EFFECTS OF A COMBINED TRENBOLONE ACETATE AND ESTRADIOL IMPLANT (REVALOR-S) ON CARCASS COMPOSITION AND BIOLOGICAL PARAMETERS OF FEEDLOT STEERS

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ABSTRACT

Implanting steers with a combined trenbolone acetate (TBA) and estradiol (E_2) implant (Revalor-S) increased ADG 21% ($P < .001$), improved feed efficiency 13% ($P < .01$), increased longissimus muscle area ($P < .05$), and caused an 82% increase in daily carcass protein deposition during the first 40 d following implantation. As compared to nonimplanted steers implantation with TBA/E_2 also increased circulating insulin-like growth factor (IGF)-1 concentrations by 40% on d 40 and 35% on d 115 ($P<.001$). Additionally, serum concentration of insulin-like growth factor binding protein-3 (IGFBP-3) was higher in implanted steers on d 21 and 40 after implantation ($P < .05$). Sera from implanted steers stimulated proliferation of cultured muscle satellite cells to a greater extent ($P < .05$) than did sera from nonimplanted steers. Steady-state hepatic IGF-1 mRNA concentrations were increased 2.5 fold in TBA/E_2-implanted sheep compared to nonimplanted animals ($P<.01$). These data suggest that liver may be the source of at least part of the increased circulating IGF-1 in steroid-implanted sheep. In serum-free medium containing IGF-1 and FGF-2, the proliferative response of muscle satellite cells isolated from TBA/E_2-implanted steers was greater ($P < .05$) than the response of satellite cells isolated from nonimplanted steers. This may be because a higher proportion of satellite cells isolated from implanted steers are actively proliferating whereas a higher proportion of satellite cells isolated from nonimplanted steers are quiescent and must be activated in culture before proliferating. The presence of more actively proliferating satellite cells in muscle of implanted steers may play a role in the enhanced muscle growth seen with steroid treatment.

INTRODUCTION

Based on approximately 40 years of experimentation and commercial use, it generally is agreed that anabolic steroids increase growth rate, feed conversion and muscle deposition by ruminants (Hancock et al., 1991). Recently, combined estrogen/androgen implants have been shown to be even more effective than either androgens or estrogens alone for stimulating growth of ruminants (Hayden et al., 1992; Hancock et al., 1991; Johnson et al., 1996a). However, despite general agreement on the effectiveness of anabolic steroids, there is no consensus concerning the biological mechanism(s) responsible for the anabolic effects of either estrogenic or androgenic steroids (Hayden et al., 1992). Potential mechanisms of action of anabolic steroids have been reviewed recently (Hancock et al., 1991). Mechanisms that have been proposed for estrogen action include increasing the circulating level of somatotropin (Gopinath and Kitts, 1984; Grigsby and Trenkle, 1986; Breier et al., 1988a), increasing hepatic somatotropin receptors and thus enhancing somatotropin binding (Breier et al., 1988b), enhancing endocrine or local (autocrine or paracrine) production of growth factors (Hongerholt et al., 1992), or interacting directly with estrogen receptors in muscle tissue (Meyer and Rapp, 1985; Sinnett-Smith et al., 1987; Sauerwein and Meyer, 1989). Mechanisms proposed for androgen action include reduction of circulating levels of corticosteroids and/or down regulation of muscle corticosteroid receptors (Mayer and Rosen, 1978), decreasing circulating thyroxine levels (Donaldson et al., 1981), or direct action of androgens on muscle androgen receptors (Sinnett-Smith et al., 1987; Sauerwein and Meyer, 1989). However, none of these mechanisms has been proven conclusively to be the mode of action of either estrogenic or androgenic anabolic steroids (Hayden et al., 1992). In an effort to increase our understanding of the potential mechanisms by which anabolic steroid implants may enhance muscle growth in feedlot cattle, we have assessed the effect of Revalor-S, a combined trenbolone acetate (TBA) and estradiol (E_2) implant (120 mg of TBA plus 24 mg of E_2), on growth rate, feed efficiency, carcass composition, and circulating concentrations of specific growth factors at various times after implantation. Additionally we have compared the growth factor responsiveness of cultured muscle satellite cells isolated from implanted or

nonimplanted steers (Frey et al., 1995; Johnson et al., 1996a; Johnson et al., 1996c); we also have measured the effect of Revalor-S on the steady-state concentration of hepatic insulin-like growth factor-1 (IGF-1) mRNA (Johnson et al., 1996b).

Effect of a Combined Trenbolone Acetate (TBA) and Estradiol (E_2) Implant on Growth and Carcass Composition of Feedlot Steers.

Implantation with Revalor-S increased average daily gain by 21% (P<.001) and feed efficiency by 13% (P<.03) as compared to nonimplanted control steers (Johnson et al., 1996a). Longissimus muscle area was larger in steroid-implanted steers than in nonimplanted steers (P<.05) and implantation resulted in increased carcass protein (P<.05; Fig. 1; Johnson et al., 1996a). Fat accumulation was not affected by implantation (Fig.1; Johnson et al., 1996a). These data are consistent with other reports on the effect of anabolic steroids on growth and composition of gain and establish the rather significant impact these hormones have on rate and efficiency of growth. Because composition data show that muscle growth was stimulated in anabolic steroid-implanted animals, we examined some of the biological mechanisms that might be responsible for this increased muscle growth.

Effect of Revalor-S Implantation on Insulin-Like Growth Factor-1 (IGF-1) Concentration, Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) Concentration, and Mitogenic Activity of Bovine Sera.

Blood sera obtained from steers on d 0, 40, 115, and 143 were analyzed for IGF-1, insulin-like growth factor binding proteins (IGFBPs), and mitogenic activity. Glycyl-glycine (GG) extraction of serum was performed to reduce IGFBP interference in the IGF-1 radioimmunoassay (RIA). Implantation with TBA/E_2 increased circulating IGF-1 concentrations by 40% on d 40 and 35% on d 115 as compared to nonimplanted steers (P<.001; Fig. 2; Johnson et al., 1996c). Implantation with TBA/E_2 also increased the serum concentration of insulin- like growth factor binding protein-3 (IGFBP-3) on d 21 and 40 after implantation (P < .05; Fig, 3; Johnson et al., 1996c). Sera from implanted steers stimulated proliferation of cultured muscle satellite cells to a greater extent than did sera from nonimplanted steers on d 21, 40, 115, and 143 after implantation (P < .05; Fig. 4; Johnson et al., 1996c). This result is consistent with the increased IGF-1 concentration observed in sera from steroid-implanted steers. It also is significant that the maximum circulating IGF-1 concentration in implanted steers occurs by d 40 which coincides with

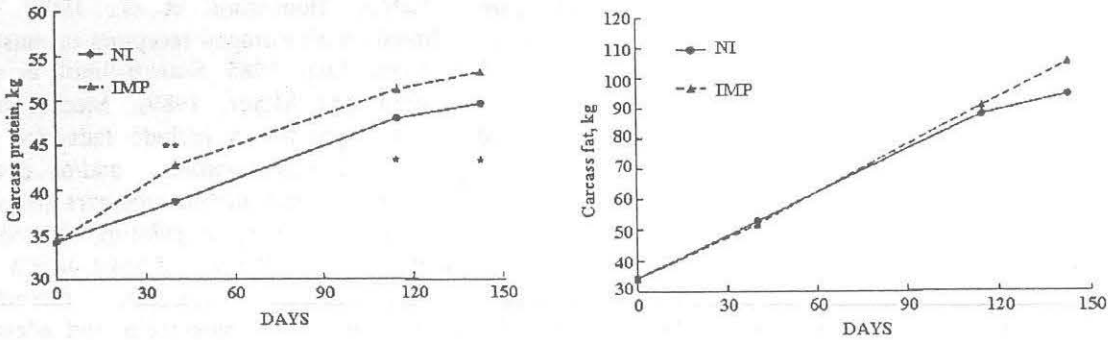

Figure 1: Accumulation of protein and fat in carcasses of implanted and nonimplanted steers calculated from 9-10-11th rib section composition (Hankins and Howe, 1946). After the initial slaughter at the beginning of the study, three serial slaughters were completed on d 40 (n = eight/treatment), d 115 (n = eight/treatment), and d 143 (n = eight/treatment). Implantation increased (P < .01) carcass protein throughout the feeding period but had no effect on carcass fat. The biggest differences between protein accretion rates in implanted and nonimplanted steers were observed between d 0 and d 40 (114 g/d in nonimplanted steers vs. 207 g/d in implanted steers, P < .004). Protein accretion rates in nonimplanted and implanted steers were not significantly different from d 40 to d 115 or from d 115 to d 143. Statistical significance of differences between nonimplanted and implanted steers within a particular slaughter group are denoted by asterisks (*P < .10 and **P < .05). Pooled SEM for protein and fat are 1.34 and 6.00, respectively.

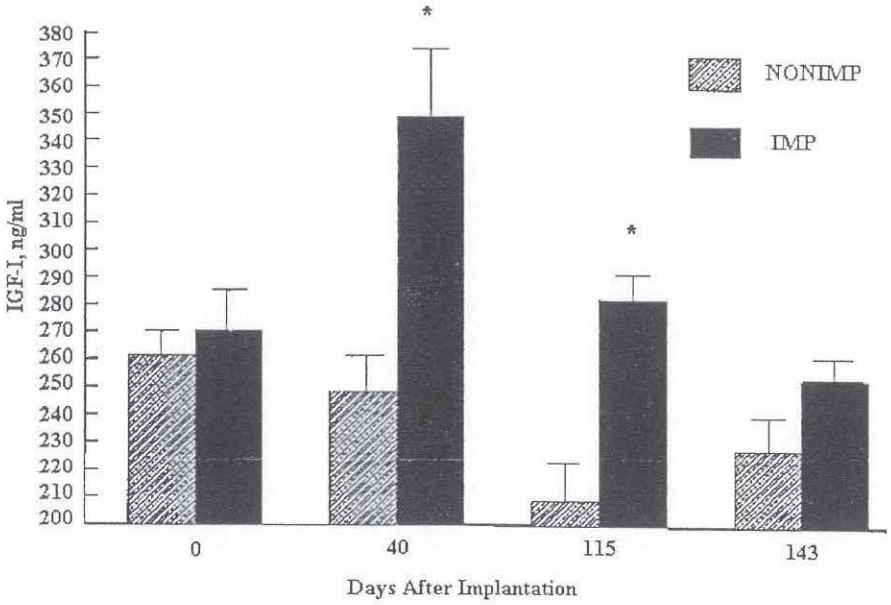

Figure 2: Serum insulin-like growth factor-I (IGF-1) concentrations (nanograms/milliliter) in steers implanted with trenbolone acetate and estradiol (TBA/E_2) compared with nonimplanted steers. Samples were taken from steers used in a serial slaughter study on d 0 (n = eight/treatment group), d 40 (n = eight/treatment group), d 115 (n = eight/treatment group) and d 143 (n = eight/treatment group). Before IGF-1 RIA, raw sera were extracted with .1M glycyl-glycine (pH 2.0; **G G**) in a 1:1 ratio at 37° C for 48 h to reduce the IGFBP interference in the IGF-1 RIA (Plaut et al., 1991). In all cases final pH of the extraction mixture was between 3.6 and 3.8. Serum IGF-1 concentrations were quantified using a heterologous RIA (Frey et al., 1994). Intra- and inter-assay coefficients of variation were 6.9 and 8.1%, respectively. Asterisks denote time points within which the IGF-1 concentrations for implanted and nonimplanted steers are significantly different ($P < .05$). Pooled SEM is 12.4 ng/ml.

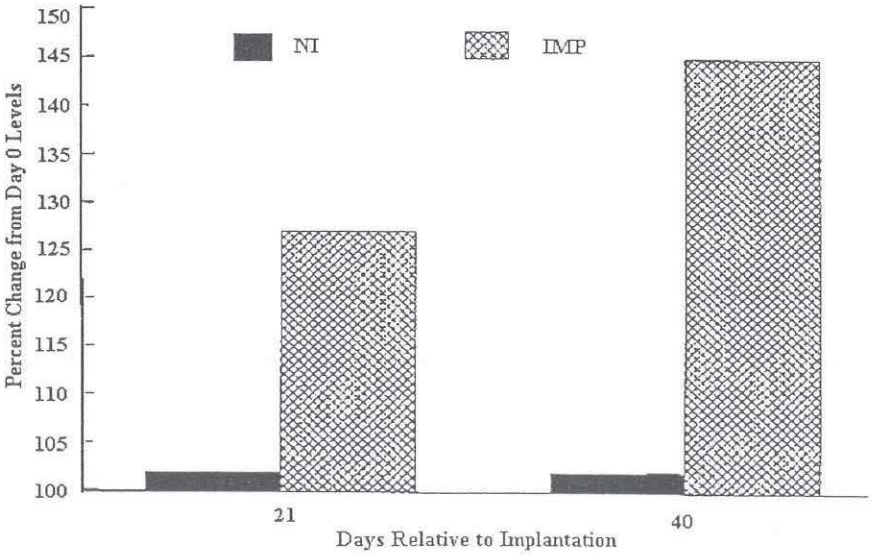

Figure 3: Relative change from d 0 concentrations of serum insulin-like growth factor binding protein-3 (IGFBP-3) in steers implanted with trenbolone acetate and estradiol (TBA/E_2) and nonimplanted steers. IGFBP concentrations in sera were determined by measuring the density of bands corresponding to individual IGFBPs on autoradiograms of ^{125}I-IGF-1 Western ligand blots. The graph represents the change in IGFBP-3 concentrations at d 21 and 40. Implantation with TBA/E_2 increased ($P < .05$) concentrations of IGFBP-3 on both d 21 and 40. Pooled SEM = 4.6 (n = 4).

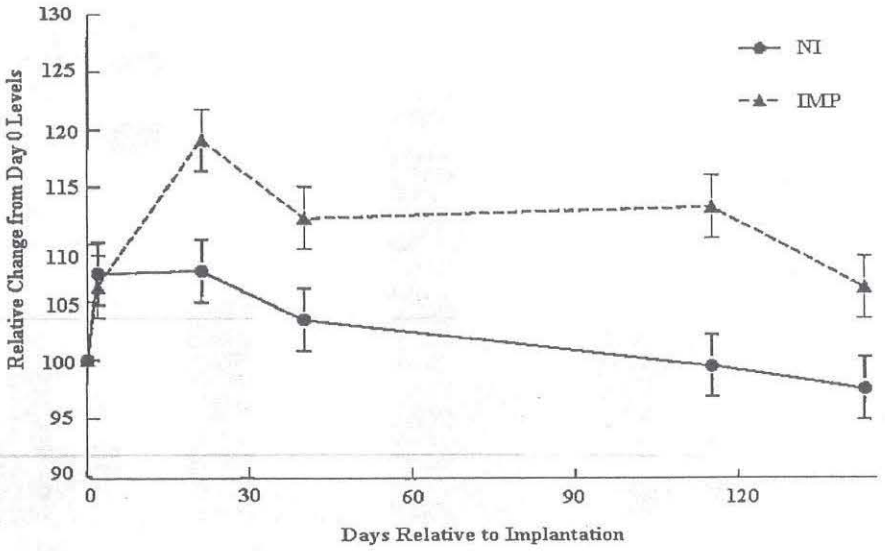

Figure 4: Mitogenic activity of the serum from steers implanted with trenbolone acetate and estradiol (TBA/E_2) and nonimplanted steers. Values are expressed as percentage of cells/cm^2 at d 0. Cloned sheep satellite cells were plated in triplicate wells, and assays were conducted in duplicate. Sera from eight steers/treatment were pooled by treatment and day. Pooled sera were added to McCoy's media at a concentration of 3%. Sera from steers implanted with TBA/E_2 increased ($P < .05$) proliferation of the cloned sheep satellite cells to a greater extent than sera from nonimplanted steers on d 21, 40, 115, and 143. Pooled SEM = 2.7 (n = 6).

the period of maximum protein accretion (Fig. 2), and that after d 40 both circulating IGF-1 concentration and protein accretion declined. These data support the hypothesis that increased circulating IGF-1 concentration may play a major role in the positive effects of TBA/E_2 implants on feedlot performance and rate of protein accretion in steers.

Implanting with a combination of TBA/E_2 (40 mg TBA and 8 mg E_2) also increased circulating IGF-1 concentration in lambs. Circulating IGF-1 concentration began to rise immediately following implantation; by d 3 and d 10 following implantation sera from wethers implanted with TBA/E_2 showed a 32% and a 51% increase, respectively, in IGF-1 concentration as compared to sera from nonimplanted wethers ($P<.001$; Johnson et al., 1996b). This steroid-induced increase in circulating IGF-1 concentration was maintained throughout the entire 24 d duration of the study. Steady-state IGF-1 mRNA concentrations were measured in liver samples collected from these animals on d 24. Northern blot analysis revealed that hepatic IGF-1 mRNA levels were increased 2.5 fold in TBA/E_2-implanted animals compared to nonimplanted animals ($P<.01$; Johnson et al., 1996b). These data suggest that liver may be the source of at least part of the increased circulating IGF-1 in steroid-implanted sheep. It is possible that TBA/E_2 implants also may increase IGF-1 mRNA levels in muscle resulting in altered local IGF-1 production in this tissue. A recent report that treatment of elderly men with testosterone increases the steady-state level of IGF-1 mRNA in muscle tissue supports this hypothesis (Urban et al., 1995). Additionally, alterations in local production of IGF binding proteins by muscle or other cells found in muscle tissue may alter the bioactivity of locally-produced or circulating IGF-1. Consequently, a complete understanding of the role of IGF-1 in anabolic steroid-induced muscle growth will require analysis of the effects of anabolic steroids on local production of IGF-1 and IGF binding proteins in muscle tissue.

Potential Role of Satellite Cells in Revalor-S-Induced Muscle Growth.

During embryonic development of muscle tissue, mononucleated muscle precursor cells proliferate, differentiate, and eventually fuse to form myotubes that mature into multinucleated muscle fibers found in postnatal muscle tissue (Dayton and Hathaway, 1989). Because the number of muscle fibers in meat-producing animals essentially is fixed at birth, postnatal muscle growth is due primarily to hypertrophy (an increase in length and diameter) of existing, multinucleated muscle fibers. This increase

in fiber size is accompanied or preceded by an increase in muscle DNA that is necessary to support the increased size of the fiber (Powell and Aberle, 1975; Harbison et al., 1976; Swatland 1977; Trenkle et al., 1978). Because the nuclei in muscle fibers are unable to divide, the source of this DNA initially was a mystery. However, it is now known that specialized, mononucleated cells known as satellite cells are responsible for providing this critically needed DNA to growing muscle fibers (Mauro 1961; Campion 1984). Satellite cells fuse with existing fibers and, in doing so, contribute their nuclei to the fiber (Moss and Leblond, 1970; Moss and Leblond, 1971). From 60 to 90% of the DNA in mature muscle fibers originates from satellite cells (Allen et al., 1979). Thus, proliferation of satellite cells and their fusion with muscle fibers to provide DNA required for fiber growth may be critical rate-limiting step in muscle growth. Thus it is significant that the number of satellite cells decreases dramatically as animals become older; and, in adult animals, the remaining satellite cells normally exist in a quiescent state in which proliferation does not occur (Dodson and Allen, 1987; Bischoff 1990a; Moss and Leblond, 1970; Campion 1984). Because fusion of a satellite cell with a growing muscle fiber results in loss of the satellite cell, satellite cells must be stimulated to proliferate in order to maintain their population in growing muscle. Because the number of satellite cells in muscle decreases substantially during normal growth (Allen et al., 1979), the balance between proliferation and fusion appears to be tipped toward fusion. Consequently, the number of active satellite cells and their rate of proliferation may limit the rate of DNA accretion and hence the potential for muscle growth at all stages of growth. Thus, increasing either the rate of satellite cell proliferation or the number of proliferating satellite cells should enhance muscle growth rate and efficiency. Because current evidence shows that IGF-1, fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF) and transforming growth factor beta-1 (TGF beta-1) play major roles in regulating proliferation and differentiation of satellite cells (Allen and Boxhorn, 1989; Allen and Rankin, 1990; Hathaway et al., 1991; Greene and Allen, 1991; Dayton and Hathaway, 1991), the response of satellite cells to these growth factors may be a key element in determining the rate and extent of satellite cell proliferation in muscle tissue.

Biological activity of IGF is regulated by a family of six IGF binding proteins (IGFBPs) (Clemmons 1991; Binoux et al., 1991; Baxter 1991; Reeve et al., 1993; Oh et al., 1993; Frost et al., 1993; Figueroa et al., 1993; Conover and Kiefer, 1993; Clemmons et al., 1993) whose activities are regulated by IGFBP-specific proteases (Fowlkes and Freemark, 1992; Davenport et al., 1992; Nam et al., 1994; Kanzaki et al., 1994; Claussen et al., 1994; Cheung et al., 1994) . Consequently, the level of IGFBPs and IGFBP-specific proteases in muscle tissue may play a significant role in regulating satellite cell response to IGF. Despite the ability of IGF-1, FGF, PDGF and TGF beta to regulate division of actively proliferating satellite cells, these growth factors are not able to activate quiescent satellite cells. Consequently, whether satellite cells are quiescent or activated may play a crucial role in their ability to respond to mitogenic growth factors and to support muscle growth. An extract obtained from crushed muscle (CME) has been shown to activate quiescent satellite cells (Bischoff 1986; Chen et al., 1994; Chen and Quinn, 1992; Bischoff 1990b). Fractionation of CME has shown that it contains transferrin, FGF-2, and PDGF-BB in addition to some unidentified mitogenic factor(s) (Chen et al., 1994). Recently, Allen and coworkers have shown that hepatocyte growth factor (HGF) is able to activate quiescent satellite cells in culture; these workers have hypothesized that HGF may be at least one of the unidentified active components of CME (Allen et al., 1995).

Because muscle satellite cells play a crucial role in postnatal muscle growth, it seemed likely that an increased proliferation of satellite cells could be involved in the increased rate of muscle growth observed in steroid-implanted steers. Consequently, we examined the growth factor responsiveness of cell cultures established from satellite cells isolated from steroid-implanted and nonimplanted steers (Frey et al., 1995). Using procedures in routine use in our laboratory (Hathaway et al., 1991; Frey et al., 1995), we isolated satellite cells from the semimembranosus muscles of nonimplanted steers and steers that had been implanted for 40 days with a combined TBA and E_2 implant. Satellite cells were stored in liquid nitrogen for use in later studies. The effects of growth factors on proliferation of bovine satellite cell cultures were assessed in a serum-free medium that was a slight modification of a medium that had previously been shown to support growth of bovine satellite cells (Greene and Allen, 1991). We have observed that satellite cells isolated from sheep that are approaching market weight under go a 24 - 48 h period of little or no proliferation (lag phase) after being placed in culture (Hathaway et al., 1991). A similar lag phase observed in satellite cells isolated from adult rat

muscle has been interpreted to indicate that the majority of satellite cells in uninjured adult muscle are in a quiescent (G_o) phase (Dodson and Allen, 1987; Johnson and Allen, 1993). This lag phase in culture is thought to represent the time required for the cells to re-enter the G_1 phase and begin to proliferate. Satellite cells isolated from rats of different ages exhibit variable lag periods (Schultz and Lipton, 1982), suggesting that their activation state and(or) their response to growth factors varies with the age of the rat from which they were isolated. Based on the preceding information, we believe it is critical to examine the properties of satellite cells from nonimplanted and implanted steers approaching market weight to determine if implantation affects the activation state of these cells or their responsiveness to growth factors that have been shown to affect satellite cell proliferation and differentiation. Isolation and culture of satellite cells from implanted and nonimplanted steers allows us to assess the effects of steroids on these parameters.

Effect of Revalor-S on Growth Factor Responsiveness of Cultured Satellite Cells Isolated from Implanted and Nonimplanted Steers

Figure 5 shows the effect of IGF-1 and FGF-2 on proliferation of cultured satellite cells from nonimplanted and TBA/E_2-implanted steers. Cells were plated on basement membrane Matrigel coated plates in DMEM containing 10% fetal bovine serum and allowed to attach for 48 h. At the end of this attachment period, cultures were washed 4 times with DMEM to remove any residual serum components. Serum-free medium containing 10 ng IGF-1 and 50 ng FGF-2/ml then was added to the cultures and incubation was continued for 72 h. Control cultures for each animal were treated with basal serum-free medium containing no IGF-1 or FGF-2. Cell counts for growth-factor-treated cultures from nonimplanted and implanted steers are shown in Figure 5. Proliferative response to growth factors for each steer was expressed as the percent change in cell number in growth factor-treated cultures relative to the cell number in control cultures from the same animal. In the presence of growth factors, the proliferative response of satellite cells isolated from TBA/E_2-implanted steers was 18% greater than the response of satellite cells isolated from nonimplanted steers ($P<.05$; Frey et al., 1995). These data are consistent with a report that satellite cells isolated from female rats treated with trenbolone were more sensitive to FGF-2 and IGF-1 than were satellite cells from control rats (Thompson et al., 1989). Although rats are not a particularly good model for large ruminants, we believe these data support our results showing that satellite cells from TBA/E_2-treated steers are more responsive to a growth factors than are satellite cells isolated from nonimplanted steers. This increased responsiveness may indicate that, even after 48 h in culture, a higher percentage of the cells isolated from nonimplanted steers are quiescent and nonresponsive to growth factors; this, suggests that there is difference in the in vivo satellite cell activation state between implanted versus nonimplanted steers. Alternatively, proliferation of satellite cells may be more responsive to IGF-1 and FGF-2 when obtained from implants than nonimplanted steers due to an alteration in the number or affinity of satellite cell receptors for IGF-1 and/or FGF-2. At this point, the biological mechanism by which anabolic steroid treatment of steers is able to influence the growth factor responsiveness of their satellite cells in culture is unclear.

All primary satellite cell cultures are contaminated by nonmuscle cells; presence of these cells complicates interpretation of data from these cultures. To assess the degree of nonmuscle cell contamination in our bovine muscle cell preparations, we analyzed the fusion percent ([nuclei in myotubes/total nuclei] x 100). Cultures of satellite cells isolated from nonimplanted or TBA/E_2-implanted steers gave approximately equal fusion (57 $\pm$ 2.3% in cultures from implanted steers and 53 $\pm$ 2.3% in cultures from nonimplanted steers) on day 8 in culture (Frey et al., 1995). Thus, it is unlikely that differences in number of myogenic cells in cultures isolated from nonimplanted and TBA/E_2-implanted steers contributed to the difference in responsiveness to IGF-1 and FGF-2.

We also assessed whether TBA and estradiol had a direct effect on proliferation rate of cultured bovine satellite cells. In these studies, bovine satellite cells in serum-free medium were treated for 72 hrs with TBA and estradiol; direct effects of these hormones as well as their effect on the responsiveness of the cells to FGF-2 and IGF-1 were monitored. Addition of TBA and estradiol to the culture medium at concentrations at least as great as those found circulating in implanted steers (10^{-7} M and 2×10^{-8} M, respectively) had no effect on the proliferation rate of cultured bovine satellite cells (Frey et al., 1995). Additionally, the presence of TBA and estradiol in the culture medium did not alter the response of satellite cells to 10 ng IGF-1 and(or) 50 ng FGF-2/ml (Frey et al., 1995). Thus, it did not appear that TBA and