Zinc supplementation mitigates its dyshomeostasis in experimental diabetic rats by regulating the expression of zinc transporters and metallothionein†

Susmita Barman, Seetur R. Pradeep and Krishnapura Srinivasan

Zinc depletion during diabetes projects a role for zinc nutrition in this condition. This study explored whether zinc supplementation annuls diabetes-induced zinc dyshomeostasis through modulation of zinc transporters and metallothionein. Groups of hyperglycemic rats were exposed for six weeks to supplemental zinc (5 or 10-times the normal level). Intracellular zinc concentration and zinc transporter and metallothionein expression levels in different tissues were analysed. Depleted zinc concentrations in different organs were restored by zinc supplementation. Zinc ions cross biological membranes with the aid of membrane proteins, belonging to zinc transporter families – ZIPs (responsible for the influx) and ZnTs (responsible for intracellular traffic/efflux). Up-regulated expression levels of zinc efflux proteins and influx proteins were beneficially modulated by zinc treatment, which also induced metallothionein expression in tissues to mitigate oxidative stress. Thus, zinc supplementation has a significant benefit in controlling zinc fluxes during diabetes, exerted through a protective influence on the modulation of the expression of zinc transporters and metallothionein.

Significance to metallomics

Significant findings of the study: The present animal study explored whether zinc supplementation protects against diabetes-induced zinc dyshomeostasis through modulation of zinc transporters and metallothionein. Depleted zinc concentrations in different organs and bone were restored by zinc supplementation. Up-regulated expression levels of the zinc transporters ZnTs and ZIPs in the diabetic condition are beneficially modulated by exogenous zinc treatment. Zinc supplementation also induced a significant increase in metallothionein expression in different tissues. What this study adds: The present study has evidenced for the first time that Zn supplementation has a significant beneficial effect in the control of zinc fluxes during diabetes. This was exerted through a protective influence on the modulation of the expression of zinc transporters and metallothionein.

Introduction

An association between zinc (Zn), pancreatic function and diabetes was proposed > 70 years ago. In this context, research on diabetes has directed an electrifying role for zinc signalling in this disease. Intracellular Zn homeostasis is firmly controlled through the regulation of the compartmentalization and availability of a family of transmembrane Zn transporter proteins for zinc efflux/sequestration (ZnT/Slc30 family) or Zn influx (ZIP/Slc39 family). The recent connotation of these two ‘Zn gene families’ and metallothionein (MT) with diabetes at the genetic level and with insulin secretion in clinical studies offers a novel way to identify new targets to modulate Zn homeostasis directly in different organs to alleviate the complications of diabetes. The predominant consequence of diabetes on body Zn homeostasis is to stimulate hypozincæmia, hyperzincuria, and reduced gastrointestinal absorption of Zn. Ionic Zn exhibits insulin ‘mimetic’ activity where it is involved in insulin receptor signal transduction and insulin storage, secretion and circulation. In pancreatic ß-cells during synthesis and in the stored condition, insulin conserves in the solid form as Zn–insulin (2 : 6) crystals. In peripheral tissues such as fat and muscle, Zn ions expedite insulin-induced glucose transport and glycaemic control through modulation of intrinsic pathways intricate in glucose homeostasis. Conversely, there is deficient information on how the concentration of free Zn in cells is controlled. The proteins that transport Zn presumably...
regulate cell signalling procedures that facilitate glycaemic control in tissue through the regulation of ionic Zn concentrations in the cytosol. However, how these transporters are regulated to effectively facilitate Zn flux, contributing to cellular metabolism, is unknown.

Several studies have documented that high-dose Zn causes stimulation of the metallothionein protein, which is an antioxidant rich in cysteine groups, and binds a wide range of metals including zinc, copper, cadmium, etc., via a sequential, non-cooperative mechanism. By binding and discharging Zn, metallothioneins (MTs) may control Zn levels within biological systems. There is indeed significant literature establishing that the elevated expression of metallothionein can be reasonably protective in diabetic rodents. For example, metallothionein in knockout mice intensifies the complications of diabetes. A general or tissue-specific intensification of metallothionein is documented to provide protection from diabetes-induced cardiomyopathy, nephropathy, and neuropathy and also defends pancreatic beta cells from oxidative damage, subsiding commencement of diabetes in some circumstances.

Accordingly, diabetes precipitates Zn dyshomeostasis; contrariwise, Zn deficiency was also found to increase the risk of diabetes and its complications. We have recently accounted that exogenous Zn supplementation pronouncedly alleviated hyperglycemia, hypoinsulinemia, and associated metabolic abnormalities, and oxidative stress in STZ-induced diabetic rats. Hence, it could be significant in inspecting the beneficial influence of supplemental Zn on the progression of diabetes-induced Zn dyshomeostasis. A significant effect of Zn supplementation on the Zn transporters including metallothionein has been reported in diabetic patients. The present study was undertaken to systematically understand how the Zn transporter gene family acts at cellular processes in diabetes. It is envisaged that understanding the probable significance of dysfunctional ionic Zn apportioning in preventing the disease progression or treating diabetes will create opportunities for deciphering basic research into clinically significant applications to develop novel therapeutic approaches. Although Zn supplementation in Zn-deficient diabetic rats might be an appropriate treatment in inherently susceptible rats, the prospective mechanisms that lead to dysfunctional intracellular zinc signalling and altered Zn subcellular distribution, rather than Zn deficit, will require the development of pioneering drugs that target Zn transporters for instance.

Materials and methods

Chemicals

o-Dianisidine, horseradish peroxidase, glucose oxidase, streptozotocin (STZ) and zinc carbonate (basic) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TSQ (N-(6-methoxy-8-quinonyl)-p-toluenesulphonamide) (CaF.M688) and fluorescein isothiocyanate (FITC) were procured from Invitrogen Thermo Fisher Scientific Inc. All other chemicals and reagents were of analytical grade procured from either Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) or Sisco Research Laboratories (Mumbai, India). A Bernhart-Tomarelli modified salt mixture was purchased from Sisco Research Laboratories (Mumbai, India). Casein was purchased from Nimesh Corporation (Mumbai, India). Maize starch, cane sugar powder and refined groundnut oil were purchased from the local market.

Experimental animals

All the experimental processes in this animal study were approved by the Institutional Animal Ethics Committee (CSIR-CPTRI, Mysore, India) and precautions were taken to reduce pain and distress to the animals. Adult 12 week old female Wistar rats (140–150 g) were obtained from the Experimental Animal Production Facility Unit of this Institute. The rats were individually housed in stainless steel cages under controlled temperature (25 ± 2 °C) and humidity (40–60%) with a 12/12 h light–dark cycle. The rats were fed ad libitum and had free access to water. Diabetes was induced by a single i.p. injection of streptozotocin (40 mg kg⁻¹ body weight dissolved in freshly prepared 0.1 M (pH 4.5) citrate buffer). The rats having fasting blood glucose levels >15 mmol L⁻¹ determined using the glucose oxidase method were considered as diabetic rats.

Diets and animal treatment

The normal basal diet (AIN-76) was amended with the addition of zinc carbonate as Zn salt and confirmed to be 5-times (0.32 g kg⁻¹ diet) and 10-times (0.64 g kg⁻¹ diet) that of normal level. Rats were distributed into six groups, among which three groups were diabetic (12 rats in each group) and three groups were normal (eight rats in each group) and were maintained on the respective diets for 6 weeks. Thus, the six animal groups were as follows: (1) normal control (N), (2) normal + Zn-dose 1 (N + Zn-1), (3) normal + Zn-dose 2 (N + Zn-2), (4) diabetic control (D), (5) diabetic + Zn-dose 1 (D + Zn-1), and (6) diabetic + Zn-dose 2 (D + Zn-2).

Food intake was measured at 24 h intervals; urine and faeces samples were collected by housing the rats in specially designed metabolic cages at the 6th week and the samples were stored at −80 °C until further analysis. At the end of the 6th week, the animals were sacrificed under euthanasia and blood samples were collected in heparinised tubes, and tissues were quickly excised, weighed and processed for various parameters and stored at −80 °C until analysis.

Zn analysis

The Zn contents in the tissue samples were determined using Atomic Absorption Spectrometry (Shimadzu AAF-6701) following an acid digestion procedure. In brief, weighed tissue samples were subjected to the cold acid digestion procedure overnight, in a mixture of concentrated nitric, sulfuric and perchloric acids (3 : 3.1 : 1, v/v/v). The digested samples were then boiled for about 4–5 hours to evaporate acid fumes, and subsequently, the volume was made up to a known volume with deionised water. Plasma and urine were acidified in 1.0 mL of 1 mol L⁻¹ HNO₃ for 48 h prior to absorption spectrophotometry. Calibration of the mineral measurements was implemented using calcium, iron
and Zn standards and appropriate acid blanks. All measurements were carried out under standard flame operating conditions as endorsed by the manufacturer.

**Activity of Zn containing enzymes**

Small intestinal alcohol dehydrogenase activity was evaluated according to Brink et al. by determining the amount of reduced nicotinamide adenine dinucleotide (NAD) at pH 9.6 in the presence of excess ethanol, spectrophotometrically (340 nm). The carbonic anhydrase activity in the intestine was assessed by computing the amount of p-nitrophenyl acetate hydrolyzed to p-nitrophenol at pH 7.6 according to Verpoorte et al.

**Real-time PCR analysis**

Total RNA extraction and mRNA expression analysis for the intestine, liver, pancreas, heart, and kidneys were performed as detailed earlier. Primers for the PCR amplifications used were acquired from Sigma-Aldrich Chemical Co., as shown in Table S1 (ESI†). Specific transporters have been studied among the tissues as all the transporters are not expressed ubiquitously in all the tissues.

**Western blotting analysis**

Western blot analyses were performed as described earlier. In brief, samples were homogenized using ice-cold lysis buffer (pH 7.5). Each lysate (50 μg of protein) was resolved on 16% urea Tricine-SDS-PAGE and the separated proteins transferred onto PVDF membranes and immunoblotted with polyclonal primary antibodies anti-metallothionein (mouse monoclonal [UC1MT] to metallothionein; Cat.#ab12228), which has specificity for both MT1 and MT2, and β-actin (Cat.#ab25894; Abcam, Cambridge, MA, USA), used as per the instructions given by the manufacturer. Immunoreactive bands were then detected by incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using enhanced chemiluminescence reagents (Sigma Chemicals, St. Louis, MO, USA). The band intensity was quantified as the ratio of targeted protein band to β-actin using GeneTools v4.01 analysis software (Syngene).

**Immunohistological and confocal laser scanning microscopy analysis**

In brief, paraffin-embedded 5 μm thickness tissue sections were deparaffinised and rehydrated, following overnight incubation with a polyclonal antibody against MT, and further incubated with the HRP-conjugated secondary antibody and then developed using a 3,3′-diaminobenzidine substrate and counterstained with haematoxylin before mounting. Images were captured using a microscope (Olympus Model: BX-5, Japan; ProgRes C-5 software).

For confocal microscopy (Carl Zeiss, LSM 700, Jena, Germany; ZEN 2009 software) study, secondary FITC was used for metallothionein detection and TSQ was used for the identification of intracellular zinc accumulation in different tissue sections. The fluorescence was excited at λ = 488 nm (FITC) and λ = 365 (TSQ) nm to observe the images. The fluorescence of the respective protein spectra emitted was analyzed using a highly sensitive T-PMT detector with 1 a.u. spectral increments, with a 30.5 μm pinhole size, and a scanning time of 11.3 s. The images were captured at a resolution of 512 × 512 pixels, with a pixel size of 1.25 μm, and a pixel dwell time of 25.6 μs.

**Statistical analysis**

The results were expressed as the mean ± SEM. The statistical significance was determined using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test using GraphPad InStat statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Values with p < 0.05 were considered to be statistically significant.

**Results**

**Dietary Zn intake and Zn excretion in the diabetic rats**

The rats in the diabetic group showed a dramatic increase in the amount of both food intake and water intake (almost doubled) (p < 0.05) compared with the control group, signifying the symptoms of polydipsia and polyphagia caused by diabetes. Compared with the diabetic group, the food intake of the Zn supplemented diabetic group was reduced as shown in Table 1. Zn excretion through urine and faeces was pronouncedly high (21- and 4-fold) in the diabetic animals compared with the normal control; but in the Zn treated condition both the normal and diabetic animals exhibited the same due to additional Zn intake. The absorption rate in the diabetic animals was reduced, which was normalised in the Zn supplementation condition.

**Disturbed Zn homeostasis in different tissues of the diabetic rats**

Zn levels in plasma and different tissues – liver, kidneys, spleen, muscle, pancreas, and bone of the diabetic animals – are

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Food intake/24 h (g)</th>
<th>Zn intake/24 h (μmol)</th>
<th>Zn excretion/24 h (μmol)</th>
<th>Zn absorption (% of intake per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control [N]</td>
<td>11.5 ± 1.6</td>
<td>6.62 ± 0.5</td>
<td>0.033 ± 0.008</td>
<td>2.74 ± 0.17</td>
</tr>
<tr>
<td>N + Zn-1</td>
<td>11.8 ± 2.1</td>
<td>33.6 ± 1.3</td>
<td>0.055 ± 0.009</td>
<td>23.2 ± 1.59</td>
</tr>
<tr>
<td>N + Zn-2</td>
<td>10.6 ± 4.2</td>
<td>62.8 ± 2.1</td>
<td>0.097 ± 0.007</td>
<td>47.9 ± 3.41</td>
</tr>
<tr>
<td>Diabetic control [D]</td>
<td>22.1 ± 1.3a</td>
<td>13.2 ± 0.9</td>
<td>0.706 ± 0.08a</td>
<td>10.9 ± 1.75</td>
</tr>
<tr>
<td>D + Zn-1</td>
<td>20.3 ± 2.4</td>
<td>60.3 ± 3.4a</td>
<td>0.794 ± 0.06</td>
<td>33.2 ± 2.14a</td>
</tr>
<tr>
<td>D + Zn-2</td>
<td>20.7 ± 1.7</td>
<td>123.1 ± 8.3a</td>
<td>0.858 ± 0.06</td>
<td>63.1 ± 3.09a</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 8 animals in each group. Significantly different from the normal control group (p < 0.05). Significantly different from the diabetic control group (p < 0.05).
given in Table 2. Most of the tissues in the diabetic group showed significantly ($p < 0.05$) depleted Zn concentration as compared to the control rats. The Zn concentration was decreased by 79%, 46%, 52%, 50%, 46%, 55% and 14%, in the respective abovementioned tissues and bones as a result of diabetes; these depletions had almost become normalised by Zn supplementation. Depleted Zn in the body tissues and bones of the diabetic animals was concomitant with higher diabetes; these depletions had almost become normalised by respective abovementioned tissues and bones as a result of diabetes; these depletions had almost become normalised by Zn supplementation. Depleted Zn in the body tissues and bones of the diabetic animals was concomitant with higher

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Table 2: Zn distribution in various tissues and bone in diabetic rats as influenced by Zn supplementation

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Normal control (N)</th>
<th>N + Zn-1</th>
<th>N + Zn-2</th>
<th>Diabetic control (D)</th>
<th>D + Zn-1</th>
<th>D + Zn-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>109.8 ± 8.1</td>
<td>144.8 ± 10.4</td>
<td>214.3 ± 12.7</td>
<td>61.3 ± 8.7*</td>
<td>83.1 ± 7.4</td>
<td>117.2 ± 11.8b</td>
</tr>
<tr>
<td>Liver</td>
<td>11.79 ± 0.89</td>
<td>14.81 ± 0.84</td>
<td>17.08 ± 1.79</td>
<td>6.34 ± 0.33*</td>
<td>9.65 ± 0.71</td>
<td>12.29 ± 1.42b</td>
</tr>
<tr>
<td>Kidneys</td>
<td>7.26 ± 0.43</td>
<td>10.14 ± 0.70</td>
<td>14.36 ± 2.67</td>
<td>3.48 ± 0.29γ</td>
<td>7.17 ± 0.37b</td>
<td>9.39 ± 0.76γ</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.29 ± 0.38</td>
<td>6.84 ± 0.99</td>
<td>8.71 ± 0.61</td>
<td>2.64 ± 0.27γ</td>
<td>5.14 ± 0.35b</td>
<td>7.46 ± 0.44b</td>
</tr>
<tr>
<td>Muscle</td>
<td>7.89 ± 0.56</td>
<td>8.97 ± 0.61</td>
<td>10.14 ± 1.09</td>
<td>4.26 ± 0.53γ</td>
<td>6.44 ± 0.72</td>
<td>8.11 ± 0.79b</td>
</tr>
<tr>
<td>Pancreas</td>
<td>13.92 ± 0.94</td>
<td>16.87 ± 1.21</td>
<td>20.76 ± 2.47</td>
<td>11.76 ± 0.91b</td>
<td>15.11 ± 1.19b</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 8 animals in each group.* Significantly different from the normal control group ($p < 0.05$). † Significantly different from the diabetic control group ($p < 0.05$).

Table 3: Effect of Zn supplementation on the activity of Zn containing enzymes in the small intestine of diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Alcohol dehydrogenase (μmol acetaldehyde formed, min⁻¹ mg⁻¹ protein)</th>
<th>Carbonic anhydrase (μmol p-nitrophenol formed, min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (N)</td>
<td>19.86 ± 1.8</td>
<td>166.7 ± 6.2</td>
</tr>
<tr>
<td>N + Zn-1</td>
<td>20.95 ± 1.4</td>
<td>212.8 ± 7.1a</td>
</tr>
<tr>
<td>N + Zn-2</td>
<td>24.34 ± 2.2</td>
<td>230.9 ± 11.8a</td>
</tr>
<tr>
<td>Diabetic control (D)</td>
<td>12.15 ± 1.5a</td>
<td>109.3 ± 6.3a</td>
</tr>
<tr>
<td>D + Zn-1</td>
<td>17.09 ± 1.4</td>
<td>196.8 ± 10.9a</td>
</tr>
<tr>
<td>D + Zn-2</td>
<td>20.29 ± 2.1a</td>
<td>211.9 ± 12.3a</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 8 animals in each group.* Significantly different from the normal control group ($p < 0.05$). † Significantly different from the diabetic control group ($p < 0.05$).

Diabetes induced Zn dyshomeostasis: Zn transporter mRNA transcriptional profiles in different tissues

Diabetes induced overexpression of genes for Zn transporters at the tissue level in various organs (Fig. 1–3). Zn supplementation to these diabetic animals was found to be accompanied by the inhibition of the mRNA transcriptions of the transporters in intestinal tissue (ZnT1, 2, 4, 5 and Zip1, 4). The liver tissue (ZnT1, 2, 4, 7 and Zip1), pancreas (ZnT8), heart (ZnT5) and kidneys (ZnT2) were also analysed in parallel using RT-qPCR.

To better understand the molecular mechanism underlying alleviation of Zn dyshomeostasis in various tissues of diabetic animals as a result of Zn supplementation, the quantitative analysis of the mRNA expression of Zn transporters was done in various tissues. Transcriptions of mRNA of the expression of the intestine for all the ZnT transporters (ZnT1, 2, 4, 5 and 7) showed elevated levels in the diabetic condition (by 24-, 5-, 19-, 22- and 5.4-fold respectively) as compared with the normal intestine (Fig. 1). Zn supplementation evidenced down-regulation of the same by 63%, 80%, 62%, 19%, and 85% and 64%, 58%, 45%, 79% (†), and 76%, respectively, in the D + Zn-1 and D + Zn-2 groups. Among the ZIP transporters Zip1 increased in the diabetic intestinal tissue (by >3.15-fold) compared to the normal control, whereas Zip4 decreased by 35% (Fig. 1). In the case of the diabetic rats with Zn supplementation, significant ($p < 0.05$) up-regulation of the ZIP transporter mRNA expression (2 and 7.7-fold in D + Zn-1 and 2.6 and 13.4-fold in D + Zn-2 respectively) was seen, indicating inhibition of Zn depletion from this tissue.

The mRNA expression levels of hepatic Zn transporters are shown in Fig. 2. Significantly ($p < 0.05$) down-regulated Zip1 expression (74%) in the diabetic rats showed that the diabetic rats were associated with a lower accumulation of Zn in hepatic store compared to the normal control group. Dietary Zn intervention significantly ($p < 0.05$) ameliorated the same (by 81% in D + Zn-1 and >3.62-fold in D + Zn-2). All ZnT transporters (ZnT2, 4 and 7) were up-regulated by 1.85, 1.90 and 1.64-fold, indicating facilitation of Zn depletion from diabetic hepatic tissue compared to the normal control condition. In the Zn fed...
diabetic rats, there was a down-regulation in mRNA expression for the same (48%, 39% and 32% in D + Zn-1; 78%, 78% and 25% in D + Zn-2, respectively) which signifies normalization in the Zn dyshomeostasis condition.
Tissue specific Zn transporters for the pancreas (ZnT8), heart (ZnT5) and kidneys (ZnT2) were evaluated as presented in Fig. 3. The pancreas of the diabetic animals showed down-regulated ZnT8 expression compared with that of the normal control group. Zn supplementation of these diabetic rats pronouncedly countered the expression of ZnT8 ($p < 0.05$) (mainly in D + Zn-1 by 1.9 and in D + Zn-2 by 3.8-fold). Cardiac and renal tissues of the diabetic animals also showed a significantly ($p < 0.05$) elevated ZnT5 and ZnT2 expression levels by 1.87 and 2.28-fold respectively. Dietary Zn supplementation in the diabetic rats inhibited the over-expression of cardiac ZnT5 and renal ZnT2. In D + Zn-1 it increased by 63% and 36%, and in D + Zn-2 by 36% and 28%. The kidney ZnT2 expression was further up-regulated in the case of D + Zn-2, which may be for flushing out the excessively accumulated Zn by the supplementation.

**Calcium transporter mRNA transcriptional profiles in different tissues of the diabetic rats**

The influence of dietary Zn supplementation on Ca$^{2+}$ transporter Trpv-5 in the intestinal and renal tissues of the diabetic animals is shown in Fig. S2 (ESI†). Activities of this transporter in the diabetic intestine increased by 2.28-fold concomitant with a diminished level (51%) in renal tissue compared with that of the normal control animals. The increased or decreased expression of the Ca$^{2+}$ transporter may be an adaptive mechanism. Elevated activity was further up-regulated in the diabetic intestine treated with zinc.
supplementation, by 7.8-fold and 9.4-fold in the respective D + Zn-1 and D + Zn-2 dose groups. Diminished expression in diabetic renal tissue was produced by Zn supplementation (by 1.9- and 2.5-fold, respectively).

Diabetes induced metallothionein (MT) mRNA transcriptional profiles and protein expression profiles in different tissues

Transcriptions of mRNA, conforming to quantitative analysis by Western blotting of the expression of intestine for MT showed elevated levels in the diabetic condition (7.0 and 5.7-fold, respectively, for MT-1 and MT-2 mRNA; 1.45-fold protein expression) compared with the normal animal (Fig. 4). Zn supplementation evidenced further up-regulation of MT-1 by 44% and by 2.8-fold in the D + Zn-1 and D + Zn-2 groups, respectively. Similarly, RNA of MT-2 was further up-regulated by 50% in the D + Zn-2 group. The expression of the MT protein was also similarly higher in the Zn supplemented diabetic animals (2.04- and 2.22-fold as compared to 1.45-fold higher expression in the diabetic control).

Increased transcription of mRNA for hepatic MT with concomitantly augmented protein expression was evident in the diabetic condition as seen by >1.7, 1.8 and 1.6-fold expression, respectively, compared with the liver of the normal animals (Fig. 5). Supplemental zinc promoted additional up-regulation of mRNA of MT by 1.9-, 1.4-, and 1.6-fold and 3-, 2.2-, and 2-fold, respectively, in the D + Zn-1 and D + Zn-2 groups. The diabetic animal group showed progressive cardiac MT expression in both the mRNA and protein expression levels (1.6- and 2.2-fold), which showed similar trends like in other tissues (up-regulated expression) by Zn supplementation (in D + Zn-1 by 56%, 4% and 10%; in D + Zn-2 by 184%, 46% and 22%) (Fig. 6A–C). Pancreatic MT also exhibited a similar propensity in diabetes like normal control, whereas supplemental Zn treatment evidenced the over-expression of MT in the diabetic pancreas (Fig. 6D–F). The kidneys of the diabetic animals showed augmented MT mRNA and protein expression levels, which were >1.3-, 1.5-, and 1.81-fold compared with that of the normal control group (Fig. 6G–6I). Zn supplementation in these diabetic rats pronouncedly regulated the expression of MT (by 59%, 71%, and 64% in the D + Zn-1 group and by 68%, 140%, and 113% in the D + Zn-2 group) (p < 0.05). Both immunohistochemical study and confocal microscopic observation were consistent with these effects of supplemental Zn in all the tissues of the diabetic animals (Fig. 7).

Discussion

Zn is indispensable for numerous aspects of living systems and human health.18 While Zn has a role in several aspects of diabetes, its precise molecular targets are not clear. Current findings of the associations of diabetes and polymorphisms in human genes suggest participation of proteins in controlling the cellular availability of Zn ions. The purpose of this study was to examine if disturbed Zn homeostasis due to streptozotocin induced diabetes complication can be curbed by Zn supplementation. The STZ-induced diabetic model studied here represents type-1 diabetes (induced by a single high dose of STZ). Deviations in the concentration of Zn in the whole body changed only at very low or very high consumption.19 In the present investigation, 5 and 10-folds of the normal level of Zn have been used as supplementation. The STZ-induced diabetic model studied here represents type-1 diabetes (induced by a single high dose of STZ). Deviations in the concentration of Zn in the whole body changed only at very low or very high consumption.19 In the present investigation, 5 and 10-folds of the normal level of Zn have been used as supplementation. The amount of Zn (5 and 10-times the normal level) used in the present study did not show any toxic effect in the normal or diabetic rats. Zn-supplemented diet containing 500 or 1000 ppm was previously shown to be well tolerated and non-toxic to mice.20

Fig. 3 mRNA expression profiles of Zn transporters in tissues of diabetic rats: (A) pancreas, (B) heart and (C) kidneys. Values are mean ± SEM of 8 animals in each group. Relative gene expressions were normalized to the β-actin level and expressed in arbitrary units as fold change compared with the normal control. a: Significantly different from the normal control group (p < 0.05), and b: significantly different from the diabetic control group (p < 0.05). N: normal control; D: diabetic control; Zn-1: Zn supplemented at dose 1; Zn-2: Zn supplemented at dose 2.
Comprising 2–3 g of the whole human body, about 60% Zn is distributed in skeletal muscle, 30% in bone, and 5% in the liver and skin. Zn homeostasis is tightly controlled by the coordinated regulation of the uptake, efflux, distribution, and storage. When the body Zn content is inadequate, its absorption increases in the small intestine, and so is its release.

Fig. 4 Expression of intestine metallothionein in diabetic rats. (A) Immunohistochemistry, 20× (a–d) and confocal, 20× (e–h). Relative mRNA expressions for (B) MT-1 and (C) MT-2 in diabetic intestine which were normalized to the β-actin level and expressed as fold change compared with the normal control intestine. Western blot analysis of MT protein abundance in lysates of the intestine tissue in diabetic rats and (D) Relative protein expressions of MT which were normalized to the β-actin level and expressed as fold change compared with the normal control. Values are mean ± SEM of 8 animals in each group. a: Significantly different from the normal control group (p < 0.05), and b: significantly different from the diabetic control group (p < 0.05). N: normal control (a and e); D: diabetic control (b and f); Zn-1: Zn supplemented at dose 1 (c and g); Zn-2: Zn supplemented at dose 2 (d and h).

Fig. 5 Expression of liver metallothionein in diabetic rats. (A) Immunohistochemistry, 20× (a–d) and confocal, 20× (e–h). Relative mRNA expressions for (B) MT-1 and (C) MT-2 in diabetic liver which were normalized to the β-actin level and expressed as fold change compared with the normal control liver. Western blot analysis of MT protein abundance in lysates of the liver tissue in diabetic rats and (D) Relative protein expressions of MT which were normalized to the β-actin level and expressed as fold change compared with the normal control. Values are mean ± SEM of 8 animals in each group. a: Significantly different from the normal control group (p < 0.05), and b: significantly different from the diabetic control group (p < 0.05). N: normal control (a and e); D: diabetic control (b and f); Zn-1: Zn supplemented at dose 1 (c and g); Zn-2: Zn supplemented at dose 2 (d and h).
from the pancreas and liver store. Meanwhile, additional Zn is assumed to be released primarily through gastrointestinal secretion from mucosal cells and the integument. 21 The absorbed Zn is commonly bound to albumin (about 84%) and tightly bound to α2-macroglobulin (about 15%) and only 1% to amino acids in the circulation and delivered to peripheral tissues. 22 Thus, albumin is the carrier for mobile Zn and the α2-macroglobulin fraction does not contribute to Zn transportation and dissemination. 23 While 50% of total Zn within the cell is dispersed in the cytosol, a portion of this cytosolic Zn pool is bound by metallothioneins. 24 Several reports have shown that diabetic patients exhibited a marked decrease in total plasma Zn and hyperzincuria, suggesting that the hyperglycemic condition may probably hinder Zn absorption in the tissue. 25 In the current study, the observed declined plasma Zn concentration might be due to microalbuminuria prompted by diabetes which is reported in our previous study. 10 These results were concomitant with the levels of Zn within tissues (spleen, muscle, kidneys, and liver).

Reduced pancreatic Zn has likewise been reported in a genetic model of T2D. 26 However, significant reduction in the ultrastructural localization of Zn was similarly noted in the section of diabetic tissue when measured with a synthetic, intracellular trappable quinoline based fluorophore TSQ which was substantiated with the improved level of intracellular Zn upon dietary supplementation. The activities of Zn-containing enzymes in the intestine, which are a stronger indicator of the Zn state, were also considerably reduced in the diabetic condition, depicting the prevalent Zn deficiency. Zn supplementation enhanced body Zn store, thus countering the deficiency induced by diabetes which was also synonymous with the increased activity of Zn containing enzymes in the small intestine.

Zn excretion was more prominent through feces than urine in this study. Studies have specified in the diabetic condition an association of augmented zincuria with polyuria because of osmotic diuresis, which was also documented in our previous study. 27 In addition to hyperzincuria, other potential mechanisms

Fig. 6 Expression of metallothionein in the heart, pancreas and kidneys of diabetic rats. Relative mRNA expressions for MT-1 (A, D and G) and MT-2 (B, E and H) in diabetic tissues which were normalized to the β-actin level and expressed as fold change compared with normal control. Western blot analysis of MT protein abundance in lysates of the respective tissues in the diabetic rats. Relative protein expressions of MT (C, F and I), normalized to the β-actin level and expressed as fold change compared with normal control. Values are mean ± SEM of 8 animals in each group. a: Significantly different from the normal control group (p < 0.05) and b: significantly different from the diabetic control group (p < 0.05). N: normal control; D: diabetic control; Zn-1: Zn supplemented at dose 1; Zn-2: Zn supplemented at dose 2.
of Zn loss include dominant fecal excretion of Zn consequent to inefficient intestinal absorption of Zn or enhanced excretion from the pancreas. In this study, Zn supplementation improved body Zn store, which may compensate for the urinary loss, by improved absorption of Zn or decreased fecal excretion. Our data documented that most of the tissues in the diabetic group had significantly depleted Zn concentration compared to the control rats. Further, these depletions of Zn in body tissues and bones of diabetic animals concomitant with its higher excretion were almost normalised by Zn supplementation. This is the novel information obtained from the current study.

Refined control over systemic and cellular Zn homeostasis is pivotal for human health. Several Zn permeable proteins, transport membrane proteins and metallothioneins are involved in the body for this control. Amongst them, transporters, belonging to ZRT/IRT-like Proteins (ZIP-Slc39A gene family) and Zn transporters (ZnT-Slc30A gene families), play crucial roles in physiological functioning and intensely affect health, as they are involved in a wide variety of diseases.27 Thus, the study of Zip and ZnT transporters is presently receiving extensive attention due to their disease/disorder mutation and the tissue specificity of their aberrant expression. In mammalians, 14 members of the Zip family and ten members of the ZnT family have been identified.28 In general, Zip proteins function in increasing intracellular Zn by the uptake into the cytosol of the cell from the extracellular space or intracellular compartments, while ZnT proteins function in the efflux of Zn from the cytosol of the cell to the extracellular space or intracellular compartments. Regulated by intracellular and extracellular Zn concentrations, many of these transporters are also controlled by hormones and cytokines.29 Such regulation, tissue expression patterns, and subcellular localization of these transporters indicate their physiological roles in pathophysiological disease states. In this context, the present study has, for the first time, evaluated diabetes-induced Zn-deprivation to understand if Zn-supplementation has a counteractive role as a regulator of Zn transporter expression in a tissue specific manner mainly in the intestinal tissue.

Although ZnT1 is ubiquitously distributed, it is highly expressed in tissues involved in Zn recycling, or relocation, such as the basolateral membrane of enterocytes of the small intestine and hepatic cells. ZnT2 mRNA has been identified in specific tissues, viz., the small intestine, liver, kidneys, placenta, pancreas, seminal vesicles, testes, and mammary glands. In our study, ZnT1 and ZnT2 were progressively abundant in tissues of diabetic animals. They may participate in Zn efflux from tissues into the circulation, leading to progression of Zn deficiency in that particular tissue. The connotation of ZnT2 with elevated cellular Zn concentrations upon Zn supplementation and its vesicular localization in the intestinal, hepatic and renal tissues proposes that this transporter may function over an exocytotic pathway, before assimilation of Zn into secreted tissue proteins to regulate endogenous losses. During exposure to supplemental...
Zn, ZnT2 could play an important role in the recovery of Zn from the glomerular filtrate to possibly restore cellular Zn. In our study, the diabetic condition exhibited elevated ZnT4 mRNA within the intestinal epithelial cells and hepatic tissue, which suggests the contribution of ZnT4 to the intracellular transport of Zn from the apical side to the basolateral one from where Zn leaches into the portal blood. The expression of ZnT4 in the liver, and the vesicular compartment of kidney cells appears to be unaffected by the Zn state. Thus, supplemental Zn intake did not induce ZnT4 in the small intestine, liver and kidneys in our study, suggesting that it is not Zn-responsive.

ZnT5 is most profusely expressed in insulin-producing β-cells which contain Zn at the highest level in the body. This transporter interacts with ZnT6 to form a complex which transports Zn into the secretory pathway. Both the up-regulated expression of ZnT5 (in Caco-2 cells exposed to zinc) and the down-regulated expression of ZnT5 (in human intestinal mucosa) exposed to Zn have been reported. Therefore, two modes of regulation of ZnT5, namely, transcriptional suppression and improved mRNA stability, appear to exist. Our study has confirmed augmented ZnT5 expression in both the heart and intestinal tissue which could be due to the excessive Zn efflux from the cytosol of the cell to the extracellular space. Intriguingly, ZnT6 has been detected only in the small intestine in this study, suggesting a post-transcription mechanism, like ZnT5, of playing a role in the tissue-specific expression of the ZnT6 transporter. Another mechanism may be a feedback regulation wherein the diabetes induced Zn deficiency condition stimulates the transcription factor XBP1 gene which in turn elevates ZnT5 transcription, leading to augmentation of the Zn supply into the early secretory pathway. Our study specified that this alteration was voided by Zn treatment, presumably due to amended mRNA stability. The ZnT7 transporter gene is expressed mainly in the small intestine, liver, and lungs, and moderately in the kidneys, spleen, heart, and brain. This transporter was seen in the same manner as ZnT5/ZnT6 in the diabetic tissue, which was modulated by supplemental Zn treatment.

ZnT8, which is a highly β-cell specific Zn transporter, has received the most attention because of its association in the aetiology of both type-1 and type-2 diabetes. ZnT8 is also shown to facilitate the accumulation of Zn from the cytoplasm into intracellular vesicles in the β-cells of the pancreas and providing Zn for the formation of zinc-insulin crystals. In our study, the diabetic condition decreased the ZnT8 gene expression, while Zn supplementation improved the expression of ZnT8, which also indicates increased secretion of insulin from β-cells and its response which was documented in our previous study.

Zip1 is identified in diverse specific cells where it is confined to the plasma membrane and facilitates energy independent Zn uptake. Zip1 is affected by endogenous uptake, representing requirement for Zip1-mediated Zn mobilization into the cytoplasm. The ubiquitous expression of Zip1 mRNA proposes its housekeeping role in many cell types. The observed up-regulation in intestinal cells and down-regulation in hepatic cells of diabetic animals signifies balancing the scarcity after facing Zn deficiency through intestinal cells and limiting Zn store in hepatic cells. Although Zn treatment increases the resource of Zn for Zip1, it leads to intensifying the accretion of tissue Zn post-absorption by the elevated expression of Zn transporter protein Zip1. Zip4 mRNA is mostly expressed in the mature enterocytes of the intestinal villi, colon, stomach and renal tissue. The duodenum and jejunum are crucial sites of Zn absorption and the Zip4 protein inhabits on the apical surface of the enterocytes, undeviating in its transferring function for Zn uptake from the intestinal lumen. In our study, the intestine of diabetic animals exhibited diminished expression of Zip4 with a subsequently lowered Zn content in this tissue, which was corrected by Zn supplementation. This could constitute a response to diabetes-induced disturbed Zn homeostasis through rectifying the Zip4 transporter.

Metallothioneins (MTs) are cysteine-rich, small molecular mass metal-binding proteins (≈14 kDa), of which MT-1 and MT-2 are the major isoforms found in most tissues. A defending role for MT in diabetes has been advocated from the observed overexpression of this protein in feedback action to Zn, in streptozotocin-induced diabetic mice. The induced synthesis of MT averts diabetes complication independent of the type of diabetes and even in a different genetically prodiabetic rodent model. MT has been proposed to possess several physiological roles including antioxidant action, homeostasis of essential metals like zinc and copper, and detoxification of potentially toxic heavy metals, like cadmium. MT, being a potent antioxidant, is highly inducible by various oxidative stresses and, being an efficient inter-reactant with different reactive oxygen and nitrogen species, perhaps can increase the oxidative defense capacity of different tissues. In this study, we have delineated, for the first time, organ specific patterns together, for diabetes induced MT expression. Diabetes induced renal and pancreatic MT expression levels were metal dependent where Zn deficiency in those respective tissues couldn’t stimulate the over-production of MT even though the pathogenic oxidative stress is high compared to the corresponding tissues of normal control. Zn treatment of diabetic animals up-regulated the MT expression, which is evident by subsequent alleviation of oxidative stress as documented in our previous report. In contrast, other tissues like the intestine, liver and heart showed metal-independent MT expression, which may be due to the response of the defense mechanism to combat oxidative stress. Furthermore, a protective role of metallothioneins induced by Zn supplementation as we observed advocates that overexpression of this protein in diabetes upon zinc treatment could attenuate streptozotocin-induced diabetic hyperglycemia, hypoinsulinemia and concomitant oxidative stress as we reported recently.

With reference to the interaction of Zn with Ca²⁺ in the diabetic condition when Zn is supplemented, we examined the transporter of Ca²⁺. From a previous study, the proposed mechanism is that Zn and Ca²⁺ are transported across the brush border membrane via a multi-divalent metal channel and Ca²⁺ has lower affinity than Zn for the brush border membrane transport protein. TRPV5 and TRPV6 are the epithelial Ca²⁺ channels belonging to a superfamily of transient receptor potential...
channels which comprise the apical Ca\(^{2+}\) entry mechanism for active Ca\(^{2+}\) transport in the kidneys and intestine.\(^6\) Our study indicated that in the kidneys and intestine of diabetic rats, the expression of this Ca\(^{2+}\) channel TRPV5 was down-regulated, while the same was improved in the tissues of Zn supplemented diabetic rats. Though supplemental Zn was anticipated to diminish the efficacy of Ca\(^{2+}\) to bind this transporter, the expression of the Ca\(^{2+}\) channel TRPV5 was down-regulated, while indicated that in the kidneys and intestine of diabetic rats, the diabetes-induced efflux.

Thus, dietary Zn supplementation has a significant beneficial effect in the control of diabetes induced Zn dyshomeostasis. This animal study has highlighted the potential ameliorative effect of supplemental zinc on hyperglycemia-induced disturbed Zn distribution in body tissues. This is achieved through regulation of the tissue specific Zn transporters along with a protective influence on the oxidative stress induced metallothionein proliferation in these organs. Thus, Zn supplementation may offer potential for effectively addressing disturbed Zn regulation, which can be translated for clinical application in managing diabetic complications. In the diabetic condition, where the Zn efflux is more concomitant with upregulated ZnTs, in order to meet the efflux-created deficiency, the influx of Zn (mediated by ZIPs) was also more. With Zn supplementation to diabetic animals, the efflux was controlled by restricting the expression of ZnTs, while ZIPs (responsible for the influx) were further higher to compensate the lost Zn store in the body by the diabetes-induced efflux.

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MT</td>
<td>Metallothionein</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>Zn</td>
<td>Zinc</td>
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<td>ZIP</td>
<td>Zinc influx protein</td>
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<td>Zinc transporter</td>
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### Conflicts of interest

There are no conflicts to declare.

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