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Chromium Picolinate did not Effect on the Proliferation and Differentiation of Myoblasts

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Abstract: This experiment is conducted *in vitro* to investigate trivalent chromium picolinate affects the proliferation and differentiation of myoblasts. A myoblasts cell line (C2C12) from rats was used in the experiment. These were randomly divided into the control group, the Pic group (50ppb picolinate) and the CrPic group (50ppb chromium picolinate). The differentiation of myoblasts reveals that the number of differentiated myotubes, creatine kinase (CK) activity and the aldolase (ALB) activity do not differ among the three groups ($P > 0.05$). The activity of hexokinase in the CrPic and Pic groups clearly exceeds that in the control group ($P < 0.05$), and the activity of myokinase in the Pic group exceeds that in the CrPic and control groups ($P < 0.05$). Besides, the transcription factor myogenin exerts no effect on any of the three groups ($P > 0.05$). Myoblast proliferation was the same across the three groups ($P > 0.05$), and the quantity of DNA in the control group exceeded that in the Pic group ($P < 0.05$). The experiment indicated that 200ppb chromium picolinate did not influence the proliferation and differentiation of myoblasts.

Key words: Myoblasts, Differentiation, Proliferation, Chromium picolinate

INTRODUCTION

Trivalent chromium has been recognized as a cofactor of insulin[1,2], and has played a prominent physiological role in humans[3,4] and domestic animals[5-8]. Chromium is considered an essential animal trace element. This element is involved in the metabolism of carbohydrates[9], lipids[4,10], amino acids and nucleic acids[11-13].

Chromium deficiency can retard growth, interfere with blood glucose tolerance, and lead to hyperglucosemia, hypercholesteromia and hyperlipidemia. Chromium deficiency is likely to cause diabetes and coronary heart and vessel diseases[14]. Those syndromes can be ameliorated through supplementation with chromium[10,15].

Some studies have indicated that a 200-400ppb trivalent organic chromium supplement can reduce the blood glucose, cholesterol and lipid of mammalian animals, and particularly can reduce bodyfat and increase muscle percentage[3,4,6,8,13,16,]. However, some reports have shown that chromium supplementation did not influence backfat and the loin-eye area in growing pigs[17-20], or the body composition of rats[21]. Therefore, conflicting results must be further investigated to elucidate whether chromium supplementation can increase muscle mass, and if so how. The experiment in this study was conducted *in vitro* to determine the effects of supplementation with chromium picolimate on the proliferation and differentiation of myoblasts.

MATERIALS AND METHODS

Trial 1: Differentiation trial: A myoblast cell line C2C12 (CCRC 60083) purchased from the Institute of the Development of the Food Industry in Taiwan was used in this study. The culturing involved two stages, proliferation and differentiation. DMEM(Dulbecco's modified Eggle's medium) was used as the basal culture medium, and 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, 5 μg/mL insulin, 5 μg/mL transferring, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 10% FBS(foetal bovine serum) were added. The culturing conditions were 37□, 5% CO_2 . When the myoblasts reached confluence, the cells were divided into three groups, control, 200ppb picolinic acid (Pic) (negative control) and 200ppb Cr as chromium picolinate (CrPic). The differentiation stage then began and involved the same differentiation medium as the proliferation stage except that FBS was replaced by 2% horse serum. The medium was changed at two day intervals, when the shape of the fibro-like myoblasts changed into long rods, indicating differentiation into multinuclear myotubes. The cells were then harvested in a buffer (1 mM NaCl, 1 mM EDTA-2Na, 1% triton x-100 and 10 mM Tris-HCl (pH 7.2)). Next, the cells were homogenized, after which they were put into liquid nitrogen for 10 min and treated ultrasonically for 10 min, followed by being centrifuged at 14000xg for 20

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min and 148000xg for 60 min. The clear phase then was taken and stored at -80□ for further analysis.

Table 1: Effect of chromium picolinate on differentiation related traits of myoblast[1]

Items	Control	Pic	CrPic
Differentiated cells, %	42.2±3.3	41.4±4.2	42.3±4.9
Creatine Kinase, unit	265.8±11.9	265.5±3.9	258.1±10.0
Aldolase, unit	9.0±5.3	8.3±3.6	11.0±1.3
Hexokinase, unit	11.5±4.7b	15.2±2.8a	15.9±3.5a
Myokinase, unit	51.9±12.0b	70.4±12.9a	55.6±20.3b

[1]Means ±SD (n=12).

a,b: Means in the same row without common superscripts differ significantly (p<0.05).

1 unit=1n mole substrate/mg protein/min.

The differentiated cells were collected and placed in a lysis solution (20mM Tris-HCl (pH7.4), 150mM NaCl, 2.5mM EDTA-2Na, 2.5mM EGTA, 01mM PMSF, 10µg/mL aprotinin and 1% triton x-100) to measured myogenin.

The differentiated cells were counted using Giemsa staining and measured using the procedure of Galvin *et al.*[22]. The activities of creatine kinase, myokinase, hexokinase and aldolase were determined as described in Young *et al.*[23]. The activities of creatine phosphokinase and myokinase were measured in reaction buffer at final concentrations of 4.0 mM $MgCl_2$, 3.0 mM ADP, 1.0 mM $NADP^+$, 5.0 mM glucose, 2 U/mL hexokinase, 2 U/mL glucose-6-phosphate dehydrogenase and 20 mM creatine phosphate (creatine phosphokinase reaction only). Aldolase activity was measured in a reaction buffer containing 0.2 mM NADPH, 2 U/mL α-glycerolphosphate dehydrogenase, 17 U/mL triose phosphate isomerase, and 20 mM fructose-1-6-phosphate. Hexokinase activity was measured in a buffer at final concentrations of 2.0 mM ATP, 0.5 mM $NADP^+$, 2 U/ mL glucose-6-phosphate dehydrogenase and 50 mM glucose. Enzymatic reaction was started by adding aliquots of cell samples, and was monitored based on the rate of change in absorbance at 340 nm. The protein content was measured by the Lowry method[24].

The transcription factor myogenin level was determined via Western blot analysis using the procedure described by Katagiri *et al.*[25]. Briefly, The cell samples were boiled for 5 min before electrophoresis. Cellular protein (200µg) was diluted in SDS-sample buffer and subjected to SDS-

Table 2: Effect of chromium picolinate on proliferation of myoblasts

Items	Control	Pic	CrPic
Proliferated cell, 104/mL	175.1±15.9	176.4±12.7	176.1±14.9
DNA, ng/µL	1.07±0.15[a]	0.73±0.35[b]	0.96±0.29[ab]

Means ±SD (n=18)

a,b: Means in the same row without common superscripts differ significantly (p<0.05).

polyacrylamide gel electrophoresis. After electrophoretic transfer to PVDF (polyvinylidene fluoride) transfer membrane, myogenin were detected using rabbit antibodies specific to myogenin (1:40, Santa Cruz). The membrane was then incubated with horseradish polypeptidase-conjugated second antibody (1:1000). The immunoreactive polypeptides were visualized by the ECL detection system. The results of Western blot were analyzed by computer-assisted image analysis using a CCD camera and image software.

Trial 2: Proliferation trial: The proliferation trial involved seeding 5×10^4 cells in each fask, a total of 18 flasks were used, and the cells were divided into control, 200ppb (µg/L) picolinic acid (Pic) and 200ppb Cr as chromium picolinate (CrPic) groups, all of which were incubated for five days in a 37□ 5 % CO_2 condition.

The cells were then harvested, counted, and the total DNA was measured using a kit.

Statistical: SAS software was used for the statistical analysis. The analysis was performed applying the general linear model. The differences among the groups were compared using the Duncan's multiple range test[26].

RESULTS AND DISCUSSION

Effect of chromium on differentiation of myoblasts: Table 1 lists the effects of chromium picolinate on the differentiation related traits of myoblasts. The number of differentiated myoblasts did not differ markedly among the three groups (P > 0.05) (Fig. 1), indicating that chromium did not influence the differentiation of myoblasts. This result was inconsistent with the results of some in vivo studies. Evans and Bowman[1] indicated that chromium picolinate could increase insulin activity and enhance the absorption and utilization of glucose and amino acid by muscle cells. Studies on pigs have

also shown that chromium enhances the loin-eye area or the percentage of lean tissue[5,16]. However, Smith *et al.*[27] noted that supplementing pigs with chromium nicotinate did not increase lean mass. In this study, CrPic did not increase the differentiation of myoblasts, but a previous study indicated that CrPic could inhibit the differentiation and proliferation of preadipocytes in vitro. Accordingly, we suspect that may be result from the ability of chromium to depress the bodyfat, leading to increased muscle mass ratio.

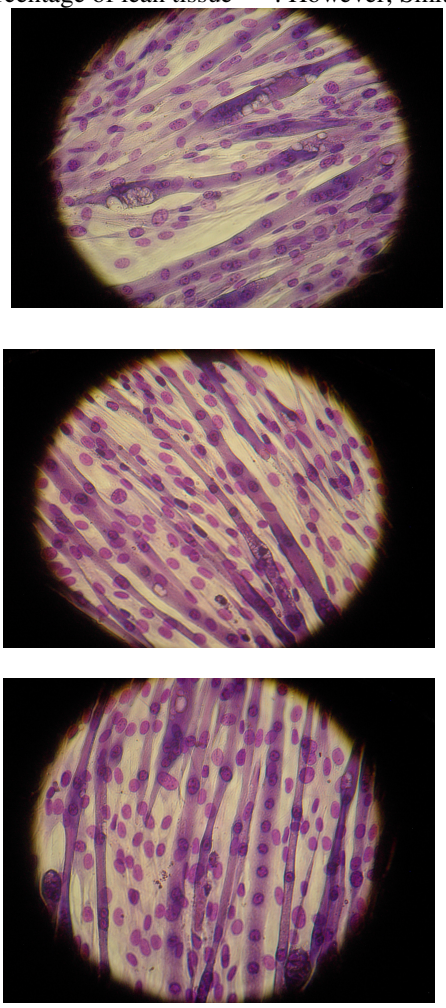

Fig. 1: Differentiated of myoblast (C2C12) (a)control (b)picolinate (c)chromium picolinate (Microscopic view at ×200 magnification).

Chromium has been recognized as a cofactor of insulin, and can increase insulin activity. Evock-Clover *et al.*[17] demonstrated that since chromium could increase insulin activity, which can enhance the effectiveness and level of the growth hormone. Consequently, stimulating muscle development. The in vivo study is governed by several factors, and therefore is more complex than an in vitro study. Therefore, the in vitro results may differ from the in vivo results.

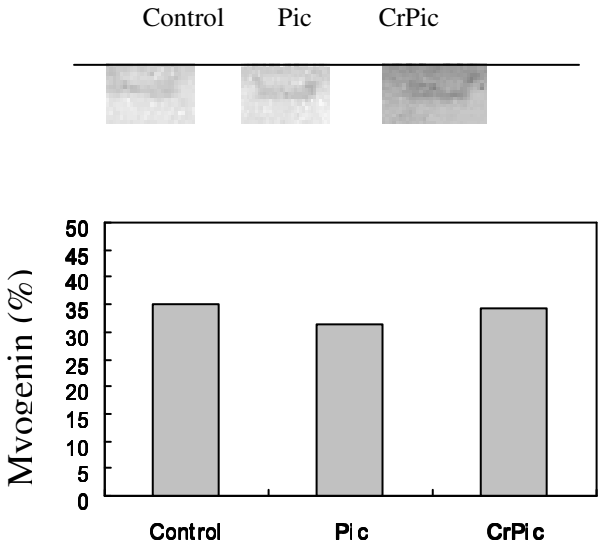

Fig. 2: Effect of chromium picolinate on myogenin expression of myoblasts (a) Western blot analysis of myogenin (b) percentage of myogenin expression.

Effect of chromium picolinate on the differentiation related enzymes activities of myoblasts : Table 1 shows that creatine kinase and aldolase did not differ significantly among the three groups ($P > 0.05$). Creatine kinase is a cytosol enzyme, catalyzing the reaction of phosphocreatine into creatine and generating ATP to provide energy for muscles. Creatine kinase generally serves as an index of myoblasts differentiation. Aldolase catalyzed the hydrolysis of fructose-1-6-diphosphate into glyceroaldehyde-3-phosphate and dihydroxyacetone, releasing ATP. Hexokinase activity in the CrPic and Pic groups exceeded that in the control group ($P < 0.05$). Hexokinase is a key enzyme involved in glycolysis, since chromium is a cofactor of insulin, which can increase the glycolysis metabolism. Therefore, chromium can increase hexokinase activity. Morris *et al.*[28] stated that supplementing the diet of diabetic rats with CrPic could restore hexokinas activity. This result is consistent with the result of present study. The myokinase activity in the Pic group was higher than in the CrPic and control groups ($P < 0.05$).

Effect of chromium picolinate on the transcript factor myogenin of myoblast: Myogenin is a vital transcription factor that regulates the differentiation of myoblasts. Western blotting was performed to determine the level of myogenin expression, which did not vary significantly among the three groups ($P > 0.05$) (Fig. 2). Myogenin play an important role in myoblasts differentiation. Myogenin can stimulate p21 protein expression (a cyclin-dependent kinase inhibitor), and then arrest myoblasts growth. Consequently, the terminal differentiation stage begins. The fibro-like myoblasts then fuse, becoming multiple nuclear myotubes[29]. When the rat knocks out the myogenin gene, result of severe defect of myoblast differentiation, and only a little myofiber is observed[30,31]. Because of the important function of myogenin in regulating myoblasts differentiation, myogenin expression can be used to measure the differentiation of myoblasts. In this study, CrPic did not stimulated myogenin expression, indicating that CrPic cannot influence myoblasts differentiation.

Effect of chromium picolinate on the proliferation of myoblasts: Table 2 displays the effect of chromium picolinate on myoblats proliferation. The number of cells did not differ markedly among the three groups ($P > 0.05$). The DNA content in the control group exceeded that in the Pic group ($P < 0.05$), but no difference was observed between the control and CrPic groups ($P > 0.05$). Thus, CrPic did not influence the proliferation of myoblasts.

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