESULTS

In control group amylin itself readily aggregated and formed a ThT-Positive material, at zero time reading gave a mean value of 55.32 a.u. which at 120 h had increased to mean value of 65.86 a.u. Added iron with concentration of 10 µM significantly ($P<0.05$) promoted amylin aggregation so that ThT fluorescence of amylin was increased by 13.1% after 120 h incubation in 37°C, while zinc and ascorbic acid significantly ($P<0.05$) inhibited amylin deposition and reduced ThT fluorescence by 9.1 and 22.6% with respect to control group at the same time (Figure 1).We more analyzed the total participation of amylin with the different agents at the incubation time point of 120 h, at which we believe that the interactions between amylin and the agents were at a plateau according to ThT results (Figure 2). The data from light scattering show almost the same results from ThT assay. It confirmed that iron enhanced amylin beta pleated sheet formation, while zinc and ascorbic acid had inverse effects (Figure 2). In order to study the possible combined effects of the agents on human islet amyloid poly peptide amyloidogenesis, the 6th, 7th, and 8th groups were designed, as mentioned in previously. ThT fluorescence assay showed that, zinc and ascorbic acid significantly reduced the toxic effect of iron on amylin aggregation by 6.8 and 9.2% respectively ($p<0.05$) (Figure 3). ThT fluorescence value was decreased by
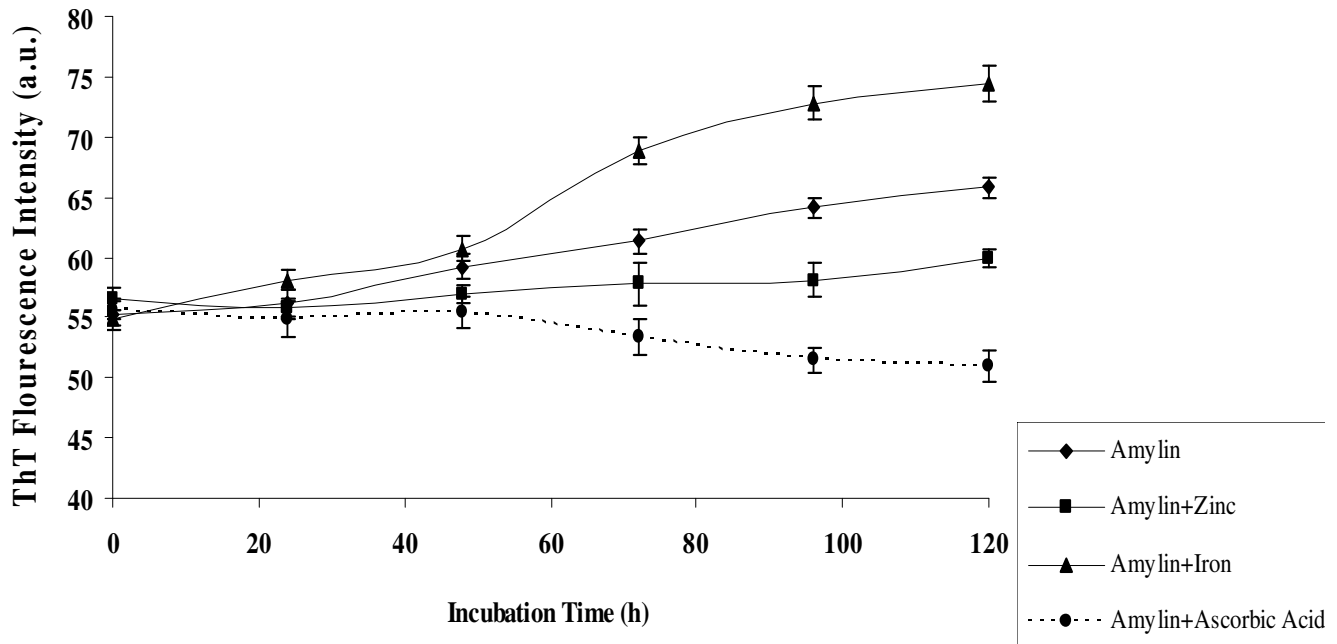
**Figure 1.** Incubation time effect on beta-sheet formation of considered groups. Amyloidal beta-sheet formation was monitored by ThT fluorescence in the absence and presence of zinc, iron and ascorbic acid for 120 h at 37°C. There were statistically significant differences of amyloid formation between treated groups and the controls (p<0.05). Data have been shown as Mean±SD, n=6.
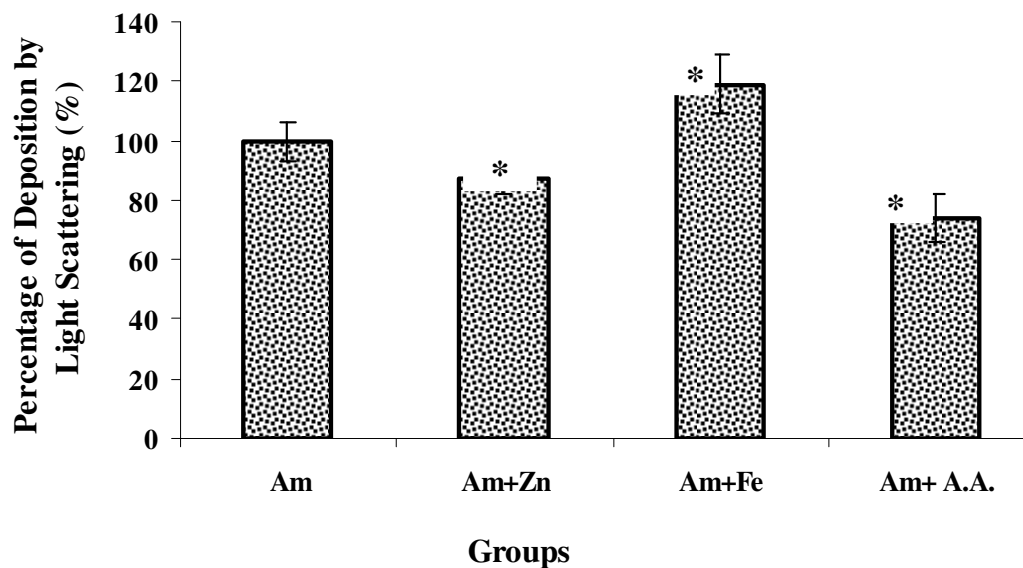


**Figure 2.** Light scattering assay of amylin aggregation of studied groups. Amylin (Am) deposition was affected by zinc (Zn), iron (Fe) and ascorbic acid (A. A.) as measured by light scattering. Amylin was selected as a positive control that gave 100% aggregation measured by light scattering. Data have been shown as Mean±SD, n=6. The Star (*) indicates statistically significance at p<0.05 relative to control.

14.7% (p<0.05) in 8th group which contained amylin, iron, zinc and ascorbic acid (Figure 3) that implicated the protective role of combined zinc and ascorbic acid. Figure 4 indicates that the addition of zinc and ascorbic acid significantly (P<0.05) reduced the intrinsic fluorescence (IF) of amylin while added iron showed a statistically
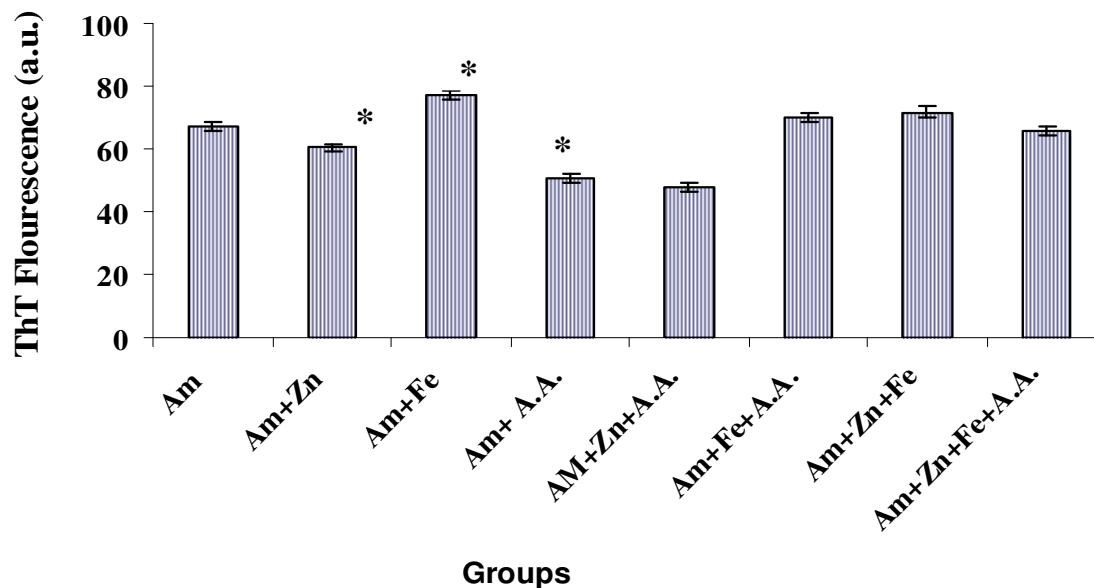
**Figure 3.** Thioflavin T fluorescence assay of protective effects of zinc and ascorbic acid on amylin deposition. All eight groups were incubated at 37°C for 168 h with shaking by a shaker incubator. The Star (*) indicates statistically significant protective roles of zinc and ascorbic acid. Data have been shown as Mean±SD, n=6.
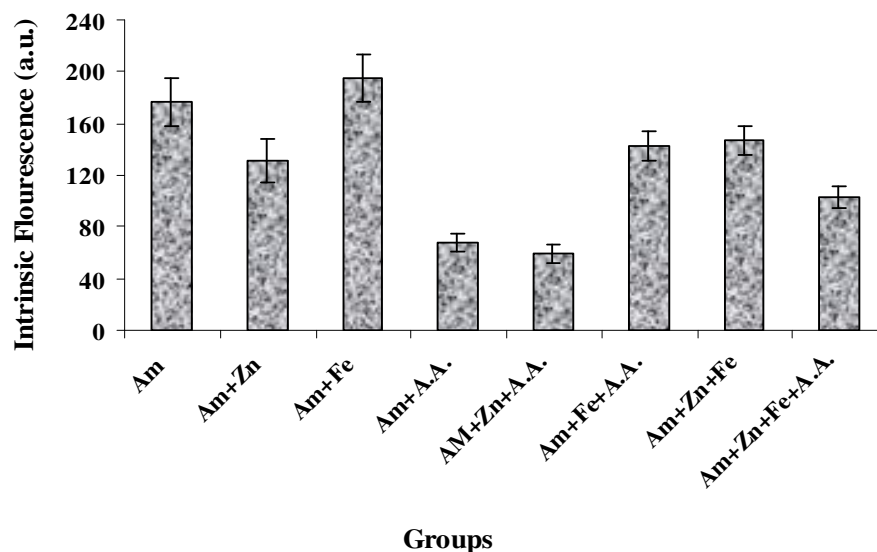


**Figure 4.** Intrinsic fluorescence of the control and treated groups. Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the agents was measured after 168 h incubation in 37°C. Data have been shown as Mean±SD, n=6.

significant (P<0.05) increase in intrinsic fluorescence relative to the control (Figure 4).

## DISCUSSION

It is now well recognized that diabetes is an epidemic disease in most countries that are undergoing socio-economic transitions. World wide, 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

Harmine, 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 type 2 DM (Konarkowska et al., 2006; Lorenzo et al., 1994 ). There are evidences that some essential and toxic elements influence the aggregation of amylin (Mirhashemi et al., 2011; Ward et al., 2008; Westermark et al., 1990) *in vitro*. Since there is no data in the literature concerning combined effects of zinc, iron and ascorbic acid on amylin amyloidogenesis, therefore, some evidences regarding correlation between zinc and amylin depositions are controversial (Ward et al.,2008; Brender et al., 2010 ) 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 and ascorbic acid on amylin amyloidogenesis. Results obtained from this study, revealed that, in control group, the 10 µM solution of amylin formed aggregates of peptide within the incubation time under physiological-like conditions. These findings showed that iron promotes abnormal folding while zinc and ascorbic acid inhibit beta-sheet formation of human islet amyloid polypeptide significantly ($p < 0.05$), (Figures 1, 2 and 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 may be other factors which promote the precipitation and deposition of amylin *in vivo* (Lomas et al., 1992). It is well established that trace elements status in diabetic patients altered as compared to healthy subjects (Aguilar et al., 2007). The disturbed metabolism of some elements has been reported and it was postulated that certain metals have specific roles in the pathogenesis and progress of diabetes (Meyer et al., 2009; Valko et al., 2005). Iron is an essential ion for life, the most important property of iron is its capacity to be reversibly oxidized and reduced, but at same time this make it highly pro-oxidant molecule. In this regard, iron is able to generate powerful reactive oxygen species (ROS) (Ciudin et al., 2010). Redox-cycling metals as iron are involved in the formation of reactive oxygen species; in particular the catalytic function of Fe(II)/Fe(III) in Fenton reaction–mediated generation of hydroxyl radical should be mentioned (Zheng et al., 2008). The role of iron in induction of amyloidogenesis may be result from stimulation of ROS production by this element. ROS may impact disulfide bond formation (Cumming et al., 2004) and subsequently influence the development of hIAPP misfolding. Disulfide bonds formed in newly synthesized proteins in the endoplasmic reticulum (ER) 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 zinc there is conflicting documents. Unlike our study, Ward et al. (2008) expressed that zinc stimulated amyloidogenesis (Ward et al., 2008), but Brender et al. (2010) reported that zinc has a dual effect on hIAPP fibrillogenesis. They expressed that zinc increases the lag-time for fiber formation and decreases the rate of addition of hIAPP to existing fibers at lower concentrations, while having the opposite effect at higher concentrations (Brender et al., 2010). Our findings indicated that zinc has inhibitory effect on amyloidogenesis. The mechanism whereby zinc 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 (Khan et al., 2004).

## Conclusion

In summary, we have characterized that iron stimulated amyloidal beta-sheet formation but zinc and ascorbic acid inhibited amylin amyloidogenesis. We further found that zinc and ascorbic acid could reduce the iron effect (Figure 4) *in vitro*. It may be concluded that if amyloidogenesis of amylin is involved in the etiology of diabetes mellitus then our results suggest that zinc supplementation and ascorbic acid might protect against the toxicity of amylin.

## ACKNOWLEDGEMENTS

**REFERENCES**

Aguilar MV, Saavedra P, Arrieta FJ, Mateos CJ, Gonzalez MJ, Meseguer I (2007). Plasma mineral content in type-2 diabetic patients and their association with the metabolic syndrome. Ann. Nutr. Metab., 51: 402-406.

Anelli T, Alessio M, Mezghrani A, Simmen T, Talamo F, Bachi A, Sitia R (2002). ERp44, a novel endoplasmic reticulum folding assistant of the thioredoxin family. EMBO. J., 21: 835-844.

Brender JR, Hartman K, Nanga RP, Popovych N, de la Salud Bea R, Vivekanandan S, Marsh EN, Ramamoorthy A (2010). Role of zinc in human islet amyloid polypeptide aggregation. J. Am. Chem. Soc., 139(26): 8973-8983.

Berg JM, Shi Y (1996). The galvanization of biology: a growing appreciation for the roles of zinc. Science. 271: 1081-1085.

Ciudin A, Hernández C, Simó R (2010). Iron overload in diabetic retinopathy: a cause or a consequence of impaired mechanisms? Exp. Diabetes. Res., p. 714108.

Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. J. Biol. Chem., 279: 21749-21758.

Falchuk KH (1998). The molecular basis for the role of zinc in developmental biology. Mol. Cell. Biochem., 188: 41-48.

Fassio A, Sitia R (2002). Formation, isomerisation and reduction of disulphide bonds during protein quality control in the endoplasmic reticulum. Histochem. Cell. Biol., 117: 151-157.

Houstis N, Rosen ED, Lander ES (2006). Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature, pp. 440: 944-948.

Khan A, Ashcroht A.E, Higenell V, Korchazkina OV, Exley C (2005). Metals accelerate the formation and direct the structure of amyloid fibrils of NAC. J. Inorg. Biochem., 99: 1920-1927.

Khan A, Ashcroht AE, Korchazkina OV, Exley C (2004). Metal-mediated formation of fibrillar ABri amyloid. J. Inorg. Biochem., 98: 2006-2010.

King H, Aubert RE, Harman WH (1998). Global burden of diabetes, 1995-2025, prevalence, numerical estimation and projections. Diabetes. Care, 21: 1414-1431.

Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJS (2006). The aggregation potential of human amylin determines its cytotoxicity towards islet beta-cells. FEBS. J., 273: 3614-3624.

Kopito RR, Ron D (2000). Conformational disease. Nat. Cell. Biol., 2(11): E207-209.

Lorenzo A, Razzoboni B, Weir GC, Yankner BA (1994). Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. Nature, 368: 756-760.

Lomas DA, Evans DL, Finch JT, Carrell RW (1992). The mechanism of Z alpha 1-antitrypsin accumulation in the liver. Nature, 357: 605-7.

Meyer JA, Spence DM (2009). A perspective on the role of metals in diabetes: past findings and possible future directions. Metallomics, 1: 32-49.

McCracken AA, Kruse KB, Brown JL (1989). Molecular basis for defective secretion of the Z variant of human alpha-1-proteinase inhibitor: secretion of variants having altered potential for salt bridge formation between amino acids 290 and 342. Mol. Cell. Biol., 9: 1406-1414.

Mirhashemi SM, Shahabaddin ME (2011). Evaluation of aluminium, copper, and selenium effects on human islet amyloid polypeptide hormone aggregation. Pak. J. Biol. Sci., 14(4): 288-292.

Naqshbandi M, Harris SB, Esler JG, Antwi-Nsiah F (2008). Global complication rates of type 2 diabetes in Indigenous peoples: a comprehensive review. Diabetes. Res. Clin. Pract., 82: 1-17.

Rhoades E, Agarwal J, Gafni A. (2000). Aggregation of an amyloidogenic fragment of human islet amyloid polypeptide. Bioch. Biophys. Acta, 1476: 230-238.

Roberts CK, Sindhu KK (2009). Oxidative stress and metabolic syndrome. Life. Sci. 84: 705-712.

Robrtson RP, Harmon JS (2007). Pancreatic islet beta-cell and oxidative stress: the importance of glutathione peroxidase. FEBS. J., 581: 3743-3748.

Sakurai H, Adachi Y (2005). The pharmacology of the Insulin mimetic effect of zinc complexes. Biometals. 18: 319–323.

Valko M, Morris H, Cronin MT (2005). Metals, toxicity and oxidative stress. Curr. Med. Chem., 12: 1161-208.

Ward B, Walker K, Exley C (2008). Copper (II) inhibits the formation of amylin amyloid in vitro. J. Inorg. Biochem., 102(2): 371-375.

Westermark P, Engstrom U, Johnson KH, Westermark GT, Betsholtz C (1990). Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation. Proc. Natl. Acad. Sci. USA., 87: 5036-5040.

Zheng Y, LiX K, Wang Y, Cai L (2008). The role of zinc, copper and iron in the pathogenesis of diabetes and diabetic complications: therapeutic effects by chelators. Hemoglobin, 32: 135-145.