PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **188**, 40–45 (1988)

Proliferation of Skeletal Muscle Satellite Cells after Castration and Administration of Testosterone Propionate[1,2] (42704)

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Abstract. Thirty-six neonatal pigs were randomly assigned to the following treatment groups: sham implanted gonadally intact males (B), sham-implanted castrated males (C), or castrated males implanted with testosterone propionate (C + TP). Four pigs from each group were sacrificed at 7, 14, or 21 days of age after a 6-hr continuous infusion of [^{3}H]thymidine. Myofibers isolated from the triceps brachii were prepared for satellite cell enumeration by light microscope autoradiography. A developmental decline in labeled myofiber nuclei occurred in all groups, however, the greatest decline occurred in C ($P < 0.01$). A treatment-by-age interaction was observed for percentage of labeled nuclei. Castration reduced total and labeled nuclei per millimeter myofiber ($P < 0.05$), and C + TP had a higher percentage of labeled nuclei than C (2.8 vs 2.2%; $P < 0.05$). Since triceps brachii muscles from 21 day B and C + TP were 120% ($P < 0.05$) of C, the results indicate that postnatal growth of skeletal muscle is dependent on satellite cell mitotic activity and that testosterone enhances this activity in neonatal pigs.

Skeletal muscle growth is dictated by number of myofibers, DNA content, and number of nuclei per myofiber (1, 2). The nuclear to cytoplasmic volume ratio or functional DNA unit (1) plays a role in limiting muscle growth as one diploid nucleus can accommodate only protein synthesis demands of a defined cytoplasmic volume (3). In most vertebrates, the number of myofibers within a muscle reaches a maximum shortly after birth. Postnatally, myofibers are incapable of DNA synthesis and division (4). However, over 80% of the DNA in skeletal muscle of adults is accumulated postnatally (5). Postnatal replication of satellite cell nuclei and their intrusion into myofibers results in increased myonuclei numbers and increased muscle fiber DNA, which endows muscle with greater protein synthetic potential (1, 5, 6). Satellite cells (5, 7, 8) are small, mononucleated, heterochromatic, spindle-shaped cells that lie between the sarcolemma and basement membrane of fully differentiated skeletal muscle fibers (7). When satellite cells are cultured *in vitro,* they divide, differentiate, and fuse into myotubes in a pattern similar to that of embryonic muscle cells (9, 10). Several factors that have been shown to activate satellite cell mitosis or recruitment and migration of satellite cells *in vivo* include localized injury, denervation, increased functional demand, elevated growth hormone, and somatomedin C concentration (11, 12).

When testosterone was added to Yaffe's L 6 myogenic cells in culture, DNA Labeling Index was enhanced 25–30% over controls (13) but direct measures of cell proliferation and protein accretion were unaffected by testosterone (13, 14). While *in vitro* studies suggest that testosterone has no direct effects on stimulating satellite cell proliferation (14), androgen-specific cytosolic receptors have been identified in pig skeletal muscle (15). Presence of androgen specific receptors supports the concept that androgens may have direct effects on skeletal muscle. In growing rabbits, testosterone administration resulted in increased total DNA in the semitendinosus muscle relative to nontreated controls (16). Administration of androgens to 15- and 74-kg castrated male pigs (17) and other meat animal species (18) increased growth rate of nonadipose tissues.

[1] Alabama Agricultural Experiment Station Article No. 4-871328; Michigan Agricultural Experiment Station Journal Article No. 12378.

[2] Partially supported by a grant from the Auburn University Grant-in-Aid Program.

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0037-9727/88 $1.50

The influence of elevated serum concentration of androgen during the neonatal period upon subsequent muscle growth has not been examined. Male pigs have elevated circulating androgens during the first few weeks of life (19) and recent data suggest that neonatal testosterone is important in imprinting of pituitary somatotrophs and altering responsiveness to growth hormone releasing factor (20–22). There have been no *in vivo* investigations of muscle satellite cell nuclei proliferative activity relative to animal sex or androgen manipulation. Consequently, the present study was designed to determine the effect of castration and administration of testosterone on satellite cell proliferative activity in neonatal pigs.

Materials and Methods. Thirty-six gonadally intact male newborn crossbred pigs from eight litters having at least three male pigs per litter were used in this study. Littermates were randomized across three age (7, 14, and 21 days of age) and treatment groups: castrated within 24 hr of birth (C = Castrate), gonadally intact (B = Boar), or castrated and implanted with 4-androsten-17β-ol-3-one 17-proprionate (C + TP). Three polydimethylsiloxane (Silastic, Dow Corning, Midland, MI) capsules each 6 cm long and containing approximately 40 mg of the androgen were implanted in each of the treated castrates. Pigs in the C and B groups received empty capsules. At implantation, pigs were anesthetized with Fluothane (Ayerst Laboratories, New York, NY) and the C groups were castrated. The implanted capsules were placed subcutaneously and parallel to the ribs with the aid of a blunted trocar. This procedure has been shown to elevate plasma testosterone concentrations in neonatal pigs to pubertal concentrations (Mulvaney, unpublished data).

Four pigs from each group were slaughtered at 7, 14, or 21 days of age. The day before slaughter, pigs were fitted with an indwelling catheter in the brachial-facial branch of the jugular vein under Fluothane anesthesia. Six hours before slaughter, each pig received a continuous infusion of tritium-labeled methyl thymidine ([^{3}H]dThd; 50 Ci/mmole; Amersham) for a total dose of 0.5 mCi/kg body wt. Infusions were initiated between 0600 and 0900 hr. All pigs remained with their mothers until infused. Infusion was used as the mode of delivery to increase labelling index and is based on the rapid metabolism of thymidine *in vivo*. Animals were killed by infusion of 0.5 cc Beuthanasia-D (Schering Corporation, Kenilworth, NJ). After exposing the triceps brachii muscle, small muscle bundles from the long head were clamped *in situ* to prevent shortening. Muscle bundles were then excised and placed in Carnoy's solution for 18 to 20 hr at 4°C. The remaining triceps muscle comprising the long, medial, and lateral heads was excised and weighed.

Myofiber segments were isolated and processed as follows (23). Briefly, muscle bundles about 0.5 mm in width were hydrated sequentially through washes containing decreasing concentrations of ethanol, and incubated in Sorenson's phosphate buffer containing collagenase (Sigma, Type 1a) at 3 mg/ml. Myofiber segments were stained with Mayers Hemalum prior to being coated with NTB-2 liquid photographic emulsion (Kodak) according to manufacturers' specifications. After 12 weeks of exposure at 4°C, slides were developed, fixed, restained and coverslips permanently mounted to enable enumeration of total and labeled nuclei (those overlaid with silver granules indicating tritiated thymidine uptake) by light microscopy. Nuclei tagged with silver grains were assumed to be satellite cells since subsarcolemmal nuclei are incapable of proliferative activity (24). At least 1000 nuclei were counted from 100 different fiber segments of the muscle from each animal. Nuclei found in shriveled or contracted fibers were not counted. Nuclei number for fiber segments was expressed per millimeter length and also per unit standardized volume (volume of a cylinder 1 mm long and 20 μm diameter). Incorporation of [^{3}H]thymidine into nuclei of satellite cells using autoradiography and electron microscopy has been confirmed (8, 9, 25, 26).

All data were subjected to analysis of variance using General Linear Model procedures of Statistical Analysis System (27). The model included treatment, litter, age, treatment by age, and residual error. Significant differences between least square means are shown for data when the probability of a greater F value is less than 0.05.

Results. Live body and triceps brachii

muscle weights of the C, B, and C + TP at slaughter are summarized in Table I. Boars and C + TP pigs were 118 and 132% heavier than littermate C pigs at 7 and 21 days, respectively. However, there were no differences observed among treatments at 14 days. No treatment differences were observed in triceps brachii muscle weight until 21 days when muscle from C weighed 82% ($P < 0.05$) of those from B and C + TP pigs.

The relationships of age of pig to myofiber diameter and nuclei density expressed on a volume and length basis are presented in Table II. Regardless of treatment, myofiber diameter increased ($P < 0.01$) with age (Table II). Castration or treatment with testosterone had no influence on fiber diameter. As fiber diameter increased, incidence of total and tagged nuclei decreased ($P < 0.01$). However, total nuclei per millimeter myofiber length was 41% greater ($P < 0.01$) at 3 weeks than for 1-week-old pigs. Number of tagged nuclei per millimeter myofiber length decreased with age.

The effects of castration at birth and testosterone treatment on total and tagged nuclei per millimeter myofiber length are presented in Table III. Total nuclei per millimeter of length were reduced by castration ($P < 0.05$) and treatment with testosterone had no effect ($P > 0.05$). Incidence of proliferating satellite cells per millimeter myofiber length also was reduced ($P < 0.01$) by castration (C vs B), and C + TP pigs had greater satellite cell proliferative activity ($P < 0.05$) than castrates.

TABLE I. EFFECT OF CASTRATION, ANDROGEN TREATMENT, AND AGE ON BODY AND TRICEPS BRACHII MUSCLE WEIGHTS[a]

Group	Age (days) 7	14	21
	Body weight (kg)		
Castrate	2.85[b]	4.77[b]	5.22[b]
Boar	3.35[c]	5.12[b]	6.85[c]
Castrate + TP	3.39[c]	4.77[b]	6.95[c]
SE	0.14	0.29	0.30
	Triceps brachii weight (g)		
Castrate	23.4[b]	39.6[b]	47.1[b]
Boar	24.6[b]	47.6[b]	56.5[c]
Castrate + TP	26.4[b]	42.5[b]	58.5[c]
SE	2.4	2.1	2.8

[a] Least squares means within columns for a trait with different superscripts differ ($P < 0.05$).

TABLE II. EFFECT OF AGE ON MYOFIBER NUCLEI DENSITY AND DIAMETER[a]

Trait	Age (days) 7	14	21
Nuclei/unit vol	266.7[e]	169.9[d]	136.7[c]
SE	6.6	6.0	6.0
Tagged nuclei/unit vol	3.8[e]	3.0[d]	2.3[c]
SE	0.2	0.2	0.2
Nuclei/mm myofiber length	43.0[c]	55.4[d]	60.9[d]
SE	3.5	3.2	3.2
Tagged Nuclei/mm myofiber length	3.8[e]	3.1[d]	2.3[c]
SE	0.2	0.2	0.2
Fiber diameter, μm	8.2[c]	10.5[d]	12.3[e]
SE	0.3	0.3	0.3

[a] Unit volume = volume of a cylindrical fiber with a diameter of 20 μm = 3.1414×10^5 μm^3. Least squares means in rows with different superscripts differ $P < 0.01$.

An age-by-treatment interaction for percentage of labeled nuclei in isolated myofibers from triceps muscle of neonatal B, C, or C + TP pigs at 7, 14, and 21 days of age is presented in Figure 1. A developmental decline in percentage of proliferating nuclei was observed for B, C and C + TP pigs. However, a more dramatic decline ($P < 0.01$) in proliferative activity occurred in castrates relative to the other groups. A higher percentage of labeled nuclei, suggesting more proliferating satellite cells, was observed in triceps muscle from B compared to the other groups. At 21 days, B and C + TP pigs did not differ ($P > 0.05$) in percentage of labeled nuclei.

Discussion. The neonatal castrates from the present study weighed 85 and 76% of that of boars at 7 and 21 days, respectively, which illustrates the effect of castration on growth of pigs. The effects of castration were not due to differences in birth weights since birth weights were similar ($P > 0.05$) among pigs in this study. Testosterone administration countered the effect of castration as live weight of these pigs was not different from

TABLE III. EFFECTS OF CASTRATION AND TESTOSTERONE TREATMENT ON MYOFIBER NUCLEI DENSITY AND PERCENTAGE TAGGED NUCLEI[a]

Trait	Treatment		
	Castrates	Boars	Cast + TP
Tagged nuclei/mm myofiber length	2.20[b]	4.16[d]	2.84[c]
SE	0.19	0.21	0.20
Nuclei/mm myofiber length	45.46[b]	61.67[c]	52.18[b]
SE	3.29	3.61	3.35

[a] Rows with different superscripts differ $P < 0.05$.

boars. This is consistent with other results in which testosterone-implanted neonatal pigs were 116% of the weight of sham-treated castrates (28). Injection of testosterone propionate into pigs at birth has also been shown to increase weaning weights and survival during the neonatal period (29). Effects of testosterone and castration have been reviewed recently (30, 31).

In a previous study (32), castration of pigs at birth had no effect ($P > 0.05$) on live weight or weight of longissimus and semitendinosus muscles of pigs at 28 days of age. However, triceps brachii muscle weights were greater ($P < 0.1$) in B compared to C. The latter is consistent with the current observations that boars and C + TP pigs at 21 days had greater triceps muscle weights. The response to androgens differs among muscles with muscles from the shoulder, back and head being affected to a greater extent than muscles in the hind limb (33, 34). Triceps brachii of 15- and 74-kg pigs increased in response to testosterone and dihydrotestosterone compared to castrated males in previous investigations (17).

It has been demonstrated clearly that the number of myonuclei increases during postnatal muscle growth (2, 35, 36–37). After administration of [^{3}H]dThd, the number of labeled nuclei in muscle increased with time (24) and labeled nuclei in muscle fibers belonged exclusively to satellite cells (38), indicating that satellite cell mitotic activity had occurred. DNA content of muscle is influenced by castration. At 100 kg, C had only 81% of the DNA per semitendinosus muscle when compared to littermate B (39). Male pigs, castrated at birth, had 19.6% less DNA in the semitendinosus muscle relative to noncastrates at 4 weeks of age (32). Exposure of cultured rat skeletal muscle cells to 10^{-8} M testosterone reduced cell cycle times by almost 9 hr (13) and the G_1 phase was reduced by approximately 20% (13). In soleus skeletal muscles of rats having elevated serum growth hormone due to secreting tumors, incidence of myonuclei was increased but satellite cell nuclei as a percentage of total was twofold greater in tumor-induced compared to normal control rats (40). However, glucocorticoid administration decreased (41) satellite cell numbers in muscle from rats, thus demonstrating that satellite cells are influenced by endocrinological manipulation (42).

Even though the present assessment of satellite cell proliferative activity does not include a measurement of total satellite cells, the proportion of total nuclei in triceps muscle of B that were undergoing proliferation was greater than that found in C at 14 and 21 days of age. This suggests that myonuclei content of castrates was restricted by satellite cell mitotic activity. All nuclei tagged with silver grains were assumed to be satellite cells

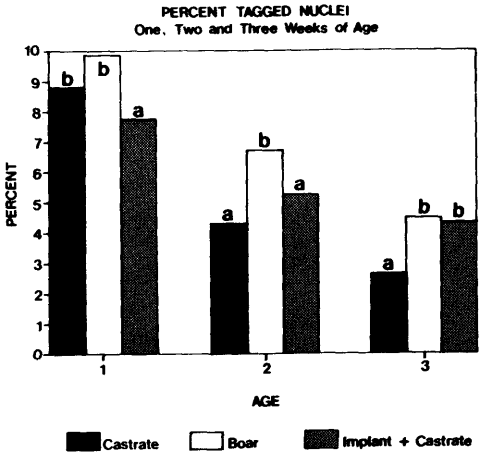

FIG. 1. Numbers of labeled nuclei (nuclei containing silver grains following autoradiography) in isolated myofibers from triceps brachii muscle of 7, 14, and 21-day-old boars, castrates or castrates implanted with testosterone propionate. Castration and implanting was performed at birth. Nuclei numbers are shown as a percentage of total myonuclei (least squares means; age effect standard error = 0.44).

since subsarcolemmal nuclei are not capable of mitosis (24).

The number of nuclei per millimeter of myofiber length is similar to the values reported for neonatal pigs (37) but lower than those found for mice (43, 44). The significant increase in nuclear numbers with age is consistent with the increases found in chickens (24), rats (45), and young pigs (37). The decrease in total and tagged nuclei per unit myofiber volume as pigs became older corresponds to the simultaneous increase in fiber diameter and is consistent with other data (11). Even though no treatment differences were found for fiber diameter, numbers of nuclei per millimeter of myofiber length of castrates and C + TP pigs were 74 and 84%, respectively, of those of B. These observations suggest that satellite cells in young B have greater proliferative activity and fusion with existing myofibers than C, which in turn favors the greater rates of protein synthesis found in B relative to C (17).

When data from all treatment groups are pooled, the percentage of nuclei that incorporated thymidine as detected by silver granules declined with age (8.8, 5.4, and 3.8% at 7, 14, and 21 days, respectively). These data are consistent with the developmental decreases in percentages of satellite cells and percentages of mitotically active cells demonstrated in pigs (37) and rodents (26, 45, 46). However, a treatment-by-age interaction resulted from the more extensive decline in percentage proliferating cells in C as compared to B and C + TP pigs.

The sensitivity of satellite cell proliferative activity to a variety of stimuli is well documented (11). Satellite cell proliferative activity also is affected by animal genotype. Lean mice have been shown to have greater muscle growth and satellite cell proliferative activity than their obese littermates at 2 and 3 weeks of age (44). However, the percentage of total muscle nuclei that were satellite cells did not differ between the two genotypes. An analysis of satellite cells in quail selected for growth also suggested that muscles from select lines had greater mitotic activity than control lines at 4 to 28 days posthatching (47).

Results from the present study are consistent with the suggestion that muscle growth is dependent on satellite cell proliferative activity. The data also suggest that perinatal serum androgen can at least indirectly modify the regulatory scheme responsible for enhancing satellite cell proliferation in skeletal muscle of newborn pigs.

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Received September 1, 1987. P.S.E.B.M. 1988, Vol. 188.
Accepted January 5, 1988.