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Chromium and Methionine Effects on Amino Acid Uptake, Gene Expression, and Protein Abundance in Bovine Satellite Cells

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Abstract: The objective of the study was to evaluate the effects of chromium (Cr) and methionine on myogenic gene expression, GLUT4 and insulin receptor (IR) synthesis, and amino acid uptake in differentiated bovine satellite cells. Cells were treated with 0, 1, or 10 μM of chromium acetate (CrAc) and 0, 1, or 10 mg/mL of methionine, with cells utilized for the amino acid assay receiving 0.1 μM instead of 1 μM of CrAc. The addition of 10 μM of Cr tended to downregulate AMPKα mRNA gene expression ($P < 0.1$). Myogenin was impacted by methionine and CrAc with high doses and low doses upregulating expression ($P < 0.01$). The ratio and protein levels of AMPKα to pAMPKα, GLUT4, and IR abundance were not affected ($P > 0.1$). Increase in time decreased concentrations of leucine, isoleucine, threonine, methionine, phenylalanine, glutamine, ornithine, lysine, and cysteine ($P < 0.01$) in conditioned media. Over time, alanine increased ($P < 0.01$). Methionine inclusion increased alanine, proline, glutamic acid, ornithine, and tryptophan concentrations, as well as decreased leucine, isoleucine, and cysteine ($P < 0.01$). CrAc only affected glutamine and tryptophan, which both increased with CrAc dose, whereas alanine decreased ($P < 0.01$). Methionine decreased over time within the 10 mg/mL group ($P < 0.01$). Tryptophan was generally unaffected but decreased in the 0 mg/mL group at 48 h. Cysteine decreased with a decreased dosage of CrAc at 24 h ($P < 0.01$). Lysine was generally unaffected by this interaction ($P > 0.1$), with the exception of the 0.1 μM treatment group at 24 h being higher ($P < 0.01$). CrAc affected the uptake of alanine, glutamine, and tryptophan ($P < 0.01$). Methionine inclusion increased alanine, proline, glutamic acid, ornithine, and tryptophan, as well as decreased leucine, isoleucine, and cystine ($P < 0.01$). Increased time saw a significant decrease in leucine, isoleucine, threonine, methionine, phenylalanine, glutamine, ornithine, lysine, and cystine ($P < 0.01$) and an increase in alanine ($P < 0.01$).

Key words: chromium, methionine, satellite cells, genes, protein
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Introduction

The micronutrient chromium (Cr) has been established to have benefits on cattle immune function and growth performance (Page et al., 1993; Bernhard et al., 2012). Chromium is one of the most abundant minerals on earth and has been utilized as a dietary supplement more so in recent years. There are many sources of Cr; however, the only source that is approved for beef cattle supplementation is chromium propionate (FDA, 2019). Chromium is also available as Cr picolinate, Cr methionine, and Cr acetate (CrAc). Chromium supplementation has been shown to increase lean tissue while decreasing fat accumulation

in hogs (Page et al., 1993; Mooney and Cromwell, 1995). Conversely, Tokach et al. (2015) reported that Cr propionate increased adipogenic gene expression and promoted adipogenic differentiation in Intramuscular (IM) adipocytes in cattle. Chromium has also been shown to affect insulin signaling and improve both glucose and amino acid uptake (Vincent, 2000). Insulin responsiveness is increased with the addition of Cr, thus increasing glucose uptake (Mertz, 1993; Hoffman et al., 2014). Chromium source can also potentially affect adenosine monophosphate (AMP)-activated protein kinase (AMPK) activity *in vitro* and *in vivo* (Penumathsa et al., 2009; Wang et al., 2009; Hoffman et al., 2014) via phosphorylation. Increased phosphorylation of AMPKα in muscle tissue can trigger metabolic changes that switch the cell from energy storage to energy-consuming pathways (Tong et al., 2009). With this shift in cell signaling, energy can be provided to muscle tissue, which could affect myogenic gene expression, facilitating muscle growth and thus improving lean tissue marketed in the live animal.

Ohh and Lee (2005) state Cr supplementation with methionine could improve feed efficiency, glucose clearance, and insulin concentration in blood in well-nourished calves. However, this chelate is widely known not to have any adverse effect on growth performance (Ohh and Lee, 2005). The combination of Cr and methionine in a bovine satellite cell model has not been thoroughly explored. Therefore, this project aims to determine if there is an effect of gene expression, protein abundance, and amino acid uptake by incorporating Cr at normal and high levels on methionine uptake in cell culture.

Materials and Methods

Bovine satellite cell isolation

The isolation and cultivation of bovine satellite cells were conducted as previously reported by Johnson et al. (1998), Kim et al. (2018), and Kim et al. (2019). Three Angus steers, aged 16 mo and weighing 475 ± 51.2 kg, were processed at the Gordon W. Davis Meat Sciences Lab in Lubbock, TX, under USDA inspection. Upon harvest, approximately 800 g semimembranosus muscle tissue was collected and transported to the Meat Science and Muscle Biology laboratory at Texas Tech University. The tissue was transported in phosphate-buffered saline (PBS) containing 0.76M NaCl, 0.3M NaH2PO4 (pH 7.2), along with Antibiotic-Antimycotic (Gibco, Waltham, MA). The muscle tissue was isolated from vascular components, adipose tissue, and connective tissue, followed by homogenization using a sterile meat grinder. The acquired muscle tissue was incubated in Earl's Balanced Salt Solution (EBSS; Sigma Aldrich, St. Louis, MO), augmented with 0.1% Pronase® (Calbiochem, LaJolla, CA) at 37°C. This mixture was subjected to intermittent agitation at 10-min intervals. After 1 h of incubation, the mixture was centrifuged at 1,500 × g for 4 min at room temperature. The resulting pellet was resuspended in PBS (Sigma Aldrich) and centrifuged at 500 × g for 10 min at room temperature. The supernatant obtained from this step was collected and centrifuged at 1,500 × g for 10 min at room temperature to isolate the mononucleated cells. The resulting mononucleated-cell preparation was suspended in Dulbecco's Modified Eagle's medium (DMEM; Gibco) at 37°C, supplemented with 10% fetal bovine serum (FBS) and 1× Antibiotic-Antimycotic (Gibco). The cells were incubated in a humidified atmosphere with 5% CO_2 at 38°C. This procedure was completed 3 separate times and cells were not pooled across animal.

Bovine satellite cell cultures

Cells were not counted but instead cultured until reaching approximately 70% to 80% confluency on a collagen matrix well plate. Induction of myogenic differentiation was achieved by replacing the existing medium with a differentiation medium containing DMEM supplemented with 2% FBS and 1× Antibiotic-Antimycotic. Both DMEM and FBS were from the same respective lots. There was no traceable level of Cr included in the DMEM formulation (Dulbecco and Freeman, 1959). Varying doses of additional methionine, 0 mg/mL (differentiation media only; negative control [NC]), 1 mg/mL, and 10 mg/mL, and additional Cr, 0 μM (differentiation media only; NC), 1.0 μM, and 10.0 μM as CrAc (Sigma Aldrich, USA) were added to the differentiation media. Cells were harvested at 24, 48, and 72 h for the remaining analysis (Kim et al., 2018; Kim et al., 2019).

Fluorescence microscopy

For immunostaining, the cells were cultured on 3-well chamber microscopy glass slides (Cat. #: 80381, Ibidi, Fitchburg, WI) and incubated with different concentrations of methionine (0 mg/mL and 10 mg/mL) and CrAc (0 μM and 10.0 μM). To fix the slides, a 4% paraformaldehyde solution (Thermo Fisher Scientific) was applied at room temperature for 10 min. To prevent nonspecific background staining, the fixed cells were incubated with 1% bovine serum albumin for 30 min.

Immunostaining was performed using a rabbit IgG polyclonal anti-GLUT4 antibody for glucose transporter 4 (GLUT4) and a mouse IgG2b polyclonal anti-insulin receptor antibody for the insulin receptor (IR). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) for 5 min, followed by 2 washes with PBS. The slides were imaged using an inverted fluorescence microscope (Nikon Eclipse, Ti-E; Nikon Instruments Inc.) at a magnification of 20×, equipped with a UV light source (Nikon Intensilight Inc.) (Kim et al., 2018; Kim et al., 2019).

RNA extractions

Following 48 h of incubation with treatments, mRNA extraction was conducted utilizing TRIzol® reagent (Invitrogen, Carlsbad, CA) as described in Kim et al. (2018). The quantification and assessment of mRNA purity were performed through spectrophotometric analysis at 260-nm and 280-nm wavelengths, employing a NanoDrop™ UV-Vis Spectrophotometer (Thermo Fisher Scientific).

Real-time quantitative PCR (7900HT Real-Time PCR System, Applied Biosystems, Foster City, CA) was used to measure the quantity of GLUT4, alpha serine/threonine-protein kinase (AKT), *AMPKα*, myoblast determination protein 1 (MyoD), myogenin, and creatine phosphokinase (CPK) relative to the quantity of ribosomal protein subunit 9 (*RPS9*) mRNA (Table 1). Primers were employed to target *AMPKα* broadly rather than distinguish between the *AMPKα*1 and *AMPKα*2 isoforms. This decision was made to focus on the general activity and expression levels of *AMPKα* to focus on *AMPKα*'s overarching contribution to the observed biological phenomena. This procedure was conducted as seen in Kim et al. (2019). Expression of *RPS9* was not different across bovine tissue samples and was used as a housekeeping gene, as noted by Janovick-Guretzky et al. (2007) and Bionaz et al. (2007). The experiments were replicated 3 times, each consisting of 45 cycles that included a 15-s duration at 95°C and a subsequent 1-min interval at 60°C.

Table 1. Primer and probe sequences for the gene expression analysis

Genes[1]	Sequence (5' to 3')
AMPKα	
Forward	ACCATTCTTGGTTGCTGAAACTC
Reverse	CACCTTGGTGTTTGGATTTCTG
TaqMan Probe	6FAM-CAGGGCGCGCCATACCCTTG-TAMRA
GLUT4	
Forward	CCTCGGCAGCGAGTCACT
Reverse	AAACTGCAGGGAGCCAAGAA
TaqMan Probe	6FAM-CCTTGGTCCTTGGCGTATTCTCCGC-TAMRA
AKT	
Forward	TTGCCCATAACTAAGCCTACATCTC
Reverse	GCAGGCGCTCTATGTACTGGAT
TaqMan Probe	6FAM-CCCAACCAGAGGCTGCCCAAAGT-TAMRA
MyoD	
Forward	AGGCCTTCGAGACGCTCAA
Reverse	TGGCGTTGCGCAGGAT
TaqMan Probe	6FAM-CGCTGCACGTCTAGCAACCCAAACC-TAMRA
Myogenin	
Forward	AGAAGGTGAATGAAGCCTTCGA
Reverse	GCAGGCGCTCTATGTACTGGAT
TaqMan Probe	6FAM-CCCAACCAGAGGCTGCCCAAAGT-TAMRA
CPK	
Forward	GCCAATGGACTGTTTTACAATGC
Reverse	CCAAAGGGATTTTGTAGATGTTTCTC
TaqMan Probe	6FAM-TCATATTCCAGACGCATCTCCACAGCTACG-TAMRA
RPS9	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA

[1]*AMPKα*: adenosine monophosphate-activated protein kinase-α; *GLUT4*: glucose transporter 4; *AKT*: alpha serine/threonine-protein kinase; *MyoD*: myoblast determination protein 1; *CPK*: creatine phosphokinase; *RPS9*: ribosomal protein subunit 9.

Western blotting

After 48 h of treatment, cellular lysates were prepared using the mammalian protein extraction (M-PER™) (Thermo Fisher Scientific). Varying doses of methionine, 0 mg/mL (differentiation media only; NC), 1 mg/mL, and 10 mg/mL, and Cr, 0 µM (differentiation media only; NC), 1.0 µM, and 10.0 µM as CrAc (Sigma Aldrich, USA) were used as treatments. The quantification of total protein in these lysates was carried out via the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) with absorbance measurements at 562 nm using a NanoDrop™ UV-Vis Spectrophotometer. Samples were subsequently separated on a Bolt 4-12% Bis-Tris Plus gel (Thermo Fisher Scientific) under 200 V for 22 min. Following this, proteins were transferred to a nitrocellulose membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). Primary antibodies used for anti-AMPKα, anti-phosphorylated AMPKα, and GLUT4 were rabbit polyclonal AB (Cell Signaling, Danvers, MA). Mouse polyclonal IgG2b (Cell Signaling,

Danvers, MA) was used for IR. Primary antibody dilutions of 1:1,000 were used for this study. The secondary antibody for all proteins in this study was goat anti-rabbit with IgG2b used for IR and IgG for the remainder of the proteins observed. A 1:10,000 dilution was used for the secondary antibody. Membranes were dried and subsequently visualized with the VersaDoc Imaging System (Bio-Rad). Protein bands were identified and quantified using Bio-Rad's Quantity One Band Analysis software. To ensure consistency, the intensity of each band was normalized against a pooled reference sample present on every blot. The mean intensity of each band was then calculated in relation to the NC to determine the level of phosphorylation.

Amino acid uptake

Cultured Bovine Satellite Cells (BSC) were allowed to reach approximately 80% confluency in a 96-well enzyme immunoassay/radioimmunoassay (EIA/RIA) plate (Corning Incorporated, Corning, NY). Custom differentiation media lacking methionine and arginine was added with varying doses of methionine, 0 mg/mL (differentiation media only; NC), 1 mg/mL, and 10 mg/mL, and Cr, 0 µM (differentiation media only; NC), 1.0 µM, and 10.0 µM as CrAc (Sigma Aldrich, USA) as treatments. There was no traceable level of Cr included in the DMEM formulation (Dulbecco and Freeman, 1959). Arginine was added at a fixed rate for all cells. Multiple wells were not seeded with cells in order to serve as blank measurements and used as a correction in final calculations. Cell media was collected from wells at 24, 48, 72, and 96 h. Cells were seeded into the 96-well plate and conditioned media was harvested only once from each well. Once media was harvested, the wells were discarded and therefore not considered a repeated measure. Free amino acids were determined using the EZ: faast amino acid analysis kit (Phenomenex, Torrance, CA) and gas chromatography-mass spectrometry at a 1:15 split ratio using helium as carrier gas on a Zebron ZB-AAA Gas Chromatography (GC) column. The GC oven was set to increase 30°C/min, starting at 110°C with a final temperature of 320°C. Values from gas chromatography are described using the assumption that a decrease in values over time corresponds to an increase in Amino Acid (AA) disappearance in the media. In this highly controlled environment, this disappearance could be attributed to AA absorption by the cell.

Equation 1. Determination of chromatography results where PA = Peak Area and IS = equal Norvaline internal standard.

$$Amino\ Acid\ Conc.\left(\frac{ng}{mL}\right) = \left(\frac{(PA_X) \times [IS]}{PA_{IS}}\right) \times Blank\ Correction$$

Statistical analysis

For all analyses, cells were isolated from steers, and animal was considered an experimental unit. Cell wells were treated independently and replicated within each animal based on power. A two-tailed power test was run using SAS (SAS Inst., Inc., Cary, NC) with $\beta \geq 0.8$. Within the gene expression experiment, wells were replicated 4 times. In the amino acid update experiment, cell wells were replicated 6 times, and protein wells were replicated 4 times. Each replication was repeated across animal. Data were analyzed using the GLM procedure of SAS. Treatment and time were included as fixed effects. If interactions were not present, they were removed from the model. For gene expression and protein abundance experiments, a two-way ANOVA was used to determine interactions with methionine and Cr. Amino acid experiments utilized a 2×2 factorial ANOVA to determine time effects. $P \leq 0.05$ was considered significant, and tendencies were defined as $P \geq 0.05$ but ≤ 0.1.

Results and Discussion

There was no observable difference in GLUT4 and IR based on immunohistochemistry staining (Figures 1–2) or Western blot (Figures 3 to 4) ($P > 0.1$). The addition of 10 µM of CrAc tended to downregulate *AMPKα* ($P < 0.1$; Figure 5) but did not produce differences in protein accumulationbut did not produce differences in protein accumulation ($P > 0.1$; Figure 6). Myogenin was not impacted by both methionine or CrAc with high doses, and low doses did not alter gene expression ($P < 0.01$; Figure 7). Treatments with varying levels of dosage, high methionine plus low CrAc and low methionine and high CrAc, downregulated myogenin expression ($P < 0.01$). Myogenin initiates terminal differentiation in satellite cells. The downregulation of myogenin seen in this study by mixed treatment groups suggests that both methionine and Cr work in tandem to promote the differentiation of satellite cells. Ohh and Lee (2005) provided support for this concept by observing that chromium methionine chelate (CrMet) can directly cross the intestinal cell membrane without the need for prior digestion. Tsa and Lien (2007) saw no effect on myogenin in myoblasts using Cr picolinate with other organically bound Cr sources.

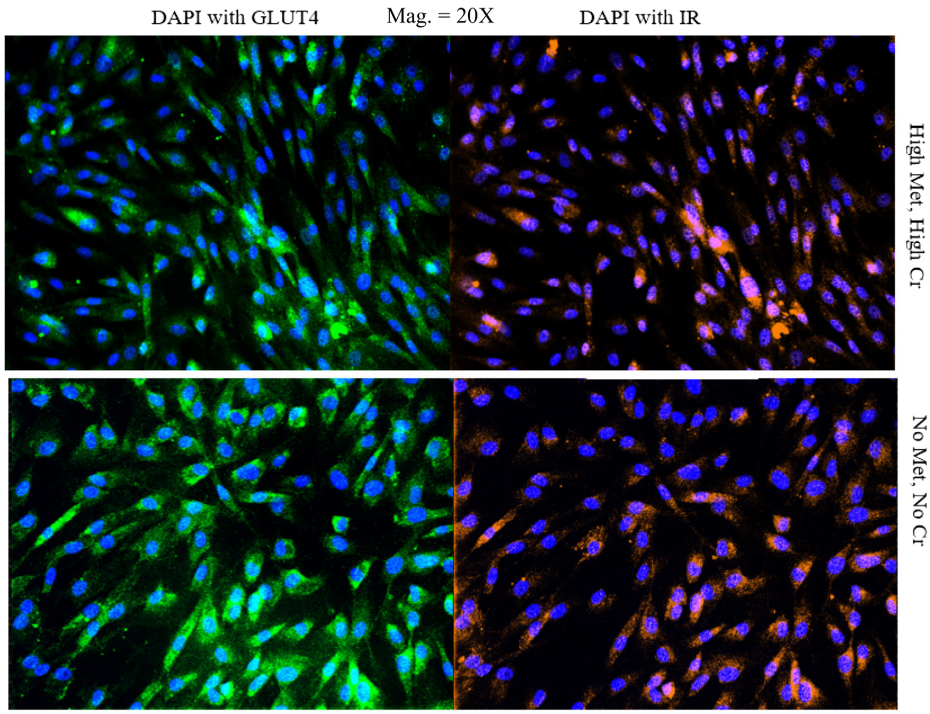

Figure 1. Immunohistochemistry (mag = 20x) staining for cells treated with 0 and 10 mg/mL of methionine and 0 and 10 µM of chromium (Cr). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). Glucose transporter 4 (GLUT4) was stained with a rabbit IgG polyclonal anti-GLUT4 (green). Insulin receptor (IR) was stained with a mouse IgG2b polyclonal anti-insulin receptor (orange).

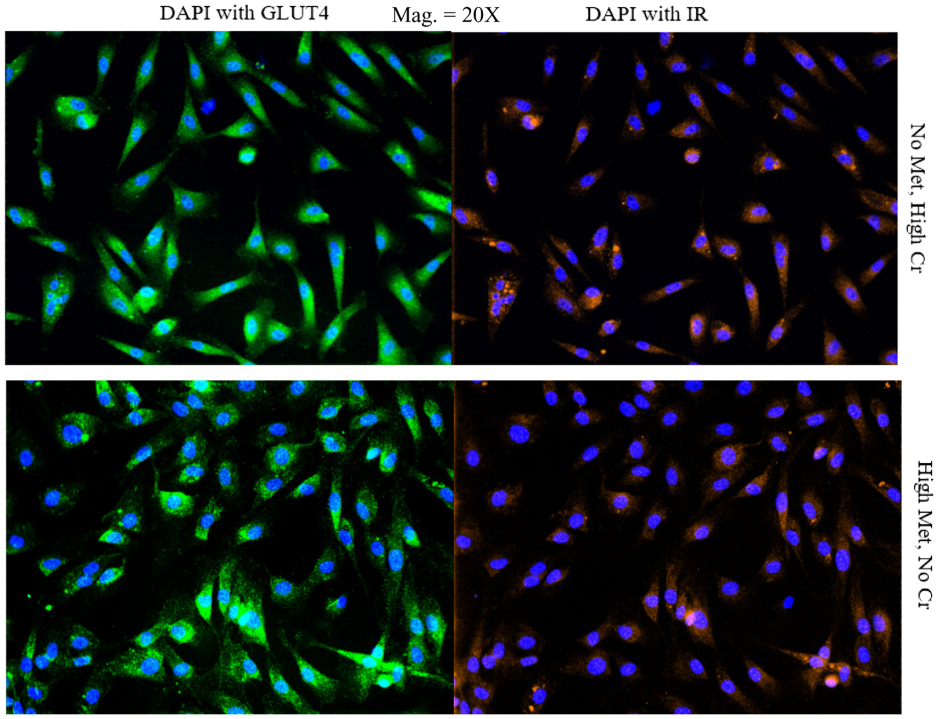

Figure 2. Immunohistochemistry (mag = 20x) staining for cells treated with 0 or 10 mg/mL of methionine and 0 or 10 µM of chromium (Cr). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). Glucose transporter 4 (GLUT4) was stained with a rabbit IgG polyclonal anti-GLUT4 (green). Insulin receptor (IR) was stained with a mouse IgG2b polyclonal anti-insulin receptor (orange).

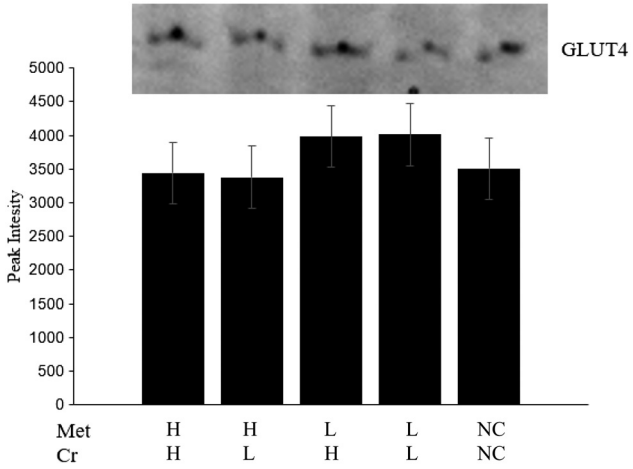

Figure 3. Effects of varying doses of methionine and chromium (Cr) on protein abundance of GLUT4 in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

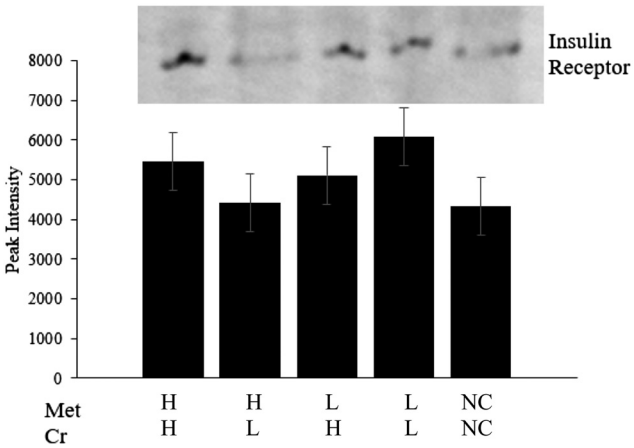

Figure 4. Effects of varying doses of methionine and chromium (Cr) on protein abundance of insulin receptor (IR) in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM chromium, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

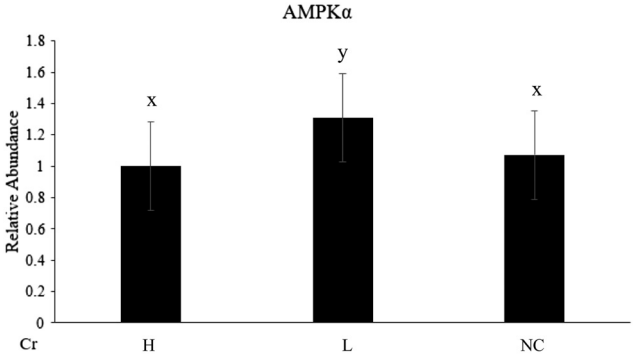

Figure 5. Effects of varying doses of chromium (Cr) on AMPKα in bovine satellite cells. Trt: "H" = 10 µM Cr, "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts tended to be different ($P < 0.1$).

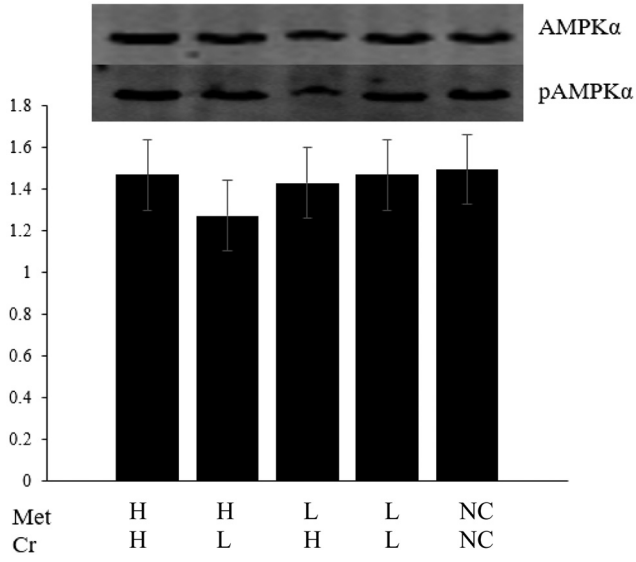

Figure 6. Effects of varying doses of methionine and chromium (Cr) on the ratio of AMPKα to pAMPKα ratio of protein abundance in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

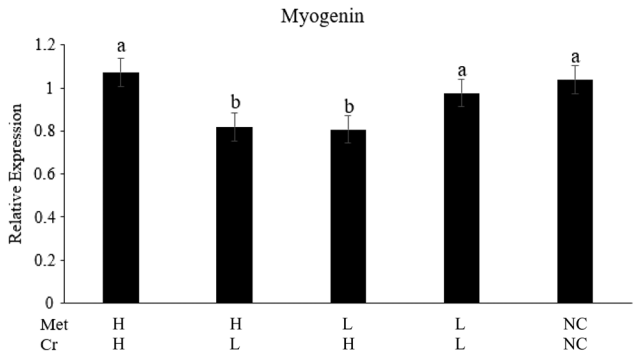

Figure 7. Effects of varying doses of methionine and chromium (Cr) on myogenin in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

All other genes measured in this study were not affected ($P > 0.1$; Figures 8–10). The ratio of AMPKα:pAMPKα, GLUT4, and IR abundance were also not affected ($P > 0.1$). Qiao et al. (2009) observed contradicting results as the inclusion of Cr in various forms upregulated the expression of GLUT4 and IR in rat L6 cells. Okada et al. (1983) demonstrated that Cr(III) specifically enhances RNA synthesis without affecting the nucleotide pool. It has been shown that Cr does have an impact on the phosphorylation of Akt (Dong et al., 2009); however, there was no difference regarding up- or downregulation ($P > 0.1$) in this study. Roos et al. (2009) also observed an unaffected

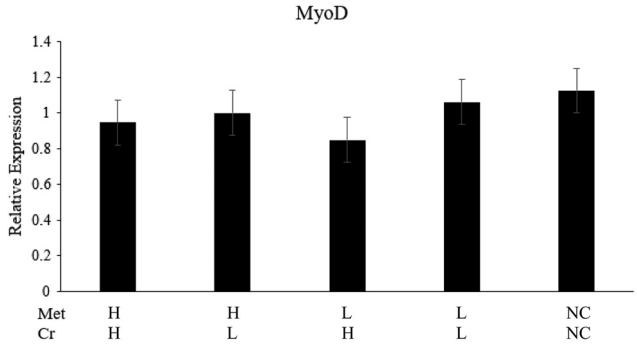

Figure 8. Effects of varying doses of methionine and chromium (Cr) on MyoD in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

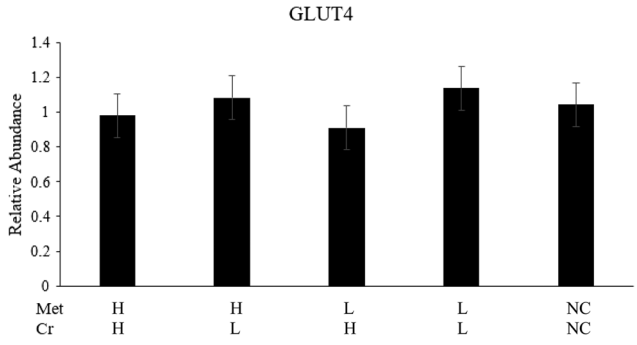

Figure 9. Effects of varying doses of methionine and chromium (Cr) on GLUT4 in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

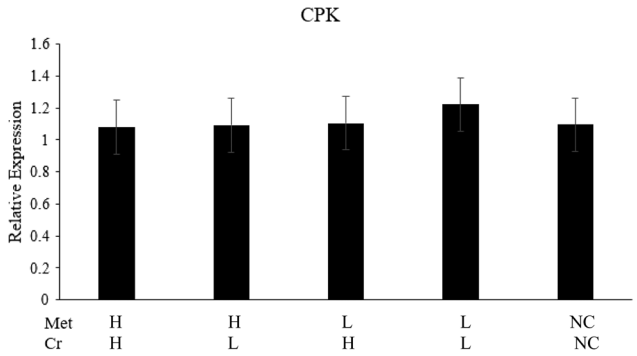

Figure 10. Effects of varying doses of methionine and chromium (Cr) on CPK in bovine satellite cells. Trt: Upper "H" = 10 mg/mL methionine, upper "L" = 1 mg/mL methionine, lower "H" = 10 µM Cr, lower "L" = 1 µM Cr, and "NC" = negative control. Bars with different superscripts were significantly different ($P < 0.05$).

AMPK pathway and expression of *S6K1* with the treatment of glucose-deprived cells. While glucose deprivation was not a treatment in the current experiment, high Cr treatments could increase glucose clearance levels (Bunting et al., 1994). Kegley et al. (2000) observed results indicating that Cr fed as chromium-L-methionine increased glucose clearance rate in steers.

Free amino acids in cultured media displayed various main effects with select interactions. An increase of time saw a significant decrease in leucine, isoleucine, threonine, methionine, phenylalanine, glutamine, ornithine, lysine, and cystine ($P < 0.01$). Over time, alanine increased ($P < 0.01$; Table 2). The inclusion of methionine as a treatment saw an increase in alanine, proline, glutamic acid, and ornithine, as well as a decrease in leucine, isoleucine, and cystine ($P < 0.01$; Table 3). Lysine was different based on methionine inclusion ($P < 0.01$). As time progressed, the need for various amino acids changes (Mary et al. 2008). New amino acids are needed for protein synthesis and therefore can be taken in by the cell via their requisite transporters. Cells also may perish and lyse their membrane, thereby releasing its contents, which can include amino acids such as the ones observed in this study. An interaction of methionine and time was observed in methionine and tryptophan concentrations ($P < 0.01$; Figures 11–12). Tryptophan concentrations in the free media decreased over time in the 0 mg/mL treatment group.

Table 2. Main effects of time on free amino acids in conditioned media from bovine satellite cells

	Time, h			
Amino Acid, ng/ml	24	48	SEM[a]	*P* Value
Alanine	17.99	32.72	1.341	<0.01
Glycine	53.27	56.90	6.725	0.69
Valine	69.94	72.56	2.296	0.42
Leucine	71.18	62.32	1.775	<0.01
Isoleucine	81.98	74.60	2.399	0.03
Threonine	95.68	52.46	7.361	<0.01
Proline	4.87	7.53	0.268	<0.01
Aspartate	1.78	2.19	0.436	0.32
Methionine	70.73	49.82	6.903	0.01
Hydroxyproline	4.61	4.01	0.422	0.37
Glutamic Acid	37.80	31.69	2.789	0.17
Phenylalanine	44.08	34.80	2.586	<0.01
Cysteine	154.3	155.63	20.00	0.65
Glutamine	0.04	0.01	0.004	<0.01
Ornithine	2.61	1.07	0.231	<0.01
Lysine	69.53	39.63	7.066	<0.01
Histidine	26.03	26.79	1.899	0.62
Tyrosine	39.04	33.62	2.442	0.09
Tryptophan	4.48	3.67	0.313	0.09
Cystine	9.03	4.93	0.567	<0.01

[a]Pooled standard error of the mean.

Table 3. Main effects of supplemental methionine on free amino acids in conditioned media from bovine satellite cells

	Methionine, mg/mL			
Amino Acid, ng/mL	0	10	SEM[a]	*P* Value
Alanine	23.42	27.30	1.341	<0.01
Glycine	52.70	57.57	6.725	0.47
Valine	75.03	67.47	2.296	0.79
Leucine	71.62	61.88	1.775	<0.01
Isoleucine	83.64	72.94	2.399	<0.01
Threonine	68.01	80.13	7.361	0.33
Proline	5.92	6.48	0.268	0.05
Aspartate	1.72	2.24	0.436	0.41
Hydroxyproline	3.78	4.84	0.422	0.07
Glutamic Acid	26.53	42.95	2.789	<0.01
Phenylalanine	39.41	39.48	2.586	0.77
Cysteine	148.85	161.13	20.003	0.97
Glutamine	0.02	0.03	0.004	0.25
Ornithine	1.06	2.69	0.231	<0.01
Lysine	6.90	102.26	7.066	<0.01
Histidine	26.56	26.25	1.899	0.81
Tyrosine	36.11	36.54	2.442	0.94
Cystine	7.42	6.55	0.567	0.07

[a]Pooled standard error of the mean.

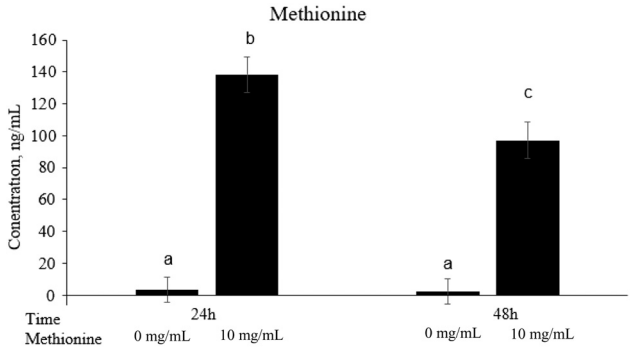

Figure 11. Effects of varying doses of methionine on free methionine in conditioned media from bovine satellite cells based on time. Bars with different superscripts were significantly different ($P < 0.05$).

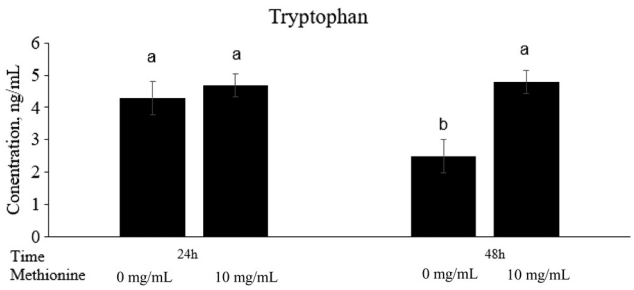

Figure 12. Effects of varying doses of methionine on free tryptophan in conditioned media from bovine satellite cells based on time. Bars with different superscripts were significantly different ($P < 0.05$).

Chromium acetate only affected 3 amino acids ($P < 0.01$; Table 4). CrAc and time interactions were present in cysteine and lysine concentrations ($P < 0.01$; Figures 13–14). Previous research has shown that Cr has effects on increasing amino acid absorption and incorporation by various cell types. Roginski and Mertz (1969) found that incorporating Cr into the drinking water of rats increased amino acid incorporation in cardiac muscle. Dong et al. (2009) showed that supplementing Cr inhibited muscle atrophy associated with muscle disuse in rat hind limbs. A potential

Table 4. Main effects of supplemental chromium (Cr) on free amino acids in conditioned media from bovine satellite cells

	Cr Dose, µM				
Amino Acid, ng/mL	0	0.1	10	SEM[a]	*P* Value
Alanine	28.21[x]	23.35[y]	24.52[xy]	1.774	0.02
Glycine	64.81	49.31	51.13	8.897	0.21
Valine	71.85	69.77	72.13	3.038	0.56
Leucine	65.95	67.49	66.80	2.348	0.83
Isoleucine	78.81	77.04	79.02	3.017	0.81
Threonine	72.42	76.51	73.28	9.738	0.81
Proline	6.47	5.99	6.13	0.355	0.40
Aspartate	1.84	2.17	1.94	0.577	0.85
Methionine	57.35	67.83	55.63	9.132	0.48
Hydroxyproline	4.08	4.79	4.06	0.558	0.49
Glutamic Acid	34.79	34.91	34.53	3.689	0.77
Phenylalanine	37.36	42.53	38.44	3.421	0.38
Glutamine	0.02[x]	0.03[x]	0.04[y]	0.005	<0.01
Ornithine	1.83	1.97	1.72	0.306	0.71
Histidine	25.46	28.28	25.48	2.513	0.53
Tyrosine	34.03	40.08	34.87	3.231	0.26
Tryptophan	3.54[x]	4.18[x]	4.52[y]	0.414	0.02
Cystine	6.24	7.81	6.88	0.750	0.15

[x–z]Within a row, means that do not have a common superscript differ ($P < 0.05$).

[a]Pooled standard error of the mean.

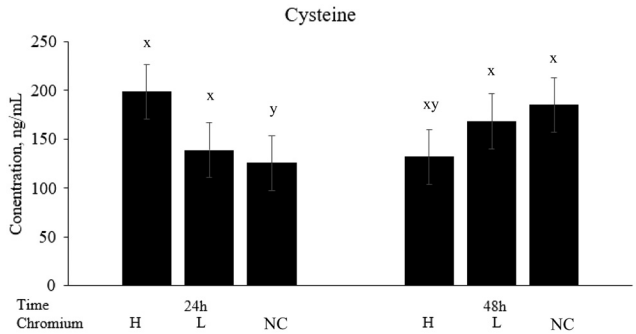

Figure 13. Effects of varying doses chromium on free cysteine in conditioned media from a bovine satellite cell. Trt: "H" = 10 µM chromium, lower "L" = 0.1 µM chromium, and "NC" = negative control. Bars with different superscripts tended to be different ($P < 0.1$).

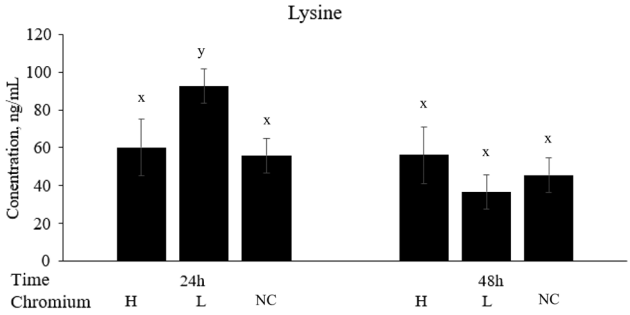

Figure 14. Effects of varying doses chromium on free lysine in conditioned media from a bovine satellite cell. Trt: "H" = 10 μM chromium, lower "L" = 0.1 μM chromium, and "NC" = negative control. Bars with different superscripts tended to be different ($P < 0.1$).

explanation of this observation is the effect Cr has on protein uptake. Chromium is known to affect the mammalian target of rapamycin (mTOR) pathway (Brautigan et al., 2006). The low-molecular-weight Cr-binding substance, known as chromodulin, potentiates the effects of insulin. Insulin is stimulated by chromodulin without altering the concentration of insulin required for half-maximal activity (Vincent, 2018). Insulin is released rapidly into the bloodstream to raise blood sugar levels. Insulin binds with the external α subunit of the transmembrane protein IR, leading to a conformational change in the receptor. This conformational change triggers the autophosphorylation of tyrosine residues on the internal portion of the receptor's β subunit, thereby activating the receptor as a kinase (Saltiel, 1994). Increasing phosphorylation of the IR is one mode of action that activates mTOR, which begins a cascade of events that ends with nutrient absorption and transcription of new proteins. This increase in protein synthesis in the cells could have increased the need for the amino acid influx in cells from this study. Free amino acids can also affect their corresponding transporters. A high concentration of methionine and lysine could affect the S and L amino acid transporter families (SAT and LAT), respectively (Hyde et al., 2003). Chromium itself could have had an effect on uptake, specifically on cysteine. The reduction of Cr(VI) to Cr(III) in natural aqueous solutions is facilitated by cysteine (Lay and Levina, 1996). By increasing Cr supplemented to the cell, the cell may have consumed cysteine to prepare for the reduction of Cr(VI) should it be necessary.

Higher levels of these amino acids in the media could increase receptor number, increasing amino acid uptake (Christie et al., 2001; Hyde et al., 2003). The SAT, which is sodium independent, can use glycine, alanine, serine, cysteine, asparagine, arginine, methionine, and proline as substrates. The LAT, which is sodium dependent, can use histidine, methionine, leucine, threonine, isoleucine, and glutamine as substrates for transport (Hyde et al., 2003). Specifically, the LAT1 (or SLC43A2) transporter also can affect mTORC1 activity by supplying leucine to the cell (Salisbury and Arthur, 2018). Milkereit et al. (2015) indicated that LAT1 associates with the cell membrane as well as mediates leucine transfer into the lysosome. Leucine is the most effective amino acid activator of mTORC1 (Saxton and Sabatini, 2017). This increase in the available substrate may have affected the amino acid transporters and allowed for an increase in amino acid absorption. The increase in leucine over time in this study could have also stimulated the mTOR pathway, thereby helping stimulate activity. Using this method in future studies would be beneficial to further elucidate the mode of action of different transporters in cultured media.

Conclusions

Chromium and methionine have been shown to affect nutrient uptake and gene expression in multiple studies. While the results from this study have not solidified the essentiality of Cr, it has been shown through novel techniques that Cr can affect specific amino acids absorbed into the cell. This study elucidates some of the effects both Cr and methionine have on bovine satellite cells. The authors of this study believe the EZ:faast quantification method on cultured media is a valuable asset to expand the quantification capabilities of researchers in the muscle biology field, but further quantification and replication are needed. Further research is needed to elucidate the full effects of these compounds in tandem and how they affect cells from other species. Live animal research can also be used to determine whether these effects are observed *in vivo*.

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