Effect of Chromium (III) -Amino Acid (1:3) Complexes on High Sucrose Induced Insulin Resistance, Lipid Abnormalities and Oxidative Stress in Male Sprague Dawley Rats

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Introduction

Type 2 diabetes (T2D) is an emerging health problem worldwide, with the number of cases projected to double to 350 million by the year 2025[1]. Chronic over nutrition, sedentary lifestyle and the lack of physical activity have contributed to the increasing prevalence of diabetes[2].

Insulin resistance (IR), an impaired responsiveness of the body to insulin, is a prediabetic stage in the transition from obesity to full-blown T2D[3]. IR is observed long before the development of diabetes and early identification and treatment of persons in prediabetic stage can delay their progression to full-blown diabetes and associated cardiovascular diseases etc[3]. Therefore, IR is an attractive target for therapeutic intervention ahead of the development of diabetes. The currently accepted therapies include pharmacological treatment, calorie restriction and physical exercise, which are effective, but none is deemed the ultimate cure. Although the currently popular medications such as thiazolidine diones and biguanides can improve insulin sensitivity, they have serious adverse effects. Therefore, new agents need to be developed that augment insulin sensitivity and help overcome the complications associated with IR and T2D.

In this context, the design and characterization of effective and safe nutritional supplements that can alleviate IR represents an attractive strategy. Among them, the micronutrient, chromium (Cr) has been gaining popularity as a dietary supplement to improve the actions of insulin under insulin-resistant conditions. The potential role of Cr in regulating blood sugar conditions is well established and the regulatory mechanisms are well documented[4]. Acting as a cofactor for insulin, Cr enhances glucose utilization by target tissues[5]. It facilitates insulin binding to its receptors, activates insulin receptor kinases and inhibits insulin receptor phosphatases[6]. Dietary deficiency of Cr is positively associated with the risk of diabetes and its complications[7] and dietary Cr supplementation lowers blood glucose concentrations and improve lipid profile in diabetic patients[8,9,10]. In a clinical trial, Martini et al[11] demonstrated improved insulin sensitivity in T2D subjects treated with Cr. In contrast, some reports claim Cr to be not an essential trace element for mammal[12] and that Cr treatment may not benefit diabetic subjects[13,14,15]. Therefore further studies are necessary to understand the potential role of Cr in treating IR and T2D[16]. Based on the better bioavailability of Cr reported from low molecular-weight (LMW) organic Cr complexes...
and the identification that the biologically active form of Cr is a complex with an oligopeptide complex, several LMW organic Cr complexes have been designed and evaluated as potential therapeutic agents to counter the diminished effect of insulin in T2D\(^{[17]}.\) In view of the foregone literature the present study aimed to investigate the impact of sub chronic treatment with binary complexes of Cr with some amino acids reported to be present in GTF of yeast, viz Gly, Cys and Lys on high sucrose (HS) induced IR and/or impaired glucose tolerance in male SD Rats. Considering the reported beneficial effects of Cr-D-(Phe), complex, we assessed the effects of the binary complexes of L- and D-Phe with Cr in this model. In addition, it assessed the effects of Cr-(AA), on HS induced lipid abnormalities and oxidative stress (OS) in this rat model.

**Materials and methods**

The micro protein assay kit was purchased from Thermo Scientific (USA) and antibodies to GLUT-4, pAkt, Akt, IRS-1 and PI3-K were from Calbiochem and Merck Millipore Company, UK. Unless stated otherwise, all other chemicals & reagents used in this study were of analytical grade obtained from Sigma Chemicals Co.

**Synthesis of Cr-(AA), complexes**

Cr-(AA), complexes were synthesized by mixing aqueous solutions of 10mM CrCl\(_3\)\(_{6}\)H\(_2\)O (50mL) and 30mM amino acid (50mL) and heating at 80°C and refluxing for 4 hours. The homogeneous green reaction mixture was freeze-dried and the greenish-violet solid obtained was washed with acetone and heated at 80°C and refluxing for 4 hours. The greenish-violet solid obtained was washed with acetone and heating at 80°C and refluxing for 4 hours.

**Animals**

The animal experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) approval (IAEC No: P42/12-2011/MR) at the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Regd. No. 154/1999/CPCSEA) and the experiment was carried out as per the animal ethical norms.

Table: 1 Composition of the diets used in the experiments.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control diet (g/kg)</th>
<th>Sucrose diet (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Starch</td>
<td>545</td>
<td>0</td>
</tr>
<tr>
<td>Oil</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>545</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mineral mix(^1)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mix(^2)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

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</table>
| 1| mineral mix composition (g/kg): calcium phosphate, dibasic, 500; magnesium oxide, 24; Potassium citrate, 220; potassium sulphate, 52; sodium chloride, 74; magnesium sulfate, 0.55; cupric carbonate, 0.3; Potassium iodide, 0.01; ferric citrate, 6; manganous carbonate, 3.5; sodium selenite, 0.01; zinc carbonate, 1.6.
| 2| Vitamin mix composition (g/kg): vitamin A (synthetic vitamin A blended in corn oil), 0.8; cholecalciferol, 1.0; vitamin E acetate (d-l-α-tocopherol acetate), 10.0; menadione sodium bisulphate, 0.08; biotin 1%, 2.0; cyanocobalamin 0.1%, 1.0; folic acid, 0.2; nicotinic acid, 3; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin,0.6;thiamine-HCL, 0.6.|

**Control and high sucrose diets**

Synthetic diets were prepared as per American Institute of Nutrition (AIN)-93G recommendations. Control diet for the rats contained starch- (carbohydrate) 54.5%, casein- 25%, oil-10%, cellulose- 5%, mineral mixture-4%, vitamin mixture-1%, L-cysteine-0.3%, and choline chloride-0.2%. The HS diet contained 54.5% sucrose instead of starch while the composition of the other ingredients remained the same (Table 1).

**Experimental design**

Rats were fed a rodent chow diet *ad libitum*, for one week initially. They were then randomly divided into six groups consisting 8 animals in each group. **Group-C**: fed control diet and DW. **Group-S**: fed HS diet and DW. **Group-S**: fed HS diet and DW containing Cr-D-(Phe). **Group-S**: fed HS diet and DW containing Cr-L-(Phe). **Group-S**: fed HS diet and DW containing Cr-(Gly). **Group-S**: fed HS diet and DW containing Cr-(AA). Based on the calculated water intake of the rats, Cr-(AA) complexes were administered through drinking water to provide a dose of ~45μg/ kg body weight/day. The daily dosage of Cr (III) to the rats was based on an earlier report on the beneficial effects of Cr (III) in a rat model\(^{[19]}\). The drinking water containing the Cr-(AA), was freshly prepared every day. These animals were maintained on their respective diets for a period of 12 weeks from the day of HS feeding and Cr-(AA), complex supplementation.

**Fasting glucose, Insulin, oral glucose tolerance and insulin resistance**

Body weights of the animals were measured at the beginning and end of experimental period. The experimental animals and their controls were subjected to the oral glucose tolerance test (OGTT) after an overnight / 12-h fasting and collection of a blood sample from retro orbital sinus. Without delay, a glucose solution (40% in DW) was administered through a gastric gavage at a dose of 2.5g/kg body weight. Three more blood samples were collected at 30, 60,120 minutes after glucose administration. All blood samples were collected in 2 ml centrifuge tubes containing 2% sodium fluoride (100μl/ml of blood) and
kept on ice till centrifugation. From the whole blood, plasma was separated and stored at -20°C until further use. Blood glucose concentrations were measured using a glucometer (Rite Check Blood Glucose Monitoring System, OK Biotech Co, Ltd., Taiwan) and plasma insulin concentration was measured using the Radio Immuno Assay (RIA) kit purchased from BRIT Mumbai, India. The area under the curve (AUC) of glucose and insulin during OGTT were computed by the trapezoidal method[20]. The indices of IR such as Homeostasis Model Assessment of IR (HOMA-IR) index and the ratio of glucose AUC to insulin AUC during OGTT were computed as described by us earlier[21].

Biochemical measurements

Plasma triglycerides[22,23], total cholesterol[24,25], and HDL-cholesterol[26,27] levels were measured using commercially available enzyme based assay kits (Biosystems, Barcelona, Spain).

Sample collection

At the end of the experimental period the animals were fasted overnight and sacrificed by cervical decapitation. Liver and skeletal muscle (Gastrocnemius) were excised quickly and frozen immediately in liquid nitrogen, and stored at -80°C until used.

Oxidative stress and antioxidant defence markers

Liver was weighed, minced and homogenized (10%w/v) in 50mM phosphate buffer (pH 7). The homogenate was centrifuged at 1000xg for 20min at 4°C and a portion of supernatant was used for the estimation of lipid peroxidation [Thiobarbituric acid reactive substances (TBARS)] & protein carbonyls using standard methods[28,29]. The remaining supernatant was further centrifuged at 12,000xg for 20min at 4°C to obtain the post-mitochondrial supernatant, which was used for the estimation of reduced glutathione[30] and to determine the activities of antioxidant enzymes: Catalase[31], Superoxide dismutase (SOD)[32] and Glutathione peroxidase (GPx)[33].

SDS-PAGE and Western blotting

Skeletal muscle was homogenised in Radio immune precipitation Assay (RIPA) buffer (150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS and 50mM Tris, pH 7.2) containing 1µM phenyl methane sulfonfluoride (PMSEF) and 1:100 dilutions of protease inhibitor cocktail. The homogenate was centrifuged at 15,000 x g for 15 min at 4°C, the supernatant was collected and its protein concentration determined by bicinechonic acid (BCA) method. Equivalent amounts of proteins were boiled in Laemmli sample buffer, resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were incubated with appropriately diluted primary antibody for IRS-1 (1:2000), GLUT-4 (1:1000), Akt (1:1000) and pAkt (1:500) in blocking buffer followed by incubation with horseradish peroxidase coupled secondary antibodies. Immuno reactive bands were visualized using chemiluminesence reagents (Bio-Rad, CA, USA). The band intensity was measured with a scanning densitometer coupled with Bio-Rad PC analysis software.

Statistical analysis

All the values are expressed as Arithmetic Mean ± SEM, except HOMA IR which is given as Geometric mean ± SEM. The differences among different groups in various parameters was analysed by one-way analysis of variance (ANOVA) using SPSS statistics package (version 17.0) followed by post-hoc least significant difference test (LSD). A probability value of p < 0.05 was considered to indicate the significance of the ratio and significant difference between means of different groups. All the results are reported as mean ± SEM.

Results

Characterization of Cr-(AA)₃ complexes

Purified Cr-(AA)₃ complexes were characterized by using following methods. UV-Visible spectra were recorded with an Elico Bio spectrophotometer, model BL198 (Figure 1 and table 2). IR spectra were recorded on KBr disks on a Perkin-Elmer FT-1605 spectrometer (Table 3). Elemental micro analysis (C, H and N) were carried out with a Perkin-Elmer 240 elemental analyser. ESI-MS mass spectra were recorded on ESI-MS Micro mass Quattro Lc triple quadruple mass spectrometer with Mass Lynx software (Manchester, UK) in m/z.

Table: 2 Uv-Vis λmax values of Cr-(AA)₃ complexes.

<table>
<thead>
<tr>
<th>Cr(AA) complexes</th>
<th>λ max(A)</th>
<th>λ max(B)</th>
<th>Δλ(A)</th>
<th>Δλ(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free chromium</td>
<td>417</td>
<td>602</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cr-D-(Phe)₃</td>
<td>400</td>
<td>547</td>
<td>17</td>
<td>55</td>
</tr>
<tr>
<td>Cr-L-(Phe)₃</td>
<td>398</td>
<td>545</td>
<td>19</td>
<td>57</td>
</tr>
<tr>
<td>Cr-(Gly)₃</td>
<td>402</td>
<td>549</td>
<td>15</td>
<td>53</td>
</tr>
<tr>
<td>Cr-L-(Cys)₃</td>
<td>412</td>
<td>558</td>
<td>05</td>
<td>44</td>
</tr>
</tbody>
</table>

Difference in λ-max indicates that complexation has taken place.

Figure: 1 A: Proposed chemical structure Cr-(AA)₃ complexes, B: Uv-Vis Spectra of Different Cr-(AA)₃ complexes
In FTIR, strong peaks for Cr-O, Cr-N indicate that complex formation has taken place. New absorption bands in the far IR region around 450 and 455 cm\(^{-1}\) can be assigned to the Cr–O and Cr–N bonds. The UV–V is spectrum of the methanolic solution of the complex registered bands at (402nm) 15,670 cm\(^{-1}\) (m1) and (549nm) 22,058 cm\(^{-1}\) (m2). The complex being greenish-violet in colour, the above two bands are due to the absorption in yellow and blue parts of the spectrum. Mitigation of the moderately sharp absorption band in the free ligand (2900–3100 cm\(^{-1}\)) to about 600 cm\(^{-1}\) may be attributed to the reorganization in intramolecular hydrogen bonding after chelation. New absorption bands in the far IR region around 458 and 455 cm\(^{-1}\) can be assigned to the Cr–O and Cr–N bonds. The UV–V is spectrum of the methanolic solution of the complex registered bands at (402nm) 15,670 cm\(^{-1}\) (m1) and (421nm) 22,073 cm\(^{-1}\) (m2). The complex being greenish-violet in colour, the above two bands are due to the absorption in yellow and blue parts of the spectrum. These absorptions are due to the spin allowed transitions 4T2g \(\rightarrow\) 4A2g (m1) and 4T1g (F) \(\rightarrow\) 4A2g (m2). The third band m3 overlaps with UV absorption of the ligand. These observations suggest a hexa-coordinate environment around chromium (III). The pH of the aqueous solution of the complex is 4.5 and the presence of chlorine demonstrates the presence of HCl in the lattice. Based on the stoichiometry, elemental analysis and spectral studies, the product obtained is a complex containing a 1:1 ratio of chromium to D-phenylalanine.

**Cr-D-(Phe)\(_3\) complex:** Elemental analysis found: C, 47.84; H, 5.60; N, 5.92. The stoichiometry Cr-D-(Phe)\(_3\) 3HCl 2H\(_2\)O requires C, 52.00; H, 5.40; N, 7.29. The ESMS (expand) of the complex in methanolic solution registers signals at 546.1 and 165.8 representing, respectively, the tris chelate and the deprotonated ligand. Formation of the complex was associated with Formation of the complex was associated with m C'O (2883 cm\(^{-1}\)) and m–H (3407 cm\(^{-1}\)) shifts in the IR-spectrum by about 17 and 20 cm\(^{-1}\) respectively. The broadening of the moderately sharp absorption band in the free ligand (2900–3100 cm\(^{-1}\)) to about 600 cm\(^{-1}\) may be attributed to the reorganization in intramolecular hydrogen bonding after chelation.

**Cr-(Gly)\(_3\) complex:** Elemental analysis found: C, 26.28; H, 4.41; N, 15.33 The stoichiometry Cr-(Gly)\(_3\) 3HCl 2H\(_2\)O requires C, 25.29; H, 4.40; N, 15.25. The ESMS of the complex in methanolic solution registers signals at 274.01 representing, respectively, the tris chelate and the deprotonated ligand. Formation of the complex was associated with Formation of the complex was associated with m C'O (2880 cm\(^{-1}\)) and m–N'H (3409 cm\(^{-1}\)) shifts in the IR-spectrum by about 16 and 20 cm\(^{-1}\), respectively. The broadening of the moderately sharp absorption band in the free ligand (2900–3100 cm\(^{-1}\)) to about 600 cm\(^{-1}\) may be attributed to the reorganization in intramolecular hydrogen bonding after chelation.

**Cr-L-(Cys)\(_3\) complex:** Elemental analysis found: C, 48.54; H, 5.60; N, 5.92. The stoichiometry Cr-L-(Cys)\(_3\) 3HCl 2H\(_2\)O requires C, 50.00; H, 5.45; N, 7.18. The ESMS of the complex in methanolic solution registers signals at 545.3 and 164.8 representing, respectively, the tris chelate and the deprotonated ligand. Formation of the complex was associated with Formation of the complex was associated with m C'O (2883 cm\(^{-1}\)) and m–N'H (3412 cm\(^{-1}\)) shifts in the IR-spectrum by about 16 and 20 cm\(^{-1}\), respectively. The broadening of the moderately sharp absorption band in the free ligand (2900–3100 cm\(^{-1}\)) to about 600 cm\(^{-1}\) may be attributed to the reorganization in intramolecular hydrogen bonding after chelation.

**Table: FTIR spectra of Cr(AA)\(_3\) complexes (strong peaks) in cm\(^{-1}\)**

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<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Free-L(Phe) alanine</td>
<td>3400</td>
<td>2875</td>
<td>1610</td>
<td>1315</td>
<td>1225</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cr-(L)Phenylalanine</td>
<td>3407</td>
<td>2883</td>
<td>1615</td>
<td>1319</td>
<td>1261</td>
<td>458</td>
<td>453</td>
<td>--</td>
</tr>
<tr>
<td>Free-D(Phe)phenylalanine</td>
<td>3405</td>
<td>2875</td>
<td>1610</td>
<td>1317</td>
<td>1228</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cr-(D)Phenylalanine</td>
<td>3410</td>
<td>2886</td>
<td>1620</td>
<td>1325</td>
<td>1267</td>
<td>460</td>
<td>455</td>
<td>--</td>
</tr>
<tr>
<td>Free Cysteine</td>
<td>3380</td>
<td>2868</td>
<td>1625</td>
<td>1328</td>
<td>1250</td>
<td>--</td>
<td>--</td>
<td>1584</td>
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<tr>
<td>Cr-(L)Cysteine</td>
<td>3385</td>
<td>2871</td>
<td>1628</td>
<td>1336</td>
<td>1256</td>
<td>462</td>
<td>452</td>
<td>1588</td>
</tr>
</tbody>
</table>

In FTIR, strong peaks for Cr-O, Cr-N indicate that complex formation has taken place.

**Cr-D-(Phe)\(_3\) complex**

<table>
<thead>
<tr>
<th>Volume 2: Issue 1www.ommegaonline.org</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>J Diabetes Obes</td>
</tr>
</tbody>
</table>
As expected, feeding HS diet increased fasting glucose and insulin levels in the SD rats compared to controls (Group-C). As a consequence, the HOMA-IR was significantly higher in group-S than group-C rats. Chronic oral administration of Cr-D-(Phe)₃ and Cr-L-(Phe)₃ (group-S) complexes but not in those given Cr-Gly₃ (group-S) or Cr-L-(Cys)₃ (group-S) complexes. HDL-cholesterol levels were comparable among the groups (Figure 3).

**Glucose tolerance and insulin sensitivity**

In line with the observations on fasting glucose, insulin and HOMA-IR mentioned above, rats fed HS diet had significantly higher (than group-C rats) values of AUC glucose and insulin during the OGTT indicating that they developed significantly higher (than group-C rats) values of AUC glucose and insulin levels in the SD rats compared to controls. Interestingly, plasma triglyceride and total cholesterol levels were significantly increased in the group-S rats compared to group C. Interestingly, plasma triglyceride and total cholesterol levels were increased significantly in rats given Cr-D-(Phe)₃ (group-S₁) and Cr-L-(Phe)₃ (group-S₂) complexes but not in those given Cr-Gly₃ (group-S₃) or Cr-L-(Cys)₃ (group-S₄) complexes. Hepatic oxidative stress markers and antioxidants

Malondialdehyde (MDA) and protein carbonyl levels were significantly increased in group-S compared to group-C. Interestingly (MDA) and protein carbonyl levels were significantly increased in group-S compared to group-C. However the levels of GSH were significantly lower in group-S compared to group-C. Interestingly, plasma triglyceride and total cholesterol levels were significantly increased in the group-S rats compared to group C. Interestingly, plasma triglyceride and total cholesterol levels were increased significantly in rats given Cr-D-(Phe)₃ (group-S₁) and Cr-L-(Phe)₃ (group-S₂) complexes but not in those given Cr-Gly₃ (group-S₃) or Cr-L-(Cys)₃ (group-S₄) complexes. HDL-cholesterol levels were comparable among the groups (Figure 3).

**Fasting plasma glucose, insulin and HOMA-IR**

As expected, feeding HS diet increased fasting glucose and insulin levels in the SD rats compared to controls (Group-C). As a consequence, the HOMA-IR was significantly higher in group-S than group-C rats. Chronic oral administration of Cr-D-(Phe)₃ and Cr-L-(Phe)₃ complexes but not Cr-(Gly)₃ and Cr-L-(Cys)₃ complexes mitigated the HS induced changes in fasting glucose, insulin and HOMA-IR (Table 4).
**Table: 5 Effect of Cr-AA complexes on hepatic oxidative stress markers and antioxidants.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>S</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>0.57± 0.025</td>
<td>1.42 ± 0.043</td>
<td>0.346± 0.043</td>
<td>0.392± 0.062</td>
<td>1.24 ± 0.025</td>
<td>1.37 ± 0.034</td>
</tr>
<tr>
<td>protein carbonyls (nmol/mg protein)</td>
<td>1.79 ± 0.21</td>
<td>2.61 ± 0.21</td>
<td>1.42 ± 0.16</td>
<td>1.38 ± 0.17</td>
<td>2.63 ± 0.31</td>
<td>2.54 ± 0.35</td>
</tr>
<tr>
<td>Reduced glutathione (GSH) (µg/mg protein)</td>
<td>4.92 ± 0.38</td>
<td>3.92 ± 0.19</td>
<td>4.75 ± 0.18</td>
<td>4.87 ± 0.24</td>
<td>4.07 ± 0.14</td>
<td>3.97 ± 0.31</td>
</tr>
<tr>
<td>superoxide dismutase (SOD) (U/mg protein)</td>
<td>40.14 ± 1.43</td>
<td>39.54 ± 0.84</td>
<td>49.61 ± 2.35</td>
<td>48.41 ± 1.32</td>
<td>40.17 ± 0.47</td>
<td>41.17 ± 0.25</td>
</tr>
<tr>
<td>glutathione peroxidase (U/mg protein)</td>
<td>7.56 ± 0.34</td>
<td>6.02 ± 0.34</td>
<td>7.48 ± 0.85</td>
<td>7.68 ± 0.31</td>
<td>5.78 ± 0.17</td>
<td>6.12 ± 0.24</td>
</tr>
<tr>
<td>Glutathione reductase (U/mg protein)</td>
<td>41.35 ± 0.84</td>
<td>28.42 ± 0.76</td>
<td>39.34 ± 0.93</td>
<td>41.57 ± 0.57</td>
<td>42.61 ± 0.34</td>
<td>41.82 ± 0.54</td>
</tr>
<tr>
<td>Catalase (U/mg protein)</td>
<td>65.46 ± 1.28</td>
<td>52.04 ± 1.58</td>
<td>63.59 ± 2.65</td>
<td>67.72 ± 1.57</td>
<td>41.07 ± 0.98</td>
<td>49.07 ± 0.98</td>
</tr>
<tr>
<td>glutathione -s-transferase (U/mg protein)</td>
<td>738.58 ± 13.65</td>
<td>609.57 ± 17.45</td>
<td>732.54 ± 14.57</td>
<td>723.5 ± 17.56</td>
<td>643.17 ± 18.20</td>
<td>621.57 ± 17.37</td>
</tr>
</tbody>
</table>

A- Amount of enzyme which gave 50% inhibition of pyrogallol autooxidation/min; B- µg of GSH consumed/min; C- µmol of NADPH oxidized/min; D- mmol of H₂O₂ decomposed/min; E- µmol of GSH-CDNB conjugate formed/min. Values are mean ± S.E.M. (n=8), values in a row bearing different superscripts are different (p<0.05) from one another by one way ANOVA followed by post hoc least significant difference (LSD) test.

### Skeletal muscle insulin signaling

The expression of IRS-1, pAkt and GLUT-4 were significantly lower in group-S compared to group-C. Interestingly these changes were mitigated in group’s S1 & S2, but not in group’s S3 & S4 rats. The amount of IRS1, phosphorylated Akt (pAkt) and GLUT-4 (translocated to membrane) were significantly higher in skeletal muscles of group’s S1 & S2, but not in group’s S3 & S4 (Figure 4).

**Figure: 4 Effect of oral supplementation of Cr-(AA)₃ complexes on insulin signaling molecules.** Panel A: Blots of GLUT4, pAkt, Akt, IRS1 and GAPDH, panel B: ratios of gel optical densities of protein bands. Values are mean ± S.E.M. (n=8). Bars without a common superscript (‘a’ and ‘b’) different at P< 0.05.

### Discussion

Several studies have shown that elemental chromium (III) may play an important role in carbohydrate and lipid metabolism[38]. The biologically active form of Cr was identified as an oligopeptide chromodulin, a LMW chromium binding substance[39]. Based on these findings, LMW chromium complexes have been synthesized and evaluated as insulin-potentiating agents. Among the chromium-complexes used, chromium picolinate has gained popularity as a nutritional supplement. However, there have been some concerns regarding the mutagenic potential of chromium picolinate, which has been attributed to the OS caused by the picolinate ligand[39].

Cr-(AA)₃ complexes used in this study were designed to mimic the activity of chromodulin, which is an oligopeptide complex of chromium with different amino acids. Compared with the picolinate ligand, the amino acid ligands used in the current study have better solubility at physiological pH and may also inhibit OS[37]. Earlier, a few studies have shown that the chronic supplementation of Cr-D-(phe), have beneficial effects in T2D[39]. Since L-amino acids are biologically active forms which are metabolised in most of the organisms including animals, whereas D-amino acids are present in only few bacteria and are not metabolised by higher organisms including animals, in this study we investigated the efficacy of Cr-(AA)₃ complexes derived from L-amino acids rather than D-amino acids. The Cr-(AA)₃ complexes synthesized and utilized in this study are in line with that of GTF reported from yeast, with glucose uptake promoting properties and reported to contain amino acids. Also some earlier studies have reported that simple binary Cr-AA complexes may be sufficient to exert beneficial effects on glucose uptake by yeast in cultures[38].

High sucrose/fructose- induced IR animal model has been recommended for assessing the therapeutic efficacy of insulin sensitizers and drugs that are likely to affect insulin sensitivity. Therefore we selected HS induced IR model to study the efficacy of synthetic Cr-(AA)₃ complexes in preventing / modulating HS induced IR in male SD rats. Results of the present study showed that HS feeding for 12 weeks resulted in fasting hyperglycemia, hypertriglyceridemia, hyperinsulinemia, glucose intolerance and IR in male SD rats. Further this was associated with impaired antioxidant potential and increased OS. These findings are consistent with those reported earlier[39,40].

The hypertriglyceridemia after HS feeding is known to result from the enhanced rate of hepatic VLDL- triglyceride synthesis[41] and/or a decrease in peripheral triglyceride clearance[42]. Further, increased delivery of triglycerides to the muscle impairs insulin action and interferes with the utilization of glucose, leading to hyperglycemia and hyperinsulinemia. That chronic oral supplementation of Cr-D/L-(Phe)₃ complexes prevented the HS induced hyperglycemia and hyperinsulinemia in group’s S3 and S4. SD rats appears to suggest that these positive effects could be attributed to the observed decrease in plasma triglyceride levels in these animals perhaps due to decreased synthesis and / or peripheral clearance.

The ability of insulin to stimulate glucose disposal under fasting condition was markedly impaired in rats fed HS diets, as evidenced by increased HOMA-IR which could be due to a decline in insulin sensitivity in peripheral tissues. That chronic oral supplementation of Cr-D/L-(Phe)₃ complexes in HS fed rats prevented the abnormal rise in HOMA-IR that too by decreasing the fasting insulin levels significantly, indicates the ability of Cr-D/L-(Phe)₃ complexes to increase insulin sensitivity of the target tissues and promote the clearance of circulating glucose. It is possible that the observed decrease in fasting glucose could...
also be due to decreased gluconeogenesis, which normally is the chief contributor to the fasting blood glucose levels. That chronic supplementation of Cr-D/L-(Phe), complexes to HS fed rats also improved oral glucose tolerance (decreased AUC glucose during OGTT) significantly indeed by decreasing the AUC insulin during OGTT suggests that Cr-D/L-(Phe), complexes improved the insulin sensitivity of the target tissues significantly. That binary complex of Cr with L. Phe (but not with Gly or L-Cys) was as effective as that with D. Phe seems to suggest binary complexes of Cr with only a few (but not all) AAs may be effective in this regard.

Defects in insulin signalling cascade have been shown to underlie impaired glucose utilization and proposed to play a key role in the pathogenesis of IR[41]. It is known that tyrosine phosphorylation of IRS 1 in response to insulin stimulation, generally increases the association of pIRS-1 with membrane bound PI-3 kinase, stimulating PI-3 kinase activity (by dissociating the 110 KDa catalytic subunit from the 85 KDA regulatory subunit) , which in turn activates serine/threonine protein kinase-B (PK-B or Akt). The active Akt ultimately leads to an enhancement of insulin stimulated glucose uptake / disposal by translocating intracellular Glut 4 to plasma membrane[44]. Our results reveal that the chronic feeding of HS diet significantly decreased the the levels of IRS 1 , pAkt (but not Akt per se) and total GLUT-4 levels, whereas chronic supplementation of Cr-D/L-(Phe), complexes (but not those with Gly or L-Cys) significantly increased the expression of IRS1 and Glut 4 and pAkt suggesting the correction of HS feeding induced impairment of insulin signalling in the skeletal muscle ,the insulin responsive target tissue important specially in post prandial clearance of circulating glucose. These findings also support the well-known concept that Cr may act by augmenting insulin signalling.

The development of OS, an imbalance between pro- and antioxidant status, has been shown to play an important role in mediating IR. Therefore, we studied whether increased OS plays a role in the HS induced IR and diabetes in this rat model and also its modulation by supplementation with Cr-AA peptides. Increased lipid peroxidation and protein carbonyl levels along with decreased hepatic GSH levels, and activities of antioxidant enzymes in HS fed rats, clearly indicate the development of OS and impaired anti-oxidant status in these animals. The increase in catalobolism of sucrose and its product fructose could be associated with the cellular energy depletion that can increase the susceptibility of cells to lipid peroxidation[45]. Furthermore, it has been postulated that fructose can accelerate free radical production similar to glucose. Reactive oxygen species (ROS) can themselves reduce the activity of antioxidant enzymes[40]. The decreased SOD activity in HS fed rats may be due to enhanced protein glycation by fructose as it is a more reactive reducing sugar compared to others (glucose and lactose)[42]. The reduced GSH levels could be due to its increased utilization to trap free radicals, and/ or decreased regeneration as evident with the lower activity of glutathione reductase enzyme. That chronic supplementation of Cr-D/L-(Phe), complexes reversed the changes induced by HS in oxidative stress and anti-oxidant enzyme activities in addition to correcting the insulin resistance, impaired glucose tolerance and lipid metabolism / profile in these rats seems not only to suggest their modulation by Cr-AA complex but also that altered oxidative stress and anti oxidant activity could underlie the HS diet induced impairment of insulin sensitivity and glucose tolerance in these rats. As OS has been suggested to be one mechanisms for the detrimental effects of sucrose/fructose, the antioxidant potential of Cr-D/L-(Phe), complexes appears to be one of the mechanisms by which these complexes prevented IR and impaired glucose tolerance in HS fed rats. Some studies have indeed linked ROS production and OS to IR[40]. Several clinical trials, have also demonstrated that treatment with vitamin-E, vitamin-C, or glutathione improves insulin sensitivity in insulin resistant individuals and/or patients with T2DM[49,50].

In summary, the present study shows that chronic administration of a new Cr-(AA) complex’s Cr-D-(Phe), and Cr-L-(Phe), alleviates HS diet induced IR and impaired glucose tolerance probably due to the correction of changes in oxidative stress and anti-oxidant status and also mediated by augmenting insulin signalling. Cr-(AA), complexes treatment also attenuates hepatic triacylglyceride levels and lipid accumulation. These results suggest that nutritional supplementation with Cr-(AA), complexes may have potential therapeutic value in the better management of the IR associated with metabolic syndrome.

Conflict of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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