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EFFECTS OF SUPPLEMENTATION OF TRIVALENT CHROMIUM AND FATTY ACIDS ON CYTOKINES AND INSULIN-SIGNALING FACTORS IN ADIPOCYTES

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ABSTRACT

Obese patients had a high blood non-esterified fatty acid (NEFA) concentration. The high NEFA concentration could induce adipocytes to secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which would inhibit insulin function and cause insulin resistance. This study, therefore, was to investigate the effects of supplementation of trivalent chromium and fatty acids on non-esterified fatty acid (NEFA), pro-inflammatory cytokines of IL-6, TNF-α and insulin-signaling factors *in vitro*. Differentiated mouse 3T3-L1 preadipocytes were randomly divided into three groups: control, trivalent chromium (Cr group, Cr), trivalent chromium + fatty acids (Cr+FA group, CrFA). The added level of trivalent chromium was 50 μg/kg, fatty acids was 0.1%. The NEFA level in the CrFA group had significantly greater than that in the control group ($P< 0.05$); however, the Cr group was significantly lower than the control ($P < 0.05$). The levels of pro-inflammatory cytokines of IL-6 and TNF-a in Cr group was significantly lower than in the control ($P< 0.05$), in CrFA group was higher than in Cr group ($P< 0.05$). The c-Jun N-terminal kinase (JNK) in CrFA group was lower than in the Cr and control groups ($P<0.05$). In insulin-signaling factors, added trivalent chromium or fatty acids had no effect on IR (insulin receptor) expression. Added trivalent chromium could increase the expression of IRS-1 (insulin receptor substrate 1), PI3K-p85a and Akt significantly ($P< 0.05$). However, in the CrFA group, the expression of IRS-1 and PI3K-p85a was reduced and increased the expression of Akt than that of the Cr and control groups ($P< 0.05$). Thus, trivalent chromium with fatty acids supplementation has no greater efficiency than chromium only in cytokines and insulin-signaling factors.

KEYWORDS

Chromium picolinate, Fatty acid, Adipocytes and Insulin-signaling factors.

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INTRODUCTION

In recent years, over nutrition has steadily increased due to refined diets. Over nutrition will induce many nutrition-related diseases, one of which is obesity. It is well-established that inflammation is responsible for the pathogenesis of obesity-associated diseases, due to cytokine production by metabolic tissues and infiltrated immune cells[1,2]. Obese patients had a high blood non-esterified fatty acid (NEFA) concentration[3]. The high NEFA concentration could

induce adipocytes and my oblasts to secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which would inhibit insulin function and cause insulin resistance, resulting in an increased blood glucose concentration in patients[4,5].

Trivalent chromium has been closely related to the metabolism of carbohydrates, lipids and proteins. Some recent studies reported that trivalent chromium could enhance insulin activity to improve the abnormal metabolism of carbohydrates and lipids[6-8]. Moreover, Wang *et al*.[9] found that obese animals also had a higher blood NEFA concentration, and trivalent chromium supplementation in obese animals could reduce blood insulin, cholesterol and triglycerides significantly as compared to lean animals. Sahin *et al*. [10] indicated that feeding high fat diet could induced type 2 diabetes as compared to normal diet in rats, high fat diet rats had greater body weight and less serum and brain chromium concentrations than normal diet rats. High fat diet caused a 32% reduction in expressions of glucose transporters in brain tissue[11]. Moreover, a high dietary fat content has been shown to increase chromium excretion and hepatic lipid content increases[12]. Striffler *et al.* [13]indicated that dietary chromium decreased insulin resistance in rats fed a high-fat diet. Therefore, the dietary fat content may also influence the efficacy of dietary chromium on glucose metabolism. Our previous study also indicated that more dietary fat the chromium was more effective[14,15] (Hung *et al*., 2015; Li *et al*., 2016). Thus, trivalent chromium supplementation in the high NEFA condition may become more efficient. This study, therefore was to investigate the effect of supplementation of trivalent chromium and fatty acids on non-esterified fatty acid (NEFA), pro-inflammatory cytokines of IL-6 and TNF-α and insulin-signaling factors *in vitro*.

MATERIAL AND METHODS

Cell culture and differentiation

Cell source

Mouse 3T3-L1 preadipocytes (cell no: CCRC 60159) was purchased from the Food Industry Research and Development Institute (FIRDI), Taiwan.

Cell incubation

3T3-L1 preadipocytes were incubated in a T175 flask in a 37℃, 95% O_2, 5% CO_2 incubator (Thermo Scientific, MA, USA). The composition of the medium was based on Dulbecco's modified Eagle's medium (DMEM) / Nutrient mixture F-12 ham medium, with an added 4.5 g/l D(+)-glucose, 1.5 g/l sodium bicarbonate, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 10% bovine calf serum (BCS), pH 7.2-7.4. During incubation, the growth status of cells was checked with an inverted microscope (Olympus, Tokyo, Japan) and the medium was changed every 2-3 days and when the cells reached confluence, then subculture.

Cell differentiation

When about 100% of the 3T3-L1 preadipocytes adhered to the flask bottom, induced cell differentiation was done in a 37℃, 95% O_2, 5% CO_2 incubator for 3 days. The composition of the induced medium was based on cultured medium and Nutrient mixture F-12 ham medium (1:1), and 5 mg/l insulin, 5 mg/l transferring, 17 μM biotin, 1 ml/l lipid mixture (6 mg/ml lecithin, 3 mg/ml cholesterol, 1 mg/ml sphingomyelin), 0.1 mM dexamethasone (DEX), 0.25 mM 1-methyl-3-isobutylxanthine (MIX), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2% BCS and 0.5% bovine serum albumin (BSA), pH 7.2-7.4.

After induction of cell differentiation, cells were then differentiated in a 37℃, 95% O_2, 5% CO_2 incubator for 10 days and the medium was changed every 2–3 days. The composition of the differentiated medium was based on cultured medium and Nutrient mixture F-12 ham medium (1:1), and an added 5 mg/l insulin, 5 mg/l transferring, 17 μM biotin, 1 ml/L lipid mixture (contain6 mg/ml lecithin, 3 mg/ml cholesterol, 1 mg/ml sphingomyelin), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2% BCS, and 0.5% BSA were supplemented(pH 7.2-7.4). When the cells became globular, the differentiation was completed.

Cell count

Some plate of cells were harvested by added EDTA-trypsin buffer, and then centrifugation at 165×g for 5 min to obtain the cells. An equal volume of cell suspension was mixed with 0.4% trypan blue, and the cell number was counted and the survival rate was calculated by hemacytometer (Marienfeld, Lauda-Konigshofen, Germany) under an inverted microscope. Cell number (cells/ml) was calculated by number of cells ÷ [counted area (mm^2) × chamber depth (mm) × dilution] ×10^4. Cell survival rate (%) was calculated by the number of non-stained cells ÷ (number of stain cells + number of non-stained cells) × 100%.

Cell differentiation check

The collected cells were washed by phosphate-buffered saline (PBS) and the stained by 1% oil red O (dissolved in isopropanol) was added to the stain for 20 min at room temperature. Cells were moved out of the stain solution and destained by 20% ethanol and de-ionized water. After that, PBS was added and the differentiated rate was calculated by hemacytometer under an inverted microscope. The differentiated rate (%) was calculated by the number of stained ÷ (number of stain cells + number of non-stained cells) × 100%. In this study, the differentiated rate was above 90%.

Cell treatment and collection

Trivalent chromium

In this study, trivalent chromium solution was made with Chromax II Chromium Picolinate (123000 μg Cr/gm) (Nutrition 21, NY, USA).

Cell treatment

The 3T3-L1 adipocytes were washed by PBS twice, and randomly divided into control, trivalent chromium group (Cr group, Cr), trivalent chromium + fatty acids group (Cr + FA group, CrFA), every group have 6 plates. The supplemented level was 50 μg/kg for Cr, 0.1% for FA (SAFC Biosciences, Kansas, USA). The experiment period was 9 days, and the medium was changed every 3 days. After treatment, the medium samples were collected for NEFA and cytokines determinations. The cells were moved out of the medium and washed by PBS twice. Then 3mL 0.05% trypsin-EDTA solution was added and put at 37℃ for 1–2 min to detach the cells. The cell suspension was mixed with fresh medium and centrifuged at 165×g for 5 min. The supernatant was removed and 20 mM KH_2PO_4 was added, then stored at -20℃ for analysis.

Cell lyses

The cell suspension was thawed, put on ice for 20 min, then broken by ultrasonic machine (Bransonic, CT, USA) for 20 min. This step was repeated 3 times. After cells were broken, they were centrifuged at 12000×g at 4℃ for 20 min. The collected suspension was stored at -20℃ for analysis.

ANALYSIS ITEMS AND METHODS

NEFA determination

The level of NEFA in medium secreted by adipocytes was determined with a free fatty acid quantification kit (Bio Vision, CA, USA) by colorimetric methods according to manufacturer's procedure. After the kit reaction was finished, measurements were taken of the level of NEFA by O.D. 570 nm in the microplate reader (Biochrom Ltd., Cambridge, UK). The fatty acids level added in medium was subtracted from the value of determined NEFA.

Protein quantification

The protein concentration of the cells was determined by the Lowry method[16].

Pro-inflammatory IL-6 and TNF-α

The level of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in medium secreted by adipocytes were determined by the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's procedures (R and D system, MN, USA).After the kit reaction was finished, measurements were taken by O.D. 570 nm in a microplate reader (Biochrom Ltd., Cambridge, UK).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

SDS-PAGE

Cell lysates were separated by SDS-PAGE in triplicates. Equivalent amounts of proteins were boiled in sample buffer [2.5 ml of 0.5M Tris-HCl (pH 6.8), 4.0 ml of 10% SDS, 2.0 ml glycerol, 1.0 ml β-mercaptoethanol (2-ME), and added 2d H_2O until 10 ml] for 5 min. Proteins 30 μg were separated on

4–8% polyacrylamide gel. The electrophoresis condition was 70 volts.

Western blot

When SDS-PAGE was finished, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MO, USA). The membranes were incubated at room temperature for 1 h in blocking buffer (5% w/v non-fat milk in PBS). After that, the primary antibodies were incubated with insulin receptor (IR) (1:500; R and D system, MN, USA), insulin receptor substrate 1 (IRS-1) (1:100; R and D system, MN, USA), phosphatidylinositol-3kinase-p85α(PI3K-p85α)(1:200; Santa Cruz Biotechnology, CA, USA), protein kinase B (Akt) (1:2500; R and D system, MN, USA), and Jun N-terminal kinase (JNK) (1:5000; R and D system, Minneapolis, MN, USA) at 4℃ overnight with gentle shaking. On day 2, they were moved off the primary antibodies and washed three times (10 min/time) by phosphate buffered saline with tween (PBST) (2.9 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.2 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.5 ml/l tween 20). Next, the secondary antibodies were incubated with donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, CA, USA), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA, USA) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, CA, USA) at room temperature for 1 hour. The blots were developed by enhanced chemiluminescence (ECL) substrates reagents (PerkinElmer, MA, USA). The fluorescent signals were transferred to X-ray film and quantified by the Image J image analysis system (version 1.46c).

Statistical analysis

The differences of each group were analyzed by ANOVA using the general linear model (GLM) procedure of SAS (version 9.1). The significant differences among the groups were determined by Duncan's new multiple-range test[17].

RESULTS AND DISCUSSION

Effect of trivalent chromium and fatty acids on NEFA level

The results indicated the groups that added trivalent chromium had significantly lower NEFA levels than the control ($P< 0.05$) (Table No.1). The groups that added chromium together with fatty acid had higher NEFA levels than the Cr and control groups ($P< 0.05$).

In this study, added trivalent chromium could decrease the level of NEFA in adipocytes. Many *in vitro*[18] and *in vivo*[6,7,19] experiments showed the same result. Chromium picolinate which was supplemented with insulin would increase the level of NEFA in adipocytes. Some studies indicated that trivalent chromium could stimulate the function of insulin and increase glucose up take in cells[20-22]. Newshome[23] and Howard *et al.*[24] indicated that trivalent chromium could stimulate lipoprotein lipase (LPL) activity. When the level of glucose in the cells increased, the activity of LPL on the cell surface was activated and caused triglyceride (TG) in TG-rich lipoprotein (VLDL) to decompose to fatty acids and glycerol. Fatty acids would then uptake into cells, resulting in the level of NEFA increasing[25]. In the present study, in fatty acid supplementation, the fatty acids we added would uptake by adipocytes, so the level of NEFA in the adipocytes would increase in fatty acid supplementation groups.

Effect of trivalent chromium and fatty acids on pro-inflammatory cytokines

The groups that added trivalent chromium showed a significantly lower IL-6 and TNF-α levels than the control ($P< 0.05$); the groups that added chromium and fatty acid shad a significantly higher IL-6 and TNF-α levels than the Cr group, but lower than the control group ($P < 0.05$).

As for the expression of JNK in 3T3-L1 adipocytes (Figure No.1). Trivalent chromium supplementation was no effect on the expression of JNK in 3T3-L1 adipocytes; However, chromium together with fatty acids would decrease its expression significantly ($P<0.05$) (Figure No.1).

IL-6 and TNF-α were secreted by adipocytes, and might involve in obesity-related insulin resistance[1,2,26]. IL-6 and TNF-α would inhibit the activity of LPL and stimulate the activity of hormone-sensitive lipase (HSL)[27,28]. HSL played an important role in the lipolysis of adipose tissue. When LPL was inhibited and HSL was stimulated, the decomposition of TG in adipocytes was increased, which could release NEFA into blood

stream. The high blood NEFA level would then cause insulin resistance[29-31]. In present study, trivalent chromium could reduce the level of IL-6 and TNF-α in adipocytes, so we suggested that trivalent chromium could prevent insulin resistance. Many recent experiments also indicated that trivalent chromium could improve insulin resistance[32-34], and could attenuate pro-inflammatory cytokine expression in both blood circulation and skeletal muscle[33]. Moreover, obesity-related insulin resistance also had hyperinsulinemia and hyperglycemia[35,36]. Bastard *et al.*[37] indicated that individuals with high blood NEFA levels also had high IL-6 and TNF-α levels. This study confirmed that fatty acids supplementation had higher NEFA levels, and also had higher IL-6 and TNF-α levels in 3T3-L1 adipocytes. The results were similar to those reported by Bastard *et al.* [37].

JNK was one of the mitogen-activated protein kinase family. Activated JNK would affect the phosphorylation of IRS-1, so JNK played an important role in insulin activation[38]. In this study, trivalent chromium supplementation alone with fatty acids could reduce the expression of JNK significantly. Chen *et al.*[34], who fed trivalent chromium to KK/HIJ mice, found trivalent chromium could decrease the expression of JNK effectively. The results were similar to our study.

Effect of trivalent chromium and fatty acids on insulin-signaling factors

In this study, the effects of trivalent chromium and fatty acids on the expression of insulin-signaling factors were determined by Western blot (Figure No.2-6).

Supplementation of trivalent chromium had no effect on the expression of the IR. But it could increase the expression of IRS-1, PI3K-p85a and Akt (P <0.05). But in the chromium and fatty acids supplementation group, the expression of IRS-1 and PI3K-p85αwas significantly reduced, meanwhile, the Akt was increased than in the Cr and control groups (P <0.05).

Numerous studies indicated that trivalent chromium could promote insulin activity[39-41]. However, the pathway was not yet consistent with the same conclusion. A clearer and more recognized insulin pathway was a tyrosine phosphorylation pathway caused by the combination of insulin and IR, called the "IR-IRS1-PI3-kinase pathway" [42]. In this study, the supplementation of trivalent chromium could increase the expression of IRS-1, PI3K-p85α and Akt, which confirmed that trivalent chromium could improve the insulin signal transduction pathway. This result was similar to Wang *et al.*[9] and Chen *et al.*[35], who also reported that trivalent chromium could increase the expression and activation of insulin-signaling factors.

When the NEFA level in cells was increased, IL-6 and TNF-α secretions were then increased, to stimulate the expression of JNK. The tyrosine phosphorylation of insulin subunit was then decreased, and insulin-signaling factors were inhibited consequently. Chromium supplementation could decrease the NEFA level. Then the IL-6 and TNF-α level will decline, which could increase the tyrosine phosphorylation of the insulin subunit, and then increase the activity of phosphatidylinositol-3 kinase (PI3-K). The serine phosphorylation of protein kinase B (Akt) will then increase, After that, the expressions of insulin-signaling factors were increased to enhance the cellular glucose uptake[9,34,43].

However, the expression of IR was not affected significant by treatments in this study. Yang *et al.*[44] and Wang and Yao[45] found that trivalent chromium did not affect the expression of IR in 3T3-L1 adipocytes. Therefore, we suggested that trivalent chromium may affect other downstream factors to stimulate the activity of insulin.

Chromium supplementation with fatty acids showed lower expressions of IRS-1 and PI3K-p85α. These results indicated that chromium and fatty acid supplementation could decrease the expression of insulin-signaling factors. Sahin *et al.*[10] indicated that feeding a high fat diet to diabetic rats PPAR-γ expression was decreased in adipose tissue and phosphorylated insulin receptor substrate 1 (p-IRS-1) expression of liver, kidney and muscle tissues. Gao *et al.*[46] treated 3T3-L1 adipocytes with 0.1% fatty acids could induce insulin resistant. Treatment of cultured differentiated my otubes with palmitic acid evoked insulin resistance and the cellular glucose up-

take was impairment, all of which were inhibited by chromium supplementation. These results suggest that chromium supplementation in insulin resistant conditions had a beneficial effect on glucose metabolism[47].

In this study, we speculate that 3T3-L1 adipocytes may develop insulin-resistant cells in CrFA group. The high fatty acid level, which was also a common feature of insulin-resistant cells, will induce insulin resistance[3].Fatty acids and their metabolites can impair insulin signaling by inhibition serine phosphorylation of insulin receptor substrates[48]. Moreover, groups of chromium with fatty acids had higher Akt expression in this study. It may associate with leptin, which is secreted by adipocytes. Ando and Aquila[49] indicated that leptin plays an important role in the regulation of cellular energy metabolism, its impact on PI3K/Akt pathway. Leptin could activate the JAK/SATA pathway. When Janus kinase 2 (JAK2) was activated in this pathway, it would stimulate PI3K to activate and increase the expression of Akt[50].

Table No.1: Effects of trivalent chromium and fatty acids on the levels of non-esterified fatty acid and pro-inflammatory cytokines in 3T3-L1 adipocytes[1]

S.No	Items[2]	Control	Cr	Cr+FA	SEM[3]
1	NEFA, pmol/well	24.67^{b}	22.53^{c}	30.15^{a}	0.27
2	IL-6, pg/mL	21.48^{a}	12.17^{c}	16.69^{b}	0.26
3	TNF-α, pg/mL	128.79^{a}	114.24^{c}	120.30^{b}	0.58

[1] n = 6.
[2]NEFA: non-esterified fatty acid; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α.
[3] SEM: standard error of means.
[a, b, c] Means in the same row without common superscripts differ significantly ($P< 0.05$).

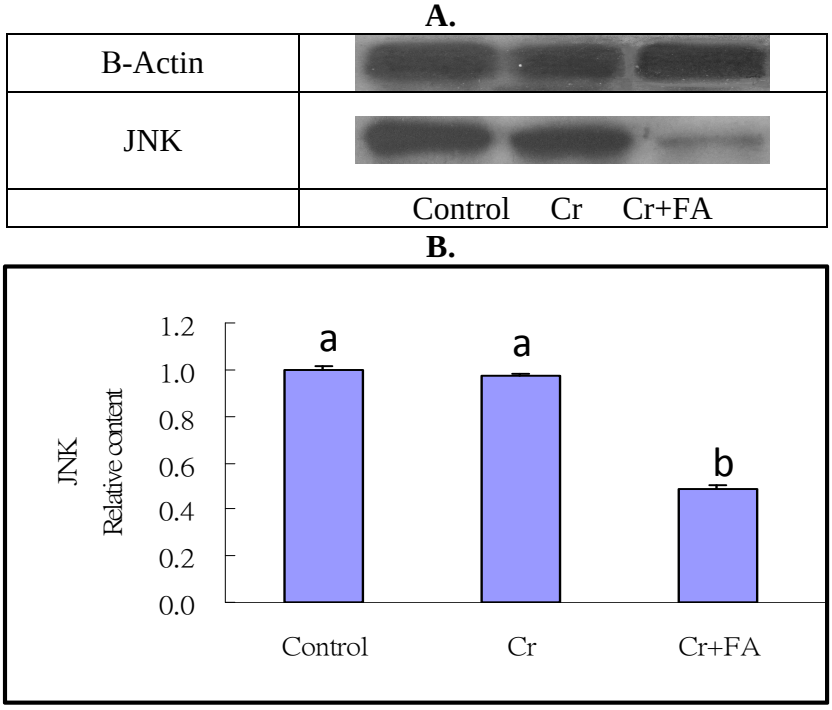

Figure No.1: Effects of supplemental trivalent chromium and fatty acids on c-Jun N-terminal kinase (JNK) in 3T3-L1 adipocytes. A: The protein content of Jnk in 3T3-L1 adipocytes assessed by Western blots. B: The relative level of different treatments expressed as folds. Values are means ± SD, n=3. Means without same letter differ significantly ($P< 0.05$)

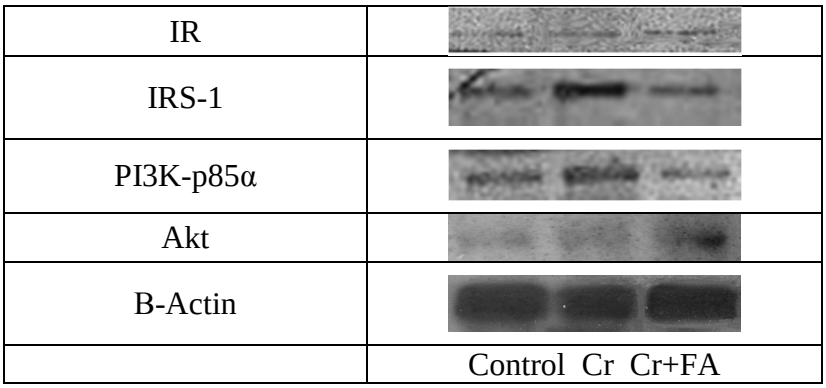

Figure No.2: Effects of supplemental trivalent chromium and fatty acids on insulin signalling factor levels in 3T3-L1 adipocytes. (IR: insulin receptor, IRS-1: insulin receptor substrate 1)

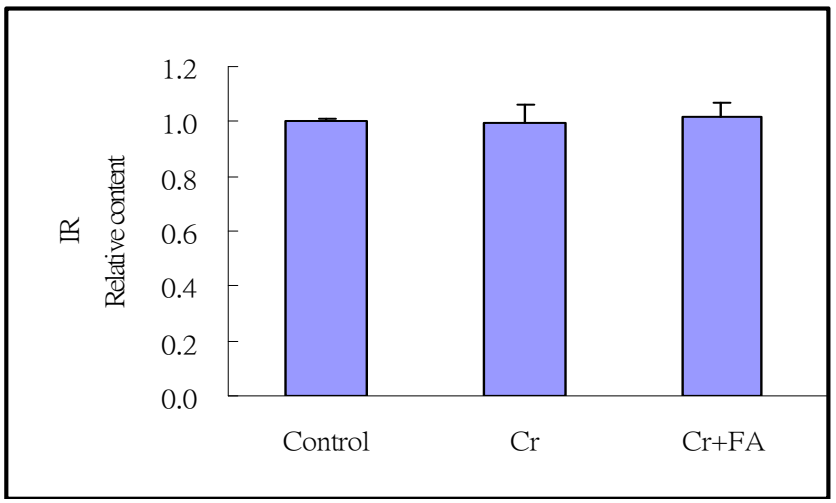

Figure No.3: Effects of supplemental trivalent chromium and fatty acids on insulin receptor (IR) in 3T3-L1 adipocytes. Values are means ± SD, n=3

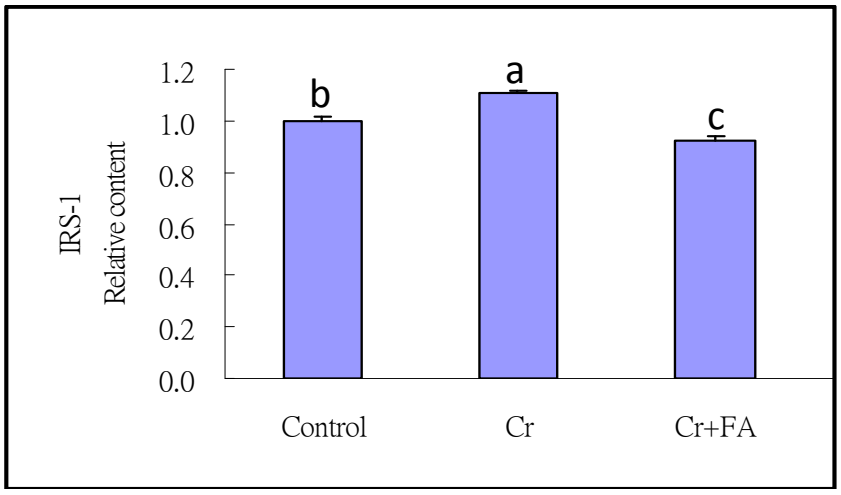

Figure No.4: Effects of supplemental trivalent chromium and fatty acids on insulin receptor substrate 1 (IRS-1) in 3T3-L1 adipocytes. Values are means ± SD, n=3. Means without same letter differ significantly ($P < 0.05$)

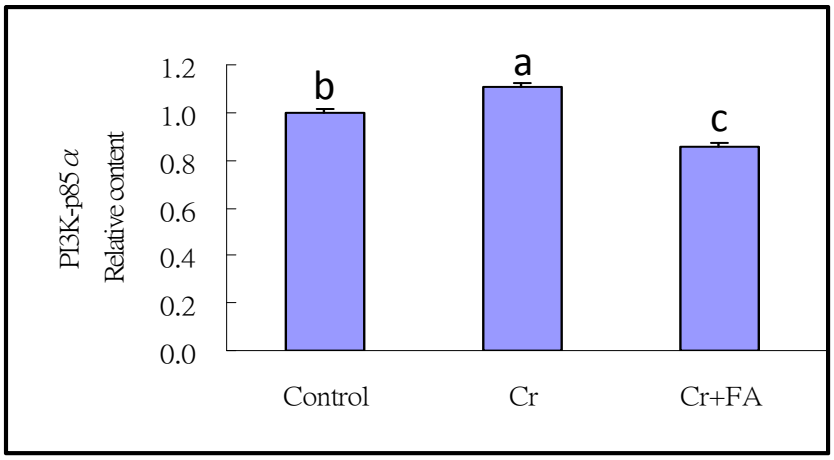

Figure No.5: Effects of supplemental trivalent chromium and fatty acids on PI3K-p85α in 3T3-L1 adipocytes. Values are means ± SD, n=3. Means without same letter differ significantly ($P< 0.05$)

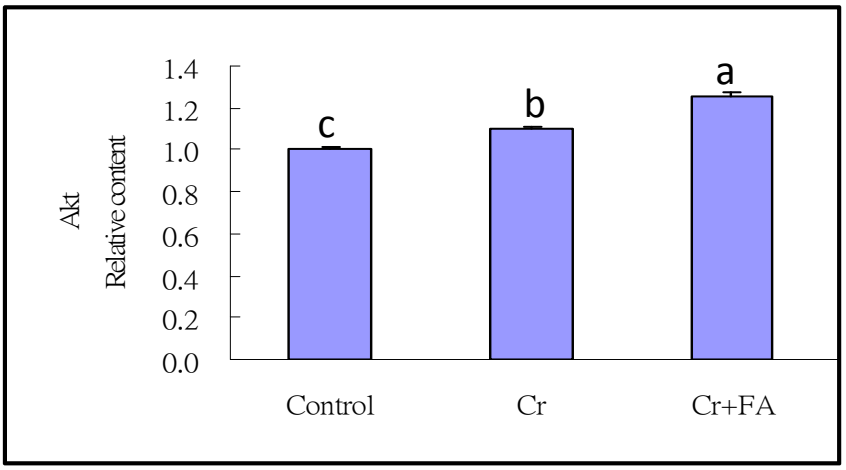

Figure No.6: Effects of supplemental trivalent chromium and fatty acids on Akt in 3T3-L1 adipocytes. Values are means ± SD, n=3. Means without same letter differ significantly ($P< 0.05$).

CONCLUSION

Trivalent chromium could reduce the levels of NEFA, IL-6 and TNF-α, and enhance the expression of insulin-signaling factors; chromium supplemented together with fatty acids would increase the levels of NEFA, IL-6 and TNF-α, and could inhibit the expression of insulin-signaling factors IRS-1 and PI3K-p85α, and increase the AKT expression. Thus, trivalent chromium supplementation with NEFA has no greater efficiency than chromium only in cytokines and insulin-signaling factors in 3T3-L1 adipocytes.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no known conflicts of interest associated with this publication.

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