ROLE OF CERTAIN AGENTS ON ZINC MODULATION AND INFLAMMATORY PATTERN IN ZINC DEFICIENT DIABETIC RATS

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ABSTRACT

Aims and Objectives: to configure the pattern of inflammation and diabetic state in alloxan diabetic rats fed on zinc deficient diet. Secondly to illustrate the effect of two selected agents, namely zinc gluconate and omega-3 fatty acid (fish oil) on modulation for diabetic state and zinc state. Materials and methods: diabetes was induced by intraperitonially administration of alloxan monohydrate (120 mg /kg) body weight; zinc deficient state was done by feeding zinc deficient diet and deionized water to rats for 28 days and monitor of serum zinc level at interval times. Thirty male albino rats were divided into 5 groups; first one was fed on normal based diet, referred to normal group. Second group is alloxan diabetic rats, received no treatment and referred to diabetic control (DC). Group 3, diabetic rats received zinc deficient diet and referred to as positive control. Group 4 diabetic and zinc deficient rats, received fish oil 50 mg/kg body weight daily for 8 weeks while group 5, diabetic zinc deficient rats, received zinc gluconate 150 mg/kg body weight daily for 8 weeks. Results: Our report showed that treatment with zinc gluconate and fish oil significantly reduced serum glucose, inflammatory markers CRP, TNF-α, IL-6, IL-1β, and IFNγ along with an increase in serum zinc and pancreatic ZNT8 and IL-1β.

KEYWORDS: Type1 diabetes mellitus; inflammatory markers; zinc deficiency; Fish oil; zinc gluconate; zinc transporter 8 (Znt8).
INTRODUCTION

Diabetes mellitus especially type I is expressed as inflammatory disease of the pancreatic islets where inflammation represent a protective mechanism for the disease progression. Interleukin 1 beta (IL-1β) is a pro inflammatory cytokine involved in auto immune process in type I Diabetes[1], while TNF-α, IL-6 are another interleukin members usually achieved significantly increase in diabetes.[2] IFN-γ is a key cytokine in the development of autoimmune diabetes and any treatment or intervention intended to protect against the disease development, usually directed to its down regulation.[3] Zinc ions are essential for insulin formation in pancreatic β cells; its flux is regulated through zinc transporter (ZNT) family.[4, 5] Over the past decade a clear and strong relationship between zinc homeostasis and pancreatic function has been established [6] where zinc acts as anti-inflammatory and anti-oxidative agent.[7] Therefore zinc deficient state usually leads to an increase in inflammatory cytokines, apoptosis and endothelial cell dysfunction.[7] Subsequent studies in the last years indicated that supplementation of omega-3 fatty acids induced an improvement of endothelial, myocardial function, triglyceride level, thrombotic state and inflammatory pattern.[8, 9]

Present study aimed mainly to illustrate the features of zinc deficient state in alloxan diabetic rats fed on zinc deficient diet. To illustrate also the effect of omega 3 fatty acid, and zinc gluconate individually and illustrate their potential as therapeutic agents.

MATERIALS AND METHODS

30 adult male albino rats weighing 170 ± 20 g were housed under environment-controlled conditions and allowed one week for acclimatization at room temperature with a 12 hours dark/light cycle before beginning the experimental work. Rats were fed rodent chow and allowed free access of drinking water. The animals were maintained and used in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (The University of Zagazig, Egypt). Rats were fed on zinc deficient diet, consist of: egg albumin (200 g/kg), Dextrin (631 g/kg), Maize oil (100 g/kg), Vitamin mixture (11.7 g/kg), Salt mixture (-zinc) (31.3 g/kg), Cellulose powder 20g/kg for 28days, deionized water was allowed as drinking water for all rats.[10] Single dose (120mg/kg) of freshly prepared solution of Alloxan Monohydrate which was purchased from the ACROS; ORGANIC CHEMICALS COMPANY (New Jersey, USA)
(Dissolved in Normal Saline, Citrate buffer, pH 4.5) was administered intraperitonially to overnight fasting rats for induction of diabetes mellitus. Control rats were similarly injected with normal saline. Fasting blood glucose level was checked after 48-72 hours. Rats which achieved a blood sugar level > 200mg/dl were selected as diabetics.\textsuperscript{[11]}

Five experimental groups (n=6) were used: normal rats (NC), alloxan-induced diabetic rats which received no treatment and served as diabetic control (DC), alloxan-induced diabetic rats which received zinc deficient diet and deionized Water and served as diabetic zinc deficient (Dz.d), (F.O group ) diabetic zinc deficient rats received daily oral dose (50 mg/kg) \textsuperscript{[12]} of fish oil ( gelatin capsules each one contains 1000 mg fish oil were obtained from the Arab Company for Gelatin and Pharmaceutical Products) for 8 weeks, (Z.G group) diabetic zinc deficient rats received daily(150 mg/serving water) \textsuperscript{[13]} zinc gluconate ( fine powder of zinc gluconate was obtained from Egyptian pharmaceutical industrial company (EPICO), Cairo – Egypt.) for 8 weeks.

During the experimental period (8 weeks), body weight, blood glucose, food and water consumption and physical examinations were determined at regular intervals. The dosage was adjusted every week according to any change in body weight to maintain the dosage state.

**Blood Sampling**

After 4 weeks and at the end of the treatment periods (8 weeks) rats were fasted overnight, blood samples were taken for each rat individually, directed to serum preparation. Samples were processed instantly for determination of glucose, IL-6, CRP, and TNF-\(\alpha\), IFN-\(\gamma\), IL1-\(\beta\) and Zn.

**Tissue Collection**

After 8 weeks blood collection was done, rats were killed by decapitation, pancreases were removed instantly. Tissues placed in cold saline solution, trimmed of adipose tissue and homogenized instantly on ice using buffer. Tissue homogenate stored at -20°C for determination of IL1-\(\beta\) and ZNT8.

**Pancreas histology**

According to \textsuperscript{[14]} a slice of pancreas was fixed in 10 % formalin for 1 week at room temperature. Then the specimens were dehydrated in a graded series of ethanol cleared
in xylene, and embedded in paraffin wax Tissue blocks were sectioned in to 4-μm thick using a rotary microtome. Sections were stained by hematoxylin and eosin. Stained sections were examined by light microscope.

Analytical Methods
Blood glucose determination was done according to the method of[15] using commercial kits supplied by Spinreact Kits, Barcelona, Spain. IL-1beta was determined using commercial ELISA kit supplied by R& D quantikine (USA). according to.[16] C-reactive protein (CRP) was determined according to the method of[17] using BD Biosciences ELISA Kit, USA.

Serum IL-6 was determined according to the method of[18] using commercial ELISA kit supplied by R&D quantikine (USA). Serum TNF-α was determined according to the method of[19] using R&D Quantikine ELISA Kit (USA). Serum INFγ was determined according to the method of[20] using Platinum ELISA Kit (USA). Serum ZN was determined by colorimetric method according to[21] using QCA–Química Clínica Aplicada S.A., Spain. Znt-8 was determined using Antibodies ELISA Kit (Aachen, Germany).

Statistical Analysis
Statistical analyses of data were done by Prism 5, Graph pad, CA, USA. Results were expressed as mean ± standard deviation. Statistical differences were sought using Student’s t-test or one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD) post-hoc test (if more than two sets of data were being compared), taking p < 0.05 as statistically significant.[22]

RESULTS
After 4 and 8 weeks of alloxan administration diabetic control group (DC) and diabetic zinc deficient control group (Dz.d) demonstrated significant increase in serum glucose, IL-6, CRP, and TNF-α, IFN-γ, IL1-β and in pancreatic IL1-β but decrease in serum zinc level and pancreatic ZNT8 in comparison to normal group (NC). Also (Dz.d) group demonstrated significant increase in serum glucose, IL-6, CRP, and TNF-α, IFN-γ, IL1-β and in pancreatic IL1-β but severe decrease in serum zinc level and pancreatic ZNT8 in comparison to diabetic group (DC) (Table1, figure 1). Treatment with fish oil and zinc gluconate for 4 and 8 weeks induced a significant decrease of serum glucose and inflammatory markers as compared to the Dz.d group (Table 2, Table 3, figure 2). It was shown that the effect of fish
oil and zinc gluconate is nearly similar but fish oil is more effective than zinc gluconate as anti-inflammatory agents.

**Zinc modulation parameters**

Treatment with fish oil and zinc gluconate for 4 and 8 weeks induced a significant increase of serum zinc and pancreatic znt8 as compared to the Dz.d group and by calculating percent of parameters change it was shown that zinc gluconate is more effective in zinc modulation in serum and pancreatic tissue.(Table 4, figure 2 ). It was found that serum zinc and pancreatic znt8 was highly significant negatively correlated with serum glucose which may confirm the relation between diabetic and zinc deficient status (Fig 3a, Fig 3b) but positively correlated with each other (Fig 3c). Percent changes of all parameters from positive control to treated group proved that treatment with fish oil is slightly effective than zinc gluconate as anti-inflammatory and anti-diabetic agent but zinc gluconate has the higher effect as zinc modulator.

**TABLE 1: Comparison of results for alloxan administration effect on serum and pancreatic biochemical parameters after 8 weeks of experiment.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(NC)</th>
<th>(DC)</th>
<th>(Dz.d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>79.5±6.8</td>
<td>487.7±41.9**</td>
<td>547.0±30.2**##</td>
</tr>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>32.9±2.2</td>
<td>48.5±4.2**</td>
<td>103.6±9.5**##</td>
</tr>
<tr>
<td>Serum TNF-α (pg/ml)</td>
<td>34.7±2.5</td>
<td>118.8±7.8**</td>
<td>124.3±8.2**</td>
</tr>
<tr>
<td>Serum CRP (pg/ml)</td>
<td>1.02±0.04</td>
<td>12.1±1.2**</td>
<td>12.7±0.8**</td>
</tr>
<tr>
<td>Serum IFNγ (pg/ml)</td>
<td>0.97±0.04</td>
<td>11.1±1.1**</td>
<td>12.2±0.7**##</td>
</tr>
<tr>
<td>Serum IL1-β (pg/ml)</td>
<td>40.2±3.8</td>
<td>120.5±7.3**</td>
<td>128.6±7.6##</td>
</tr>
<tr>
<td>Serum ZN (µg/ml)</td>
<td>0.55±0.2</td>
<td>0.19±0.06**</td>
<td>0.02±0.006***##</td>
</tr>
<tr>
<td>Pancreatic ZNT8 (µg/ml)</td>
<td>1.002±0.06</td>
<td>0.69±0.06**</td>
<td>0.27±0.03**##</td>
</tr>
<tr>
<td>Pancreatic IL1-β (pg/ml)</td>
<td>93.4±3.6</td>
<td>116.4±8.6**</td>
<td>124.4±4.98**</td>
</tr>
</tbody>
</table>

NC = normal rat group, DC = alloxan diabetic rat group, Dz.d = alloxan diabetic zinc deficient rat group, n=6 in each case. Values are expressed as mean ± S.D. Significant differences are shown: *p<0.05, **p<0.001 vs. NC group, #p<0.05, ##p<0.001 vs. DC group.
TABLE 2: effect of fish oil and zinc gluconate treatment in diabetic zinc deficient rats for 8 weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D Z.d</td>
<td>547.0±30.2</td>
</tr>
<tr>
<td>F.O</td>
<td>96.5±8.4</td>
</tr>
<tr>
<td>% change</td>
<td>-82.36%</td>
</tr>
<tr>
<td>Z.G</td>
<td>120.2±7.4</td>
</tr>
<tr>
<td>% change</td>
<td>-78.03%</td>
</tr>
</tbody>
</table>

Dz.d= alloxan diabetic zinc deficient rat group, F.O = fish oil treated diabetic zinc deficient rat group, Z.G= zinc gluconate diabetic zinc deficient rat group, n=6 in each case. Values are expressed as mean ± S.D. Significant differences are shown: ᵃp<0.001 vs. D Z.d group, ᵇp<0.05, ᵇᵇp<0.001 vs. F.O group.

TABLE 3: effect of fish oil and zinc gluconate treatment in diabetic zinc deficient rats for 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>IL-6 (pg/ml)</th>
<th>CRP (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>Serum IL-1β (pg/ml)</th>
<th>Pancreatic IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D Z.d</td>
<td>103.6±9.5</td>
<td>12.7±0.8</td>
<td>124.3±8.2</td>
<td>12.2±0.8</td>
<td>128.6±7.6</td>
<td>124.4±4.9</td>
</tr>
<tr>
<td>F.O</td>
<td>59.3±5.8</td>
<td>4.5±0.4</td>
<td>64.3±6.4</td>
<td>3.2±0.2</td>
<td>62.5±5.5</td>
<td>60.6±3.7</td>
</tr>
<tr>
<td>% change</td>
<td>-42.8%</td>
<td>-64.6%</td>
<td>-48.27%</td>
<td>-73.8%</td>
<td>-51.4%</td>
<td>-51.3%</td>
</tr>
<tr>
<td>Z.G</td>
<td>57.4±5.2</td>
<td>5.3±0.4</td>
<td>65.8±4.8</td>
<td>3.9±0.3</td>
<td>74.3±6.5</td>
<td>78.5±4.6</td>
</tr>
<tr>
<td>% change</td>
<td>-44.6%</td>
<td>-58.3%</td>
<td>-47.1%</td>
<td>-68.03%</td>
<td>-42.2%</td>
<td>-36.9%</td>
</tr>
</tbody>
</table>

Dz.d= alloxan diabetic zinc deficient rat group, F.O= fish oil treated diabetic zinc deficient rat group, Z.G= zinc gluconate diabetic zinc deficient rat group, n=6 in each case. Values are expressed as mean ± S.D. Significant differences are shown: ᵃp<0.001 vs. D Z.d group, ᵇp<0.05, ᵇᵇp<0.001 vs. F.O group.

TABLE 4: effect of fish oil and zinc gluconate treatment for 8 weeks on zinc modulation in diabetic zinc deficient rats.

<table>
<thead>
<tr>
<th></th>
<th>serum ZN (µg/ml)</th>
<th>pancreatic ZNT8 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D Z.d</td>
<td>0.02±0.001</td>
<td>0.3±0.03</td>
</tr>
<tr>
<td>F.O</td>
<td>0.6±0.05</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>% change</td>
<td>2900%</td>
<td>266.7%</td>
</tr>
<tr>
<td>Z.G</td>
<td>0.9±0.04</td>
<td>1.1±0.06</td>
</tr>
<tr>
<td>% change</td>
<td>4400%</td>
<td>266.7%</td>
</tr>
</tbody>
</table>

Dz.d= alloxan diabetic zinc deficient rat group, F.O= fish oil treated diabetic zinc deficient rat group, Z.G= zinc gluconate diabetic zinc deficient rat group, n=6 in each case. Values are
expressed as mean ± S.D. Significant differences are shown: *p<0.001 vs. D.Z.d group, ᵇp<0.05, ᵇᵇp<0.001 vs. F.O group.

Figure 1: Comparison of results for alloxan administration effect on serum biochemical parameters after 4 weeks of experiment
Figure 2: Comparison of results for fish oil and zinc gluconate treatment effect on serum biochemical parameters after 4 weeks of experiment
Figure 3: correlation study of fish oil and zinc gluconate administration effect on zinc modulation in diabetic zinc deficient rats.

Histological changes
Pancreatic tissue of normal control arrayed showed normal distribution of islet of Langerhans within the exocrine part, islets were regular with well-defined boundaries. Their cells had oval or rounded nuclei (Fig 4). On the other hand, alloxan –diabetic pancreatic tissue showed shrinkage of islets Langerhans in size, signs of necrosis of β-cell destruction and reduction of number of islets (Fig 5). Pancreatic tissue of diabetic zinc deficient rats showed pathological changes of both exocrine and endocrine part of the pancreas represented by acinar cells damage and very small sized islets cell compared to normal and diabetic ones (Fig6).Diabetic zinc deficient pancreatic tissue treated with fish oil showed a large irregularly shaped islet of Langerhans, aggregation of inflammatory cells and
acinar cells damage are slightly better than diabetic and Dz.d ones (Fig7). Diabetic zinc deficient pancreatic tissue treated with zinc gluconate showed a mild increase in the size of pancreatic cell and acinar cell returned to normality (Fig8).

Fig 4: Control pancreatic tissue, showed rounded normal sized islet of Langerhans (L), cells were arranged normally with prominent nuclei (A). (H& E x 200).

Fig 5: Alloxan-Diabetic pancreatic tissue showed vacuolation (↑), marked decrease of β-cells (L) with an adjacent dilated rounded inter lobular duct (d) and focal acinar damage (A). (H& E x 200).

Fig 6: Alloxan-Diabetic zinc deficient pancreatic tissue showed very small sized islets cell (↑) and acinar cells damage represented by cytoplasmic vacuolation. (H& E x 200).
Fig 7: Fish oil treated pancreatic tissue showed a large irregularly shaped islet of Langerhans (↑) with an adjacent dilated irregularly rounded inter lobular duct (d), cytoplasmic vacuolation and acinar cells damage are slightly better than diabetic one (H& E x 200).

Fig 8: Zinc gluconate treated pancreatic tissue showed mild increase in the size of pancreatic cell (↑) with an adjacent dilated irregularly rounded inter lobular duct (d), acinar cell returned to normality and presence of hyperplasia. (H& E x 200).

DISCUSSION

As reported before diabetes mellitus represent an autoimmune, metabolic and genetic disorders that share one major characteristic, hyperglycemia which enhanced inflammation and cell apoptosis.[23,24] Our results demostrated an increase in serum and pancreatic inflammatory markers (CRP, IL-6, TNF-α, IL-1β, IFNγ). Previous clinical studies demonstrated an increase in CRP and IL-6.[25,26] TNF-α behaved similarly and mostly expressed as a useful biomarker for the early detection of diabetes.[27] Increased IL-1β levels in the peripheral blood monocytes have been linked to the development of T1D in humans[28] and diabetic animal models.[29] Additionally increased levels of IFN gamma may be suggestive of its destructive role in the patho physiology of type I autoimmune diabetes.[30] These cytokines stimulate hepatocytes to increase the synthesis and release of positive acute-phase proteins, including CRP.[31]
Zinc as a trace element possess a wide range of functions, antioxidant properties,[32] and has a role in the synthesis, storage, secretion of insulin, in turn has a great benefit in diabetic state.[33] Its homeostasis is regulated by the ZnT (SLC30A gene family).[34] Zinc transporter 8 (ZnT8) is specifically expressed in the pancreatic β-cells and has been identified as a novel target auto antigen in patients with type 1 diabetes.[35] Its auto antibodies (ZnT8A) are the most recently identified islet auto antibodies and are expressed as risk markers in relatives at low genetic risk of diabetes and older individuals.[36] Present study showed that serum zinc and pancreatic ZnT8 were negatively correlated with serum glucose where acute decrease in ZnT8 levels impairs β-cell function, Zn homeostasis and may contribute to the inflammatory cytokine-induced alterations in β-cell function and increased oxidative stress.[37,38] The effects of zinc deficiency on peripheral glucose metabolism could be related to the action of zinc on glucose transporter translocation inside cells or modification of the glucose transporter structure. While Zinc deficiency significantly decreases the response of tissue to insulin,[39] Zinc gluconate supplementation significantly decreased blood glucose level, in agreement with reported clinical and experimental studies.[40-42] This may be attributed to blocking of NFκβ activation in pancreatic tissue since the latter is sensitive to ROS.[43] Later studies indicated that zinc supplementation improved also Th1 cells cytokines production, decreased generation of inflammatory cytokines, additionally oxidative stress.[7, 38] Omega-3 fatty acids have a potential effect to protect pancreas in diabetic state.[44] The present study indicated that administration of fish oil significantly decreased, pancreatic inflammatory markers along increased serum zinc level and pancreatic ZnT8. DHA content of fish oil is a long-chain polyunsaturated fatty acid which exert many effects on platelet function, lipid levels, oxidation, glycemic control, and immune function.[45] Anti diabetic effect of fish oil was discussed before where EPA and DHA content help to regulate glucose homeostasis through the relationship and interaction with the Peroxisome-proliferator-activated receptor gamma (PPARγ) the latter represent an important transcription factor that helps to regulate fat cell differentiation and has also been known to play a vital role in maintaining glucose homeostasis.[46] The peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes.[47]

Treatment with different sources of omega-3 as fish oil induced an improvement in zinc levels of diabetic rats.[44] Previously it was reported that Eicosapentaenoic acid (EPA) and
docosahexaenoic acid (DHA found in fish oil supplements are able to inhibit partly a number of aspects of inflammation.[48] In agreement with other studies fish oil supplementation in the present study decreased serum TNF-α, IL-1β, CRP.[49-51] This may be modulated through up-regulation of PPARγ target for controlling inflammation where it directly regulates inflammatory gene expression and interferes with the translocation of NFκB to the nucleus.[48] Pancreatic tissue of zinc deficient diabetic rats demonstrated pathological changes of both exocrine, endocrine represented by acinar cell damage, small sized islets in comparison to normal and diabetic groups. Fish oil supplementation induced aggregation of inflammatory acinar cells slightly better than diabetic and diabetic zinc deficient groups. Zinc gluconate administration induced mild increase in sizes of pancreatic cells while acinar cells had returned to normality.

CONCLUSION

Individual administration of fish oil and zinc gluconate induced marked improvement in the biomarkers studied as compared to control group. Histopathological examination demonstrated certain attenuation to islets, acinar cells and damage induced and offered support to the biochemical data. This may be of value as therapeutic agent for diabetes either individually or in combination form.

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