

# Zinc Alters Ractopamine HCl Response in Cultured Skeletal Muscle Cells

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## Abstract

The objective of this study was to determine if zinc, when added in combination with ractopamine hydrochloride (RH), would stabilize the interaction of RH with the  $\beta$ -adrenergic receptor, as indicated by altered cAMP concentrations, mRNA quantity, or protein abundance. Cultured bovine skeletal muscle cells were established and treated after 120 h for 6, 24, and 96 h with differentiation media of specific treatments. Treatments were applied in a factorial arrangement with two levels of zinc (0  $\mu$ M or 1  $\mu$ M) and two levels of RH (0  $\mu$ M or 10  $\mu$ M) in differentiation media. cAMP levels were measured at 6, 24, and 96 h, while mRNA and protein were measured at 24 and 96 h. At 6 h, no differences ( $P > 0.05$ ) were detected in cAMP levels between the treatments. However, at 24 h the 10  $\mu$ M RH, 1  $\mu$ M zinc treatment had the greatest concentrations of cAMP ( $P < 0.05$ ). At 96 h the 10  $\mu$ M RH, 0  $\mu$ M zinc treatment had a lower concentration of cAMP ( $P = 0.05$ ) compared to the control. No differences were detected in mRNA ( $\beta_1$ -adrenergic receptor,  $\beta_2$ -adrenergic receptor, AMPK $\alpha$ , myosin heavy chain I, myosin heavy chain IIA, and myosin heavy chain IIX) concentrations between treatments. Protein quantity of the  $\beta_1$ -adrenergic receptor and  $\beta_2$ -adrenergic receptor did not differ between treatments. These results indicate that zinc, in combination with RH, may help sustain the RH response during prolonged exposure as indicated by increased cAMP concentrations.

## Keywords

$\beta$ -Agonist, cAMP, Muscle Hypertrophy, Ractopamine Hydrochloride, Zinc

## 1. Introduction

$\beta$ -adrenergic agonists ( $\beta$ -agonists) are growth promotants commonly used in the beef cattle industry.  $\beta$ -agonists elicit a response by increasing lean tissue deposi-

tion, increasing rate of gain, and improving feed efficiency through an interaction with the  $\beta$ -adrenergic receptors [1] [2] [3]. After binding of a  $\beta$ -agonist to a  $\beta$ -adrenergic receptor, a downstream enzymatic cascade is initiated and the effects of the growth promotant will be carried out [4] [5]. The overall effect of  $\beta$ -agonists causes a shift in the nutrient partitioning of an animal from lipogenesis to muscle accretion [5] [6]. Ractopamine hydrochloride is a widely used  $\beta$ -agonist in the swine and beef cattle industries that bind with the  $\beta$ -adrenergic receptor, specifically the  $\beta_1$ -adrenergic receptor ( $\beta_1$ AR) and possibly the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR), and initiates cAMP production [7]. The increase in cAMP production initiates an enzyme cascade that results in protein accretion through increased protein synthesis and reduced protein degradation [5].

The  $\beta$ -adrenergic receptor loses functionality as total time of stimulation with an agonist increases. Pippig *et al.* [8] showed that in cell culture studies, desensitization leads to a decrease in cAMP production as a result of down-regulation of adenylyl cyclase activity. Another form of desensitization has been reported that causes the receptor to sequester within an intracellular vesicle, thus, losing its functionality to bind extracellular agents and couple with intracellular mechanisms necessary for normal activity [9] [10].

It has been shown that the  $\beta_2$ -adrenergic receptor, which is the predominant  $\beta$ -adrenergic receptor found in beef cattle skeletal muscle and adipose tissue, potentially has multiple allosteric binding sites for zinc [11]. There are two main binding sites on the  $\beta$ -adrenergic receptor; one affects the agonists ability to bind to the receptor, while the other affects the antagonists ability to bind the receptor causing an increase in cAMP production [12]. It has been postulated that zinc binding to the receptor causes a conformational change that affects receptor functionality. However, it is not known how the combination of RH and Zn would influence the  $\beta$ -adrenergic receptors ability to produce cAMP, and its regulation of downstream mRNA and protein synthesis. Our objective was to determine if Zn, when administered in combination with RH, resulted in a prolonged, extended production of cAMP that could be verified by downstream altered mRNA and protein expression.

## 2. Materials and Methods

### ***Bovine muscle satellite cell isolation.***

Satellite cell isolation was performed following previous protocols outlined by Johnson *et al.* [13]. Muscle tissue samples were extracted from the semimembranosus muscle of market age cattle at harvest using sterile techniques. The tissue was then subjected to satellite cell extraction procedures in order to isolate satellite cells from muscle tissue. Under a sterile hood, connective and adipose tissue was removed from the muscle, followed by passing the muscle sample through a sterile meat grinder that was soaked in ethanol the night before. The ground muscle is then incubated in a solution consisting of 0.1% pronase (Calbiochem, La Jolla, CA) and Earl's Balanced Salt Solution (EBSS) (Sigma, St.

Louis, MO) for 1 h at 37°C. Every 10 minutes, the samples in solution were shaken vigorously. After incubation differential centrifugation was performed on the cell solution. The solution mixture was centrifuged at  $1500 \times g$  for 4 minutes at room temperature. The supernatant was poured off and the pellet was suspended in PBS (Invitrogen, Grand Island, NY; 140 mM NaCl, 3 mM Na<sub>2</sub>H-PO<sub>4</sub>) and then centrifuged at  $500 \times g$  for 10 minutes. The supernatant was poured into another container and centrifuged at  $15,000 \times g$  for 10 minutes. The supernatant was poured into another container and centrifuged at  $15,000 \times g$  for 10 minutes to form a pellet consisting of mononucleated cells. Differential centrifugation and the PBS wash were repeated a total of two additional times. The mononucleated cells were then suspended in cold Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) that had 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY) and 10% dimethylsulfoxide (Sigma, St. Louis, MO). These cells were then stored in liquid nitrogen.

#### ***Satellite Cell Culture.***

Bovine satellite cells were cultured in either 6 well (RNA and Protein analysis) plates or 24 well (cAMP analysis) plates coated with reduced growth factor matrigel (Matrigel; BD Biosciences, Bedford, MA). The concentration of cells was approximately 0.2 g/cm<sup>2</sup>. Cells were incubated for 24 h in 10% FBS/DMEM featuring 3X antibiotic-antimycotic (Invitrogen, Grand Island, NY) and 3X gentamycin (Sigma, St. Louis, MO; growth media). After 24 h, cells were rinsed in serum-free DMEM and then the growth media was reapplied. At 120 h the cells were rinsed 3 times with serum-free DMEM and the medium was changed from proliferation to differentiation medium (3% Horse Serum [Sigma, St. Louis, MO]/DMEM-3 X antibiotic 3 X gentamycin; differentiation media) plus one of the four treatments. Preliminary data were collected from dose titrations of ZnCl and RH to establish the optimum biological effect of each molecule; these data were used to derive treatment concentrations. Treatments were arranged in a 2 × 2 factorial including control (0 μM RH/0μM Zn), 10 μM RH/0μM Zn, 0 μM RH/1μM Zn, and 10 μM RH/1μM Zn. Cells that were designated for cAMP harvest were treated for a total of 6, 24, or 96 h in differentiation media, while cells destined for mRNA and protein quantification were treated for a total of either 24 or 96 h in differentiation media.

#### ***cAMP isolation and ELISA.***

After treating for appropriate times, cells were rinsed 3 times in PBS and 0.1 M HCl was used to lyse bovine satellite cells. The cells were incubated for 5 minutes, shaken during incubation, and then the HCl solution was removed from the wells and placed into microcentrifuge tubes. The solution was removed from the wells with a pipette and was scraped vigorously to ensure all cells were removed from the bottom of the well. An enzyme-linked immunosorbent assay (ELISA; Sigma, St. Louis, MO) was performed on cell suspensions of HCl to determine cAMP concentration, following instructions provided by the manufacturer. The results were read with a plate reader (SpectraMax 340PC<sup>384</sup>, Molecular Devices, LLC, Sunnyvale, CA).

***RNA isolation.***

After 24 or 96 hours of treatment, cells from 6-well plates were harvested for mRNA analysis. Total RNA was isolated with Tri-reagent (Sigma, St. Louis, MO). After cells were rinsed 3 times in PBS, they were then subjected to incubation for 5 minutes in Tri-reagent. Total mRNA concentration was determined at an absorbance of 260 nm using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE). Following RNA quantification, all samples were DNAsed. One  $\mu\text{g}$  of RNA was reverse transcribed from each sample into cDNA by use of TaqMan Reverse Transcription Reagents and MultiScribe (Applied Biosystems, Foster City, CA). Random hexamers were used as primers for cDNA synthesis.

***Real-time quantitative PCR.***

Real-time quantitative PCR (RTQ-PCR) was performed to measure relative mRNA abundance of the  $\beta_1$ -adrenergic receptor, the  $\beta_2$ -adrenergic receptor, AMPK $\alpha$ , myosin heavy chain I (MHCI), myosin heavy chain IIA (MHCIIA) and myosin heavy chain IIX (MHCIIX). Ribosomal protein S9 (RPS9) was used an endogenous control to provide baseline values for mRNA. The solution used in the RTQ-PCR machine comprised TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM of the appropriate forward and reverse primers, 200 nM of the appropriate TaqMan forward and reverse primers, 200 nM of the appropriate TaqMan detection probe, (Table 1) and 1  $\mu\text{L}$  of cDNA mixture. The cDNA was amplified by use of the ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA) at the appropriate thermal cycling variables recommended by the manufacturer. These cycling variables were 40 cycles of 15 s at 95°C and 1 min at 60°C.

***Protein extraction.***

After either 24 or 96 h of treatment, total protein was extracted from bovine satellite cells by use of Mammalian Protein Extraction Reagent (M-PER; Pierce Biotechnology, Rockford, IL). Cells were first rinsed with PBS 3 times and then M-PER was administered to each well. Cells were incubated for 5 minutes in M-PER while shaking. The solution was removed from the wells with a pipette and was scraped vigorously to ensure all cells were removed from the bottom of the well. After collection, the cells were then centrifuged for 5 min at 14,000  $\times g$ . Once centrifuged, the protein concentration was then measured with the NanoDrop 1000 Spectrophotometer.

***Western blot analysis.***

Protein concentrations of samples were all normalized. The addition of sodium dodecyl sulfate (SDS)- $\beta$ -mercaptoethanol (Invitrogen, Grand Island, NY) was used to denature proteins and the samples were incubated in this solution for 2 min at 95°C. To separate proteins based on molecular weight, samples were run through Novex 10% - 20% Tris-Glycine Gels (Invitrogen, Grand Island, NY) for 120 min at 120 V. After separating proteins, they were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) for 120 min at 4°C. Once transfer was completed, the membranes were blocked in a

**Table 1.** Forward and reverse primers for RTQ-PCR for myogenic gene expression.

Item	Sequence (5' to 3')
AMPK $\alpha$ (accession # NM_001109802)	
Forward	ACCATTCTTGGTTGCTGAAACTC
Reverse	CACCTTGGTGTGGATTTCTG
TaqMan Probe	6FAM-CAGGGCGCGCCATACCCTTG-TAMRA
IGF-I (accession # X15726)	
Forward	TGTGATTTCTTGAAGCAGGTGAA
Reverse	AGCACAGGGCCAGATAGAAGAG
TaqMan Probe	6FAM-TGCCCATCACATCCTCCTCGCA-TAMRA
MHC-I (accession # AB059400)	
Forward	CCCACTTCTCCCTGATCCACTAC
Reverse	TTGAGCGGGTCTTTGTTTTTCT
TaqMan Probe	6FAM-CCGGCACGGTGGACTACAACATCATAG-TAMRA
MHC-IIa (accession # AB059398)	
Forward	GCAATGTGAAACGATCTCTAAAGC
Reverse	GCTGCTGCTCCTCCTCTTG
TaqMan Probe	6FAM-TCTGGAGGACCAAGTGAACGAGCTGA -TAMRA
MHC-IIx (accession # AB059399)	
Forward	GGCCCACTTCTCCCTCATTC
Reverse	CCGACCACCGTCTCATTC
TaqMan Probe	6FAM-CGGGCACTGTGGACTACAACATTACT-TAMRA
$\beta$ 1-AR (accession # AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan Probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
$\beta$ 2-AR (accession # NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan Probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
RPS9 (accession # DT860044)	
Forward	GAGCTGGGTTTGTGCGAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA

blocking solution for 1 h and primary antibody (Abcam) against the  $\beta$ <sub>1</sub>-AR and  $\beta$ <sub>2</sub>-AR were then applied. The membrane was incubated in primary antibody overnight at 4°C. After overnight incubation, the membrane was brought to room temperature (25°C) for 30 min and then rinsed 3 times in 1X concentra-

tion TBS-Tween. After rinsing was completed, the appropriate secondary antibodies (Abcam, Cambridge, MA; Fluorescent) were placed on the membrane for 1 h. After the 1 h incubation in secondary antibody, the membranes were rinsed 3 times again in 1X concentration TBS-Tween and then dried. After drying, the membranes were then viewed with QuantityOne software.

#### ***Statistical methods.***

Data were analyzed using the PROC GLM function in SAS (SAS Inst., Cary, NC). The statistical model had cAMP dependent on the fixed effects of RH, Zn, and an interaction of RH and Zn. The LSMeans procedure of SAS was used to determine significance between treatments ( $P < 0.05$ ).

### **3. Results**

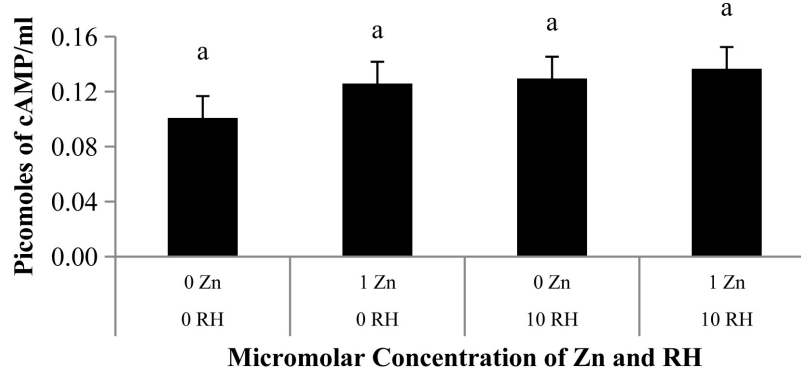
#### ***cAMP Analysis***

The study was designed to look at both acute and sustained effects of Zn and RH on stimulated cAMP production. Early on, production of cAMP at 6 hours showed no difference between treatments (**Figure 1(A)**). However, at 24 and 96 h of treatment, differences were observed among treatments (**Figure 1(B)** and **Figure 1(C)**). Cells that were provided treatments for 24 h revealed that cultures administered a combination of 10  $\mu\text{M}$  RH/1 $\mu\text{M}$  Zn produced more ( $P < 0.05$ ) cAMP than other treatments (**Figure 1(B)**). At 96 hours, more variation among treatments were found; the control group (0  $\mu\text{M}$  RH/0 $\mu\text{M}$  Zn) produced more ( $P < 0.05$ ) cAMP than cells treated with just RH (10  $\mu\text{M}$  RH/0 $\mu\text{M}$  Zn) (**Figure 1(C)**). Depicted in **Figure 1(C)** as well are the values for cAMP production by 0  $\mu\text{M}$  RH/1 $\mu\text{M}$  Zn and 10  $\mu\text{M}$  RH/1 $\mu\text{M}$  Zn which were not different ( $P > 0.05$ ) from other treatments. Compiled data over the entire 96 h period showed that in all cells administered RH, the addition of Zn sustained cAMP production relative to control (**Figure 2**). At 6 h of treatment cAMP production was nearly identical for the two treatments, however, at 24 h cells that were treated with Zn in combination produced more ( $P < 0.05$ ) cAMP than those treated with only RH. The cAMP levels grew closer together at 96 h and no difference ( $P > 0.05$ ) was observed between treatments at 96 hours of treatment.

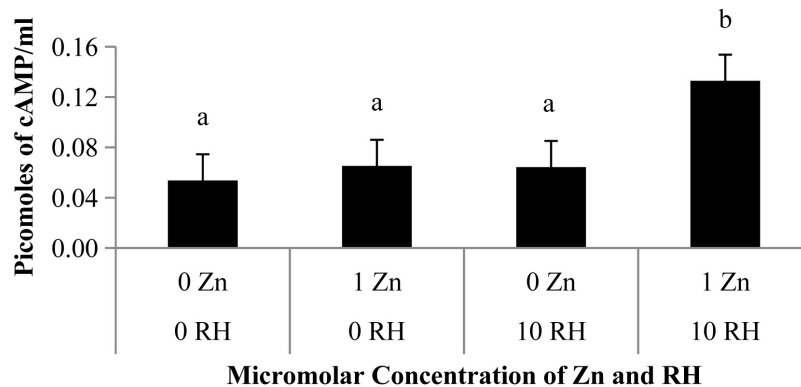
#### ***RNA and protein***

RNA quantification was performed by RTQ-PCR techniques were used to determine if effects mediated by RH would alter transcription. Genes that were quantified included the  $\beta_1\text{AR}$ , the  $\beta_2\text{AR}$ , AMPK $\alpha$ , myosin heavy chain I (MHCI), myosin heavy chain IIA (MHCIIA), and myosin heavy chain IIX (MHCIIIX). Cells were harvested after either 24 or 96 h of treatment with varying levels of RH and Zn. Different treatment levels of RH and/or Zn did not influence RNA levels, as relative quantities of genes did not differ ( $P > 0.05$ ) between treatments at 24 or 96 h (**Figure 3** and **Figure 4**, respectively).

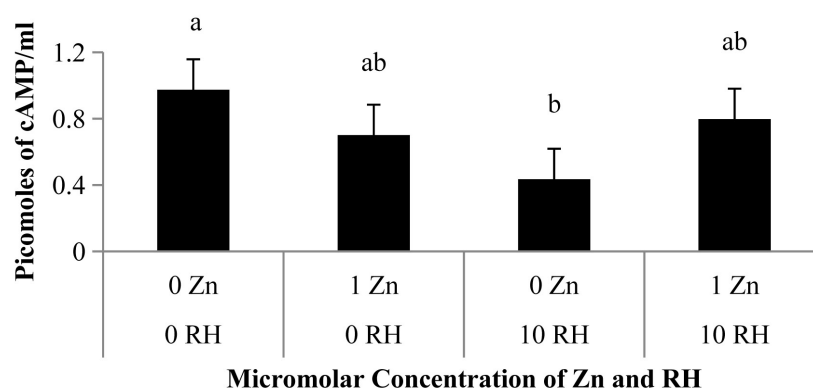
Protein analysis was performed to determine if cAMP dependent pathways were affected by RH and Zn administration. Cells were treated with varying levels of RH and Zn and harvested after either 24 or 96 h of treatment. Western



(A)

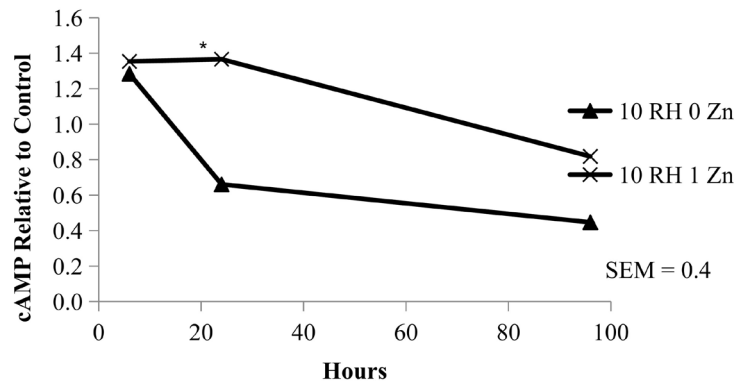


(B)

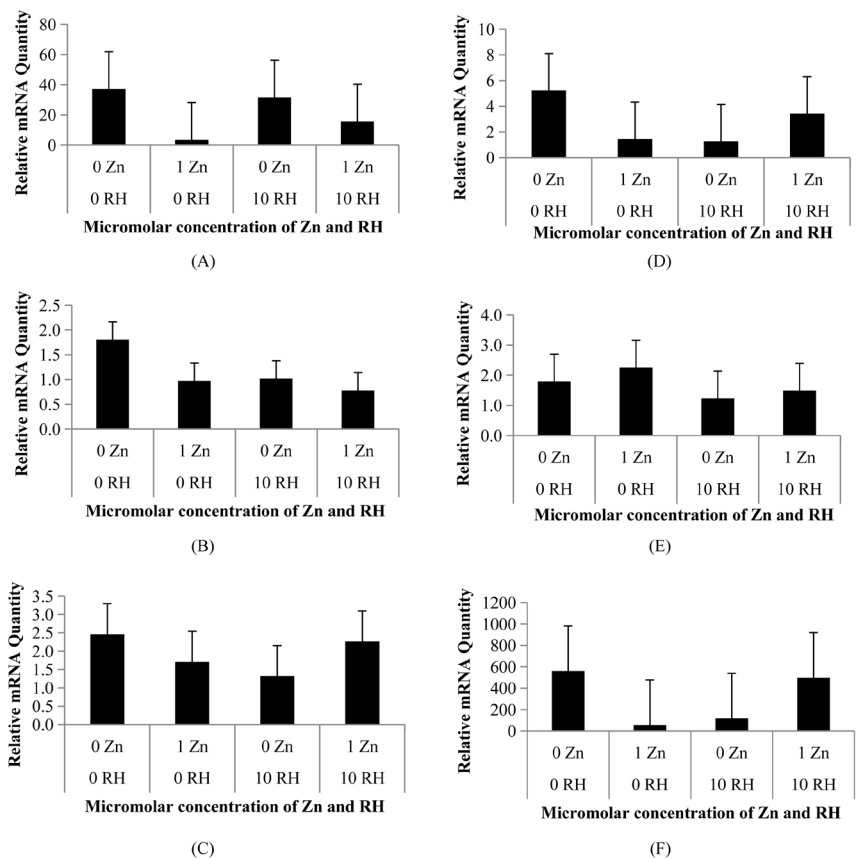


(C)

**Figure 1.** Effect of ractopamine hydrochloride (RH) and zinc (Zn) on cyclic AMP (cAMP) production in bovine satellite cells. Cells were administered RH and Zn after reaching 80% confluence. Treatments were provided along with 3% Horse Serum/Dulbecco's Modified Eagles Medium that contained 3% Antibiotic-Antimiotic and 0.3% Gentamycin. cAMP production was measured by use of an enzyme linked immunosorbent assay after (A) 6 hours of treatment, (B) 24 hours of treatment, and (C) 96 hours of treatment. Bars represent pooled standard error of the mean. There was no effect ( $P > 0.05$ ) of treatment on cAMP production after 6 hours of treatment administration.

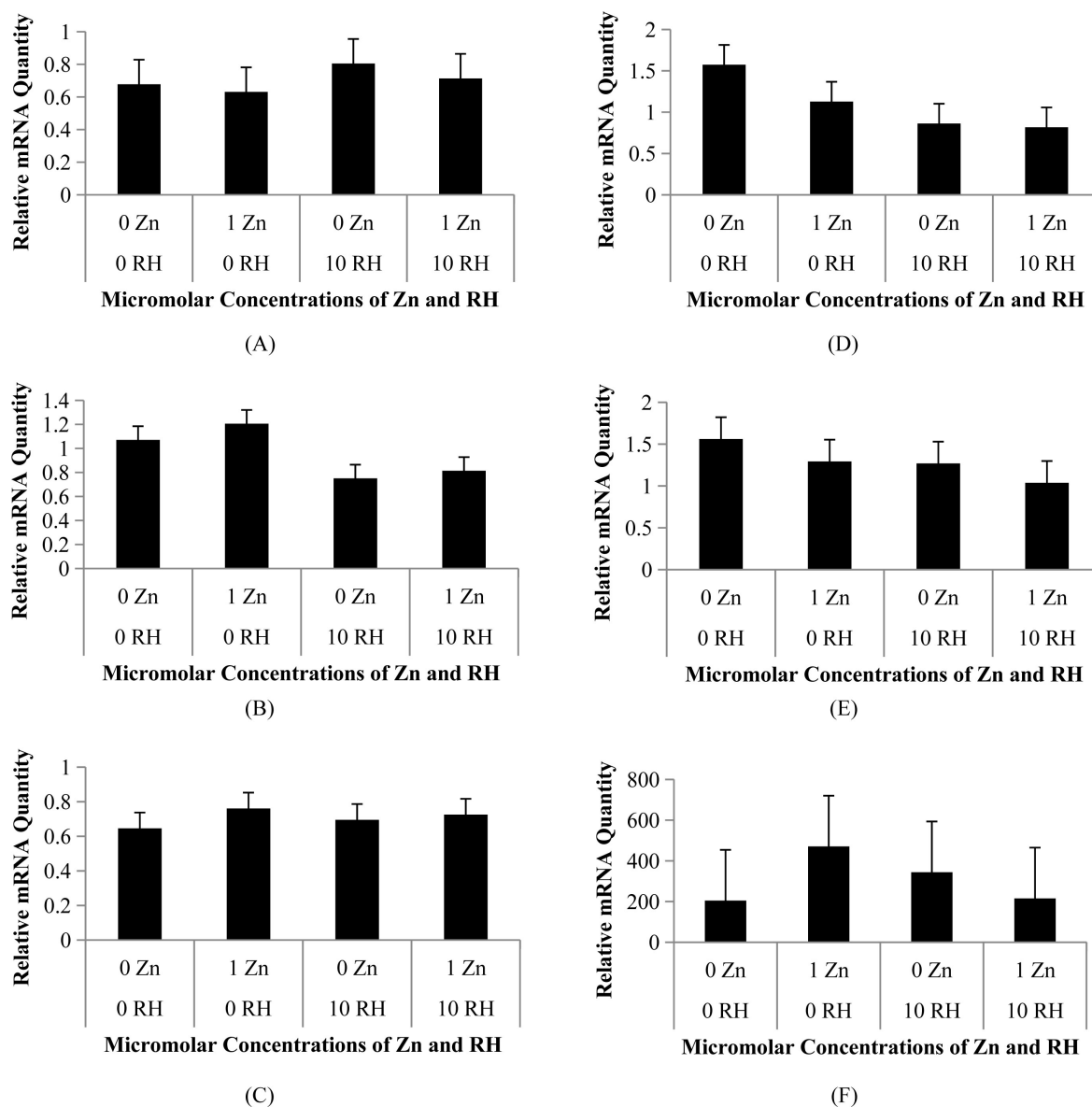


**Figure 2.** Effect of ractopamine hydrochloride (RH) and zinc (Zn) on cyclic AMP (cAMP) production in bovine satellite cells. Cells were administered RH and Zn after reaching 80% confluence. Treatments were provided along with 3% Horse Serum/Dulbecco’s Modified Eagles Medium that contained 3% Antibiotic-Antimiotic and 0.3% Gentamycin. cAMP production was measured by use of an enzyme linked immunosorbent assay after 6, 24, and 96 hours of treatment which are represented by points on the graph. Standard error of the mean (SEM) represents the largest value. Points represented by an asterisk differ ( $P < 0.05$ ) between treatments at a common time-point.



**Figure 3.** Effect of varying micromolar concentrations of zinc (Zn) and ractopamine-hydrochloride (RH) on mRNA levels of (A) the  $\beta_1$ -adrenergic receptor, (B) the  $\beta_2$ -adrenergic receptor, (C) AMPK $\alpha$ , (D) the myosin heavy chain I isoform, (E) the myosin heavy chain IIA isoform, and (F) the myosin heavy chain IIX isoform after 24 hours of treatment. Bars represent the standard error of the mean.



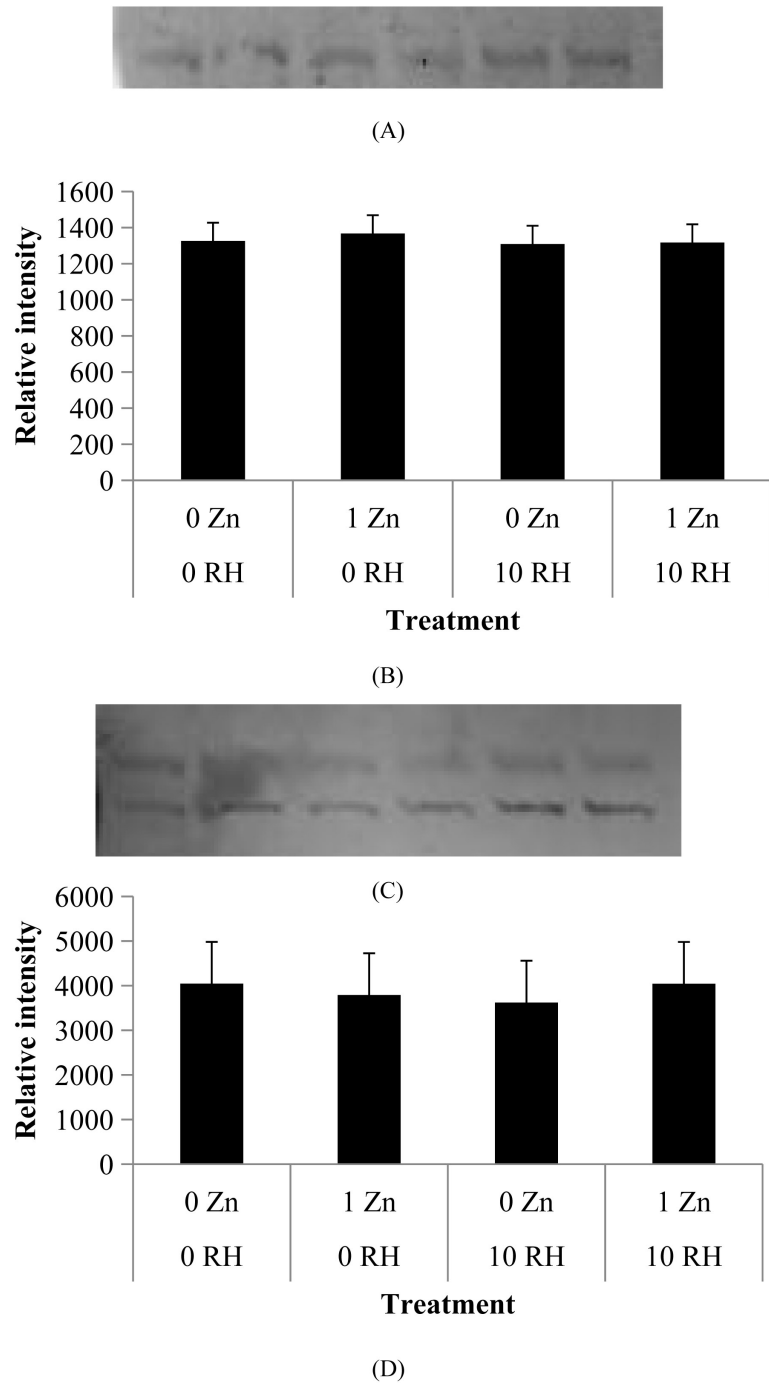


**Figure 4.** Effect of varying micromolar concentrations of zinc (Zn) and ractopamine-hydrochloride (RH) on mRNA levels of (A) the  $\beta_1$ -adrenergic receptor, (B) the  $\beta_2$ -adrenergic receptor, (C) AMPK $\alpha$ , (D) the myosin heavy chain I isoform, (E) the myosin heavy chain IIA isoform, and (F) the myosin heavy chain IIX isoform after 96 hours of treatment. Bars represent the standard error of the mean.

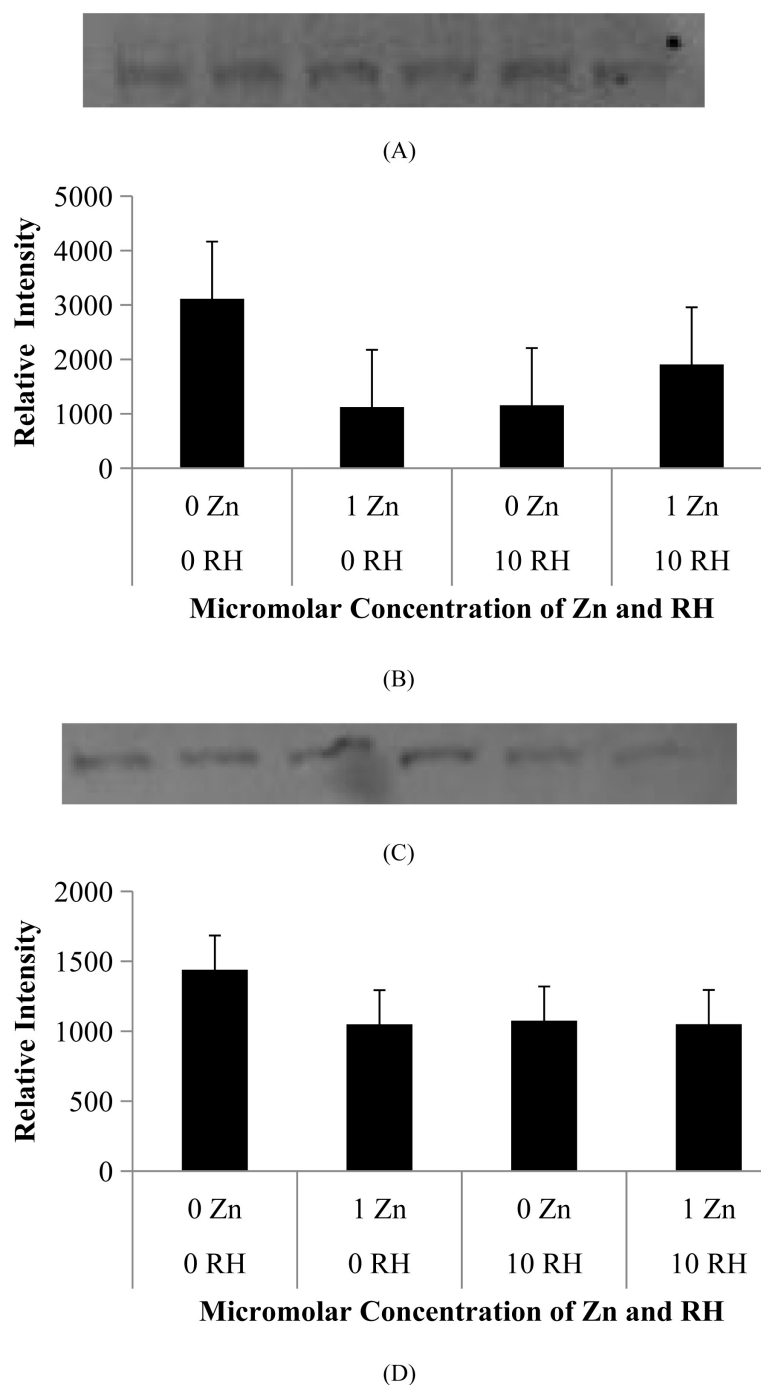
blotting procedures were conducted to quantify the  $\beta_1$ AR receptor and  $\beta_2$ AR. Protein analysis results showed that the  $\beta_1$ AR protein did not differ ( $P > 0.05$ ) depending on treatment at neither 24 nor 96 h of treatment (Figure 5(B) and Figure 6(B), respectively).  $\beta_2$ AR protein quantity was also not altered ( $P > 0.05$ ) by differing treatments after 24 or 96 h of treatment (Figure 5(D) and Figure 6(D), respectively).

#### 4. Discussion

Ractopamine hydrochloride (RH) is a widely used growth promotant in beef cattle



**Figure 5.** (A) A representative Western blot of the  $\beta_1$ -adrenergic receptor. (B) Effect of ractopamine hydrochloride (RH) and zinc (Zn) on  $\beta_1$ -adrenergic receptor protein intensity in bovine satellite cells. (C) A representative Western blot of the  $\beta_2$ -adrenergic receptor. (D) Effect of ractopamine hydrochloride (RH) and zinc (Zn) on  $\beta_2$ -adrenergic receptor protein intensity in bovine satellite cells. Cells were administered RH and Zn after reaching 80% confluence. Treatments were provided along with 3% Horse Serum/Dulbecco's Modified Eagles Medium that contained 3% Antibiotic-Antimiotic and 0.3% Gentamycin. Cyclic AMP production was measured by use of an enzyme linked immunosorbent assay after 24 hours of treatment. Bars represent pooled Standard error of the mean.



**Figure 6.** (A) A representative Western blot of the  $\beta_1$ -adrenergic receptor. (B) Effect of ractopamine hydrochloride (RH) and zinc (Zn) on  $\beta_1$ -adrenergic receptor protein intensity in bovine satellite cells. (C) A representative Western blot of the  $\beta_2$ -adrenergic receptor. (D) Effect of ractopamine hydrochloride (RH) and zinc (Zn) on  $\beta_2$ -adrenergic receptor protein intensity in bovine satellite cells. Cells were administered RH and Zn after reaching 80% confluence. Treatments were provided along with 3% Horse Serum/Dulbecco's Modified Eagles Medium that contained 3% Antibiotic-Antimiotic and 0.3% Gentamycin. Cyclic AMP production was measured by use of an enzyme linked immunosorbent assay after 96 hours of treatment. Bars represent pooled Standard error of the mean.

that increases lean muscle accretion by redirecting nutrients away from lipogenesis and towards muscle hypertrophy [4] [5]. Reports by Swaminath *et al.* [11] showed that Zn binds to the  $\beta_2$ AR and provided evidence that Zn not only binds to the receptor, but that it also causes an increase in agonist affinity, resulting in greater production of the secondary messenger cAMP. In 2003, a second study by Swaminath *et al.* [12] identified multiple possible binding sites for Zn, with the most prominent one causing an increase in agonist binding affinity and a decrease in antagonist affinity. At 6 h of treatment, results were inconclusive on whether cAMP production was affected by Zn administration with RH; however, at 24 h of treatment, an increase ( $P < 0.05$ ) in receptor activity was shown in those cells provided a combination of both RH and Zn. At 96 h of treatment, a possible desensitization response was observed. Cells administered RH alone produced less ( $P < 0.05$ ) cAMP than those that were not treated with RH or Zn. Cells treated with Zn, or a combination of RH and Zn, did not differ from any other treatments at 96 h, concluding that cAMP production may be sustained due to the potential allosteric binding effect of Zn, or other potential downstream regulation. Furthermore, when data is compiled from the three different time period treatments and cAMP production is compared over time, activity of the  $\beta$ AR appears to be maintained by Zn when comparing the 10  $\mu$ M RH/0  $\mu$ M Zn treatment to the 10  $\mu$ M RH/1  $\mu$ M Zn treated cells. This sustained production of zinc, along with data reported by Sawminath *et al.* [11] [12] leads to the conclusion that it is possible that zinc's interaction with the  $\beta$ AR stabilizes it and keeps it from becoming insensitive to agonist binding.

The mRNA and protein data results were not as conclusive as the cAMP results. Genes measured by RTQ-PCR were the  $\beta_1$ AR, the  $\beta_2$ AR, AMPK $\alpha$ , MHC I, MHC IIA, and MHC IIX; protein levels quantified by Western blotting were the  $\beta_1$ AR and the  $\beta_2$ AR. In order to determine if there was a short-term effect of cAMP, gene transcription and protein quantity were measured after 24 h of treatment. There were no differences ( $P > 0.05$ ) in gene relative quantity between treatments and no differences ( $P > 0.05$ ) in protein intensity. To determine if there were long term effects of cAMP on gene or protein levels, RTQ-PCR and Western blots were performed at 96 h of treatment as well. The same genes and proteins were measured at 96 h as were measured at 24 h. Consistent with the short-term data, genes measured at 96 h of treatment did not differ ( $P > 0.05$ ) depending on treatment.

Miller *et al.* [14] showed similar  $\beta$ AR mRNA results in bovine satellite cells treated with zilpaterol hydrochloride (another  $\beta$ -agonist). In a dose titration of zilpaterol hydrochloride, Miller *et al.* did not see a difference in  $\beta_1$ -AR or  $\beta_2$ -AR mRNA levels, however, in another trial the group compared how zilpaterol hydrochloride and ICI (a  $\beta_2$ AR antagonist) treatments affect bovine satellite cells. The results indicated that RH showed an increase in mRNA abundance of the  $\beta_2$ AR, but no difference in  $\beta_1$ AR mRNA abundance when compared to control steers [15]. In separate study, Winterholler *et al.* [16] showed that there was a

tendency for RH to increase  $\beta_1$ AR mRNA. In exception to the study performed by Winterholler *et al.* [16], a live animal study looking at the efficacy of RH in feedlot heifers showed that there was no effect of RH on  $\beta_1$ AR mRNA, however,  $\beta_2$ AR mRNA tended to increase with RH feeding [17]. Further data has supported a decrease in the expression of  $\beta_1$ AR mRNA in steers fed RH in differing muscles [18] [19]. Walker *et al.* [19] also observed a reduction in  $\beta_2$ AR mRNA in steers when fed RH for 14 days, however, after feeding RH for 28 days an increase in  $\beta_2$ AR mRNA was observed.

While our findings did not show any differences in RH driven myosin heavy chain isoforms, it has been reported that RH had differing effects when fed at varying levels on myogenic mRNA in the semimembranosus muscle (SM) of cull cows fed RH [20]. When fed at 100 mg/head/day, RH decreased  $\beta_2$ AR, MHC I and MHC IIX mRNA content in the SM, while when fed at 200 mg/head/day, RH tended to increase  $\beta_2$ AR and MHC IIX mRNA concentration in the SM [20]. Myosin heavy chain IIA mRNA expression has been shown to decrease in steers fed RH [18]. RH's effects on MHC IIA mRNA are not seen only in cattle, but in pigs as well. Depreux *et al.* [21] demonstrated that pigs fed RH expressed lower levels of MHC IIA than pigs that were not fed RH.

In contrast to the mRNA results, Miller *et al.* [14] showed protein levels for the  $\beta_2$ AR in bovine satellite cells treated with zilpaterol hydrochloride were increased when compared to the control cells. Both of these changes in mRNA were seen with administration of zilpaterol hydrochloride, a different  $\beta$ AR agonist than RH. Protein analysis performed on porcine muscle showed that RH did not affect protein  $\beta$ AR number in skeletal muscle [22].

Zinc has been reported to increase performance in cattle supplemented with various concentrations of the mineral [23] [24]. Performance variables that were affected by zinc supplementation included increased ADG, decreased feed to gain, heavier hot carcass weights, and greater dressing percentage [23] [24]. Zinc has not been associated with increased performance in cattle that were fed RH with supplemental Zinc [25]. Steers fed RH and supplemented with zinc at 159.73 mg/kg (DM basis) versus steers fed RH and not supplemented with zinc at 49.82 mg/kg (DM basis) did not differ in performance characteristics [25]. However, the bioavailability of zinc at the muscle fiber level is unknown and a larger difference in DM zinc concentration may have been required to elicit an effect of zinc on performance.

From our results, the  $\beta$ AR appears to be affected by zinc treatment in combination with RH. Zinc's interaction with the  $\beta$ AR causes a maintained production of cAMP over 96 h of treatment. Since the receptor's function is mediated through use of this secondary messenger, an increase in mRNA and protein levels that are affected by the receptor would be expected as other research has shown that [14] [15] [16] [22]. It is possible that our results did not show this because it takes longer than the maximum length trial (96 h) to elicit a response in mRNA and protein abundance. It is also possible that an unknown mechan-

ism is responsible for a change in mRNA and protein levels.

As our *in vitro* results indicated, the use of Zn in combination with RH could have benefits for enhanced growth response with RH feeding. An increase in total cAMP production over the RH feeding period could prove to have many positive effects *in vivo*. A reduction in  $\beta$ AR desensitization may lead to a new, and possibly better, feeding program for RH in beef cattle.

## Supported

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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