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Steroid Effects on Cell Proliferation, Differentiation and Steroid Receptor Gene Expression in Adult Bovine Satellite Cells

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ABSTRACT : The present study was conducted to establish primary bovine muscle satellite cell (MSC) culture conditions and to investigate the effects of various steroid hormones on transcription of the genes involved in muscle cell proliferation and differentiation. Of three different types of proteases (type II collagenase, pronase and trypsin-EDTA) used to hydrolyze the myogenic satellite cells from muscle tissues, trypsin-EDTA treatment yielded the highest number of cells. The cells separated by hydrolysis with type II collagenase and incubated on gelatin-coated plates showed an enhanced cell attachment onto the culture plate and cell proliferation at an initial stage of cell growth. In this study, the bovine MSCs were maintained *in vitro* up to passage 16 without revealing any significant morphological change, and even to when the cells died at passage 21 with decreased or almost no cell growth or deformities. When the cells were incubated in a steroid-depleted environment (DMEM(-)/10% CDFBS (charcoal-dextran stripped FBS)), they grew slowly initially, and were widened and deformed. In addition, when the cells were transferred to an incubation medium containing steroid (DMEM(+)/10% FBS), the deformed cells resumed their growth and returned to a normal morphology, suggesting that steroid hormones are crucial in maintaining normal MSC morphology and growth. The results demonstrated that treatments with 19-nortestosterone and testosterone significantly increased AR gene expression ($p<0.05$), implying that both testosterone and 19-nortestosterone bind with AR and that the hormone bound-AR complex up-regulates the genes of its own receptor (AR) plus other genes involved in satellite cell growth and differentiation in bovine muscle. (**Key Words :** Bovine Muscle Satellite Cell, Steroid, Primary Cell Culture)

INTRODUCTION

The muscle satellite cell (MSC) is a myogenic stem cell in skeletal muscle tissue (Campion 1984; Chen and Goldhamer, 2003). The MSCs are in a quiescent state after birth and begin to proliferate in response to regulatory factors during development and in cases of muscle injury (Allen et al., 1979; Allen and Rankin 1990; Cornelison and Wold 1997). It has been known that MSCs are able to fuse with closely located existing myotubes or with other satellite cells to form new myotubes (Chen and Goldhamer, 2003). A culture system for these muscle satellite cells would provide an excellent model system in which to investigate the effects of various muscle growth stimulating agents and factors. Since the primary culture of rat satellite cells was first performed by Bischoff (1974), isolation and culture methods for satellite cells of domestic animals has been developed by several research groups (Dodson et al., 1986; Dodson et al., 1987; Doumit et al., 1992; Duclos et al., 1993). The primary culture system for myogenic satellite cells have provided an outstanding tool for investigation of the molecular mechanisms for MSC activation, proliferation and differentiation (Seale et al., 2000; Bonavaud et al., 2002; Kamanga-Sollo et al., 2004). In addition, increasing knowledge obtained from MSC culture systems will allow us to better understand the mechanisms involved in postnatal skeletal muscle growth in humans as well as other animal species.

The muscle growth rate in cattle, is an important performance determinant among various beneficial factors for evaluating profits, such as daily gain and feed efficiencies among others. Since muscles are mainly composed of myofibers differentiated from the MSCs,

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muscle growth is thought to be associated with increased numbers of myofibers and MSCs (Allen et al., 1979). The majority of the hormonal growth promoters, contain estrogenic, androgenic or a combination of these steroids, and have been used to improve muscle production and meat quality. Steroid hormones are known to play a potential role in muscle growth through multiple mechanisms, influencing both hypertrophy of muscle fibers and the proliferation of satellite cells. The administrations of androgenic and estrogenic steroids reportedly stimulated muscle growth in animals (Apple et al., 1991; Herschler et al., 1995; Oh et al., 2005). The muscle protein deposition can be increased by the implantation of an estrogen and progesterone mixture in particular with cattle (Apple et al., 1991; Hunt et al., 1991; Johnson et al., 1996). However, the exact mechanisms by which anabolic steroids enhance muscle growth are still not fully understood. In addition, there has been no satisfactory agreement on the *in vitro* study results showing an anabolic effect of steroids on muscle cells (Thompson et al., 1989; Kahler et al., 1997). Thus, the aims of the present study were to establish and optimize culture conditions of primary satellite cells isolated from the muscle of adult native Korean cattle (Hanwoo) and to evaluate the effects of various steroids on the transcription of nuclear receptor genes.

MATERIALS AND METHODS

Chemicals and reagents

Protease, bovine serum albumin (BSA), 17β-estradiol (E2), testosterone (TES) and 19-nortestosterone (NOR) were purchased from Sigma Chemicals Co. (St Louis, USA). DMEM, phosphate buffered saline (PBS), KRB (Kreb's Ringer bicarbonate) medium, trypsin-EDTA solution, penicillin/streptomycin (P/S), fungizone (FZ) and Ham's F12 nutrient mixture (F12) were obtained from Gibco™ (Invitrogen Co., Carlsbad, USA). Collagenase type II (Worthington Biochemicals Co., Lakewood, USA) was dissolved in KRB digestion medium (307 unit/ml) containing 1 mg/ml BSA, 10% P/S and 0.1% FZ. Trypsin-EDTA solution contained 0.25% trypsin and 1 mM EDTA·4Na. Protease (0.17% w/v) was dissolved in Ham's F12 medium containing 10% P/S and 0.1% FZ.

Cell culture

Muscle tissues were collected from the neck region of a native Korean steer at a local slaughter house and sustained in PBS (pH 7.4) with 10% P/S and 0.1% FZ at 37°C until use for enzyme digestion. Before treatment with the enzyme the muscle samples were rinsed in Ham's F-12 medium containing 1% P/S and 0.1% FZ, and assigned to each treatment with three different digestion enzymes including collagenase, trypsin-EDTA and protease, respectively. The hydrolyzed cells were filtrated through 40 μm mesh after digestion with each enzyme for 2 h, and were then centrifuged (350×g, 20 min) at room temperature. After removal of the supernatant, the cell pellets were washed by resuspending in 10 ml of Ham's F-12 containing 1% P/S and 0.1% FZ. After one more washing step as described above, the cells were resuspended in 10 ml of DMEM supplemented with 10% FBS, 1% P/S and 0.1% FZ and transferred onto a culture plate for incubation in a humidified incubator with 5% CO_2/95% air at 37°C. Differentiation of the muscle satellite cells was induced by incubating with 2% FBS in DMEM prior to being washed with F-12 containing 1% P/S and 0.1% FZ when the cells reached full confluence.

Hormone treatments

The muscle cells were grown in phenol-red free DMEM supplemented with 10% charcoal-stripped FBS (CDFBS), prepared according to the previously described protocol of Choi et al. (2000), for at least 3 days to allow maximization of the steroid hormone responsiveness. When cell confluence reached at least 60-70%, individual steroid preparations (10 nM) were administered directly into the medium. The cells were harvested after incubation at different time points and immediately used for total RNA extraction.

RNA isolation and real-time RT-PCR analysis

Total RNA was isolated from the cells using TRIzol™ reagent (Invitrogen Co., Carlsbad, USA) based on the manufacture's description. The amount of RNA was quantified by using a spectrophotometer (ND-1000, Nanodrop Technologies Inc., Wilmington, USA) and the quality of RNA was evaluated by separation using agarose gel electrophoresis. The first strand of cDNA was synthesized using 1 μg of total RNA as a template, oligo-dT primer and SuperScript™ IIRNase H$^-$ Reverse Transcriptase (Invitrogen Co.) according to the manufacturer's instructions. Real-time PCR analysis was performed with Exicycler™ (Bioneer Co., Daejeon, Korea) using enzyme premix (Accupower Greenstar PCR premix, Bioneer Co.) containing SYBR green I (Molecular Probes) according to the manufacturer's instructions. PCR primers used for real-time PCR were selected by Primer 3 software (http://frodo.wi.mit.edu) from each gene sequence obtained from GenBank and the following are the nucleotide sequences for each primer: GAPDH; 5'-gggtcatcatctctgcacct-3' and 5'-acagtcttctgggtggcagt-3', ER-α; 5'-caggtgccctattacctgga-3' and 5'-gcctgaggcatagtcattgc-3', and AR; 5'-tctcccaagaatttggatgg-3' and 5'-ggagcttggtgagctggtag-3', respectively. The cDNA product was amplified by PCR with Taq DNA polymerase (5 U/μl) (Bioneer Co.). A melting curve was analyzed to check the absence of mispriming. Amplification of the expected size

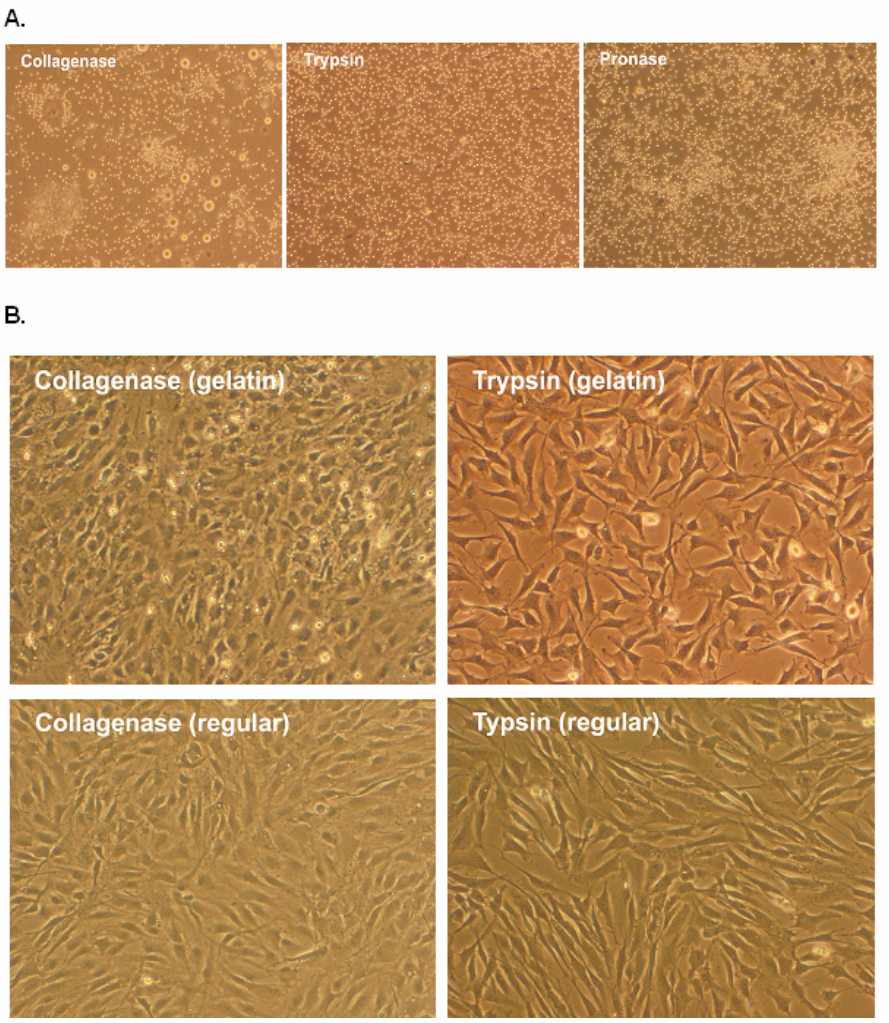

Figure 1. Effects of enzyme digestion and culture plates on the isolation and growth of MSCs. MSCs were isolated from muscle tissue by different hydrolysis procedures and incubated on regular culture plates in the presence of DMEM and 10% FBS; the pictures were taken after 24 h enzyme digestion (A). MSCs were isoated by collagenase or trypsin-EDTA, and grown on regular culture or gelatin-coated plates in cultures supplemented with DMEM and 10% FBS. Pictures were taken at 7 days incubation after enzyme digestion (B).

of PCR products was confirmed by gel electrophoresis. The calculation used for measuring fold change was $2^{-\Delta\Delta Ct}$ formula (Lay et al., 2002).

Statistical analysis

All values were represented as means±SEM. Data were analyzed by ANOVA according to the general linear model procedure. The means were compared by Tukey's Studentized Range (HSD) test to detect significant differences at $p<0.05$. All statistical procedures were done with the SAS® software package (Release 8.02, 2001).

RESULTS AND DISCUSSION

Optimal cell culture conditions for bovine muscle satellite cells

To investigate and develop an efficient method to isolate bovine myogenic satellite cells, collected muscle tissues were digested by the three different enzyme treatments of type II collagenase, pronase, or trypsin-EDTA, and grown in DMEM with 10% FBS. When the same amounts of muscle tissues were used, trypsin-EDTA treatment was shown to yield the highest number of cells (Figure 1A). When regular cell culture plates and gelatin-coated cell culture plates were compared to test for the optimal attachment of the cells onto the surface of the plates and initial cell proliferation, the gelatin-coated plate yielded the superior results. This was especially the case for cells separated by hydrolysis using type II collagenase which showed an earlier initial attachment onto the gelatin-coated plates in comparison to regular plates (Figure 1B). It is known that the proteases are effective for muscle satellite cell liberation in animals (Bischoff, 1974; Dodson et al., 1987; McFarland et al., 1988; Doumit and Merkel, 1992). It is likely that the proteases digest components of the basal lamina and sarcolemma membrane in muscle tissue, and

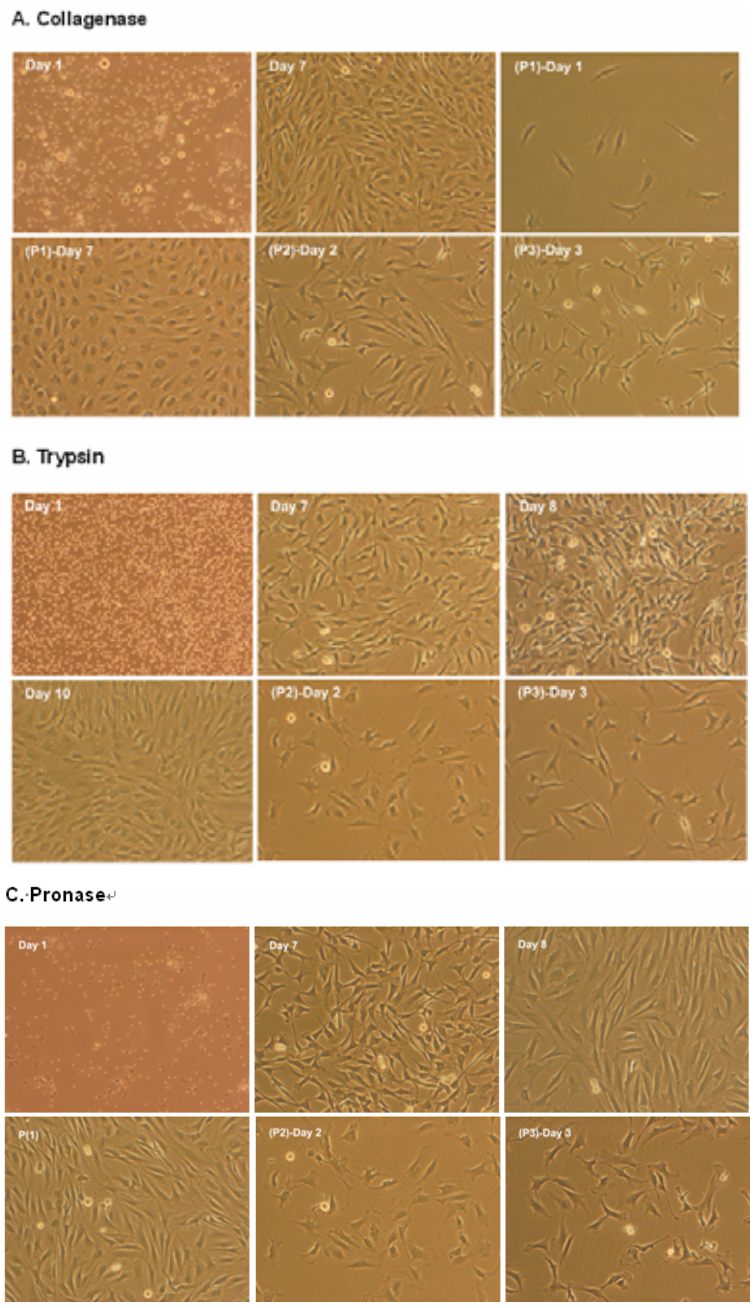

Figure 2. MSCs at different stages of incubation after enzyme digestion. MSCs were separated from muscle tissue by collagenase (A), trypsin-EDTA (B) and pronase (C), respectively, and incubated on regular culture plates supplemented with DMEM/10% FBS. Incubation period (Days) after enzyme digestion and the passage number after primary culture are indicated (by white letters) at the top left of each picture.

then liberate satellite cells. However, since these enzymes also destroy satellite cells, the duration of enzyme digestion is a critical factor for determining the success of subsequent primary culture. Thus, a shorter period (10 to 90 min) of enzyme treatment was employed to release satellite cells from muscle in the previous cited research. We have demonstrated that two hours of digestion under the conditions described in this report, are adequate to release a high number of satellite cells without showing reduced efficiencies of initial cell attachment and proliferation. During the early incubation, the cellular growth in collagenase treated culures was more rapid than for pronase and trypsin. Increased initial attachment of satellite cells in the gelatin-coated plate as compared with the regular plate indicates that the extracellular matrix-like substratum provided a better environment for satellite cells to adhere, at least at the early stage of primary cell culture. Previous reports demonstrated that different substrata can have an effect on cell proliferation and myotube formation in animal satellite cells (Dodson et al., 1987; Dodson et al., 1990;

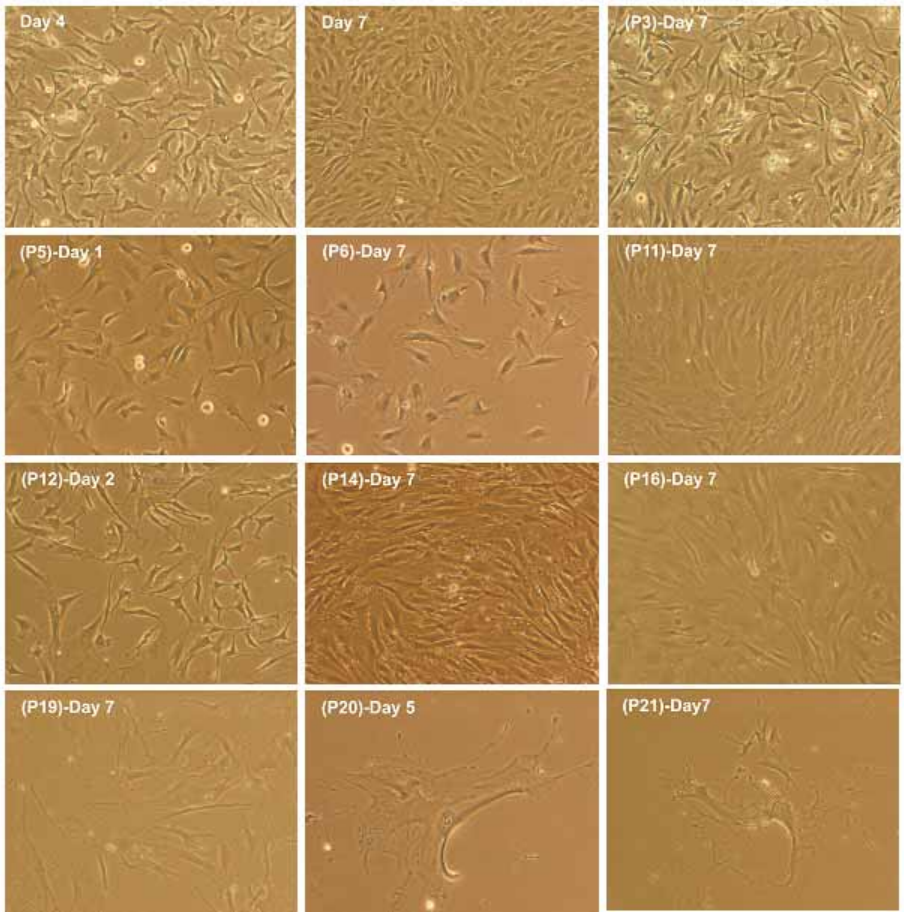

Figure 3. Morphological changes of cells at different passages. MSCs were isolated by collagenase and grown on regular cell culture plates in the presence of DMEM/10% FBS. Incubation period (Days) and passage number (P) after initial digestion are indicated (by white colored letters) at the top left of each picture.

Doumit and Merkel 1992). Whichever method described above is employed, 3 to 4 days are required for complete attachment of the muscle cells onto the surface of the culture plate. In general, there were no significant differences in separating satellite cells and the efficiency of cell attachment onto the surface of the cell culture plate at the initial stage of muscle satellite primary cell culture.

To further investigate a long-term effect of different enzyme digestion conditions on cell morphology and cell proliferation, the cells were incubated up to passage 3 (Figure 2). Although the satellite cells isolated by collagenase treatment required less time to become confluent, likely due to an initial higher yield of cell number and improved attachment at an earlier stage of cell culture compared with the other methods, the growth rate became similar to what was observed in the other methods after multiple passage. In addition, there was no significant difference in the apparent morphology of the cells. Considering the more expensive cost of gelatin-coated plates compared with regular plates and the use of collagenase with trypsin-EDTA, isolating satellite cells by hydrolysis with trypsin-EDTA with growth on regular plates is adequate for routine bovine muscle cell culture studies. However, we cannot completely eliminate the possibility that growing bovine cells on the gelatin-coated culture plates after collagenase treatment is a more efficient method of inducing the differentiation of satellite cells into myotubes than the other methods.

Morphological changes of the cells at different passage numbers

The isolated muscle satellite cells were maintained on a regular culture plate in the presence of phenol-red containing DMEM with 10% FBS from primary cell stage to passage 21, and their morphology and cell proliferation rates were observed (Figure 3). On average, 6 to 7 days elapsed before satellite cells became confluent in the flask in each passage and sufficient to be split (1:5 to 1:7 ratio) into another new flask at each passage up to passage 16. Moreover, there was no noticeable difference in cellular morphology and proliferation rates, as observed by microscope, during this period. However, the cells suddenly

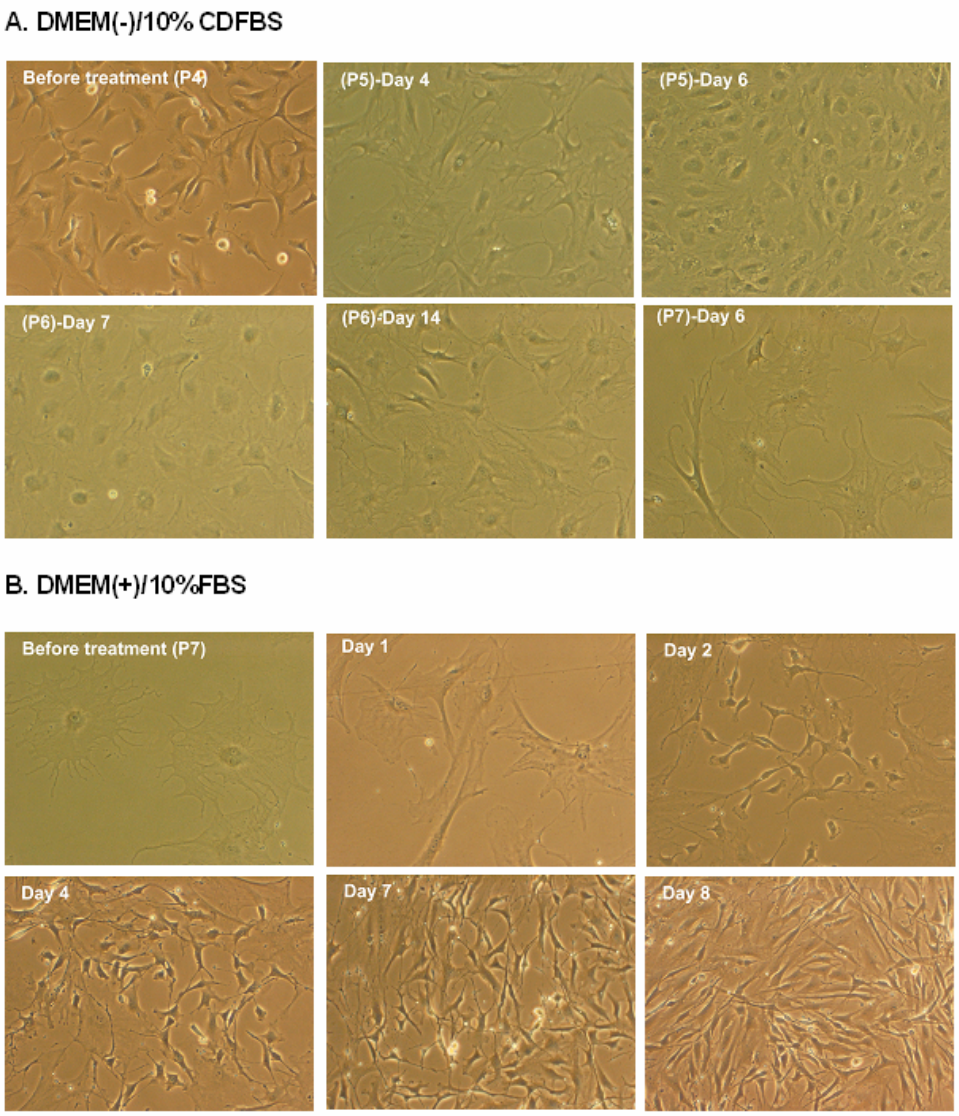

Figure 4. Effects of steroids on MSC morphology and proliferation. MSCs were isolated by the use of collagense enzymes in KRB medium and grown on regular culture plates. Cells had been maintained in regular phenol-red contained DMEM with 10% FBS (DMEM(+)/10% FBS) until passage 4 and then started to grow in phenol-red free DMEM with 10% charcoal-dextran stripped FBS (DMEM(-)/10% CDFBS) until passage 7 (A). The culture medium (DMEM(-)/10% FBS) was switched to DMEM(+)/10% FBS at passage 7 and the cells were sustained for an additional 8 days (B). Day and passage number (P) at the time the picture was taken are indicated at the top left in each picture.

began to change in their appearance and proliferation rate, and eventually died. It is interesting that bovine myogenic satellite cells can be maintained up to passage 16, without noticeable morphological change, during the *in vitro* cell culture condition.

Steroid(s) effect on bovine muscle cells

Steroids are presumed to have some anabolic effects in animals (Dodson et al., 1996), however, little data has been obtained through *in vitro* studies to demonstrate anabolic effects on animal satellite cells. Thus, to investigate possible effects of steroids on the morphology and proliferation rate of the muscle cells, cells were incubated in a steroid-depleted condition (DMEM (-)/10% CDFBS). Since phenol-red supplementation in the medium, as a pH indicator, has been known to have some estrogenic effect (Katzenellenbogen et al., 2000) and bovine serum contains high amounts of endogenous steroids, a phenol-red free DMEM medium (DMEM(-))with 10% charcoal-dextran stripped serum (CDFBS) was chosen for use in this study. When the satellite cells were grown in DMEM with 10% FBS (DMEM (+)/10% FBS), there was no significant morphological or cellular growth change observed up to passage 3. However, after the medium was switched from one containing steroids (DMEM (+)/10% FBS) to the steroid-depleted medium (DMEM (-)/10% CDFBS), cells

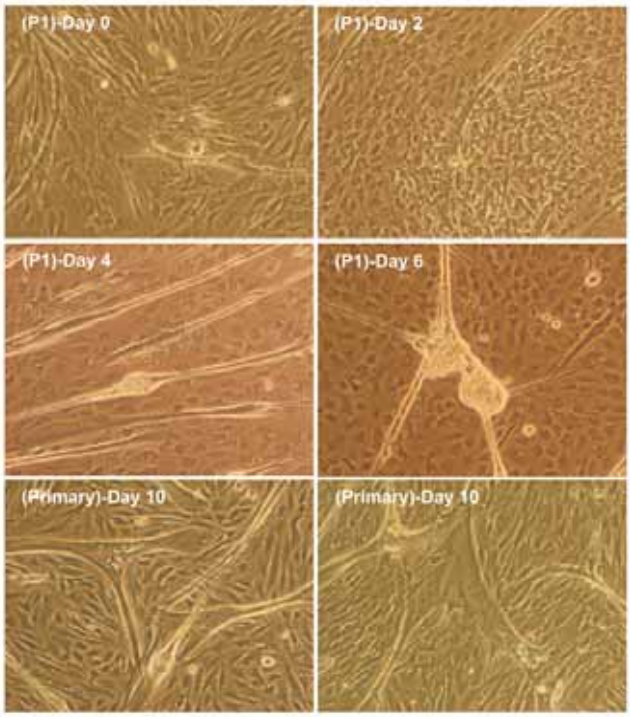

Figure 5. Induction of muscle satellite cell differentiation. MSCs were incubated in DMEM/10% FBS until confluence was reached and began to grow in DMEM containing 2% FBS for the induction of cell differentiation (upper and middle panels). Induction of cell differentiation was carried out by growing the satellite cells in DMEM with 10% FBS/10% horse serum (bottom left panel) and DMEM with 10% FBS (bottom right panel), respectively. Days of incubation after switching 10% FBS to 2% FBS (upper and middle panels) and the days of incubation time in 10%FBS/10% horse serum or 10% FBS, and the passage number for each cell cultureare indicated at the top left of each picture.

grew slowly and took 14 days from one passage to the next. In addition, the satellite muscle cells widened and became deformed over time (Figure 4A). The morphology of the cells at passage 7 differs markedly compared to pretreatment (passage 4). In contrast, when incubation was resumed in medium containing steroid (DMEM (+)/10% FBS) the deformed cells started to grow and regain their normal morphology (Figure 4). It has been demonstrated that charcoal-stripping of serum eliminates not only a variety of steroids, but others agents such as fatty acids and to a lesser extent, growth factors (Lindquist et al., 1987). As a result, it is likely that in our studies, steroid hormones and to a lesser extent fatty acid components and growth factors are crucial in maintaining the normal morphology of satellite cells and their growth.

Differentiation of myogenic stem cells

Bovine muscle satellite cells were grown in DMEM/10% FBS until reaching 90% confluence at about 7 days of incubation before being switched to DMEM/2% FBS, DMEM with 10% FBS/10% horse serum, or DMEM with 10% FBS media for subsequent continuous culture and differentiation. Satellite cells started to change in their morphology and form myotubes after 2 days of incubation with 2% FBS (top panel in Figure 5). When the concentration of FBS was reduced from 10% to 2%, the growth rate of the cells was reduced and the shape of the cells become more rounded. Myotube formation was also observed when the cells were grown either in DMEM/10% FBS or in DMEM/10% horse serum. However, compared with DMEM/2% FBS, a relatively longer time interval (10 days) was required to form the myotubes (bottom panel in Figure 5). It is worth mentioning that while there was no signifcant change of either cell shape or cell growth, myotube formation was not observed in bovine satellite cells after passage 2, suggesting that the characteristics of bovine satellite cells may have changed with increaseing cell passage. Horse sera have been used for muscle cell differentiation in several studies (Dodson et al., 1990; Doumit and Merkel 1992; Greene and Raub 1992), and it is possible that the DMEM with 2% fetal bovine serum is adequate for the induction of satellite cell differentiation. In our MSC culture system, MSCs retained a normal morphology up to passage 19. The MSCs experienced induced cellular differentiation with the reduction of FBS content from 10% to 2% in media; MSCs formed myofiber 4 days after the reduction of FBS content in the media (Figure 5).

Effects of steroid hormones on transcription of the AR genes

The satellite cells were grown in phenol-red free DMEM supplemented with 10% charcoal-stripped FBS (CDFBS) for at least 3 days before steroid hormone treatment was initiated in order to maximize the sensitivity to steroid hormone. The mRNA expression of the androgen receptor (AR) gene in bovine muscle cells was analyzed by real-time RT-PCR approach. Significant expression of the AR mRNA was shown for the bovine satellite cells as reported previously (Doumit et al., 1996; Kamanga-Sollo et al., 2004; Sinha-Hikim et al., 2004). There have been reports indicating that expression of the internal control gene used in this study for normalization of real-time PCR data may be altered depending on cell types, species, and hormone treatment (Tricarico et al., 2002). In this report, the data obtained both before and after normalization using internal controls are as shown in Figure 6. Both the 19-nortestosterone (nandrolone) and testosterone treatments increased androgen receptor mRNA expression, and there was no significant change in fold difference after normalization by GAPDH gene expression (Figures 1A and B). The 19-nortestosterone gradually induced AR mRNA expression with prolonged hormone treatment and

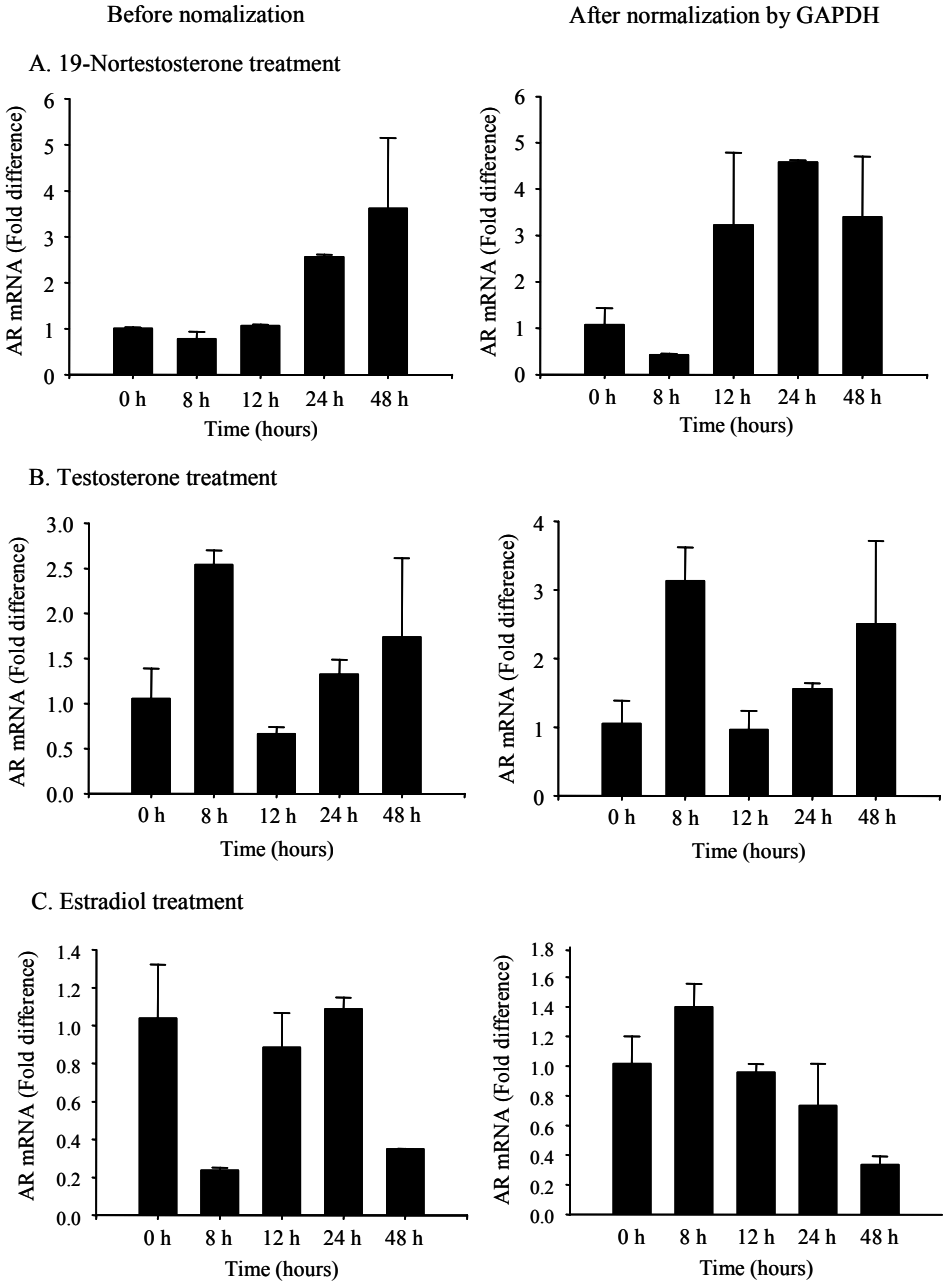

Figure 6. Androgen receptor (AR) mRNA expression. Muscle satellite cells were treated with 19-nortestosterone (A), testosterone (B), or 17β-estradiol (C). The cells were collected at different time points (0 h, 8 h, 12 h, 24 h, and 48 h, respectively) for RNA extraction. Changes of mRNA expression for the AR gene were analyzed by real-time RT-PCR. The predicted size of the amplified DNA was confirmed by agarose gel eletrophoresis (picture not shown). Data represent the means±SEM, expressed as fold difference, compared to the initial state (0 h) and normalized to the GAPDH mRNA as an internal control gene.

maximally up to 4 fold over control (no treatment, $p<0.05$). On the other hand, transcription of AR mRNA peaked at 8 h after testosterone treatment, dramatically declined by 12 h, and thereafter resumed a gradually rise ($p<0.05$). However, 17β-estradiol did not stimulate and even may have down-regulated the AR gene expression. Interestingly, the expression of the AR gene was about 5-fold lower after 8 h of treatment compared with that of control (0 h) before normalization by GAPDH, whereas the expression of the AR gene is slightly higher after 8 h of estradiol treatment using normalized data. This result indicates that the GAPDH gene expression in bovine satellite cells is influenced by estradiol. It will be of interest to further analyze the mechanism by which estrogen regulates GAPDH gene expression in bovine myogenic cells. In addition, relatively lower levels of the estrogen receptor- α

(ER-α) gene and the progesterone receptor (PR) gene but not the estrogen receptor-β (ER-β) gene were detectable in the bovine satellite cells (data not shown). It has been thought that androgen-induced muscle growth is associated with androgen receptors predominantly located in the satellite cells of muscle tissue (Sinha-Hikim et al., 2004). The expression of AR (androgen receptor) gene was enhanced by testosterone in both bovine and porcine satellite cells. Both androgen and estrogen treatments increase the number of proliferating satellite cells in bovine muscle tissue (Johnson et al., 1998). The androgen receptor is a member of a nuclear receptor superfamily and plays an important physiological role in many target cells, including muscle cells (Shen and Coetzee, 2005). Thus, it may be speculated that both testosterone and 19-nortestosterone bind the AR and that the hormone bound-AR complex then up-regulates the genes of its own receptor (AR) as well as other genes involved in satellite cell growth and differentiation in bovine muscle.

Taken together, an optimal *in vitro* condition for the bovine satellite muscle cell culture has been developed and described in this report. The results from the experiments indicate that steroids are critical factors in maintaining the morphology of myogenic satellite cells, cell proliferation, and probably myotube formation. The androgen receptor is up-regulated by testosterone and 19-nortestosterone and may be responsible for myogenic satellite cell growth and the differentiation of bovine muscle as indicated by these *in vitro* studies.

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