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Effects of Creatine Monohydrate Supplementation on Glucose Homeostasis in Skeletal Muscle of Broilers

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Abstract: The object of the study was to investigate the effects of Creatine Monohydrate (CMH) supplementation on glucose absorption, glucose oxidation and glycogen synthesis in skeletal muscle of broilers. Nine hundred, 42 days old broilers were randomly assigned to four treatments with five replicates per treatment and 45 birds per replicate. The birds received either a no-CMH diet (0 mg kg^{-1}), a low (250 mg kg^{-1}), a moderate (500 mg kg^{-1}) or a high-CMH diet (1000 mg kg^{-1}) for 21 days. The results showed that CMH supplementation did not affect serum glucose concentration but did increase lactate concentration (linear and quadratic, $p<0.001$) as CMH supplementation increased. The concentration of lactate in pectoralis major showed a quadratic effect ($p = 0.036$) in response to increasing CMH supplementation. For the thigh muscle, the concentration of lactate presented a decreasing effect (linear, $p = 0.042$). Pyruvic acid in the pectoralis major increased markedly (linear, $p<0.004$; quadratic, $p<0.003$) with increasing CMH comcentration but an opposite effect to that in the thigh muscle. The HK1 mRNA abundance in the pectoralis major was decreased by CMH supplementation (linear and quadratic, $p<0.001$) but the HK1 mRNA abundance in the thigh muscle was increased with a quadratic effect. CMH supplementation had increased Lactate Dehydrogenase (LDH) activity in pectoralis major (linear, $p = 0.003$; quadratic, $p<0.001$) while no significant effect on thigh muscle as CMH concentration increased. Thus, it was inferred that CMH supplementation may improve glucose oxidation with distribution difference in skeletal muscle. Additionally, CMH supplementation had no effect on the Glycogen Synthase (GYS), Glucose Transporter 1 (GLUT1) and Glucose Transporter 3 (GLUT3) mRNA abundance, macroglycogen or total glycogen concentration of the breast and thigh muscles. CMH supplementation significantly increased the AMPKα phosphorylation of the pectoralis major and demonstrated a quadratic effect ($p = 0.002$) as CMH supplementation increased. CMH supplement did not affect the AMPKα phosphorylation of the thigh muscle but presented the same effect as the pectoralis major. The results suggested that CMH supplementation did not affect glucose uptake and glycogen storage of skeletal muscle but it accelerate the glucose oxidation in the thigh muscle or decelerate glycolysis in the pectoralis major, possibly regulated by AMPKα phosphorylation.

Key words: Creatine, broiler, glucose uptake, oxidation, glycogen synthesis

INTRODUCTION

Creatine is a nitrogenous organic acid that occurs naturally in vertebrates and is mainly stored in muscle. The amount of creatine naturally generated is inadequate for the body's requirement as the break-down rate at 1.7% per day. Studies have demonstrated that creatine supplementation can increase the muscular creatine or phosphocreatine concentration (Op't Eijnde *et al*., 2006; Del Favero *et al*., 2012). Currently, creatine supplementation is not only used in athlete and medical treatment to improve skeletal muscle performance (MacNeil *et al*., 2011) but is also applied to animals to make better meat quality (Rosenvold *et al*., 2003). The benefits of dietary creatine supplementation on muscle performance mostly resulting in an increase in the muscle phosphocreatine concentration which is generated from creatine with catalysis of creatine kinase (Feldman, 1999). Additionally, some studies have shown that creatine supplementation plays an important role in the carbohydrate metabolism of muscle, leading an increased glucose uptake and glycogen storage in muscle (Van Loon *et al*., 2004; Alves *et al*., 2012). Moreover, creatine supplementation postpones the ultimate pH decline in muscle at 24 h postmortem by reducing lactic accumulation (Nissen and Young, 2006). However, lactic

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acid is generally produced by transformation of the substrate glycogen, glucose and glucose-6-phosphate through glycolysis (Bendall, 1979). It is reported that creatine supplementation can increase glycogen re-synthesis and glucose transporter protein (GLUT4) expression of rat skeletal muscle after exercise (Eijnde *et al*., 2001). Additionally, an *in vitro* study showed that creatine supplementation increased glucose oxidation and AMP-Activated Protein Kinase (AMPK) phosphorylation as well as reducing lactate production in L6 rat skeletal muscle cells (Ceddia and Sweeney, 2004). Therefore, it has been speculated that creatine supplementation may alter skeletal muscle glucose uptake, oxidation and glycogen synthesis, induced by AMPK phosphorylation.

AMPK is considered an energy sensor that controls the whole body glucose homeostasis by regulating the metabolism of glucose in multiple peripheral tissues such as skeletal muscle, liver, adipose tissues and pancreatic β cells. Furthermore, AMPK integrates signaling transduction between peripheral tissues and the hypothalamus to regulate food intake and whole body energy expenditure (Long and Zierath, 2006). Once AMPK phosphorylation is activated by a nutrient-deficit status, it promotes glucose uptake and oxidation to produce energy through catabolic and ATP-generating pathways. Magnoni *et al*. (2012) found that effective stimulators of muscle AMPK activity in mammals such as 5-Aminoimidazole-4-Carboxamide 1-β-D-Ribofuranoside (AICAR) and metformin can stimulate AMPK activity and increase glucose transport and the expression of key genes involved in ATP generation from glucose through glycolysis in trout myotubes. Thus, AMPK plays a potentially important role in stimulating glucose uptake and utilization in the skeletal muscle of both mammalian and non-mammalian vertebrates.

Hence, this study was conducted to determine whether creatine supplement could affect glucose absorption, glucose oxidation and glycogen synthesis of broilers and this process trigerred AMPK phosphorylation and reached glucose homeostasis eventually.

MATERIALS AND METHODS

Animals and treatment: Nine hundred, 42 days old broilers (Lingnan yellow broilers, a quality meat-type chicken reaching finishing age after 21 days experimental period) with a mean initial BW of 0.73±0.01 kg were randomly assigned to four treatments with five replicates per treatment and 45 birds per replicate. The birds received either a no-CMH diet (0 mg kg^{-1}), a low (250 mg kg^{-1}), a moderate (500 mg kg^{-1}) or a high-CMH diet (1000 mg kg^{-1}) for 21 days (i.e., 42-63 days old).

Different levels of CMH were administered as supplementation in basal diet, respectively. All procedures were approved by South China Agricultural University Animal Care and Use Committee. The birds were kept in floor pens (2×3 m each) under 24 h of light and a mean temperature of 25°C. Feed and drinking water were available *ad libitum* throughout the study. Basal diet composition and nutrient concentrations are presented in Table 1.

At the end of 21 days of treatment, blood samples (5 mL) were collected before slaughter. Blood was later centrifuged at 1200×g for 10 min at 25°C and the plasma removed and stored at -80°C for further analysis. Then, the entire left pectoralis major and thigh muscle peeled off the skin were immediately removed. Samples from left pectoralis major and thigh muscle were taken for mRNA and protein expression analysis as well as metabolites measurement and frozen in liquid N_2 and stored at -80°C.

Measurement of metabolites: The serum samples were assayed for glucose comcentration and lactate. The serum glucose concentration was analyzed by a Glucose Oxidase Method and the serum lactate was measured by the lactate oxidase assay using a lactate kit (Nanjing Jiancheng Bioengineering Institute, China).

Samples of pectoralis major and thigh muscle (Approximate 200 mg) were weighed, macerated in liquid N_2 and homogenizsed in 1 mL saline. Then, the homogenate was centrifuged at 1200×g for 10 min at 4°C and supernatant was used for analysis. Lactic dehydrogenase activity was determined as described by Briand *et al*. (1981). Na^+-stimulated Na^+-K^+-ATPase activity was determined by measuring the hydrolysis of ATP as previously described by Kristensen and Juel

Table 1: Ingredients and nutrient concentrations of basal diet

Ingredients (%)	Values	Nutrient concentration (%)	Values
Corn	71.73	Metabolisable energy (MJ kg^{-1})	13.17
Soybean meal	14.30	Crude protein[2] (g kg^{-1})	170.00
Corn gluten meal	6.40	Dry matter[2]	86.94
Mixed grease	2.98	Ether extract[2]	5.50
Dicalcium phosphate	1.62	Calcium	0.80
Limestone	1.14	Total phosphorus	0.57
Salt	0.33	Available phosphorus	0.38
DL-Methionine (98%)	0.02	Salt	0.35
Lysine (65%)	0.42	Lysine	0.85
Threonine (98%)	0.06	Methionine+Cysteine	0.69
Premix[1]	1.00	Threonine	0.68
Total	100.00	Tryptophan	0.16

[1]Added per kg of diet; DL-methionine:1 200 mg; Retinylpalmitate: 5.5 mg; cholecalciferol: 0.05 mg; DL-α-tocopheryl acetate: 40 mg; Menadione: 3 mg; Thiamin: 3 mg; Riboflavin: 4.5 mg; Pyridoxine: 7 mg; Cyanocobalamin: 0.03 mg; Nicotinic acid: 50 mg; Ca-pantothenate: 8 mg; Folic acid: 1.5 mg; Choline chloride: 600 mg; Mn: 80 mg as $MnSO_4.H_2O$, Fe: 80 mg as $FeSO_4.H_2O$, Zn, 50 mg as $ZnSO_4.H_2O$; Cu: 10 mg as $CuSO_4.5\ H_2O$; Co: 0.4 mg as $CoSO_4$; Iodine: 0.35 mg as KI; Se: 0.25 mg as $NaSeO_3$; [2]Crude protein dry matter and ether extract were determined value and other nutrient concentration were calculated according to Feed Composition and Nutritive Values in China, 2009 (20th Edn.)

(2010). Lactate and pyruvic acid were measured by oxidase assays using biochemical kits (Nanjing Jiancheng Bioengineering Institute, China). Proglycogen and macroglycogen in lyophilized samples of muscle tissues were determined according to the method by Rosenvold *et al*. (2003).

Determination of gene expression in glucose uptake, glucose metabolism and glycogen synthesis by real-time quantitative PCR: After treatment with TRIzol reagent (Invitrogen, USA), total RNA was extracted from the frozen muscle tissues (pectoralis major and thigh muscle) of broilers and purified with rnase-free DNasel (Takara, Japan) to remove contaminated genomic DNA. Total RNA was quantified using a spectrophotometer (BioRad, USA) at an optical density of 260 nm (OD_{260}) and the purity was assessed by the ratio of OD_{260} to OD_{280}. The ratio ranged from 1.8-2.0 for all samples. Then, each total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions.

Quantitative real-time PCR analysis of GLUT1, GLUT3, HK1, GYS, HPRT1, GAPDH and β-actin mRNA were performed with the MXPro 3500 System (Stratagene, La Jolla, CA). Primers for GLUT1, GLUT3, HK1, GYS, HPRT1, GAPDH and β-actin were designed with Primer Premier 6.0 (Table 2). The 20 μL final PCR volume contained 1 μL reverse-transcribed product, 1 μL 10 μmol L^{-1} forward and reverse primers and 10 μL 2×concentrated SYBR Green Master mix (Takara, Japan). The PCR cycle was set at 95°C for 1 min followed by forty cycles of denaturing, annealing and extension at 95°C for 15 sec, 60°C for 15 sec and 72°C for 40 sec (except annealing temperature at 63°C for GLUT1 and 64°C for HK1). After the forty cycle was completed, threshold Cycle (Ct) value of each gene PCR amplification was obtained by system. Then, the software of geNorm win 3.5 was used to screen the appropriate endogenous reference gene to normalize the amount of starting RNA among HPRT1, GAPDH and β-actin, referring to the method by Erkens *et al*. (2006). As described by Livak and Schmittgen (2001), the following equation was used to calculate the relative expression ratio: $R = 2^{-\Delta Ct}$ (ΔCt = Ct of the target gene Ct of the housekeeping gene). Each sample was analyzed in triplicate.

Table 2: Primer parameters of genes by real-time quantitative PCR

Genes	Sequence of primers (5'→3')	Fragement size (bp)	Genbank accessionNo.
GLUT1	F: CATTGGCTCTGGGGTGGT	184	L07300
	R: GGCAACGATGCTGAGGTAG		
GLUT3	F: ACTGTTCCTGGTAGAGCGTG	100	NM_205511
	R: CAGAGCCAAAGCAATAGTCATA		
HK1	F: GTGCCTGACTCGGATGTGCG	125	NM_204101
	R: TCAGCCAGCGTCTCGTCGAT		
GYS	F: GGCACGCACCAACAACTTCAAC	125	AB090806
	R: CCACCAGCAGCGACTCATAGAG		
HPRT1	F: TTGTTGGATACGCCCTCGACTAC	117	NM_204848
	R: CCATAGCACTTCAACTGTGCTTTCA		
GAPDH	F: TGTCCGTGGAAGCCCTGAA	189	NM_204305
	R: CGTCTTGTTGTCGTTGTGCC		
β-actin	F: CCCCAGCCATGTATGTAGCC	199	NM_205518
	R: TCTGTCAGGATCTTCATGAGGTAG		

HK1 = Hexokinase 1; GYS = Glycogen Synthase; HPRT1 = Hypoxanthine Phosphoribosyltransferase 1; GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase

Determination of phosphorylation of the alpha subunits of AMPK: Western blot analysis was used to determine the effects of creatine on the abundance of AMPKα and phospho-AMPKα of pectoralis major and thigh muscle. Samples of muscle tissue (100 mg) was homogenized in 1 mL lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 0.5 mM sodium vanadate, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 50 mM b-glycerophosphate, 1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride) on the ice. Next, lysates were centrifuged at 10,000×g for 10 min at 4°C and the supernatant fluid was used for determination of the protein concentration following the BCA Method as described by Redinbaugh and Turley. The 50 μg protein sample of each was made and heated in a 75°C water bath for 10 min. After cooling on ice, aliquots (25 μL) diluted with 2×Sodium Dodecyl Sulfate (SDS) sample buffer (0.63 mL 0.5 M Tris-HCl at pH 6.8, 0.42 mL 75% glycerol, 0.125 g SDS, 0.25 mL β-mercaptoethanol, 0.2 mL 0.05% solution of bromophenol blue and 1 mL water to a final volume of 2.5 mL) were subjected to SDS-PAGE (10% resolving gels) and then transferred to PVDF membranes. The phosphorylation of AMPKα was determined using phospho-AMPKα (Thr172) specific (1:1000 dilution, Cell Signalling Technology) and AMPKα (1:200 dilution, Santa Cruz Biotechnology, Inc.) antibodies as described by Ceddia and Sweeney (2004).

Statistical analysis: The data are presented as means with standard errors. The replicate was used as the experimental unit. The statistical analysis was carried out with SAS 9.1 (SAS Institute, Cary, North Carolina, USA). Data from the measurement of metabolites, determination of gene expression and phosphorylation of the alpha subunits of AMPK were analyzed by ANOVA. A Student-Newman-Keuls (SNK) multiple range test was applied to compare treatment means. The linear and quadratic effects of CMH supplementation were analyzed using a contrast statement. A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effects of creatine monohydrate supplementation on glucose uptake of skeletal muscle: GLUT1 and GLUT3 mRNA abundance were used to indicate the glucose

Table 3: Effect of Creatine Monohydrate (CMH) on yellow-feathered broilers growth performance

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Initial BW (kg)	0.727	0.727	0.734	0.727	0.016	-	-
Final BW (kg)	1.560	1.569	1.550	1.565	0.014	0.937	0.977
ADG (g/birds)	39.600	40.100	38.800	39.900	0.300	0.882	0.898
ADFI (g/birds)	109.800	108.000	107.200	107.800	0.900	0.423	0.596
F:G	2.770	2.700	2.760	2.710	0.030	0.693	0.918

BW = Body Weight; ADG = Average Daily body weight Gain; ADFI = Average Daily Feed Intake; F:G = The ratio to ADFI and ADG; [1]SE = Standard Error of mean; [2]Linear and quadratic effects of creatine monohydrate

Table 4: Effects of CMH supplementation on relative GLUT1 and GLUT3 mRNA expression of skeletal muscle

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Pectoralis major							
GLUT1	0.0036	0.0039	0.0055	0.0069	0.0007	0.085	0.214
GLUT3	0.0945	0.1312	0.0962	0.0863	0.0115	0.568	0.516
Thigh muscle							
GLUT1	0.0029	0.0028	0.0028	0.0030	0.0004	0.995	0.964
GLUT3	0.0680	0.0689	0.0656	0.0640	0.0115	0.664	0.901

[1]SE = Standard Error of mean; [2]Linear and quadratic effects of creatine monohydrate

uptake of skeletal muscle (Table 3). There were no significant differences in the GLUT1 and GLUT3 mRNA abundance in the thigh muscle and pectoralis major, respectively with CMH supplementation but a numerical increasing was presented in GLUT1 mRNA abundance in the pectoralis major as CMH supplementation increased (Table 4).

Effects of creatine monohydrate supplementation on glucose biochemical metabolism of broilers: CMH supplementation at concentrations of 250, 500 and 1000 mg kg^{-1} did not affect serum glucose concentration but it demonstrated an increasing effect (linear and quadratic, $p<0.001$) as CMH supplementation increased (Table 5). However, the lactate concentration of the pectoralis major showed a quadratic effect ($p = 0.036$) with increasing CMH supplementation while decreasing at the level of 500 mg kg^{-1} CMH supplementation. For the thigh muscle, the concentration of lactate presented a decreasing effect (linear, $p = 0.042$). Pyruvic acid in the pectoralis major increased significantly (linear, $p<0.004$; quadratic, $p<0.003$) as CMH supplementation increased while the opposite effect was observed in the thigh muscle. There was no significance in the LDH activity of the thigh muscle but it displayed a quadratic effect ($p = 0.026$) as CMH supplementation increased. Interestingly, LDH activity of the pectoralis major presented an increasing effect (linear, $p = 0.003$; quadratic, $p<0.001$) by increasing CMH supplementation. Meanwhile, the Hexokinase 1 (HK1) mRNA abundance in the pectoralis major decreased with CMH supplementation (linear and quadratic, $p<0.001$) but the HK1 mRNA abundance in the thigh muscle increased with a quadratic effect (Table 6). Still, CMH supplementation at the level of 1000 mg kg^{-1} only increased the Na^+-K^+-ATPase activity of the thigh muscle ($p<0.05$) although, no significance was observed among CMH supplementation treatments (Table 7).

Effects of creatine monohydrate supplementation on glycogen synthesis of broilers: Glycogen concentrations of skeletal muscles were summarized in Table 8. It was shown that CMH supplementation did not affect proglycogen, macroglycogen or total glycogen concentration of thigh muscle (Table 8). Additionally, for pectoralis major there was no significant difference in the macroglycogen and total glycogen concentration. However, higher proglycogen concentrations in the pectoralis major were observed at the CMH supplementation level of 500 mg kg^{-1} than that of control group ($p<0.05$). CMH supplementation did not affect Glycogen Synthase (GYS) mRNA abundance in either pectoralis major or thigh muscle (Table 9).

Effects of creatine monohydrate supplementation on AMPKα phosphorylation of skeletal muscle: In Fig. 1, researchers found that CMH supplementation significantly increased AMPKα phosphorylation in the pectoralis major at 250 mg kg^{-1} ($p<0.001$) and 500 mg kg^{-1} concentration levels ($p<0.05$) but the effect disappeared at 1000 mg kg^{-1} concentration level. This result indicated a quadratic effect (quadratic $p = 0.002$) as CMH supplementation increased. While CMH supplementation did not affect AMPKα phosphorylation of the thigh muscle, it did assume a similar increase of that in pectoralis major.

Being an essential energy source for all cells in animals, glucose enters cells via glucose transporters (GLUTs) and is routed into the glycolysis pathway for ATP synthesis. Thus, glucose transporters play an important role in cellular glucose uptake and glucose homeostasis in animals. GLUT4 is one of the major insulin-responsive glucose transporters in mammals. Other than mammals, chickens have higher glycemia and are markedly resistant to insulin injections (Shiraishi *et al*.,

Table 5: Effects of Creatine Monohydrate (CMH) supplementation on glucose biochemical metabolism

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Serum							
Glu (mmol L^{-1})	11.900	11.420	12.030	11.850	0.160	0.750	0.840
Lac (mmol L^{-1})	1.450^{B}	1.440^{B}	1.690^{A}	1.630^{A}	0.020	***	***
Pectoralis major							
Lac (mM g^{-1} protein)	1.490^{ab}	1.450^{ab}	1.290^{b}	1.560^{a}	0.030	0.618	*
PA (µM mg^{-1} protein)	0.009^{B}	0.010^{B}	0.016^{A}	0.013^{AB}	0.001	**	**
LDH (U g^{-1} protein)	2200.800^{B}	2196.500^{B}	2109.100^{B}	2774.300^{A}	67.800	**	***
Thigh muscle							
Lac (mM g^{-1} protein)	1.240^{ab}	1.390^{a}	1.090^{ab}	1.050^{b}	0.050	*	0.078
PA (µM mg^{-1} protein)	0.021^{a}	0.021^{a}	0.014^{b}	0.016^{ab}	0.001	**	*
LDH[1] (U g^{-1} protein)	3428.200^{ab}	3887.600^{a}	3415.400^{ab}	2851.700^{b}	134.600	0.067	*

Glu = Glucose; Lac = Lactate; PA = Pyruvic Acid; LDH = Lactic Dehydrogenase. Means belong to ANOVA analyses and means within a row with different superscripts differ; (^{A,B}p<0.01; ^{a,b}p<0.05); [1]SE = Standard Error of mean; [2]Linear and quadratic effects of ceatine monohydrate, *p<0.05; **p<0.01; ***p<0.001

Table 6: Effects of CMH supplementation on relative Hexokinase 1 (HK1) mRNA expression of skeletal muscle

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Pectoralis major							
HK1	0.50^{A}	0.29^{B}	0.23^{B}	0.07^{B}	0.04	***	***
Thigh muscle							
HK1	0.17^{b}	0.28^{ab}	0.34^{a}	0.22^{ab}	0.02	0.656	0.093

Means belong to ANOVA analyses and means within a row with different superscripts differ (^{A,B}p<0.01; ^{a,b}p<0.05). [1]SE = Standard Error of mean; [2]Linear and quadratic effects of creatine monohydrate, ***p<0.001

Table 7: Effects of creatine monohydrate supplementation on Na^+-K^+-ATPase activity of skeletal muscle

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Pectoralis major							
Na^+-K^+-ATPase (U mg^{-1} protein)	1.79	2.11	2.01	1.98	0.07	0.459	0.368
Thigh muscle							
Na^+-K^+-ATPase (U mg^{-1} protein)	1.30^{b}	1.49^{ab}	1.51^{ab}	1.85^{a}	0.07	**	*

Means belong to ANOVA analyses and means within a row with different superscripts differ (^{a,b}p<0.05); [1]SE = Standard Error of mean; [2]Linear and quadratic effects of creatine monohydrate, *p<0.05; **p<0.01

Table 8: Effects of CMH supplementation on glycogen concentration of skeletal muscle

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Pectoralis major							
PG (mM kg^{-1} protein)	5.77^{b}	5.44^{b}	7.95^{a}	6.49^{ab}	0.29	0.074	1.280
MG (mM kg^{-1} protein)	27.99	28.12	23.98	31.69	1.16	0.510	0.217
Total glycogen (mM kg^{-1} protein)	33.75	33.56	31.93	38.12	1.27	0.320	0.281
Thigh muscle							
PG (mM kg^{-1} protein)	4.71	5.71	5.15	4.52	0.25	0.773	0.127
MG (mM kg^{-1} protein)	23.00	27.35	26.24	24.34	1.20	0.602	0.183
Total glycogen (mM kg^{-1} protein)	27.71	33.06	31.31	28.86	1.19	0.643	0.075

PG = Proglycogen; MG = Macroglycogen; Total glycogen = PG+MG. Means belong to ANOVA analyses and means within a row with different superscripts differ; (^{a,b}p<0.05); [1]SE = Standard Error of mean; [2]Linear and quadratic effects of creatine monohydrate

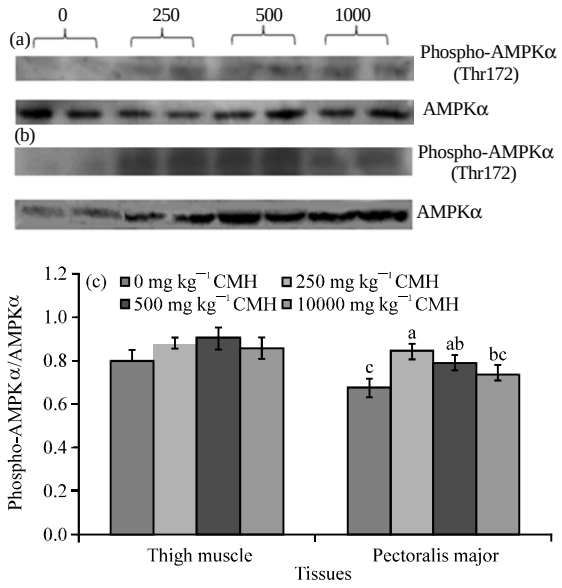

Fig. 1: Effects of creatine monohydrate supplementation on glucose homeostasis in muscle of broilers; representative blots and their respective densitometric quantification of the effects of creatine monohydrate on AMPK phosphorylation. a) AMPKaphosphorylation of thigh muscle; b) breast muscle were presented by ratio; c) phospho-AMPKa (62 kD) to AMPKa (63 kD) protein concentration. Sampling dates with different letters differ (p<0.05). Linear and quadratic effects of creatine monohydrate (linear p = 0.161, quadratic p = 0.042; linear p = 0.404, quadratic p = 0.002) for thigh muscle and pectoralis major, respectively

2011). GLUT isoforms 1, 3 and 8 were mainly expressed in chicken while intrinsically lacking GLUT4 (Kono *et al*., 2005). GLUT8 is expressed ubiquitously, higher in kidney and adipose tissue but lower in muscle thus researchers

Table 9: Effects of CMH supplementation on relative Glycogen Synthase (GYS) mRNA expression of skeletal muscle

	CMH (mg kg^{-1})					p-value[2]	
Parameters	0	250	500	1000	SE[1]	Linear	Quadratic
Pectoralis major							
GYS	0.16	0.27	0.25	0.22	0.01	0.261	*
Thigh muscle							
GYS	0.14	0.23	0.20	0.19	0.01	0.224	*

[1]SE = Standard Error of mean; [2]Linear and quadratic effects of creatine monohydrate, *p<0.05

only determined GLUT1 and GLUT3 mRNA abundance in skeletal muscle. The present study showed that CMH supplementation did not affect glucose uptake in skeletal muscle as there were no significance in GLUT1 and GLUT3 mRNA abundance in the thigh muscle and pectoralis major. These may be due to the fact that firstly, glucose is an important energy source for muscle cells. To some extent, broilers can easily keep glucose relatively stable because high levels of plasma glucose concentrations do not appear to lead to significant oxidative stress which could be proposed that broilers have the more normal pattern of metabolism than that of mammals. Secondly, dietary creatine supplementation has been shown to increase the total creatine concentration in muscle (Op't Eijnde *et al*., 2006) which may be accompanied by a corresponding augmentation in the phosphocreatine concentration of muscle (Del Favero *et al*., 2012). The energy state of the muscle can be determined by the muscle phosphocreatine concentration. High phosphocreatine suggests that the cell is energetic and that it may be not necessary to increase glucose uptake to provide energy.

Meanwhile, a high concentration of phosphocreatine would be expected to increase glycogen synthesis in muscle. Lomako *et al*. (1991) first discovered two forms of glycogen: macroglycogen and proglycogen. The well-recognized macroglycogen has a molecular weight of approximately 10^4 kDa, containing 0.35% glycogenin by weight and proglycogen, the second form of glycogen which is approximately 400 kDa, contains almost 10% glycogenin. Proglycogen can be converted into macroglycogen mainly by the catalysis of glycogen synthase (Lomako *et al*., 1993). In the present study, glycogen synthase mRNA abundance in the pectoralis major and thigh muscle showed no significant difference between the CMH treatment and the control groups which implies that macroglycogen in muscle might be not affected by CMH supplementation. The total glycogen in muscle depends on the storage of proglycogen and macroglycogen. The current study has shown that CMH supplementation did not increase the total glycogen concentration of muscle which was in accord with the result in human by Rico-Sanz *et al*. (2008). The present results do not support the hypothesis that creatine loading increases muscle glycogen storage. One possibility is that regulatory factors and key enzymatic activities within the glycogen synthesis pathway were not properly activated by CMH supplementation. Roach (2002) depicted that glycogen synthase and branching enzymes was essential to complete glycogen synthesis. Danforth (1965) was interesting to speculate that increased glycogen may serve as a signal for increasing phosphorylase afer studying glycogen synthase activity ratio and the amount of glycogen in muscle. Thus, further research on phosphorylation of glycogen synthase as a key enzyme of glycogen synthesis in muscle analysis will further elucidate the effect of CMH supplementation on the glycogen concentration of muscle.

Glucose oxidation is an important metabolic pathway for glucose to provide energy in muscles. HK1 is one of the first key enzymes in glucose oxidation and pyruvate is the main product of glycolysis. Lactate is produced from conversion of pyruvate, catalyzed by LDH under anaerobic conditions in the muscle. Therefore, researchers utilized the HK1 mRNA abundance as well as the concentrations of pyruvate and lactate to assess glycolysis in muscles with CMH supplementation. The results showed that CMH supplementation down regulated HK mRNA abundance in the pectoralis major with a dose-dependent decreasing effect. Conversely, CMH supplementation upregulated HK1 mRNA abundance in the thigh muscle with a quadratic effect as the supplementation increased, indicating that CMH supplementation may alter glucose utilization by decelerating the glycolysis of the pectoralis major and accelerating the glucose oxidation of the thigh muscle. Actually, CMH supplementation did not affect the lactate concentration in the pectoralis major and thigh muscle. High LDH activity can be beneficial to the conversion of pyruvate to lactate (Wilkie *et al*., 2001). However, lactate cannot be resynthesized to carbohydrate in skeletal muscles (Krebs and Woodford, 1965) and high LDH activity normally leads to a higher lactate accumulation in the muscle which is in agreement with the present finding. Lactate concentration of serum being an important parameter of glycolysis was higher with CMH supplement above 500 mg kg^{-1} so researchers suggest that high concentration levels of CMH supplementation may improve the whole body glycolysis in broilers. In contrast, the high concentration levels of CMH supplementation decreased lactate production in the thigh muscle. Solomon and Dunn (1988) stated that the thigh muscle of broilers comprises three fiber types including slow-twitch red, fast-twitch red and fast-twitch white myofibers. Sams and Janky (1990) added that there is only αW

myofiber exhibited in the breast muscle. The response to high-dose creatine supplementation is greater in oxidative than in glycolytic muscle (Eijnde *et al*., 2001) thus CMH supplementation is expected to increase glucose oxidation in the thigh muscle. The Na^+/K^+-ATPase as an energy transducing ion pump has been studied extensively since its discovery in 1957. The Na^+-K^+-ATPase activity of the thigh muscle in the 1000 mg kg^{-1} CMH supplementation treatment was significantly higher than that of the control group. From this result, researchers speculate that CMH supplementation increases glucose oxidation due to an increase in the slow-twitch red muscle fiber ratio. This hypothesis has been proven by the previous results of the study. More slow-twitch red muscle fibers in the muscles are more beneficial to glucose oxidation for providing energy. To some extent, CMH supplementation could improve the meat quality of broilers by reduction of lactate production in thigh muscles.

AMPK is a highly evolutionarily conserved serine/threonine kinase and is composed of a catalytic subunit, α and two regulatory subunits, β and γ which can be activated via phosphorylation at Thr172 of subunit-α. In fact, AMPK should be allosterically activated by AMP. Nonetheless, increased AMP is always associated with low ATP (Lage *et al*., 2008). Thus, the AMP/ATP ratio is applied to determine the energetic state of a cell and is expected to alter AMPK activation. Accumulating evidence has demonstrated that AMPK activity is increased with glucose metabolic changes (Priebe *et al*., 2011; Kim *et al*., 2012). Briefly, as energy expenditure exceeds energy intake, AMPK is activated (phosphorylated) to modulate the energy balance within the cell. As a gauge, activated AMPK switches off ATP-consuming process and turns on catabolic processes to produce ATP in the muscle. From observation of AMPK phosphorylation in the present study, researchers found that CMH dietary supplementation significantly increased AMPK phosphorylation in the pectoralis major with a quadratic effect both in the thigh muscle and the pectoralis major (Fig. 1). This result indicates that CMH dietary supplementation alters energy metabolism in muscles with AMPK activation. AMPK is activated by an increase in the AMP/ATP and Pcr/Cr ratios (Goodyear, 2000). It is known that the total creatine pool in muscle is composed of creatine and phosphocreatine. The total creatine pool in muscle would be enhanced when broilers are treated with CMH supplementation as evidence has demonstrated in human (Steenge *et al*., 1998) and rat (Op't Eijnde *et al*., 2006). Additional creatine taken up by muscles may be partly converted to phosphocreatine which is not a counterpart increase in the total creatine pool. Thus, the PCr/Cr ratio reflected in the cellular energetics actually decreases before CMH supplementation increases up to the maximum creatine storage of muscle. A low PCr/Cr ratio to some extent implies that ATP is not in a relative surplus in the muscle. As a result, AMPK is activated at low and middle concentration levels of CMH supplementation but has no effect at high concentration levels. A small difference is found between thigh muscles and pectoralis majors, possibly arising from the discrepancy in the creatine content stored in muscle. Young and Young (2002) suggested that a low dose of creatine supplementation (300 mg/kg/day) for 5 weeks may significantly increase the creatine content in fast-twitch muscles. pectoralis major is mainly constituted by fast-twitch muscle fiber in broilers (Sams and Janky, 1990). Thus, increasing creatine content in the pectoralis major leads to the promotion of AMPK phosphorylation.

CONCLUSION

Creatine supplementation did not affect the glucose uptake of muscle from blood and glycogen synthesis in muscle. However, it had different effects on glucose oxidation between the thigh muscle and the *Pectoralis major*. On one hand, it is beneficial to improve the glucose oxidation of the thigh muscle and this parameter presented a quadratic effect as CMH supplementation increased. On the other hand, researchers found a CMH supplementation dose-dependent decreasing effect of glycolysis in the pectoralis major. The study on the AMPKα phosphorylation of muscle also indicated that CMH supplementation can increase AMPKα phosphorylation with a quadratic effect and can possibly result in modulation of the glucose oxidation of muscle. Therefore, the relation between AMPK activation and glucose metabolism in muscles needs to be investigated using stimulators or inhibitors of AMPK. Meanwhile, further research on the signaling pathway of AMPK and its stimulation by CMH should be performed.

IMPLICATIONS

Creatine supplementation plays an important role in muscle carbohydrate metabolism by increasing glucose uptake and glycogen storeage in muscle which can affect glucose homeostasis. Meat quality of animal at postmortem such as pH, color and water holding capacity can be altered by glucose homeostasis in muscle. As means to control glucose homeostasis are key importance to maintain meat quality, study of glucose biochemical

metabolism in muscle in respond to creatine supplementation would have facilitated to improve meat quality.

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REFERENCES

Alves, C.R., J.C. Ferreira, M.A. de Siqueira-Filho, C.R. Carvalho, A.H. Lancha Jr. and B. Gualano, 2012. Creatine-induced glucose uptake in type 2 diabetes: A role for AMPK-α? Amino Acids, 43: 1803-1807.

Bendall, J.R., 1979. Relations between muscle pH and important biochemical parameters during the postmortem changes in mammalian muscles. Meat Sci., 3: 143-157.

Briand, M., A. Talmant, Y. Briand, G. Monin and R. Durand, 1981. Metabolic types of muscle in the sheep: II. Lactate dehydrogenase activity and LDH isoenzyme distribution. Eur. J. Applied Physiol. Occup. Physiol., 46: 359-365.

Ceddia, R.B. and G. Sweeney, 2004. Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells. J. Physiol., 555: 409-421.

Danforth, W.H., 1965. Glycogen synthetase activity in skeletal muscle. interconversion of two forms and control of glycogen synthesis. J. Biol. Chem., 240: 588-593.

Del Favero, S., H. Roschel, G. Artioli, C. Ugrinowitsch and V. Tricoli *et al*., 2012. Creatine but not betaine supplementation increases muscle phosphorylcreatine content and strength performance. Amino Acids, 42: 2299-2305.

Eijnde, B.O., E.A. Richter, J.C. Henquin, B. Kiens and P. Hespel, 2001. Effect of creatine supplementation on creatine and glycogen content in rat skeletal muscle. Acta Physiologica Scandinavica, 171: 169-176.

Erkens, T., M. van Poucke, J. Vandesompele, K. Goossens, A. van Zeveren and L.J. Peelman, 2006. Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and longissimus dorsi muscle and evaluation with PPARGC1 A. Biol. Med. Chem. Biotechnol. Vol. 6. 10.1186/1472-6750-6-41.

Feldman, E.B., 1999. Creatine: A dietary supplement and ergogenic aid. Nutr. Rev., 57: 45-50.

Goodyear, L.J., 2000. AMP-activated protein kinase: A critical signaling intermediary for exercise-stimulated glucose transport. Exercise Sport Sci. Rev., 28: 113-116.

Kim, M.S., H.J. Hur, D.Y. Kwon and J.T. Hwang, 2012. Tangeretin stimulates glucose uptake via regulation of AMPK signaling pathways in C_2C_{12} myotubes and improves glucose tolerance in high-fat diet-induced obese mice. Mol. Cell. Endocrinol., 358: 127-134.

Kono, T., M. Nishida, Y. Nishiki, Y. Seki, K. Sato and Y. Akiba, 2005. Characterisation of Glucose Transporter (GLUT) gene expression in broiler chickens. Br. Poult. Sci., 46: 510-515.

Krebs, H.A. and M. Woodford, 1965. Fructose 1,6-diphosphatase in striated muscle. Biochem. J., 94: 436-445.

Kristensen, M. and C. Juel, 2010. Na^+,K^+-ATPase Na^+ affinity in rat skeletal muscle fiber types. J. Membr. Biol., 234: 35-45.

Lage, R., C. C. Dieguez, A. Vidal-Puig and M. Lopez, 2008. AMPK: A metabolic gauge regulating whole-body energy homeostasis. Trends Mol. Med., 14: 539-549.

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_T$ method. Methods, 25: 402-408.

Lomako, J., W.M. Lomako and W.J. Whelan, 1991. Proglycogen: A low-molecular-weight form of muscle glycogen. FEBS Lett., 279: 223-228.

Lomako, J., W.M. Lomako, W.J. Whelan, R.S. Dombro, J.T. Neary and M.D. Norenberg, 1993. Glycogen synthesis in the astrocyte: From glycogenin to proglycogen to glycogen. FASEB J., 7: 1386-1393.

Long, Y.C. and J.R. Zierath, 2006. AMP-activated protein kinase signaling in metabolic regulation. J. Clin. Invest., 116: 1776-1783.

MacNeil, L.G., S.K. Baker, I. Stevic and M.A. Tarnopolsky, 2011. 17 β-estradiol attenuates exercise-induced neutrophil infiltration in men. Am. J. Physiol. Regul. Integr. Comp. Physiol., 300: R1443-R1451.

Magnoni, L.J., Y. Vraskou, A.P. Palstra and J.V. Planas, 2012. AMP-Activated protein kinase plays an important evolutionary conserved role in the regulation of glucose metabolism in fish skeletal muscle cells. PLoS One, Vol. 7. 10.1371/journal.pone. 0031219.

Nissen, P.M. and J.F. Young, 2006. Creatine monohydrate and glucose supplementation to slow-andfast-growing chickens changes the postmortem pH in pectoralis major. Poult. Sci., 85: 1038-1044.

Op't Eijnde, B., H. Jijakli, P. Hespel and W.J. Malaisse, 2006. Creatine supplementation increases soleus muscle creatine content and lowers the insulinogenic index in an animal model of inherited type 2 diabetes. Int. J. Mol. Med., 17: 1077-1084.

Priebe, A., L. Tan, H. Wahl, A. Kueck, G. He, R. Kwok, A. Opipari and J.R. Liu, 2011. Glucose deprivation activates AMPK and induces cell death through modulation of AKT in ovarian cancer cells. Gynecol. Oncol., 122: 389-395.

Rico-Sanz, J., M. Zehnder, R. Buchli, G. Kuhne and U. Boutellier, 2008. Creatine feeding does not enhance intramyocellular glycogen concentration during carbohydrate loading: An *in vivo* study by ^{31}P- and ^{13}C-MRS. J. Physiol. Biochem., 64: 189-196.

Roach, P.J., 2002. Glycogen and its metabolism. Curr. Mol. Med., 2: 101-120.

Rosenvold, K., B. Essen-Gustavsson and A.J. Anderson, 2003. Dietary manipulation of pro-and macroglycogen in porcine skeletal muscle. J. Anim. Sci., 81: 130-134.

Sams, A.R. and D.M. Janky, 1990. Research note: Simultaneous histochemical determination of three fiber types in single sections of broiler skeletal muscles. Poult. Sci., 69: 1433-1436.

Shiraishi, J., K. Yanagita, R. Fukumori, T. Sugino and M. Fujita *et al*., 2011. Comparisons of insulin related parameters in commercial-type chicks: Evidence for insulin resistance in broiler chicks. Physiol. Behav., 103: 233-239.

Solomon, M.B. and M.C. Dunn, 1988. Simultaneous histochemical determination of three fiber types in single sections of ovine, bovine and porcine skeletal muscle. J. Anim Sci., 66: 255-264.

Steenge, G.R., J. Lambourne, A. Casey, I.A. Macdonald and P.L. Greenhaff, 1998. Stimulatory effect of insulin on creatine accumulation in human skeletal muscle. Am. J. Physiol., 275: E974-E979.

Van Loon, L.J., R. Murphy, A.M. Oosterlaar, D. Cameron-Smith, M. Hargreaves, A.J. Wagenmakers and R. Snow, 2004. Creatine supplementation increases glycogen storage but not GLUT-4 expression in human skeletal muscle. Clin. Sci., 106: 99-106.

Wilkie, M.P., P.G. Bradshaw, V. Joanis, J.F. Claude and S.L. Swindell, 2001. Rapid metabolic recovery following vigorous exercise in burrow-dwelling larval sea lampreys (Petromyzon marinus). Physiol. Biochem. Zool., 74: 261-272.

Young, J.C. and R.E. Young, 2002. The effect of creatine supplementation on glucose uptake in rat skeletal muscle. Life Sci., 71: 1731-1737.