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Understanding the influence of trenbolone acetate and polyamines on proliferation of bovine satellite cells

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ABSTRACT

Approximately 90% of beef cattle on feed in the United States receive at least one anabolic implant, which results in increased growth, efficiency, and economic return to producers. However, the complete molecular mechanism through which anabolic implants function to improve skeletal muscle growth remains unknown. This study had 2 objectives: (1) determine the effect of polyamines and their precursors on proliferation rate in bovine satellite cells (BSC); and (2) understand whether trenbolone acetate (TBA), a testosterone analog, has an impact on the polyamine biosynthetic pathway. To address these, BSC were isolated from 3 finished steers and cultured. Once cultures reached 75% confluency, they were treated in 1% fetal bovine serum (FBS) and/or 10 nM TBA, 10 mM methionine (Met), 8 mM ornithine (Orn), 2 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Initially, a range of physiologically relevant concentrations of Met, Orn, Put, Spd, and Spe were tested to determine experimental doses to implement the aforementioned experiments. One, 12, or 24 h after treatment, mRNA was isolated from cultures and abundance of paired box transcription factor 7 (*Pax7*), Sprouty 1 (*Spry*), mitogen-activated protein kinase-1 (*Mapk*), ornithine decarboxylase (*Odc*), and S adenosylmethionine (*Amd1*) were determined, and normalized to 18S. No treatment $\times$ time interactions were observed ($P \geq 0.05$). Treatment with TBA, Met, Orn, Put, Spd, or Spe increased ($P \leq 0.05$) BSC proliferation when compared with control cultures. Treatment of cultures with Orn or Met increased ($P \leq 0.01$) expression of *Odc* 1 h after treatment when compared with control cultures. Abundance of *Amd1* was increased ($P < 0.01$) 1 h after treatment in cultures treated with Spd or Spe when compared with 1% FBS controls. Cultures treated with TBA had increased ($P < 0.01$) abundance of *Spry* mRNA 12 h after treatment, as well as increased mRNA abundance of *Mapk* ($P < 0.01$) 12 h and 24 h after treatment when compared with 1% FBS control cultures. Treatment with Met increased ($P < 0.01$) mRNA abundance of *Pax7* 1 h after treatment as compared with 1% FBS controls. These results indicate that treatments of BSC cultures with polyamines and their precursors increase BSC proliferation rate, as well as abundance of mRNA involved in cell proliferation. In addition, treatment of BSC cultures with TBA, polyamines, or polyamine precursors impacts expression of genes related to the polyamine biosynthetic pathway and proliferation.
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1. Introduction

Meat animals are raised for their skeletal muscle, which ultimately yields the marketable product in meat animal livestock production [1–3]. Muscle fiber number is predominantly fixed at birth and fiber nuclei have exited the cell cycle, making hypertrophy of existing fibers the only mechanism of postnatal muscle growth [4–6]. Because fiber nuclei do not divide, additional nuclei facilitating hypertrophy must be acquired from satellite cells [4–7]. Satellite cells are muscle precursor cells that aid in postnatal muscle growth and repair in mammals [5]. Subsequently, an increased number of satellite cells in growing muscle results in an increased capacity for skeletal muscle growth [7].

Putrescine (Put), spermidine (Spd), and spermine (Spe) are polyamines beneficial for normal cell growth and differentiation [8–13]. Polyamine concentrations in a cell are regulated by biosynthesis, interconversion, catabolism, and cellular uptake [14]. Methionine (Met), ornithine (Orn), and arginine are the major substrates utilized in the polyamine biosynthesis pathway (Fig. 1) to produce Put, Spd, and Spe [14]. In this pathway, there are 2 rate limiting steps: (1) the production of Put from Orn, catalyzed by ornithine decarboxylase (*Odc*); and (2) the production of decarboxylated S-adenosylmethionine, catalyzed by S-adenosylmethionine decarboxylase (*Amd1*) [14]. Increased polyamine concentrations have been associated with stimuli induced muscle hypertrophy [14–16]. In addition, previous research suggests androgens may play a role in the polyamine biosynthesis pathway [14,17,18], including early research showing that when castrated mice are supplemented with testosterone, the mice have increased levels of polyamines [19].

In the United States, approximately 90% of beef animals in feedlots receive at least one anabolic implant [20]. Implants increase the efficiency of muscle growth and economic return of beef cattle on feed [21,22]. Animals that receive a combined implant containing trenbolone acetate (TBA), a synthetic testosterone analog [22], and estradiol-17β (E2) have an increased number of proliferating satellite cells [21,23]. It is well established that bovine satellite cell (BSC) cultures treated with TBA or E2 have increased proliferation rates and protein synthesis in addition to decreased protein degradation [7,24,25]. Androgens also potentially play a role in satellite cell differentiation [26]. However, the mechanism through which anabolic implants improve growth of skeletal muscle is currently not understood [22]. An improved understanding of the mechanism through which anabolic implants increase satellite cell numbers is important to help develop more efficient strategies to improve beef production.

The impact that polyamines have on proliferation of BSC is currently unknown. In addition, the relationship between TBA and the polyamine biosynthetic pathway has not yet been studied in BSC. As such, the goal of this research was to determine the impact of polyamines on proliferation rates of BSC, and to also determine whether TBA impacts expression of genes involved in polyamine biosynthesis.

2. Materials and methods

2.1. Bovine satellite cell isolation

Bovine satellite cells were isolated from three different steers that were raised under Utah State University IACUC #10216. These animals had not previously received any implants and were between 18 and 20 mo of age and weighed approximately 590 kg at harvest. Animals were euthanized by captive bolt followed by exsanguination and satellite cell isolation was performed as previously described [22,27–30], with minor modifications. Using sterile techniques, approximately 1 kg of the *sternocephalicus m.* was collected and transported approximately 12 km to the laboratory. Approximately 45 m elapsed from exsanguination to initiation of satellite cell isolation. The following was conducted using aseptic techniques in a tissue culture hood. Adipose and connective tissue were

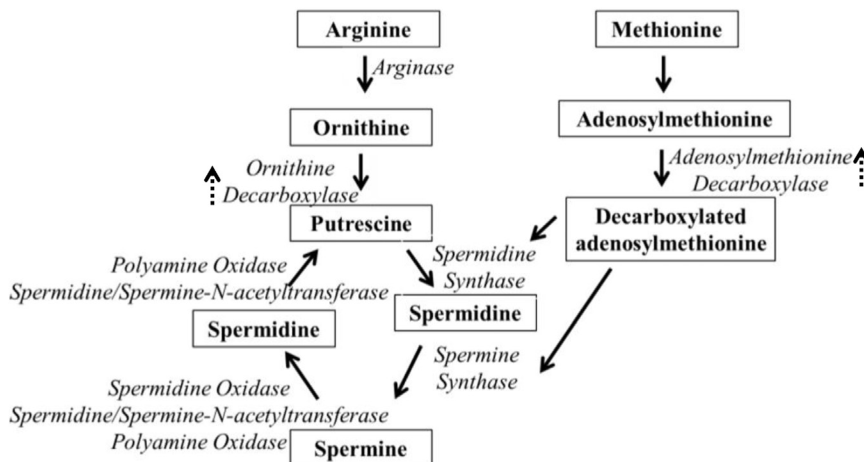

Fig. 1. Overview of the polyamine biosynthesis and interconversion pathway. Polyamines and the polyamine precursor molecules are shown in bold and enzymes are shown in italic font. The hypothesized impact of TBA is demonstrated with dashed arrows pointing up (positive impact) or down (negative impact). TBA, trenbolone acetate. Adapted from the study by Pegg et al [8].

removed, and the muscle was passed through a sterile meat grinder. The ground muscle was then incubated with a 0.1% pronase in Earl's balanced salt solution for 1 h at 37°C and mixed every 10 min. The mixture was then centrifuged at 1,500 × g for 4 min and the resultant pellets were resuspended in phosphate-buffered saline solution (PBS: 140 mM NaCl, 1.0 mM KH_2PO_4, 3.0 mM KCl, 8.0 mM Na_2HPO_4) and centrifuged again at 500 × g for 10 min. The recovered supernatant was centrifuged at 1,500 × g for 10 min to pellet the mononucleated cells. The PBS wash and centrifugation were repeated 2 more times. The mononucleated cell preparation was suspended in 4°C Dulbecco's modified Eagle medium (DMEM), containing 10% fetal bovine serum (FBS), and 10% dimethylsulfoxide, and then frozen at −80°C. The cells were stored in liquid nitrogen until subsequent use.

2.2. Bovine satellite cell culture

Bovine satellite cells were plated as previously described [27]. Briefly, BSC cultures were plated in 4 cm^2 wells that were precoated with reduced growth factor basement membrane Matrigel (Corning, Tewksbury, MA) diluted 1:50 (vol/vol). Cultures were plated at a density of 2 g/cm^2, which yields cultures that are approximately 70% confluent after 72 h in culture. Cells were plated in DMEM containing 10% FBS and incubated at 37°C with 5% CO_2 in a water saturated environment [27]. At 72 h, cultures were rinsed twice with DMEM and fresh 10% FBS was added. Cultures were grown to 75% confluency, at which time they were treated with DMEM containing 1% FBS and/or TBA, polyamines, or amino acids known to function as polyamine precursors.

2.3. Treatment of BSC cultures

Initially, a range of polyamine concentrations were selected based on previous research to determine which concentrations impact proliferation of BSC [8–10,31]. Bovine satellite cells were plated in a 96-well plate and treated with varying concentrations of polyamines or polyamine precursors in DMEM with 1% FBS. Cultures were treated with Orn (2, 4, 6, 6, 18, 12 mM), Put (1, 2, 3, 4, 5, 6, mM), Spd (0.25, 0.5, 0.75, 1.0, 1.25, 1.5 mM), or Spe (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mM). All polyamines were initially suspended in 1% PBS. Control cultures treated with 1% FBS had an equal amount of 1% PBS added. Cultures were plated as described in section 2.2. After approximately 96 h in culture, BSC were treated with polyamines or their precursors in DMEM containing 1% FBS and proliferation rates of cultures were measured as described in section 2.4.

In subsequent experiments, cultures were treated with 10 nM TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Trenbolone acetate was diluted in ethanol, as such; the 1% FBS control cultures had an equal amount of ethanol added to maintain consistency. The cells were treated once they reached 75% confluency after approximately 96 h in culture. Proliferation and mRNA abundance of these cultures were then assessed as described further in sections 2.4 and 2.5, respectively.

2.4. Analysis of proliferation rate of BSC cultures

Proliferation was assessed in cultures using a commercially available proliferation assay (DELFIA, PerkinElmer, Waltham, MA) following the manufacturer's protocol. Cells were plated in a 96-well culture plate and at 21 h after treatment, the pyrimidine analog bromodeoxyuridine (BrdU) was diluted 1:100 and added to cultures. The BrdU was allowed to incubate in culture for 3 h to label proliferating cells. The cells were then fixed and anti-BrdU was added to the plate. Cultures were incubated with the anti-BrdU for 1 h. The cells were then fluoresced and read on a BioTek all-in-one microplate reader using Gen5 2.0 all-in-one microplate reader software (BioTek Instruments, Winooski, VT).

2.5. Ribonucleic acid isolation, quantification, and cDNA synthesis

Total RNA was extracted from BSC cultures using the Absolutely RNA Microprep Kit (Agilent Technologies, Cedar Creek, TX) as per the manufacturer's protocol. Cells were lysed 1 h, 12 h, and 24 h after treatment. In brief, lysis buffer was added directly to the culture dish and a cell scraper was used to further lyse the cells. The cell lysate was then vortexed and an equal volume of 70% ethanol was added. The mixture was then centrifuged and filtered. A series of wash buffers were added and then the RNA was eluted and stored at −80°C. Isolated RNA was quantified using a Nanodrop ND-1000 UV-visible spectrophotometer (Nanodrop Technologies, Wilmington, DE), and quality was determined using the 260/280 ratio. Samples with a ratio greater than 2.0 were deemed high enough quality for cDNA synthesis. All RNA samples were treated with deoxyribonuclease (Ambion, Foster City, CA) before beginning cDNA synthesis using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol.

2.6. Quantification, cDNA synthesis, and quantitative real-time PCR

Real-time PCR quantification of mRNA was assessed using the TaqMan MGB primer/probe system and following previous described procedures [2,32]. Primer express 3.0 software (Applied Biosystems) was used to design the primers and probes for all genes. A list of primers can be found in Table 1. An ABI 7500 real-time PCR system (Applied Biosystems) was used to detect relative mRNA abundance of ribosomal 18S (*18S*), ornithine decarboxylase (*Odc*), S-adenosylmethionine decarboxylase (*Amd1*), paired box transcription factor 7 (*Pax7*), mitogen-activated protein kinase-1 (*Mapk*), and Sprouty1 (*Spry*). Ribosomal 18S was used as the housekeeping gene [2,32,33].

2.7. Statistical analysis

Statistical analysis was performed using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc, Cary, NC). All data are presented as the least square mean ± SEM. Data from multiple assays performed on cells isolated from different animals were combined. Preliminary analyses

Table 1
Primer and probe sequences used in real-time PCR.

mRNA	GBA number	Primers and probe sequences, 5' - 3′
Ribosomal 18S (*18S*)	AF243428	FP: CCACGCGAGATTGAGCAAT RP: GCAGCCCCGGACATCTAA TP: ACAGGTCTGTGATGCC
Paired box transcription factor 7 (*Pax7*)	XM_616352.4	FP: AGGACGGCGAGAAGAAAGC RP: CCCTTTGTCGCCCAGGAT TP: AAGCACAGCATCGAC
Sprouty 1 (*Spry*)	NM_001099366.1	FP: GCTTGGTCAAGGGCATCTTC RP: CCGAGTAGGAATCCCCTTCAT TP: CACTGCTCCAATGAC
Mitogen activated protein kinase (*Mapk*)	NM_175793.2	FP: GGGCTCCAGAAATCATGTTGA RP: CGACGGACCAGATGTCGAT TP: TTCCAAGGGCTACACCAA
Ornitihine decarboxylase (*Odc*)	NM_174130	FP: CTGTACTGATCCTGAGACCTTTG RP: GCTTTACATCCTCTGATCCAGG TP: ATCTCTGATGCCCGCTGTGTCTTT
S-adenosylmethionine decarboxylase (*Amd1*)	NM_173990	FP: TGCTGGAGGTTTGGTTCTC RP: TCAAAAGTATGTCCCACTCGG TP: TTGGTTTGCGTCGGGTTGCTG

Forward primer (FP), reverse primer (RP), and TaqMan probe (TP) sequence along with gene bank accession (GBA) number for the genes analyzed by using TaqMan primer probe system of real-time PCR.

indicated that there were no effects ($P > 0.05$) observed for either the assay number or the different animals, and as such, these two factors were included as random variables in the model. As samples were analyzed over time, a repeated measures analysis was used to assess treatment, time, and treatment*time interactions. The model included treatment, time, and treatment*time as fixed effects and assay number and the animal BSC were isolated from as random effects. No interactions ($P \geq 0.05$) were observed; as such all data discussed subsequently refer specifically to treatment differences. When treatment differences were found to be significant ($P < 0.05$), least square means were separated using Tukey-Kramer adjustments ($P < 0.05$). This analysis was performed for data obtained from BSC culture proliferation assays, and quantitative real-time PCR. Proliferation data are presented as the fold change of the treated cultures relative to the 1% FBS–treated control cultures set at a value of one. Gene expression analysis using TaqMan quantitative real-time PCR was performed by analyzing the relative expression of each sample calculated as $2^{-\text{relative threshold cycle } (\Delta Ct)}$.

3. Results

3.1. Proliferation rates of BSC treated with varying concentrations of polyamines

Bovine satellite cells were treated with various concentrations of polyamines and their precursors to determine which concentrations affect proliferation rate (Figs. 2-5). Cultures treated with 2, 4, 6, 10, or 12 mM Orn did not ($P \geq 0.05$) show altered proliferation rate of BSC cultures when compared with control cultures (Fig. 2). However, treatment with 8 mM Orn increased ($P < 0.05$) proliferation of BSC when compared with control cultures (Fig. 2). Treatment of BSC cultures with 1, 4, 5, or 6 mM Put had no effect ($P \geq 0.05$) on proliferation rate when compared with control cultures (Fig. 3). Cultures treated with 2 or 3 mM Put exhibited increased ($P \leq 0.05$) proliferation rate when compared with the control (Fig. 3). Exposure to 0.25, 0.5, 0.75, 1, or 1.25 mM Spd did not alter ($P \geq 0.05$) proliferation rate of BSC cultures relative to the control, but treatment with 1.5 mM Spd did result in an increased ($P < 0.05$) proliferation rate relative to the control (Fig. 4). Treatment with 0.1, 0.2, 0.3, 0.4, or 0.6 mM Spe did not affect ($P \geq 0.05$) proliferation rate of BSC cultures relative to the control (Fig. 5). However, treatment of Spd at a concentration of 0.5 mM increased ($P < 0.05$) proliferation rate relative to control cultures.

3.2. Proliferation of BSC cultures used for mRNA expression analyses

After the concentrations of polyamines and polyamine precursors necessary to affect proliferation rate of BSC cultures were determined, additionally proliferation assays were run congruently with collection of mRNA to ensure that these cultures were proliferating at the time of RNA isolation. These assays demonstrated that proliferation rates of BSC cultures treated with 10 nM TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe were increased ($P \leq 0.05$) relative to control cultures (Fig. 6), indicating that treatments did enhance proliferation rates in cultures used for analysis of mRNA abundance.

3.3. Abundance of mRNA involved in the polyamine biosynthesis pathways

Relative mRNA abundance of 2 different rate-limiting enzymes in the polyamine biosynthesis pathway, *Odc* and *Amd1*, were analyzed in proliferating BSC cultures 1, 12, and 24 h after treatment with 10 nM TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd or 0.5 mM Spe. There was no treatment*time interaction ($P \geq 0.05$) noted for any of the treatments described previously, as such only treatment differences within each time point are stated subsequently. Treatment with 8 mM Orn or 10 mM Met resulted in increased ($P \leq 0.01$) *Odc* abundance 1 h after treatment

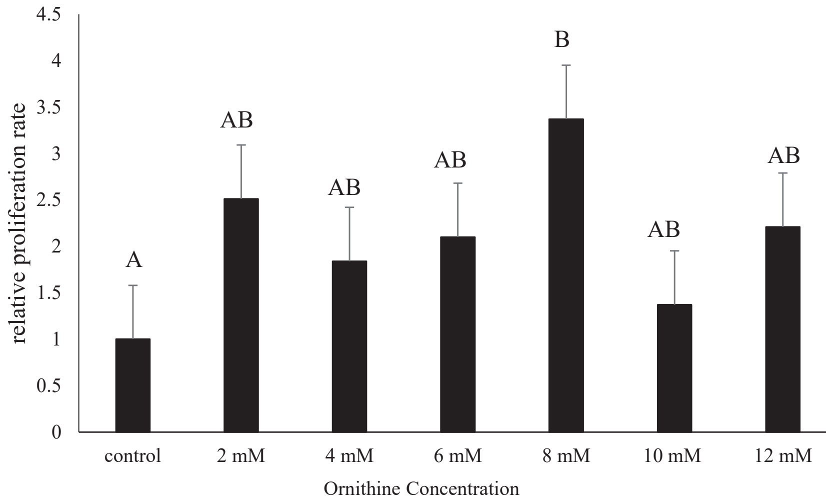

Fig. 2. Effects of increasing concentrations of ornithine (ORN) on proliferation rate of primary bovine satellite cells cultures. Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum (FBS)). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 2 mM, 4 mM, 6 mM, 8 mM, 10 mM, or 12 mM ORN. Proliferation was measured 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative proliferation rate compared with the control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

when compared with control cultures (Fig. 7). In addition, treatment with 8 mM Orn increased ($P < 0.01$) abundance of *Odc* relative to control cultures 12 h after treatment (Fig. 7). None of the other treatments, 10 nM TBA, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe had an effect ($P \geq 0.05$) on abundance of *Odc* 1, 12, or 24 h after treatment when compared with control cultures (Fig. 7). Abundance of *Amd1* was increased ($P \leq 0.05$) in BSC cultures treated with 1.5 mM Spd or 0.5 mM Spe when compared with control cultures 1 h after treatment (Fig. 8). In addition, treatment with 10 mM Met decreased ($P < 0.05$) *Amd1* abundance relative to the control cultures 24 h after treatment (Fig. 8). Treatment with 10 nM TBA, 8 mM Orn or 2 mM Put did not have an effect ($P \geq 0.05$) on *Amd1* abundance at 1, 12, or 24 h after treatment when compared with control cultures (Fig. 8).

3.4. Abundance of mRNA related to satellite cell activation

Abundance of 2 different mRNA involved in activation of satellite cells, *Spry* and *Pax7*, were also examined at 1, 12,

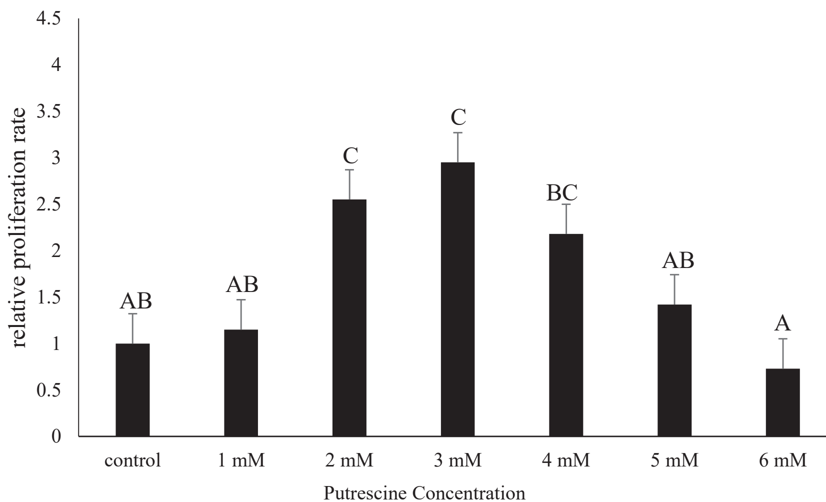

Fig. 3. Effects of increasing concentrations of putrescine (PUT) on proliferation rate of primary bovine satellite cells cultures. Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum (FBS)). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, or 6 mM PUT. Proliferation was measured 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative proliferation rate compared with the control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

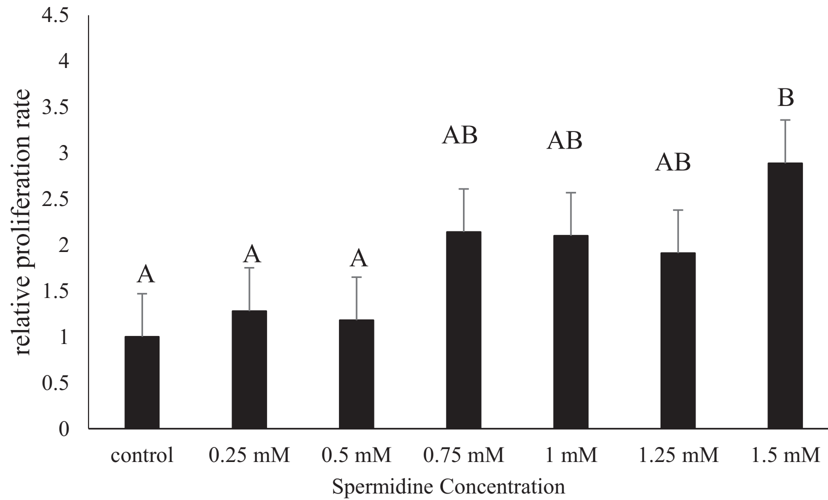

Fig. 4. Effects of increasing concentrations of spermidine (SPD) on proliferation rate of primary bovine satellite cells cultures. Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum (FBS)). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, 1.25 mM, or 1.5 mM SPD. Proliferation was measured 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative proliferation rate compared with the control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

and 24 h after treatment. There was no treatment*time interaction ($P \geq 0.05$) noted for any of the treatments described previously, as such only treatment differences within each time point are stated subsequently. No differences ($P \geq 0.05$) in *Spry* expression between any of the treatments and the control cultures were found at 1 or 24 h after treatment (Fig. 9). At 12 h after treatment, cultures treated with 10 nM TBA had increased ($P < 0.05$) abundance of *Spry* when compared with control cultures. In addition, treatment of BSC cultures with 10 mM Met increased ($P < 0.01$) *Pax7* abundance 1 h after treatment when compared with the 1% FBS control; however, at 12 and 24 h after treatment, 10 mM Met had no effect ($P \geq 0.05$) on *Pax7* abundance (Fig. 10). Treatment with 10 nM TBA, 8 mM Orn, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe did not change ($P \geq 0.05$) abundance of *Pax7* when compared with control cultures at 1, 12, or 24 h after treatment (Fig. 10).

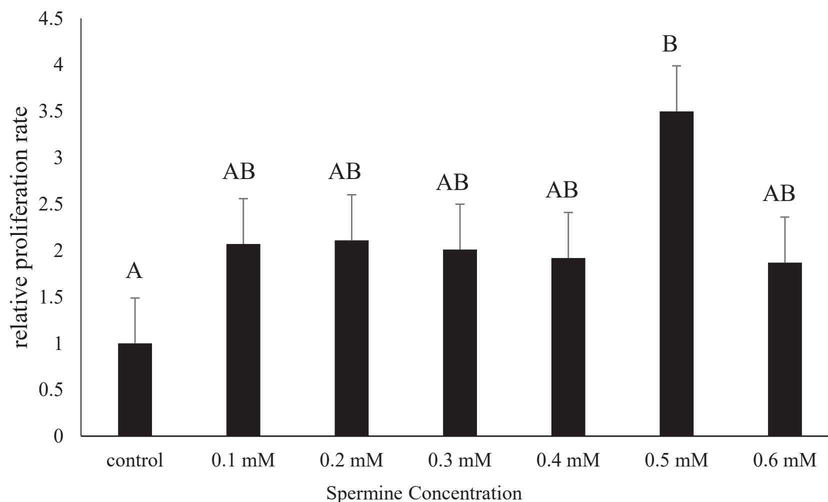

Fig. 5. Effects of increasing concentrations of spermine (SPE) on proliferation rate of primary bovine satellite cells cultures. Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum [FBS]). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, or 0.6 mM SPE. Proliferation was measured 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative proliferation rate compared with the control and are presented as LS mean ± SEM. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

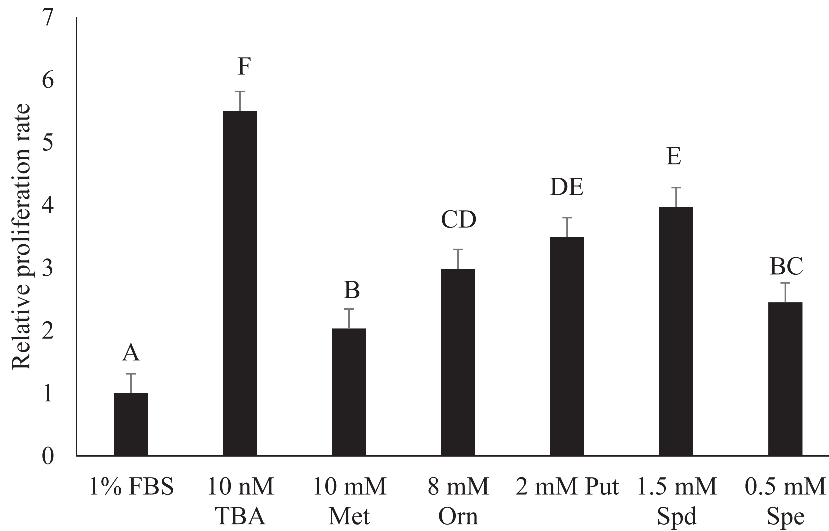

Fig. 6. Effects of 1% fetal bovine serum (FBS) and 10 nM trenbolone acetate (TBA), 10 mM methionine (MET), 8 mM ornithine (ORN), 2 mM putrescine (PUT), 1.5 mM spermidine (SPD), or 0.5 mM spermine (SPE) on proliferation rate of primary bovine satellite cells cultures. Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% FBS). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 10 nM TBA, 10 mM MET, 8 mM ORN, 2 mM PUT, 1.5 mM SPD, or 0.5 mM SPE. Proliferation was measured 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative proliferation rate compared with the control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

3.5. Messenger RNA abundance of Mapk

Abundance of *Mapk*, a marker of cell proliferation, was also analyzed in BSC cultures after treatment. There was not a treatment × time interaction ($P \geq 0.05$). When BSC were treated with 10 nM TBA, abundance of *Mapk* was increased ($P \leq 0.01$) 1 and 24 h after treatment when compared with 1% FBS control cultures; however, no differences ($P > 0.05$) were observed between control cultures and cultures treated with 10 nM TBA 12 h after application

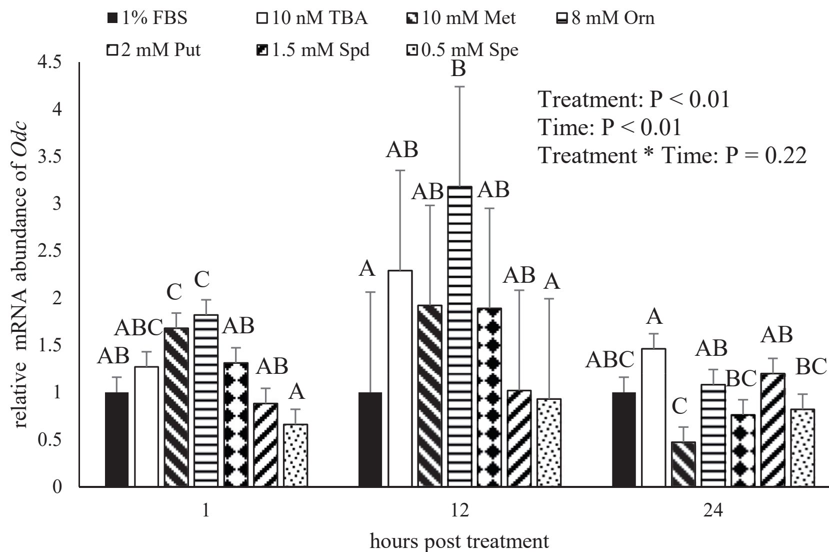

Fig. 7. Relative mRNA abundance of *Odc* from primary bovine satellite cells cultures. Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum [FBS]). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 mM ornithine (Orn), 2 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Abundance was measured 1, 12 and 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$) within each time point. Data represent relative abundance compared with the 1% FBS control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

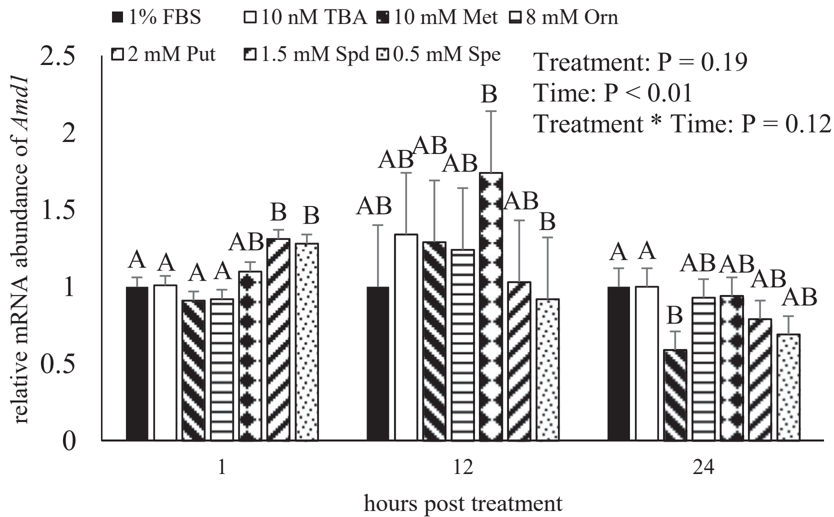

Fig. 8. Relative mRNA abundance of *Amd1* from primary bovine satellite cells cultures after treatment with 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 mM ornithine (Orn), 2 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum [FBS]). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 10 nM TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Abundance was measured 1, 12, and 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$) within each time point. Data represent relative abundance compared with the 1% FBS control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals.

of treatment (Fig. 11). However, BSC cultures treated with 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe resulted in no change ($P \geq 0.05$) in abundance of *Mapk* when compared with control cultures (Fig. 11).

4. Discussion

Despite roughly 90% of beef on feed receiving some form of anabolic implant during their lifetime [20], over 50% of consumers are concerned with exogenous hormones being used in beef production [34]. Implants cannot currently be removed from the beef industry without negatively impacting the economic and environmental sustainability of the industry [21,34]. Determining the molecular mechanism through which TBA improves the efficiency of skeletal muscle growth of beef cattle is imperative to developing improved, more efficient, consumer-accepted strategies to improve beef production, as the molecular

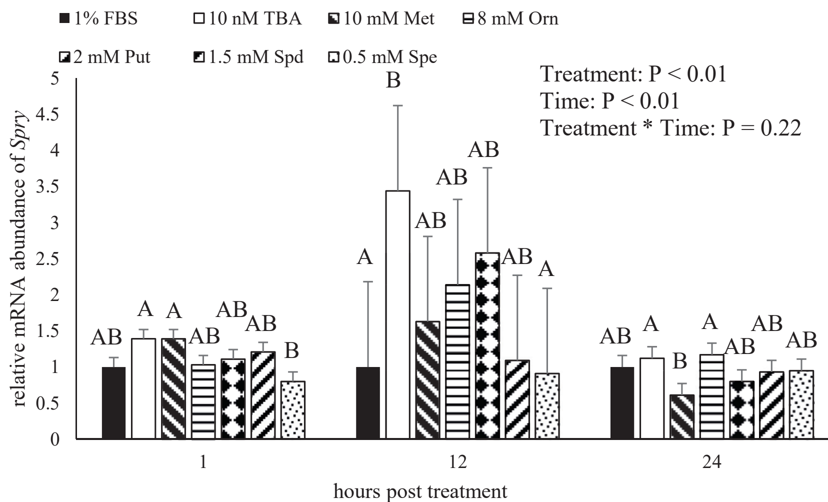

Fig. 9. Relative mRNA abundance of *Spry* from primary bovine satellite cells cultures after treatment with 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 mM ornithine (Orn), 2 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum [FBS]). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 10 nm TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd or 0.5 mM Spe. Abundance was measured 1, 12 and 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$) within each time point. Data represent relative abundance compared with the 1% FBS control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

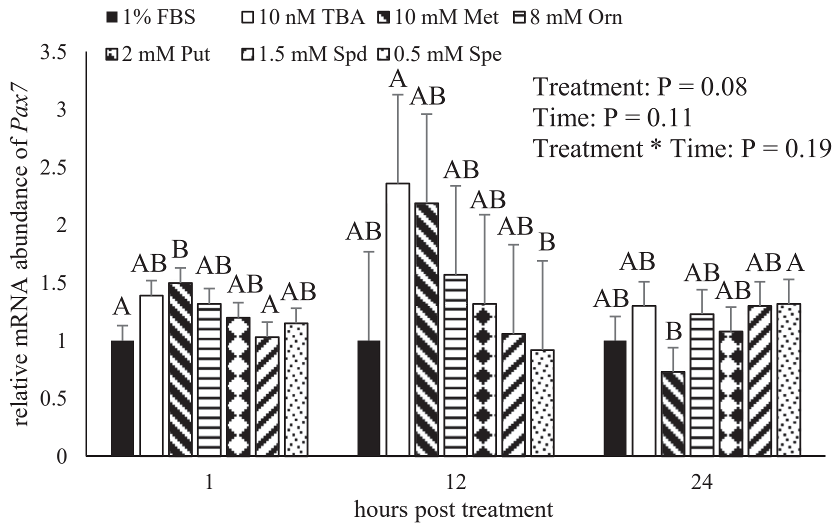

Fig. 10. Relative mRNA abundance of *Pax7* from primary bovine satellite cells cultures after treatment with 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 mM ornithine (Orn), 2 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum [FBS]). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 10 nM TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Abundance was measured one, 12, and 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative abundance compared with the 1% FBS control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

mechanism through which TBA operates is currently unknown [22]. Previous research demonstrates that testosterone may influence the polyamine biosynthetic pathway to augment growth [35–38]. A better understanding of the relationship between TBA, polyamines, and the polyamines biosynthetic pathway is necessary as polyamines are naturally occurring amino acid derivatives that increase cell proliferation [9,39,40]. If TBA increases cell proliferation by augmenting the polyamine biosynthetic pathway, then polyamines may be able to be used as an alternative means to improve growth of feedlot cattle. The research presented here investigates the interactions that TBA has with the

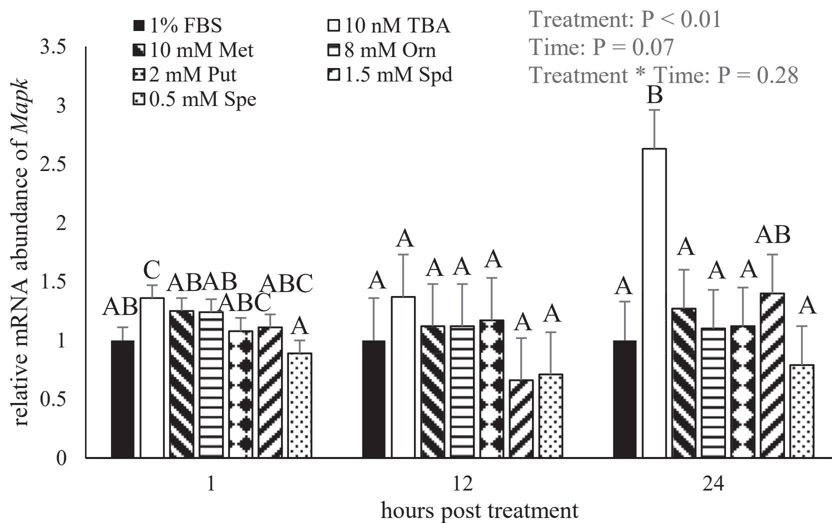

Fig. 11. Relative mRNA abundance of *Mapk* from primary bovine satellite cells cultures after treatment with 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 mM ornithine (Orn), 2 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed with DMEM and provided new growth media (DMEM +10% fetal bovine serum [FBS]). Cultures were grown to 75% confluency and treated with DMEM/1% FBS and/or 10 nM TBA, 10 mM Met, 8 mM Orn, 2 mM Put, 1.5 mM Spd or 0.5 mM Spe. Abundance was measured 1, 12 and 24 h after treatment as described in the Materials and Methods. Bars with different letter designations are significantly different from each other ($P < 0.05$). Data represent relative abundance compared with the 1% FBS control and are presented as LS mean ± SEM from 9 separate assays utilizing BSC isolated from at least 3 different animals. DMEM, Dulbecco's modified Eagle medium; BSC, bovine satellite cells.

polyamine biosynthesis pathway to improve skeletal muscle growth through proliferation of BSC and characterize abundance of mRNA related to the polyamine biosynthesis pathway, activation of satellite cells, and proliferation over time of BSC cultures which were treated with TBA, polyamines, or selected amino acids known to function as polyamine precursors.

Polyamines are small, positively charged ions [8,11], that are produced from amino acid precursors by the amino acid being decarboxylated [10,11]. Polyamines can come from both the diet, as they are orally active [10–12], and from endogenous production in tissues through the polyamine biosynthesis pathway (Fig. 1) [8,10]. The polyamine biosynthesis pathway has 2 rate-limiting steps associated with the enzymes *Odc* and *Amd1* (Fig. 1). The polyamine biosynthesis pathway produces Put, Spd, and Spe which are important regulators of cellular growth and differentiation [8–11]. In the present study, we observed that treatment of primary BSC cultures with optimal concentrations of polyamines or their amino acid precursors increased proliferation rates when compared with control cultures. To the knowledge of the authors, this is the first report of polyamines increasing proliferation rates in primary BSC cultures. The increase in proliferation suggests that polyamines do play a role in increasing skeletal muscle hypertrophy, which aligns with research showing that polyamine requirements are higher during times of extensive growth [9,12,14]. It has been well established that polyamines play an important role in cell proliferation. In fact, one of the first events that occur in proliferating cells is the induction of polyamine biosynthesis, which precedes both nucleic acid and protein synthesis [41]. However, it is important to know that optimal concentrations of polyamines must be used to have a positive impact on cell growth [42]. Research conducted in mouse mammary carcinoma FM3A cells and rat brain tumor cells demonstrates that decreasing the level of polyamines significantly inhibits cell growth [43,44]. Furthermore, several studies have found that a common feature of the effects of various growth factors and hormones on muscle cell proliferation and differentiation appears to be activation of the polyamine biosynthesis pathway [45,46].

Treatment of primary BSC cultures with TBA increases proliferation and protein synthesis rates and decreases protein degradation rates [22,24,27,28,47,48]. Similarly, in the present study, it was demonstrated that treatment of BSC with 10 nM TBA increased proliferation rate. Emerging research demonstrates that testosterone is involved in the polyamine biosynthetic pathway by modulating the enzymatic activities of *Odc* and *Amd1* [46]. When mice are castrated, they show a decrease in *Odc* activity, as well as a decrease in plasma levels of Put, Spd, and Spe [19]. When these mice are subsequently supplemented with testosterone, *Odc* activity in the skeletal muscle and concentration of polyamines in the blood increase; indicating that testosterone alters polyamine concentrations and biosynthesis [19]. Testosterone has also been shown to stimulate *Odc* and *Amd1* expression in the seminal vesicles of castrated rats [37]. When the androgen receptor is knocked out in mice, the resulting mice have decreased muscle mass, and decreased expression of *Odc* and *Amd1* [14], suggesting androgens play a role in the polyamine biosynthesis pathway. In addition, previous research demonstrates that rates exposed to resistance or endurance exercise increases endogenous testosterone production and activity of *Odc* and *Amd1* in the skeletal muscle [49].

In the present study, Orn and Met increased abundance of *Odc,* whereas Spe and Spd increased abundance of *Amd1.* This is significant as these are the 2 rate-limiting enzymes in the polyamine biosynthesis pathway. To the best of our knowledge, this is the first study in BSC to demonstrate the impacts of Orn, Met, Spe, and Spe on abundance of *Odc* and *Amd1.* However, TBA did not alter abundance of either rate-limiting step of the polyamine biosynthesis pathway. The results of this research suggest that TBA does not improve growth of skeletal muscle by altering abundance of genes that are known to be involved in the polyamine biosynthesis pathway at the time points that were tested. In the future, it could be beneficial to analyze abundance of genes involved in the polyamine biosynthesis pathway at additional time points, especially as *Odc* is known to have a rapid turnover rate, [10] which could perhaps explain some of the differences. Future research also needs to analyze activity of *Odc* and *Amd1* in primary BSC cultures. In addition, this study utilized TBA, rather than testosterone. Trenbolone acetate has a relative androgenic and anabolic activity of 3–5 and 5–8 times higher, respectively, when compared with testosterone and TBA does not get aromatized into estrogen in the body the way that testosterone can [50]. Previous research has demonstrated that estrogen regulates ornithine decarboxylase activity in several different estradiol responsive tissues/cells [51]. However, other studies demonstrate that testosterone and the androgen receptor are involved in regulation of *Odc* and thus, the polyamine biosynthesis pathway [52,53]. As such, additional research is needed to determine whether TBA is involved in the regulation of *Odc* or *Amd1* in BSC.

The present study also analyzed the effects of treating cells with TBA, polyamine precursors or polyamines on expression of genes involved in activation of satellite cells. Activation of satellite cells is closely regulated, as there are numerous states that satellite cells can be in ranging from quiescence, proliferation, continuity of the cell cycle, to differentiation [6]. Satellite cells also need to be able to maintain a reserve pool to allow for self-renewal of the satellite cell populations [5,6]. Expression of *Pax7* is involved in satellite cell activation [54–56], and *Pax7*-positive satellite cells divide in an asymmetric way for both self-renewal and production of a *Pax7*+/*Myf5*+ daughter cell. The *Myf5* daughter cell then becomes a *Myf5*+/*MyoD* + muscle progenitor cell that are capable of undergoing the differentiation process and subsequently fusing with an existing myotube to support hypertrophy of skeletal muscle [5]. In the present study, Met was the only treatment that increased expression of *Pax7.* In future studies, it would be interesting to determine whether *Myf5* expression is also increased by Met, as proliferation rates of BSC were increased when treated with polyamines, suggesting polyamines do play a role in skeletal muscle hypertrophy. Previous research in both C2C12 and L6 myoblast cells demonstrates that depletion of polyamines inhibits formation of myotubes and myofibers [57,58]. Recent research

has found that there is a decreased ratio of *Pax7:Myf5* in older animals [5], and as an animal approaches maturity, most of remaining satellite cells become quiescent and do not proliferate or differentiate unless stimulated to do so by injury or exercise [7]. The BSC used in this study were isolated from mature steers, which possibly could result in more satellite cells being in a quiescent state, resulting in TBA not having as much of an effect on expression of *Pax7*. In the future, it could be beneficial to repeat the study in younger steers to see if there is an age interaction of the dependent variables, which is a limitation of the present study. Additional research needs to be conducted to determine the role that polyamines have in activation of muscle satellite cells.

Sprouty1 is responsible for the self-renewal of quiescent *Pax7* satellite cells [59], while a general marker of cell proliferation, including satellite cells, is *Mapk* [56]. *Sprouty*1 is required for the proliferating satellite cells to return to a quiescent state, to allow for the *Pax7+* satellite cells to renew [60], thus an increase of *Spry* expression indicates a decrease in proliferating satellite cells. Therefore, *Spry* expression and *Mapk* expression should be inversely related in a pure population of cells; however, satellite cells are a heterogenic population of cells. This correlates to the results of the present research, as TBA caused increased expression of *Mapk* 1 h and 24 h after treatment, while *Spry* expression was increased 12 h after treatment by TBA. This suggests that the BSC were in a proliferative state 1 h after treatment, but by 12 h after treatment, the satellite cells entered a quiescent state to allow for the cells to renew. By 24 h after treatment, the cells were once again able to enter a proliferative phase in response to TBA. A thorough search of relevant research suggests that no previous research has analyzed expression of *Spry1* in satellite cells after treatment with androgens or polyamines. Further research needs to be completed to better understand how androgens and polyamines impacts proliferation and self-renewal of satellite cells.

To the best of our knowledge, this is the first study that has analyzed the effects of TBA in the polyamine biosynthetic pathway and the resulting effects on proliferation of primary BSC. The overall findings of this research suggest that polyamines increase proliferation rates of BSC, which in turn helps increase skeletal muscle hypertrophy; and that the involvement of TBA in the polyamine biosynthesis pathway remains unclear, as mRNA abundance of enzymes involved in the polyamine biosynthetic pathway were not altered. This research also establishes the concentrations of polyamines required to enhance proliferation in primary BSC cultures. Additional work is still needed to determine the mechanism by which polyamines impact proliferation and whether TBA is involved in this process, in hopes of understanding how implants increase skeletal muscle growth to lead to the development of more efficient and consumer accepted strategies to improve beef production.

CRediT authorship contribution statement

C.C. Reichhardt: Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **A. Ahmadpour:** Methodology, Investigation, Data curation. **R.G. Christensen:** Investigation, Data curation, Writing - review & editing. **N.E. Ineck:** Investigation, Data curation, Writing - review & editing. **G.K. Murdoch:** Conceptualization, Formal analysis, Writing - review & editing. **K.J. Thornton:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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