Original Article

Combined chromium and magnesium decreases insulin resistance more effectively than either alone

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Background and Objectives: Peroral supplementation with trivalent-chromium (Cr) or magnesium (Mg) has been shown to improve insulin resistance (IR). The objective of this study was to determine whether combined peroral supplementation with Cr and Mg improves IR more effectively than Cr or Mg alone. **Methods and Study Design:** Subjects (n=120, age range 45-59 years old) and diagnosed with IR were randomly divided into four groups and monitored for a period of 3 months: group 1 (the placebo control group), group 2 (160 μg/d Cr), group 3 (200 mg/d Mg), and group 4 (160 μg/d Cr plus 200 mg/d Mg). Fasting blood glucose (FBG), fasting insulin (FIns), erythrocyte Cr and Mg content, and glucose-transporter-4 (*GLUT4*) and glycogen-synthase-kinase-3β (*GSK3β*) mRNA levels in activated T-lymphocytes were measured, and insulin resistant index (IRI) was calculated. **Results:** Significant decreases between the baseline and study conclusion values of FBG (0.37 mmol/L, $p<0.01$), FIns (2.91 μIU/mL, $p<0.01$) and IRI (0.60, $p<0.01$) were observed in group 4, but not groups 1-3. Similarly, compared with baseline, significant changes in *GLUT4* (2.9-fold increase, $p<0.05$) and *GSK3β* (2.2-fold decrease, $p<0.05$) mRNA levels in activated T-lymphocyte were observed at the study's conclusion in group 4, but not in groups 1-3. **Conclusions:** Our results indicate that combining peroral supplementation with Cr and Mg improves IR more effectively than Cr or Mg alone, and this may be attributable to increased induction and repression, respectively, of *GLUT4* and *GSK3β* expression.

Key Words: insulin resistance, trivalent chromium, magnesium, glucose transporter 4, T lymphocytes

INTRODUCTION

Type 2 diabetes (T2D) is a growing public health burden, the number of global cases of which is projected to double to 350 million by the year 2030.[1,2] The pathogenesis of T2D and other metabolic diseases involves insulin resistance,[3] a prediabetic stage in the transition from obesity to full-blown T2D that exists long before the development of diabetes.[4] As early identification and treatment of individual with insulin resistance can delay the progression to diabetes and related cardiovascular disease,[5] there exists an urgent need to develop effective strategies for improving insulin resistance.

T lymphocytes (T-cells) are important components of the peripheral blood cell population that, in the steady state, do not contain insulin receptors and are insulin insensitive. It has been reported, however, that expression levels of insulin receptor (*INSR*) and glucose transporter 4 (*GLUT4*) in phytohemagglutinin-activated T cells are 50% lower in individuals with T2D than in controls, which is similar to insulin sensitive muscle cells.[6] Accordingly, the expression levels of genes in insulin-sensitive tissues can be extrapolated by detecting their expression levels in readily available activated T cells.

Chromium (Cr) is one of the important micronutrients that is required for glucose, protein and lipid metabolism, and whose deficiency causes hyperglycemia, hyperlipidemia and insulin resistance.[7] Cr supplementation has been shown to improve carbohydrate metabolism by increasing insulin sensitivity and decreasing indices of insulin resistance, lipid variables and serum insulin levels.[8,9] Similarly, low magnesium

Magnesium (Mg) intake has been associated with insulin resistance and increased risk of metabolic syndrome,[10,11] and recent studies have suggested that increased Mg intake is associated with lower fasting glucose and insulin[12]

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and decreased risk of T2D.[13]

Although supplementation with either Cr or Mg alone has been shown to improve insulin resistance, there have been no studies investigating the effect on insulin resistance of combination Cr and Mg supplementation, nor the effect of such therapy on expression in activated T-cells of factors in insulin-regulated signaling pathways. Therefore, we hypothesized that a combined supplementation of Cr and Mg would be more helpful to attenuate the disorders of insulin resistance; we further hypothesized that the improvement may be associated with the expression of *GLUT4* and glycogen synthase kinase 3β (*GSK3β*) by Cr in combination with Mg. Here, we compared the effect of single and combined Cr and Mg oral supplementation in 120 insulin resistant individuals with respect to indices of glucose metabolism. These indices included fasting blood glucose (FBG) and fasting insulin (FIns), erythrocyte Cr and Mg levels, and activated T cell levels of mRNAs encoding *GLUT4* and *GSK3β*. We anticipated that combination Cr and Mg supplementation would significantly improve insulin resistance more effectively in insulin resistant individuals. Based on this expected information, we can carry out further research on the metabolic changes of Cr combined with Mg in larger sample clinical trials.

METHODS AND MATERIALS

Subjects

All study subjects underwent physical examination and clinical laboratory tests, including FBG, Fins, erythrocyte Cr and Mg levels. Inclusion in the study was based upon body mass index >24 kg/m^2, and insulin resistance index >1.2. Exclusion criteria were diabetes, hypermagnesemia, cancer, coronary heart disease, and individuals taking drugs for the treatment of any condition. Based on these criteria, 120 subjects (average age =54.8, age range 45 to 59 years old) were recruited in Qingdao, China between 2011 and 2013. All selected subjects were given guidance regarding their diet and physical activity such as low-sugar and low-fat diet and walking 6,000 steps every day, and were required to abstain from smoking or excessive drinking for the duration of the study. The supplements were to be taken after eating once a day.

Study design

Computer-generated random numbers were used to assign participants to one of four groups who received daily supplementation for 3 months as follows: group 1 (n=30) was the placebo control group, group 2 (n=30) received 160 μg supplemental Cr (Chromium Yeast), group 3 (n=30) received 200 mg supplemental Mg (Magnesium Carbonate), and group 4 (n=30) received 160 μg supplemental Cr plus 200 mg supplemental Mg. Cr and placebos were provided by Angel Yeast Co. LTD (Yichang, China), and Mg was purchased from By-Health (Guangdong, China). The trial participants and the research team were unaware of the treatment assignment. The trial was deblinded after analysis of its primary outcomes.

Blood sample collection

Venous blood samples (10 mL) were obtained from participants at the beginning and at the end of the trial. In all cases, blood samples were taken between 6:00-8:00 am after a 12 hour fasting period. Serum was separated from whole blood by centrifugation at 3,000 × g for 10 mins to determine FBG and FIns. Red blood cells (RBCs) were isolated as follows: 3 mL anticoagulated blood of each sample was mixed with equivalent normal saline, after centrifugation at 2,000 × g for 5 mins, the supernatant was removed and the sediment was washed 3 times with Hank's solution without Ca^{2+} or Mg^{2+}, and centrifuged at 2,000 × g for 10 mins each time. The pellet was retained and immediately stored at -80℃.

Isolation of T-lymphocytes

Four mL anticoagulated blood of each sample was diluted with equivalent phosphate-buffered saline (PBS). Four mL lydroxypropylmethyl cellulose was added to a 15 mL centrifuge tube, after which diluted blood was added to the centrifuge tube slowly to avoid the blood entering the lydroxypropylmethyl cellulose. After centrifugation at 2,000 × g for 15 mins, the band of blood mononuclear cells was collected and washed 2 times with PBS. The supernatant was removed, leaving a pellet containing lymphocytes. Because T lymphocytes constitute the majority in this population, the pellet was considered as containing T cells.

Measurement of FBG and Fins

FBG was measured using an Automatic Biochemistry Analyzer (Hitachi 7600, Japan). FIns was measured by radio-immunoassay according to the reagent kit instructions (Jingma, Shanghai, China). To generate a standard curve, 100 μL of an insulin standard preparation (0, 10, 20, 40, 80, 160 μU/mL) was mixed with 100 μL analytical reagent (0.05 mol/L PBS plus 1% normal rabbit serum), incubated at 37℃ for 2 h, then I^{125}-insulin was added to the mixture and incubated at 37℃ for another 1 h, after which time 100 μL second antibody was added to the mixture and incubated at 37℃ for 30 mins. After centrifugation at 3,000 × g for 30 mins at room temperature, the supernatant was collected and the radioactive intensity of the sediment was detected by count monitoring. Assuming that the radioactive intensity of 0 μU/mL insulin standard preparation was B0, and the other concentration of insulin standard preparation was B, the combined rate (B%) of the different concentration was calculated using the formula: B% = B/B0 × 100%. The standard curve was drawn using B% as the ordinate, different concentrations of insulin standard preparation as the abscissa. The FIns content of the sample was obtained from the standard curve according to its B%. The insulin resistance index (IRI) was calculated as follows: FIns × FBG/22.5.

Determination of RBC Cr and Mg levels

A microwave digestion was employed for total Cr and Mg determination in RBCs. Approximately 0.05 g of RBCs were placed into a polytetrafluoroethylene digester (Milestone Ethos A, Italy), to which was added 6 mL nitric acid, and soaked for 1 h. After the hour had elapsed, 1 mL hydrogen peroxide was added. The vessels were immediately closed after the addition of the reagents, after which they were placed in the microwave digestion system (Milestone Ethos A, Italy). A microwave program

for biological material analysis was applied as described by González-Iglesias et al.[14] At the end of the digestion, the resulting solutions were transferred to 25 mL volumetric flask, brought up to the specified using ultrapure water, prior to mixing and analysis by ICP-MS (Agilent 7500cx, Santa Clara, CA, USA).

Activation of T cells and isolation of RNA

Isolated T-cells were activated by placing into a tissue culture flask containing 5 mL RPMI 1,640 medium and 2 μL PHA (Solarbio, China). The cells were incubated in a carbon dioxide incubator (BNP-310, Sanyo, Japan) with 5% CO_2 for 72 h, prior to RNA extraction using Trizol reagent (Invitrogen, Grand Island, NY, USA).

Detection of *GLUT4* and *GSK3β* mRNA levels by real-time fluorescent quantitative PCR *GLUT4* and *GSK3β* mRNA levels were detected by real-time fluorescent quantitative PCR (Light Cycler 480 II System, Roche Diagnostics, USA). cDNA preparation and real-time fluorescent quantitative PCR were performed as previously described.[15] Gene-specific primers for amplification were as follows: *GLUT4* (forward: 5′-CAGCCATGAGCTA CGTCTCCA-3′, reverse: 5′-GA AACCCATGCCAA TGATGAA-3′) and *GSK3β* (forward: 5′-GGCTACCAT CCTTATTCCTCCT-3′, reverse: 5′-GT CTGTCCACGG TCTCCAGTAT-3′). The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (forward: 5′-GCGAGATCCCTCCAAAATCAA-3′, reverse: 5′-ACTT CTCATGGTTCACACCCA TG-3′) was used as a reference for internal standardization. The PCR amplification was each performed in triplicate, under the following conditions: 95℃ for 30 s, followed by 40 cycles of amplification (95℃ for 5 s, 60℃ for 30 s), 1 cycle of melting (95℃ for 5 s, 65℃ for 15 s, 95℃ 0 s), and 50℃ for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected.

Statistical analysis

The statistical software SPSS 17.0 (Chicago, IL, USA) was used for data analysis. Baseline differences between groups with respect to height, weight, age, waist circumference (WC), hip circumference (HC), IRI and RBC Cr and Mg levels were evaluated using one-way ANOVA. Differences in values for FBG, FIns, IRI, and RBC Cr and Mg levels at baseline and study conclusion were compared within each group using *t*-test. One-way ANOVA was used to evaluate the changes in variables among groups after the intervention. $p \leq 0.05$ was considered statistically significant.

RESULTS

Of the 120 participants, 119 completed the study; one participant in group 4 withdrew from the study after being diagnosed with lung cancer. Table 1 shows baseline characteristics of the subjects including weight, height, BMI, age, WC, HC, FBG, FIns, IRI, RBC Cr and Mg levels, and *GLUT4* and *GSK3β* mRNA levels in activated T lymphocytes. Subjects in the four groups were matched for age and BMI. No significant differences were found between the four groups with respect to baseline values of any parameter ($p>0.05$).

Combination Cr and Mg supplementation decreases insulin resistance more effectively than either Cr or Mg alone

At study conclusion, significant decreases from the baseline values were observed in FBG (0.37 mmol/L; $t=6.28$, $p<0.01$), FIns (2.91 μIU/mL; $t=3.89$, $p<0.01$) and IRI (0.60; $t=2.96$, $p<0.01$) in group 4, but not groups 1-3 (Table 2). These data indicate that combination Cr and Mg supplementation improves three key indices of insulin resistance more effectively than Cr or Mg alone.

Supplementation of Cr or/and Mg has no effect on RBC Cr and Mg levels

With the exception of an increase in RBC Mg levels in group 3 ($t=2.11$, $p<0.05$), no significant differences were observed in any group between baseline and study conclusion RBC Cr and Mg levels (Table 3).

Table 1. Basic characteristics of study subjects in different groups

Measurements	Group 1 (n=30)	Group 2 (n=30)	Group 3 (n=30)	Group 4 (n=29)
Weight (kg)	70.5±7.78	70.1±7.69	72.0±9.70	72.5±8.71
Height (cm)	162±3.60	162±8.19	166±8.64	165±6.53
BMI (kg/m^2)	26.8±2.98	26.5±2.74	26.1±1.90	26.6±2.39
Age (years)	55.6±3.36	55.3±3.30	54.8±3.03	54.8±2.83
WC (cm)	92.7±7.40	93.6±8.42	93.2±6.75	93.1±7.71
HC (cm)	99.4±8.89	98.5±8.71	98.2±6.88	99.9±8.32
FBG (mmol/L)	5.42±0.62	5.42±0.61	5.41±0.63	5.41±0.59
FIns (μIU/mL)	12.6±5.33	12.5±5.53	12.6±5.29	12.5±5.63
IRI	3.09±1.38	3.03±1.46	3.02±1.23	3.05±1.57
Cr (μg/L)	188±24.8	189±15.0	188±30.7	188±45.6
Mg (mg/L)	35.4±9.29	34.9±8.01	34.1±11.9	33.4±9.23
GLUT4 mRNA	0.94±0.66	0.97±1.01	0.98±1.28	0.94±1.10
GSK3β mRNA	1.39±1.23	1.46±1.39	1.36±1.30	1.34±1.42

Data are presented as mean±SD.

FBG was measured using an automatic biochemistry analyzer. FIns was detected by radio-immunoassay, and IRI was calculated as: FIns×FBG/22.5. Determination of RBC Cr and Mg levels was achieved by ICP-MS. The mRNA expression of *GLUT4* and *GSK3β* were detected by real-time fluorescent quantitative PCR.

Table 2. Differences between baseline and study conclusion values for FBG, FIns and IRI in the four groups

Parameter/Group	n	Baseline	Changes†	*p*-value
FBG (mmol/L)				
1	30	5.42±0.62	-0.03±0.56	0.773
2	30	5.42±0.61	-0.09±0.64	0.454
3	30	5.41±0.63	-0.08±0.40	0.316
4	29	5.41±0.59	-0.37±0.32*	0.000
FIns (μIU/mL)				
1	30	12.6±5.33	0.11±5.72	0.919
2	30	12.5±5.53	-1.16±6.06	0.346
3	30	12.7±5.29	-0.49±5.50	0.667
4	29	12.5±5.63	-2.91±3.96*	0.001
IRI				
1	30	3.09±1.38	-0.04±1.34	0.877
2	30	3.03±1.46	-0.18±1.72	0.607
3	30	3.02±1.23	-0.13±1.33	0.633
4	29	3.05±1.57	-0.60±1.13*	0.006

Data are presented as mean±SD.
FBG was measured using an automatic biochemistry analyzer. FIns was detected by radio-immunoassay, and IRI was calculated as: FIn s × FBG/22.5.
†Changes = study conclusion - baseline.
*p<0.05 compared with the baseline.

Significant changes in GLUT4 and GSK3β mRNA levels in activated T lymphocytes after combination Cr and Mg supplementation

Significant differences between baseline and study conclusion in activated T lymphocyte mRNA levels of *GLUT4* (2.9-fold increase, p<0.05) and *GSK3β* (2.2-fold decrease, p<0.05) were observed in group 4, but not groups 1-3 (Table 4). These findings indicate that combination Cr and Mg supplementation may exert its beneficial effects on insulin resistance by regulating the expression of genes encoding important components of insulin-regulated signal transduction pathways.

Table 3. Differences between baseline and study conclusion RBC Cr and Mg levels in the four groups

		Changes†	
Group	n	Cr (μg/L)	Mg (mg/L)
1	30	-7.34±51.8	0.87±8.66
2	30	-13.8±21.9	-0.39±12.1
3	30	-15.3±39.4	4.59±12.3*
4	29	-28.9±81.5	2.24±9.30

Data are presented as mean±SD.
Determination of RBC Cr and Mg levels was achieved by ICP-MS.
†Changes = study conclusion - baseline.
*p<0.05 compared with the base level.

DISCUSSION

In the present study, we compared the effects of combined or individual supplemental Cr and Mg on FBG, FIns, IRI, and activated T-lymphocyte *GLUT4* and *GSK3β* mRNA levels in an insulin resistant population over a 3-month period. We detected significant decreases in FBG, FIns and IRI only in the group receiving combined Cr and Mg, and this effect was accompanied by induction of *GLUT4* and repression of *GSK3β* mRNAs in activated T lymphocytes in individuals in this group.

Cr and Mg play critical roles in glucose metabolism and insulin metabolism. For example, Cr and Mg deficiency have been reported to cause insulin resistance, hyperglycemia and hyperlipidemia,[7,11,16] and are characteristic of patients with diabetes.[17,18] Moreover, hair Cr levels have been shown to correlate negatively with the fasting blood glucose and triglyceride levels.[19] Further-

Table 4. Differences between baseline and study conclusion of activated T-lymphocyte *GLUT4* and *GSK3β* mRNA levels in the four groups

Parameter/Group	n	Baseline	Changes†	*p*-value
GLUT4 mRNA				
1	30	0.94±0.66	0.12±1.37	0.810
2	30	0.97±1.01	0.68±2.09	0.333
3	30	0.98±1.28	0.28±1.93	0.489
4	29	0.94±1.10	2.91±5.86*‡	0.023
GSK3β mRNA				
1	30	1.39±1.23	-0.06±1.34	0.865
2	30	1.46±1.39	-0.20±1.52	0.738
3	30	1.36±1.30	-0.15±1.71	0.686
4	29	1.34±1.42	-0.45±0.95*	0.041

The mRNA levels in *GLUT4* and *GSK3β* in activated T-lymphocytes were detected by real-time fluorescent quantitative PCR. Data were presented as means±SD.
†Changes = study conclusion − baseline.
*p<0.05 compared with the baseline level, ‡p<0.05 compared with group 3.

more, Cr or Mg supplementation reportedly exert beneficial effects on insulin resistance by ameliorating insulin sensitivity[8,20,21] and improving carbohydrate and lipid metabolism.[3,9,13,22-24] Our results suggest that combined Cr and Mg supplementation improves critical indices of insulin resistance more effectively than Cr or Mg alone.

GLUT4 and *GSK3β* are important components in an insulin-induced signal transduction pathway that plays a key role in glucose metabolism. Increased expression of *GLUT4* has been associated with enhanced glucose translocation from the exterior to the interior of cells in insulin-sensitive tissues and repression of *GSK3β* has been shown to enhance insulin receptor activity by relieving inhibition of its tyrosine kinase activity by GSK. The induction of *GLUT4* and repression of *GSK3β* mRNA levels in activated T lymphocytes by combined Cr/Mg observed in this study is consistent with previous reports[25-28] and suggests a possible mechanism by which combined Cr/Mg therapy exerts its beneficial effects. PI3-K/Akt signal transduction may be more reactive by combined use of Cr and Mg than separate Cr or Mg, which may be the possible underlying mechanism.

To observe the intracellular changes of Cr and Mg levels after the 3-month supplementation, the RBC Cr and Mg concentrations were detected. With the exception of an increased RBC Mg level in group 3, no significant differences of RBC Cr and Mg levels were observed in any other groups after the supplementation, and a high variation of RBC Cr after the supplementation was found. Previous study showed that Fe and Cr are competitive, and Fe can inhibit the absorption of chromium.[29] It is not easy for Cr to accumulate because of high level of Fe in RBCs, so the RBC Cr did not elevate significantly. The high variation of RBC Cr after the supplementation might be due to less detection and small sample size. We will increase the detection times and enlarge the sample size in future research to verify the result.

The strength of this study was a comparison of the effect of combined Cr/Mg supplementation with those of Cr or Mg alone in an insulin resistant population. Weaknesses in our study include that guidance with regard to diet and physical activity that was required to comply with the ethical requirements of the study. Given the well-characterized effect of diet and exercise in improving insulin resistance, the results our study may not exclusively reflect the effect of Cr and Mg on insulin resistance, but provide a valuable reference nonetheless. Other limitations of the present study are its short duration and small sample size, both of which we plan to address in future studies.

In conclusion, as anticipated, we found that combined Cr/Mg supplementation ameliorated insulin resistance more effectively than Cr or Mg alone, and this effect was likely related to the regulation by combined Cr/Mg of the expression of *GLUT4* and *GSK3β*. The results of the present study suggest the therapeutic potential of combined Cr/Mg therapy in insulin resistant individuals.

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AUTHOR DISCLOSURES

The authors declare that they have no conflict of interest. This research was supported by the National Natural Science Foundation of China (No: 81001296).

ETHICAL STANDARDS

Ethical permission for all studies was obtained from the Ethic Review Committees of Qingdao Municipal Center for Disease Control & Prevention and the study was registered in the Chinese Clinical Trial Registry (No: ChiCTR-TRC-14004863). Studies were conducted according to Declaration of Helsinki principles. Potential study subjects were informed about the study content and design via a formal presentation and an individual interview, and gave written, informed consent prior to participation.

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Original Article

Combined chromium and magnesium decreases insulin resistance more effectively than either alone

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铬和镁联合补充比单独补充更能有效降低胰岛素抵抗

背景与目的：现有研究表明口服三价铬或镁可有效改善胰岛素抵抗。本研究旨在确定铬和镁联合补充是否较单独补充铬或镁更能有效地改善胰岛素抵抗。**方法与研究设计**：本研究纳入了 120 名诊断为胰岛素抵抗的病人（45-59 岁），随机将其分为 4 组，每组 30 人，分别口服安慰剂、铬（160 μg/d）、镁（200 mg/d）、镁+铬（铬 160 μg/d+镁 200 mg/d），持续 3 个月。测定空腹血糖和胰岛素浓度、红细胞中铬和镁的含量，以及激活的 T 淋巴细胞中葡萄糖转运载体 4（*GLUT4*）和糖原合成酶激酶 3β（*GSK3β*）的 mRNA 水平，并计算胰岛素抵抗指数。**结果**：与干预前相比，铬和镁联合补充可以显著降低空腹血糖 （0.37 mmol/L，$p<0.01$）和胰岛素 （2.91 μIU/mL，$p<0.01$）浓度，以及胰岛素抵抗指数（0.60，$p<0.01$）；同时铬和镁联合补充显著上调了活化的 T 细胞 *GLUT4* mRNA 水平（升高 2.9 倍，$p<0.05$），并显著下调了 *GSK3β* mRNA 水平 （降低 2.2 倍，$p<0.05$）。对照组、铬或镁单独补充组与干预前相比改善效果不明显。**结论**：铬和镁联合补充较单独补充铬或镁能更好地改善胰岛素抵抗，可能与铬和镁联合补充使胰岛素信号传导通路中 *GLUT-4*mRNA 上调和 *GSK3β* mRNA 下调有关。

关键词：胰岛素抵抗、三价铬、镁、葡萄糖转运载 4、T 淋巴细胞