Nutrient restriction and migration of turkey satellite cells

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ABSTRACT Post hatch muscle growth and the repair or regeneration of muscle after myofiber injury is mediated by satellite cells. Satellite cells proliferate, migrate, differentiate, and fuse with growing or regenerating myofibers. The proliferation and differentiation of satellite cells are affected by nutrition, but it is unknown how nutrition impacts satellite cell migration. The objective of the study was to determine the effect of a nutrient restriction on satellite cell migration. Satellite cells from the pectoralis major muscle of 1 and 49-day-old Randombred Control Line 2 turkeys were grown in culture, and migration was measured using a wound healing assay. Nutrient restrictions of 0, 5, 10, and 20% of the standard culture medium were applied starting immediately after scratch or 24 h prior to scratch. Nutrient restrictions of 5 and 20% increased 1 D satellite cell migration at 6 h post scratch compared to 1 D satellite cells with standard culture medium but had no effect after 12 h post scratch. Nutrient restrictions started 24 h prior to scratch increased 1 D satellite cell migration at 6 and 12 h post scratch compared to nutrient restrictions started immediately after scratch. The migration of 49 D satellite cells was not affected by the percentage or timing of the nutrient restriction. These data suggest that nutrition has only a minor effect on the migration of turkey pectoralis major muscle satellite cells. Therefore, the influence of nutrition on satellite cell migration is likely not an important factor for evaluating poultry diet formulations to optimize muscle growth and structure for improved meat protein and fat content as well as meat texture.

Key words: migration, nutrition, satellite cell, skeletal muscle, turkey

INTRODUCTION Skeletal muscle growth and regeneration, post hatch, is mediated by the resident muscle stem cell, the satellite cell. Because myofibers exit the cell cycle post hatch (Stockdale and Holtzer, 1961), satellite cells are the critical means for which myofibers grow (Moss and Leblond, 1971) and regenerate after injury or disease (Relaix and Zammitt, 2012). Satellite cells, located between the myofiber sarcolemma and basal lamina (Mauro, 1961), proliferate and migrate to sites of growth or damage (Schultz et al., 1985, 1986; Schultz and McCormick, 1994). At the site of growth or injury, satellite cells fuse together to form myotubes and fuse with the growing or injured myofiber (Stockdale and Holtzer, 1961; Moss and Leblond, 1971; Pallafacchina et al., 2013). Each of these processes is vital to satellite cell mediated muscle growth and regeneration.

Satellite cell migration is an essential part of skeletal muscle growth and muscle regeneration after injury or disease. To mediate muscle hypertrophy and regeneration, satellite cells migrate from undamaged regions of muscle to damaged regions (Schultz et al., 1985; Hurme and Kalimo, 1992) and can migrate throughout the entire muscle (Morgan et al., 1987, 1990; Alameddine et al., 1989). Additionally, if satellite cell migration is inhibited, then myotube formation and size are decreased, and muscle regeneration after damage is obstructed (Cornelison et al., 2004; Mylona et al., 2006; Bae et al., 2008). Muscle damage due to myofiber necrosis and degeneration occurs in both broiler chickens (Kuttappan et al., 2013; Sihvo et al., 2014) and turkeys (Sosnicki and Wilson, 1991; Sosnicki et al., 1991). Furthermore, recent studies suggest that some myopathies with myofiber necrosis have insufficient satellite cell-mediated regeneration to repair the muscle fibers to their original state (Papah et al., 2017; Velleman et al., 2018b).

Satellite cell activity is affected by nutrient availability leading to changes in muscle gain and morphological structure. Nutrient availability decreases satellite cell proliferation and differentiation (Halevy et al., 2000, 2003; Powell et al., 2013; Harthan et al., 2014; Velleman et al., 2018a), leading to decreased BW and pectoralis major (p. major) muscle weight (Halevy et al., 2000, 2003; Mozdziak et al., 2002; Moore et al., 2005; Velleman et al., 2014a). In addition to muscle mass accretion changes, muscle composition and morphology
are also affected. Nutrient restrictions during the first week post hatch have been shown to lead to increased myofiber necrosis, increased adipose deposition, and altered myogenic gene expression controlling satellite cell proliferation and differentiation at market age (Velleman et al., 2010a, 2014a,b). Velleman et al. (2014a,b) showed if the administration of a feed restriction was moved to the second week post hatch, the effects on myogenic gene expression, intramuscular adipose deposition, and muscle morphological structure were not present. Thus, the closer nutrient restrictions are to hatch, the greater the impact on muscle growth, market age weights, muscle morphology, and composition (Halevy et al., 2000; Velleman et al., 2014a,b).

Nutrient availability is altered as part of poultry industry management practices, including feed deprivations and restrictions. Feed deprivations immediately after hatch occur during the processing and transportation of chicks and poults to grower facilities (Noy and Sklan, 1999). Feed restrictions are used to minimize metabolic diseases such as ascites (Arce et al., 1992) or skeletal issues such as tibial dyschondroplasia (Lilburn et al., 1989; Su et al., 1999). Recently, feed restrictions have shown promise as a potential management strategy to minimize the incidence and severity of degenerative myopathies in poultry (Troccino et al., 2015; Cruz et al., 2016; Radaelli et al., 2016; Meloche et al., 2018a). However, feed restrictions used to minimize degeneration while at the same time optimizing muscle growth has been shown to be difficult (Meloche et al., 2018b).

Given the importance of satellite cell migration in skeletal muscle growth and regeneration and the prevalence of feed restrictions in poultry management, the objective of the current study was to examine the effect of nutrient availability on satellite cell migration. A further objective of the current study was to investigate if the response of satellite cell migration to nutrient availability was age dependent. This objective was pursued because bird age has been shown to influence satellite cell migration (Collins-Hooper et al., 2012) as well as the proliferation and differentiation of satellite cells in response to nutrient availability (Powell et al., 2013; Harthan et al., 2014; Velleman et al., 2018a). The results of this study will provide information as to the effect of nutrient availability on satellite cell migration including age effects. This information could assist in the formulation of diets to optimize skeletal muscle growth and minimize degenerative myopathies, leading to a higher quantity and quality of poultry meat.

**MATERIALS AND METHODS**

**Turkey Myogenic Satellite Cells**

Satellite cells were previously isolated from the p. major muscle of 1 and 49 D post hatch male randombred control 2 (RBC2) line turkeys as described in Velleman et al. (2000, 2010b). Isolated cells were expanded and stored in liquid nitrogen until use for experiments. The RBC2 line represents a 1967 commercial turkey that has been maintained at The Ohio State University, Ohio Agricultural Research and Development Center Poultry Research Unit without conscious selection for any traits (Nestor et al., 1969). To avoid the effect of sex (Velleman et al., 2000; Song et al., 2013), only male satellite cells were used in the current study.

**Satellite Cell Culture**

**Plating of Satellite Cells** Satellite cells were plated and cultured for 24 h prior to experimental treatments to allow cell attachment to the plate surface. Satellite cells were plated in 48-well cell culture plates (Greiner Bio-One, Monroe, NC) coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) at 30,000 cells per well. Cells were plated in medium composed of Dulbecco’s Modified Eagle's Medium (Sigma-Aldrich) with 10% chicken serum (Sigma-Aldrich), 5% horse serum (Sigma-Aldrich), 1% antibiotic antimycotic solution (Sigma-Aldrich), and 0.1% gentamicin (Omega Scientific, Tarzana, CA), and cultured at 38°C in a 95% air/5% CO2 incubator (Thermo Fisher Scientific).

**Nutrient Restrictions** After allowing 24 h of cell attachment, the plating media was replaced with growth media composed of McCoy’s 5A medium (Sigma-Aldrich) with 10% chicken serum, 5% horse serum, 1% antibiotic antimycotic solution, and 0.1% gentamicin. Growth media was replaced every 24 h of proliferation. To test if the timing of nutrient restriction affected cell migration, 2 nutrient restriction regimens were performed. The first regimen was started 24 h prior to creating the wound (24 h prior to scratch), and the second was started immediately after wounding (0 h prior to scratch). For nutrient-restricted growth media, the McCoy’s 5A medium and the serum were replaced with Dulbecco’s phosphate-buffered saline with calcium and magnesium (Thermo Fisher Scientific) by 5, 10, or 20% of their concentration in the standard medium. Nutrient-restricted media was replaced every 24 h until the experiment was completed.

**Wound Healing Assay**

Cell migration was measured using a wound healing assay based on Shin et al. (2013). After 72 h of proliferation in growth media, the wells had 95 to 100% cell confluence. At this time, a single scratch in the cell monolayer of each well was created using a 200 μl pipet tip. Cell migration was imaged using an Olympus IX70 microscope (Olympus America, Center Valley, PA) with a QImaging digital camera (QImaging, Burnaby, BC, Canada) at a magnification of 100× at 0, 6, 12, 24, and 30 h post scratch. To create a reference point for repeated imaging of cell migration, a scratch was made on the bottom of the plate on the outside of the well, before plating the cells. This way the same region could
be imaged over all the sampling times. Five to twelve images per well were taken at each imaging time point. Two independent experiments were performed at each age with 4 replicate wells per percent nutrient restriction and timing condition.

**Wound Width Measurements** The wound width at each imaging sampling time was measured manually using ImageJ (National Institutes of Health, Bethesda, MD). The wound width was determined using crude integration, by measuring the wound area (i.e., plate surface not containing cells) and dividing by the length of the straight line along the length of the wound (i.e., parallel to the direction of the scratch wound). For each image, the migration distance was determined by subtracting the wound width at sampling time, \( t \) (6, 12, 24, and 30 h) from the initial wound width (0 h). Migration distance was calculated for each image and then averaged for each well.

To minimize human error and possible plate artifacts, a randomized plate design was used. The location within the plate of each treatment condition was determined by a random number generator (Haahr, 2019). Each treatment location within the plate was blinded during imaging and wound width measurements.

**Statistical Analysis**

Data from replicate experiments were combined and analyzed. An individual well was defined as the experimental unit. Statistical analysis was performed on the average migration distance at each sampling time as well as on the average width of the initial wound using the MIXED procedure of SAS Version 9.4 (SAS Institute Inc., Cary, NC). The model included the main effects of percentage nutrient restriction and timing of nutrient restriction as well as their interactions. Means and standard error of the mean were determined with the least square means (lsmeans) statement. The pdiff option was used to separate the interaction means. A \( P \) value of 0.05 or less was defined to be statistically significant.

**RESULTS AND DISCUSSION**

In the current study, p. major muscle satellite cells from 1-day-old RBC2 poults were subjected to either standard nutrient conditions or a 5, 10, or 20% nutrient restriction during a wound healing assay (Figure 1). Because satellite cell migration during a wound healing assay has been shown to be correlated with the initial wound width (Marom et al., 2019), statistical analysis was performed to determine if there were any differences in initial wound width with nutrient conditions. The initial wound width was not significantly different with percentage or timing of nutrient restriction (data not shown). Therefore, any differences in satellite cell migration between nutrient restriction conditions were not due to differences in initial wound width.
After the wound was created, satellite cells migrated into the vacant space to close the wound over a 30 h period post scratch (Figure 1; arrows). At 12 h post scratch, the wound closure appeared to be dominated by collective cell migration, as the satellite cells were highly aligned and closely associated. By 30 h post scratch, satellite cells appeared to separate and have reduced alignment with their neighbors, leading to more individual satellite cell migration (Figure 1; arrowheads). These 2 types of migration have been observed in satellite cells on isolated myofibers, in which both sister and unrelated satellite cells were observed migrating individually or in clusters (Siegel et al., 2009; Otto et al., 2011).

Quantitative analysis of wound closure showed that 1 D satellite cell migration was altered by nutrient availability at 6 h post scratch, but not during later sampling times (Figure 2). At 6 h post scratch, the percentage of nutrient restriction had an effect \( (P = 0.0480) \) on the 1 D satellite cell migration. Specifically, the 1 D satellite cells migrated a greater distance with 5\% \( (P = 0.0545) \) and 20\% \( (P = 0.0066) \) nutrient restriction compared to 0\% restriction. By 12 h post scratch and afterward, this difference was no longer observed. These data suggest that the early stages of wound closure were affected by nutrition, but by 12 h post scratch, nutrition had no significant impact on the wound closure process.

Satellite cell activity is not only influenced by the percentage of a nutrient restriction but also by the timing of a nutrient restriction (Powell et al., 2013; Velleman et al., 2018a). Velleman et al. (2018a) showed that 1 D satellite cells, nutrient restricted during differentiation, had a decrease in differentiation compared to unrestricted cells, but when restricted during proliferation, the satellite cell differentiation increased compared to unrestricted cells. In the current study, the effect of the timing of a nutrient restriction was examined by starting the nutrient restriction immediately after scratching or 24 h prior to scratching. The timing of nutrient restriction affected \( (P = 0.0527) \) the migration of 1 D satellite cells at 6 and 12 h post scratch (Figure 2). Specifically, the 1 D satellite cells migrated a greater distance at 6 h \( (P \leq 0.0424) \) and 12 h post scratch \( (P \leq 0.0527) \) when the nutrient restriction was started 24 h prior to scratching than when started immediately after scratching. By 24 h post scratch, this difference was no longer observed. Taken together, these data suggest that despite effects during early stages of wound closure, nutrient restriction likely has only a minor effect on 1 D satellite cell migration.

In addition to its impact immediately post hatch, nutrient availability also influences the activity of satellite cells of older birds. Older birds have satellite cells with decreased proliferation and differentiation compared to the satellite cells of younger birds (Velleman et al., 2010b; Harthan et al., 2013). Satellite cells respond to nutrient restrictions in an age-dependent manner (Harthan et al., 2014; Velleman et al., 2018a). In the current study, migration of p. major muscle satellite cells from 49-day-old RBC2 turkeys was investigated under nutrient-restricted conditions exactly like the p. major muscle satellite cells from 1-day-old Random-Bred Control Line 2 pouls. Satellite cells were nutrient restricted by 0, 5, 10, or 20\% of the standard culture media started 24 h prior to scratch (24 h pS) or immediately after scratching (0 h pS). Statistical analysis was performed with each sampling time to evaluate the effect of the percentage and timing of nutrient restriction as well as their interaction. Letters indicate statistically significant difference \( (P \leq 0.05) \) for effects of percentage of nutrient restriction \( (R) \), timing of nutrient restriction \( (T) \), and their interaction \( (RT) \). Error bars represent standard error in the mean.

The data from the current study suggest that the migration of satellite cells from the p. major muscle of 1 and 49-day-old turkeys was not affected by nutrient restriction.
availability. However, nutrient availability strongly affects satellite cell proliferation and differentiation, and recent studies suggest that satellite cell migration may respond to nutrient availability differently depending on other factors, such as heparan sulfate proteoglycan expression. Velleman et al. (2018a) showed that satellite cell differentiation by 72 h decreased with increased percentage of nutrient restriction during differentiation, but interestingly at 24 h of differentiation, 5, 10, and 20% nutrient restriction increased the differentiation of satellite cells from 1 and 49-day-old p. major muscle. This increase in differentiation during the early stages of differentiation could be due to changes in satellite cell migration (Mylona et al., 2006). Mylona et al. (2006) showed that if satellite cell migration is inhibited during differentiation, then satellite cell differentiation decreased at 24 h, but by 48 h of differentiation, there was no difference compared to control satellite cells without inhibited migration. Taken together, these data suggest that satellite cell migration is important for early stage differentiation of satellite cells and that nutrition may play a role. A possible mechanism for this interaction with nutrition could be mediated through the transmembrane heparan sulfate proteoglycan, syndecan-4. Syndecan-4 is highly expressed by satellite cells (Cornelison et al., 2001; Cho and Doles, 2017), important for satellite cell migration (Longley et al., 1999; Bass et al., 2007; Shin et al., 2013), and essential for myofiber regeneration after injury (Cornelison et al., 2004; Tanaka et al., 2009). The hypothesis that syndecan-4 is involved in nutrition and migration is supported by a recent study (Velleman et al., 2019). Velleman et al. (2019) showed at 24 h of differentiation, the response of p. major muscle satellite cell differentiation to nutrient restriction was dependent on syndecan-4 expression.
Therefore, syndecan-4 may be involved in the satellite cell response to nutrient availability and may have important implications on poultry muscle growth and regeneration.

Nutrition is a valuable management tool for the poultry industry to optimize muscle gain and morphological structure. Part of nutritional management strategies include feed restrictions, which have been shown to minimize degenerative myopathies. Designing the precise diet formulations optimizing both muscle gain and minimizing degenerative myopathies can be difficult (Meloche et al., 2018b). Given the strong connection between satellite cell activity and p. major muscle growth and structure, it is important to optimize satellite cell activity with nutrition by determining the nutritional effects on satellite cell activity. The data in the current study suggests that the migration of p. major muscle satellite cells is not affected by nutrient restriction, and thus not an important factor in formulating poultry diets. Factors that have been shown to be important with nutrient restriction are satellite cell proliferation and differentiation. Therefore, it is important to evaluate the effect of diet formulations on satellite cell proliferation and differentiation to effectively use nutrition as a means to minimize degenerative myopathies while optimizing muscle gain and meat quality.

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