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**The effect of trenbolone on skeletal muscle satellite cells**

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**The University of Arizona, 1987**

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**300 N. Zeeb Rd.  
Ann Arbor, MI 48106**



THE EFFECT OF TRENBOLONE  
ON SKELETAL MUSCLE SATELLITE CELLS

by

Steven Howard Thompson

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A Thesis Submitted to the Faculty of the  
DEPARTMENT OF ANIMAL SCIENCES  
In Partial Fulfillment of the Requirements  
For the Degree of  
MASTER OF SCIENCE  
In the Graduate College  
THE UNIVERSITY OF ARIZONA

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December 14, 1987  
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DEDICATION

To my wife, Kimberley, and  
my little girls, Andrea and Emily.

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The completion of my Masters Degree has given me an incredible sense of happiness. I am grateful to those who have contributed to the completion of this work. I wish to express my appreciation to my fellow students for their friendship and support. I would like to thank Linda Boxhorn and Michael Dodson for teaching me lab techniques necessary for my success. Special thanks to Cindy Rankin for her support and excellent assistance in preparing this thesis. Most importantly I wish to thank Dr. Ronald Allen for his support while generating the data, assistance during the editing of the thesis and most of all for giving me the opportunity to realize this goal.

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## ABSTRACT

Young female rats treated with trenbolone demonstrated an increase in weight gain per day and overall weight increase during the treatment period. Trenbolone treated rats also experienced improved feed efficiency.

Muscles removed from the lower hind limb of trenbolone treated rats had a greater DNA to protein ratio than muscles from control animals. However, there was no significant difference in wet muscle weight between trenbolone treated and control muscles.

Satellite cells from untreated female rats were not responsive to trenbolone added in vitro. In studies utilizing serum free medium, trenbolone alone, and in the presence of growth factors, could not stimulate proliferation above controls. In similar serum free medium studies, satellite cells from trenbolone treated rats were more responsive to growth factors than cells from control rats.

## CHAPTER 1

### INTRODUCTION

Anabolic agents have been used in farm animals to enhance the efficiency of meat production. A great deal of work has gone into the development of sex steroid derivatives which promote protein anabolic activities on protein activation but do not promote of secondary sex characteristics. These compounds may be either estrogenic, such as diethylstilbesterol, or androgenic like trenbolone (TBOH).

Trenbolone acetate (TBA) is rapidly metabolized to trenbolone, the active metabolite, in the blood. The mode of action of TBOH is not well understood. It could act by altering the endogenous hormone activities of the animal, by direct action after binding to the target tissue receptor, by antagonizing the binding of endogenous hormones to their receptors, or by a combination of these processes.

The current theory of TBA action is based on the results of a few studies in which TBA-treated animals experienced a decrease in both muscle protein synthesis and degradation. However, treated animals gained significantly more weight during the treatment period than the non-treated animals. To explain this disparity, researchers concluded that protein degradation must have been reduced more than synthesis, resulting in net protein accretion.

Relatively little research has been conducted to probe the cellular action of TBOH as it relates to animal growth. The goal of

this study was to examine the effects of TBOH in both the whole animal and culture. Several pertinent questions to be addressed were; (1) does TBOH-treatment have a direct or indirect effect upon satellite cells, (2) does TBOH enhance the actions of other growth factors, and (3) does TBOH treatment elicit an effect upon muscle cells in vivo that persist in vitro.

## REVIEW OF LITERATURE

### Mechanisms of Muscle Growth

The growth of muscle tissue is accomplished by two fundamental biological processes, protein accretion and myogenic cell proliferation. Protein accretion is a function of both protein synthesis and protein degradation. Cell proliferation is involved in prenatal and postnatal phases of growth, each of which has a great influence over the eventual growth of the muscle.

#### Myogenic Cell Proliferation

Cell Cycle. The life of a cell can be subdivided into several periods in an attempt to analyze processes controlling progression from one division to the next. The first period following a division is known as the presynthetic gap ( $G_1$ ). This is followed by periods of DNA replication (S), postsynthetic gap ( $G_2$ ) and mitosis (M). The S phase lasts about 4 hours, the  $G_2$  phase, during which cells prepare for mitosis and cytokinesis, takes about 2 1/2 hours, and mitosis last about an hour. After mitosis, two daughter cells are produced and each enter  $G_1$ . From  $G_1$  a cell can continue to divide or enter a mitotically

quiescent state that is often referred to as  $G_0$  (Pardee et al., 1978; Allen, Merkel and Young, 1979).

Certain hormones are thought to function as competence or progression factors in the regulatory scheme of cell replication. A competence factor stimulates cells to progress to a restriction point at the  $G_1$ S interface (Pardee, 1974). This restriction point regulates the reentry of a cell into a new round of the cell cycle. Progression factors stimulate cells which are competent to undergo a new round of division by moving cells past the restriction point into the S phase of the cell cycle.

Stem Cells. In the early embryonic stages of cellular development, stem cells have the ability to divide without differentiating. Stem cells are, by definition, not terminally differentiated. Stem cell progeny have two options available to them: (1) remain a stem cell like the parent, (2) embark on a different course that ultimately leads to irreversible terminal differentiation. Stem cells are not limited to embryonic tissue. Certain short lived tissues in adult vertebrates such as skin, intestinal epithelium, and blood are continually replaced by a population of regenerative cells or stem cells (Darnell, Lodish and Baltimore, 1986). There are still several unresolved questions regarding stem cells: (1) what determines whether stem cells exercise their ability to divide or stay quiescent? (2) what governs the choice that a daughter cell must make between terminal differentiation or continuing as a stem cell? (3) what range of possibilities does a daughter cell have when it embarks on a pathway leading to terminal differentiation?

Embryonic Development. Muscle develops from the mesodermal layer of the embryo. As the embryo develops, multipotent stem cells undergo proliferation, quantitatively increasing the myoblast stem cell population. The differentiation of embryonic myocytes into multinucleated myofibers is very important because myofiber number is fixed in most higher animals at birth (Rowe and Goldspink, 1969). Embryologically, myoblasts fuse, forming a multinucleated myotube which normally develops into the mature fiber. Furthermore, nuclei within myotubes or muscle fibers do not replicate their DNA or divide (Stockdale and Holtzer, 1961).

Satellite Cells. While investigating the deleterious effects of malnutrition on animals Winick and Noble (1966), made an interesting observation: rat muscle DNA increased 8.6 fold from 21 to 133 days of age. They also observed that rats that were starved during the critical growth phase never attained the DNA content of their control counterparts upon subsequent refeeding. Moss (1970) demonstrated similar results in chickens. Chickens were deprived of food for 48 hours, beginning 7 days after hatching. After 7, 8, 9, 10, 11, 13, 16, 20, and 27 days of age, various measurements were made. These included: (1) pectoral weight, (2) muscle weight per nucleus, and, (3) muscle nuclei number. Moss found that starvation, as expected resulted in a 30% decrease in muscle weight. The number of nuclei remained constant. Therefore, the total weight to nucleus ratio was reduced. After 18 days of refeeding the muscle weight and nuclei had both increased but still remained lower than the nonstarved chickens. The starved

chickens had fewer nuclei and lower muscle weight but equal total muscle weight:nuclei ratios, when compared to control chickens. These results suggest that the total muscle weight:nuclei ratio is constant. Since the starved chickens had fewer nuclei they were unable to accumulate more protein. Therefore, the ultimate size of the muscle was determined by the total nuclei content.

The existence of satellite cells, once thought to be a peripherally located myofiber nuclei, was discovered by Mauro (1961). The advent of the electron microscope and its improved resolving power allowed histologist to identify this independent mononucleated cell population located between the sarcolemma and the basement membrane. Satellite cells are fusiform in shape and have been observed in skeletal muscle in many species (Muir, 1970). After the identification of satellite cells it was many years before investigators elucidated their physiological significance in postnatal muscle.

The discovery that satellite cells were indeed responsible for the increase was made by Moss and Leblond (1971). Rats, 14-17 days old, received a single intraperitoneal injection of  $^3\text{H}$ -thymidine. Rats were sacrificed at times ranging from 1-72 hours. Two techniques were employed to determine the source of the new muscle nuclei. The first utilized radioautographs which were evaluated with a light microscope to identify the presence of newly synthesized DNA. In addition, adjacent sections were examined with the electron microscope to identify satellite cells and muscle fiber nuclei. The second approach involved examination of an muscle fiber digest which were prepared for autoradiography one hour after  $^3\text{H}$ -thymidine injection in order to

calculate the total number of labeled nuclei per unit length of fiber. The results suggested that as much as 65.2% of tibialis anterior muscle nuclei content was from satellite cells.

In summary, during embryonic development muscle is formed from several sub-populations of myogenic stem cells in the mesodermal layer. Several sub-populations of multi-potential stem cells undergo proliferation and differentiation resulting in myoblast stem cells which further differentiate into embryonic muscle fibers. At least one sub-population of myogenic cells, satellite cells, is thought to enter a quiescent period. The fact that satellite cells do not differentiate into embryonic muscle fibers suggest that they have different properties and will play a different role: post-natal muscle development. Upon some signal, in the post-natal animal, quiescent satellite cells proliferate and differentiate. Satellite cell terminal differentiation results in the fusion of the satellite cell with the muscle fiber and donation of its nuclear material. This additional nuclear material appears to increase the capability of the muscle fiber to synthesize muscle specific proteins.

Satellite cells also appear to have a role in muscle repair, when muscles are damaged, satellite cells proliferate and differentiate to form new myotubes (Schults, Jaryszak and Valliere, 1985). The regeneration of muscle fibers is similar to the formation of muscle fibers in embryonic muscle.

### Protein Accretion

During postnatal growth, the net accumulation of muscle protein occurs as a result of an imbalance between the rates of protein synthesis and breakdown, with synthesis exceeding breakdown. With increasing age, this imbalance gradually narrows, the ultimate mature rate depending on the type of muscle. When growth has effectively ceased, the synthesis of new proteins is counterbalanced by the degradation of existing proteins. There also appears to be a gradual lengthening of the half-life values of both contractile and sarcoplasmic proteins with increasing age (Waterlow, Garlick, and Millward, 1978). In a particular strain of rats at weaning, 20% of the gastrocnemius and quadriceps muscle proteins are degraded and replaced everyday, corresponding to half-lives of less than 3.5 days. Replacement rates in adult muscle are much slower with half-lives of approximately 20 days (degradative processes). As an animal increases in age, there is a decrease in the efficiency of synthesis (protein synthesis per unit DNA) when growth proceeds under adequate nutrition and management conditions (Rannels, Hajlmarson, and Morgan, 1974). The more rapid turnover of proteins in muscle of young animals may give the tissue a greater adaptive capacity with respect to its differentiation into different muscle fiber types and its ability to change in response to changing hormonal or environmental factors.

It is generally accepted that the number of muscle fibers does not increase after embryonic differentiation has been completed (Rowe and Goldspink, 1969). Regulation of myotube numbers and hence the number of muscle fibers eventually formed is not clearly understood but

is almost certainly under genetic control.

Post-embryonic increases in muscle size results from an increase in length and diameter of existing muscle fibers. This increase in girth can be accounted for by an increase in myofibril number (the contractile machines) within the fiber (Goldspink, 1970). The stimuli which cause muscle fibers to synthesize and assemble more myofibrils are unclear but appear to be correlated with increased work load. Muscle fibers increase in length as well as cross-sectional area. Longitudinal growth of muscle fiber is associated with an increase in the number of sarcomeres in series along the myofiber (Williams and Goldspink, 1971).

#### Protein Hormones

##### Function

The function of peptide hormones has been studied extensively, however, many cellular responses remain unclear. Although many peptide hormones have been shown to cause activation of a particular second messengers, it is not understood how a single effector system is translated into a number of individual target cell response. For example, a rise of cAMP in adipose tissue decreases amino acid uptake, but a rise of cAMP in muscle increases amino acid uptake (Sutherland, 1972). Moreover, many peptides function as trophic factors with long term effects, or acute regulators of short term activities.

Peptide hormones have been shown to act through one of three different systems. The best understood system employs cAMP as a second messenger. The effects of increasing levels of cAMP, due to hormone stimulation, varies considerably in cells of different types. However,

all the effects associated with elevated cAMP levels are thought to be mediated in a similar manner. Cyclic AMP modifies the activity of a specific group of enzymes through cAMP-dependent enzymes called protein kinases. Protein kinase transfers the terminal phosphate group of ATP to an amino acid on the substrate protein. Phosphorylation can produce an enzyme which is much more active than the unphosphorylated form or deactivate an enzyme and reduce activity.

A second system through which protein hormones can function involves a breakdown of phosphatidylinositol 4,5-bisphosphate (Ptd Ins 4,5 P<sub>2</sub>). The breakdown of Ptd Ins 4,5 P<sub>2</sub> results in the formation of diacylglycerol and inositol triphosphate (Ins 1,4, 5 P<sub>3</sub>). Ins 1,4,5P<sub>3</sub> then acts as a second messenger that causes the release of calcium from intracellular stores which increases activation of Ca<sup>2+</sup> dependent protein kinases resulting in enhanced protein phosphorylation. Another pathway stimulated by increasing levels of diacylglycerol activate protein kinase C also resulting in enhanced protein phosphorylation which initiates an enzyme cascade modulating cellular action.

The third system involves receptor kinases. These receptor kinases catalyze the phosphorylation of tyrosine residues in proteins. It has been suggested that the tyrosine receptor kinases utilize serine kinases as substrates and thereby regulate phosphorylation and dephosphorylation of a wide variety of proteins, resulting in diverse metabolic effects. (Krebs, 1984; Dornell et al., 1986)

#### Insulin-Like Growth Factors I and II

In the late 1950's a systemic factor, derived from human serum,

was found to facilitate the incorporation of  $^{35}\text{S}$ -sulfate into cartilage (Daughaday, Salmon and Alexander, 1959). This factor was given the term sulfation factor. Studies (Murphy, Daughaday and Hartnett, 1956; Salmon and Daughaday 1958) resulted in the eventual identification of the factor. It was shown that serum from normal rats stimulated  $^{35}\text{S}$ -sulfate incorporation into cartilage while serum obtained from hypophysectomized animals decreased incorporation. The decreased incorporation by serum from hypophysectomized animals was reversed upon in vivo supplementation of growth hormone. Attempts to repeat the experiment in vitro had mixed results. Growth hormone had no effect on  $^{35}\text{S}$ -sulfate incorporation into cartilage when added directly in vitro. An increase in label incorporation was observed when cartilage was incubated in serum from normal rats and serum from hypophysectomized rats which received replacement growth hormone. No increase in incorporation of  $^{35}\text{S}$ -sulfate was observed when cartilage was incubated in serum from hypophysectomized rats not receiving growth hormone replacement. These results suggested the presence of a serum growth factor which is under hypothalamic/pituitary control.

These early studies led to the isolation of what is now known as Somatomedin-C (Van Wyk et al. 1974), which is identical to insulin-like growth factor I (IGF-I) (Li et al., 1983). IGF-I is a single polypeptide chain containing 3 intrachain disulfide bonds and a molecular weight of 8389 daltons (Klapper, Svoboda and Van Wyk, 1983).

A second insulin-like growth factor was originally isolated from the conditioned medium of rat liver cells (Dulak and Temin 1973a,

1973b). It was referred to as multiplication stimulating activity (MSA). MSA is a single chain polypeptide of 67 amino acids and three intra chain disulfide bonds. MSA has a molecular weight of 7484 daltons and is 93% structurally homologous to human IGF-II (Nissley et al. 1983).

Evidence available to date suggest that circulating somatomedin activity may result in part from the release of somatomedins from the liver. It has been shown that patients with severe liver disease have low circulating somatomedin activity (Takano et al., 1977). Levels of somatomedin activity were found to be significantly higher in the hepatic vein in normal anesthetized dogs when compared to samples drawn from four other veins (Schimpff et al., 1976). In studies where partial hepatectomy was performed on fetal rat livers, circulating levels of somatomedins were reduced when compared to controls. But levels of circulating somatomedins gradually returned to normal in parallel with the regeneration of liver mass (Uthne and Uthne, 1972). These data certainly suggest the liver has a role in somatomedin production.

Attempting to isolate a large source of somatomedin from the liver met with failure. Indeed, when expressed as units/mg of protein, extracts of muscle liver, kidney, pituitary, thymus and brain from the rat consistently have revealed concentrations which were less than concentrations in the serum (Clemmons and Van Wyk, 1981). These data would suggest that somatomedins are not stored, rather synthesized on demand. Evidence supporting this theory was provided in studies observing the effect of hGH on somatomedin deficient and normal humans (Copeland, Underwood and Van Wyk, 1980). Thirty hypopituitary patients and normal subjects were given graded doses of hGH in a sequence of 0.1,

0.2, 0.4, and 0.8 units/kg (im). After a lag of 6 hours a staircase pattern of IGF-I was observed in response to the graded dose of growth hormone as determined by radioimmunoassay techniques. It was concluded that IGFs are synthesized de novo rather than released from a storage pool.

Some of the early experiments which contributed to the eventual elucidation of somatomedins utilized hypophysectomized rats. These studies led to the theory that somatomedins mediated, in part, some of the effects of growth hormone. Attempts were made to demonstrate a direct effect, on the liver, by growth hormone on the release of somatomedins using a perfused liver model. McConaghey and Sledge (1970) using a liver perfusate system were the first to report increased somatomedin-like activity following introduction of growth hormone into the perfusion medium. Similar increases in somatomedin-like activity in liver perfusates in response to growth hormone infusion have been observed (Hintz, Clemmons and Van Wyk, 1972; Phillips, et al., 1976). However, in each case where growth hormone stimulation has been observed, supraphysiological concentrations of growth hormone (>50 ng/ml) have been used.

Other studies have cast doubt on the specificity of growth hormone induced somatomedin increase in vitro. It has reported that insulin and ovine prolactin produced a greater increase in somatomedin production by perfused rat livers than did growth hormone (Daughaday, Phillips and Herington, 1976). Another study found that fetal liver explants released significant amounts of a MSA-like peptide into the

medium. What is interesting is that the explants were in serum-free medium without the presence of exogenous hormones. These studies indicate that several factors may play a role in somatomedin regulation. Nevertheless, further study is needed to understand the almost certain complex nature of in vivo somatomedin control.

Receptors. It has been suggested that insulin, IGF-I and II have closely related effector pathways as they have been shown to elicit similar biological responses in vitro. These similarities are probably best explained by their closely related receptors. The insulin receptor has two alpha subunits with a molecular weight between 120,000 and 130,000 and two beta subunits weighing 90,000 daltons. The alpha subunits are joined together by disulfide bond. Each beta subunit is attached to the alpha subunit pair by disulfide bonds. The IGF-I receptor has a secondary structure that is similar to the insulin receptor but with slightly different subunit weights. The IGF-I receptor alpha and beta subunits weigh 130,000 and 95,000 daltons respectively. The IGF-II receptor consists of only one 250,000 molecular weight molecule (Nissley et al., 1980).

Evidence suggests that both IGF-I and insulin can bind to the same receptor (Marshall et al., 1974; Florini, Nicholson and Dulak, 1977). However, superphysiological levels of insulin were needed to effect this cross-over with the IGF-I receptor (Florini et al., 1977). IGF-I and II can also cross react at the receptor level, but insulin probably does not bind to IGF-II receptor (Florini et al., 1977). These observations led to the concept that the growth-promoting actions of somatomedins are mediated through specific somatomedin receptors. The

the insulin-like actions of these hormones are mediated through their cross-reactivity with the insulin receptor (Van Wyk et al., 1974).

Transport. Both IGF-I and IGF-II are transported in the plasma via plasma binding proteins (Zapf, Waldregel and Froesch, 1975). Two binding proteins have been identified. The first is the major carrier synthesized in liver cells in response to growth hormone (White et al., 1981). The second is the minor carrier and is much smaller, approximately 60,000 daltons (Daughaday et al., 1982a) and probably functions in a scavenger role for unbound somatomedin (Smith, 1984). Both carriers have receptors for both IGF forms (Smith, 1984).

Function. A large volume of evidence suggest that concentrations of IGF-I in the human fetus and newborn are lower than those found in adult serum (D'Ercole, Underwood and Van Wyk, 1977; Takano et al., 1976; Hall, 1971). IGF-I levels rise dramatically during the rapid prepubertal growth phase (Luna et al., 1983) and appear to be correlated with postnatal growth velocity of both sexes (Rosenfield, Furlanetto and Beck, 1983). Using radioimmunoassay for SM-C/IGF-I Furlanetto et al, (1977) observed serum concentrations of IGF-I in humans from late gestation through 82 years of age. Levels in late gestational and cord blood were lowest and rose progressively through the next 13 years. Peak values were reached at puberty followed by slowly tapering of levels in successive decades. Low levels of IGF-I during the time of life when growth rate is maximal appears paradoxical. However, it must be remembered that only the concentrations of IGF-I were assayed. Similar studies for IGF-II are necessary to gain a

complete understanding of activity during growth.

\* Nutritional status appears to play a role in the regulation of IGF activity. Studies have shown that nutritionally deprived children, exhibiting impaired growth, have blood levels of growth hormone which may be normal or slightly elevated (Raghuramulu and Rao, 1974). It would appear then that inadequate nutrition could regulate IGF independent of growth hormone levels. In fasted rats, serum levels of IGF activity were decreased (Daughaday and Kipnis, 1966). Phillips and Young (1975) observed that in normal rats fasted for 72 there was a significant fall in IGF activity in spite of normal growth hormone levels. This decrease in IGF activity is thought to be due to the production of macromolecular IGF inhibitory factors. In rat liver perfusate studies, it has been shown that the liver can produce IGF inhibitor during inadequate nutritional states (Vassilopoulou-Sellin, Phillips and Reichard, 1980). Further study is necessary to identify the mechanism of control for the IGF inhibitor. The presence of an inhibitor would explain the reduction in IGF activity in the presence of elevated growth hormone levels.

IGF-I and -II are thought to play a role in fetal growth and development. Furlanetto et al., (1975) demonstrated that maternal IGF-I levels were elevated throughout gestation. IGF-I in maternal circulation also appears to have some influence on the duration of gestation (Wilson et al., 1982). Maternal IGF-II levels rise dramatically during the third trimester in humans (Cooke and Nicoll, 1983; Wilson et al., 1982) and throughout gestation in rats increasing again just prior to gestation (Daughaday et al., 1982b). Both IGF-I and IGF-II have been shown to positively influence fetal development (Sara,

Gennser and Persson, 1982; Bennett et al., 1983). After birth, maternal levels of IGF-II drop off immediately (Moses et al, 1980).

Insulin-like growth factors have been shown to exert an effect on muscle tissue as well as muscle satellite cells. Salmon and DuVal (1970), observed increased  $^3\text{H}$ -leucine incorporation into protein and tritiated thymidine into DNA in the rat diaphragm following exposure to a partially purified somatomedin preparation. Rat diaphragm muscle have also been shown to increase glucose transport, amino acid uptake and protein synthesis when exposed to partially purified somatomedin A preparations. In cell culture both IGF-I and II have demonstrated an ability to stimulate myogenic cells (Allen, Dodson and Luiten, 1984, Allen et al., 1986; Florini et al., 1977). These observations are consistent with the theory that IGFs mediate some of growth effects of growth factor.

Pledger et al. (1977) provided a model for explaining the mechanism by which somatomedins might interact with other factors to stimulate proliferation. Using quiescent Balb/C 3T3 cells it was observed that platelet derived growth factor (PDGF) was required before cells would respond to serum growth factors contained in plasma. This initial conditioning of the quiescent Balb/C 3T3 cells by PDGF was called competence. Therefore those growth factors which stimulate cells to leave the quiescent state are referred to as competence factors (Pledger et al., 1978). The 12 hour lag phase required before cells were responsive to serum factors was termed progression. Hence, those serum factors were referred to as progression factors (Vogel et al.,

1978). Some of the serum progression factors were later identified (Styles et al., 1979). Following exposure to PDGF, Balb/C 3T3 cells were exposed to plasma from hypophysectomized rats or hypopituitary humans. These cells experienced limited progression, probably due to small amounts of IGF-I in the serum. The addition of IGF-I was required for optimal stimulation of DNA synthesis.

In summary insulin-like growth factors are generated and released from the liver in response to growth hormone and possibly several others as indicated by in vitro studies. IGFs then exert insulin-like growth promoting activities on muscle, cartilage, as well as other tissues. At the cellular level IGFs appear to function as progression factors, initiating progression through the cell cycle of competent cells.

#### Fibroblast Growth Factor

Fibroblast growth factor (FGF) consist of two moieties, very closely related peptides, which are present in many mesoderm and neuroectoderm derived tissues.

Basic FGF (bFGF) was identified because it was active in the stimulation of DNA synthesis in BALB-c fibroblast cells (Gospodarowicz, 1974a). Experiments involving extracts from bovine pituitary glands, suggested the presence of a growth factor which stimulated fibroblast proliferation. This factor was subsequently isolated by carboxymethyl-sephadex gradient elution chromatography, sephadex G-50 chromatography, and polyacrylamide gel electrophoresis.

The presence of acidic FGF (aFGF) was first suggested when it

was discovered that brain or pituitary extracts accelerated the proliferation of myoblast (Gospodarowicz, Weseman and Maran, 1975). When comparing the mitogenic effects of the extracts after removal of bFGF, it was observed that the remaining extract was effective in increasing the proliferation of cells. Through further purification the growth factor was isolated using a DEAE-Sephadex column and was found to be a neutral protein.

Studies have demonstrated the similarity of acidic and basic FGF. These growth factors share a 55% total sequence homology, have about the same mass (16,000 to 17,000) and most likely descended from the same ancestral gene (Gimenez-Gallego et al., 1985). Abraham et al. (1986), compared bovine and human bFGF found a 98.7% overall amino acid sequence homology. Human aFGF and bovine bFGF had a 92% overall amino acid sequence homology. bFGF is a 146 amino acid single chain peptide. A truncated form lacking a 15 amino acid sequence from the amino-terminal also exist. The native and truncated form demonstrate equivalent biological activities.

Receptor. As might be expected from their similar structural homology aFGF and bFGF both interact with the same cell surface receptors. One research group used affinity cross-linking studies to identify two receptors with molecular weights 145,000 and 125,000 daltons that bound both mitogens (Gospodarowicz, Neufeld and Schweigerer, 1986) . Acidic FGF and basic FGF displayed different affinities for each receptor which might explain why aFGF is many fold less potent than bFGF. Neither FGF appears to bind to other growth factor receptors and other growth factors do not bind to FGF the

receptor (Gospodarowicz et al., 1986). Olwin and Hauschka (1986) identified a single 165,000 dalton receptor in Swiss 3T3 cells. This receptor was highly specific for FGF as other growth factors were unable to inhibit FGF binding. From these results they concluded that only a single FGF receptor existed. Clearly more investigation is necessary to determine the exact nature of the FGF receptor.

Function. FGF has been isolated from many different tissues (Gospodarowicz et al., 1986). The ubiquitous nature of this hormone suggests that it is representative of a class of hormones which are locally produced and exert control on self (autocrine) or neighboring cells (paracrine). Evidence supporting such a hypothesis can be found in a couple of studies. A dose response study utilizing horse serum with or without 200 ng/ml of pituitary FGF was done on primary rat satellite cells cultures (Allen, et al., 1984). An additive effect on cell proliferation was seen at every concentration where FGF was included. This data would imply that FGF is not a constituent of normal serum. Another study provides evidence which suggest a relationship between FGF and macrophages. Peritoneal exudate cells collected from thioglycollate stimulated mice were examined for the presence of FGF (Baird, Mormede, and Bohlen, 1985). The extract produced a dose dependent inhibition of binding response in a highly specific radioimmunoassay of FGF. Macrophages have long been thought to produce macrophage derived growth factor (MPGF) necessary for injury repair (Baird et al., 1985). MDGF, never characterized, has demonstrated mitogenicity for fibroblast, smooth muscle and endothelial cells

(Martin et al., 1981; Takemura and Werb, 1984). FGF may be similar to MDGF or closely related moiety possessing mitotic activity.

Basic FGF has been shown to act as a competence factor in a cloned muscle cell line (Lathrop, Thomas and Glaser, 1985). The addition of bFGF to BC<sub>3</sub>HI resulted in the suppression of the muscle form of creative phosphokinase (M-CPK). Quiescent cells, G<sub>0</sub> portion of cell cycle, normally produce M-CPK. The addition of bFGF repressed synthesis of M-CPK resulted in the accumulation of the cells at a restriction point in the G<sub>1</sub> of the cell cycle. Removal of bFGF resulted in the return of the cells to their original position. In this case bFGF appears to exert control at the level of gene transcription.

In summary FGF seems to be part of a class of hormones which function through autocrine or paracrine mechanisms. They may therefore be the local signal for growth, and could possibly play a important role in the regeneration capabilities of muscle following injury.

#### Steroid Hormones

##### Steroid Hormone Action

Generally, it is thought that steroids cross the cell membrane and bind to a receptor located in the cytosol and the newly formed hormone-receptor complex migrates into the nucleus. Once the complex is in the nucleus it is thought to interact with an acceptor, perhaps a protein in the chromatin. Steroid hormones act in target cells which possess a specific receptor to which they can bind: a glucocorticoid receptor is found in cells responsive to glucocorticoids ect.,.

## Dexamethasone

There are many naturally occurring steroids which chemists have been able to synthesize analogs to that are biologically more active. Dexamethasone (DEX), a synthetic analog of cortisol, is more potent than the natural steroid. The greater potency of DEX is probably due to DEX's higher affinity for glucocorticoid cytosolic receptors and a longer half-life (Ganong, 1981). Since DEX is an analog of cortisol, evidence obtained from studies using cortisol will be reviewed.

Cortisol is produced and released from the outer portion of the adrenal gland called the adrenal cortex. Cortisol is released in response to adrenocorticotropic hormone (ACTH) which acts via adenylate cyclase (cAMP) and protein kinase to increase free cholesterol. Free cortisol enters the mitochondria where it is converted to pregnenolone. cAMP is also thought to increase a regulatory protein which also aids in the conversion of cholesterol to pregnenolone which is converted (through a series of steps) to cortisol and secreted.

Transport. Steroids are often bound to binding proteins in the circulation. Cortisol binds to transcortin, corticosteroid-binding globulin and to a minor degree, albumin (Ganong, 1981).

Function. Cortisol as well as other glucocorticoids are thought to increase protein catabolism and exert an anti-insulin effect (Goldberg, 1969; Rannels et al., 1978b). In adrenalectomized animals, skeletal muscle is fatigued more easily than in normal animals. This suggests a regulator or permissive action, similar to the requirement of the presence of glucocorticoids for catecholamines to exert some of their metabolic actions (Ganong, 1981).

There have been many cell culture studies performed attempting to elucidate the mode of action of glucocorticoid. Mayer and Rosen (1975) postulated that the effects of glucocorticoids could be reduced by competition at the receptor level by androgens. They based their hypothesis on the fact that a specific cytosolic receptor for androgens had yet to be elucidated and androgens could bind the glucocorticoid receptor. Specific androgen and glucocorticoid receptors in the cytosol were later identified through cross-linking studies which demonstrated little cross-reactivity (Snochowski, Dahlberg and Gustafsson, 1980; Snochowski et al., 1981). Cristofalo and Rosner (1979) observed an increase in cell binding of DEX preceding proliferation when DEX was added to cultures of quiescent cells. They suggested that glucocorticoid receptor concentration is under cell control. They also observed an age-dependent decrease in response to DEX.

DEX has been shown to influence growth factor response and receptor concentration. Using cultured rat bone, Bennett et al. (1984) observed that dexamethasone increased IGF-I receptors. In 3T3 cells DEX was shown to up-regulate insulin receptors (Knutson, Ronnett and Lane, 1982). Conover et al. (1983) observed that dexamethasone enhanced the actions of IGF-I in human fibroblast and Levenson et al. (1985) observed that additions of DEX Swiss 3T3 cells improved the response of cartilage derived growth factor but not platelet-derived growth factor. Gospodarwicz and Moran (1974b) found that dexamethasone greatly enhanced the proliferative effect of FGF by up-regulating receptor number.

## Trenbolone

Testosterone is a sex steroid which is responsible for increasing growth rate and the development of secondary sexual characteristics. These two actions are referred to as anabolic and androgenic characteristics, respectively. The use of castrate male in animal production systems, has prompted investigators to search for a factor which could increase the growth rate but not the development of secondary sexual characteristics. This led to the development of synthetic anabolic compounds which have anabolic activity but possess little or no significant androgenic activity. Trenbolone acetate (TBA) is a 270 dalton synthetic anabolic compound (3 oxy-17-b-hydroxy-4,9,11, estratriene acetate) and has been shown to increase feed efficiency and rate of growth in many animal models. TBA implants have increased growth rate in cattle (Best, 1972; Galbraith and Watson, 1978; Heltzman and Chan, 1974; Chan and Heltzman, 1975) veal calves, bulls, castrate male lambs, castrate pigs and female and male castrate lambs (Sinnott-Smith, 1983) and rats (Vernon and Buttery, 1976, 1978; Martinez, Buttery, and Pearson, 1984; Thomas and Rodway, 1983). The anabolic response elicited by TBA apparently reflects a simple hypertrophy of muscle fibers as there are no changes in structure or composition of skeletal muscle (Venable, 1966).

Function. Although the effect of TBA, in many animals is well documented, the mechanism of action is still unclear. Vernon and Buttery (1976) performed the first experiments in an attempt to elucidate the mode of action of TBA. Female rats were treated with 80 ug per 100

grams of body weight. A significant increase in weight was observed among rats receiving TBA over rats receiving only the oil carrier. Fractional synthetic rates were measured by continuous infusion of L-[U-<sup>14</sup>C] tyrosine, while fractional degradation rates were determined by Na<sub>2</sub><sup>14</sup>CO<sub>3</sub> injections. Decrease in both synthesis and degradation rates were observed. Since an increase in weight was attained Vernon and Buttery concluded that degradation must have been reduced more than synthesis resulting in overall protein accretion. Sinnett-Smith et al. (1983) drew similar conclusions from data they generated in studies utilizing female and male castrate lambs. Fractional synthesis rate was determined by constant infusion of L-[4, 5-<sup>3</sup>H]leucine. The rate of synthesis was determined to be lower in TBA-treated animals than control animals. The rate of protein degradation, however, was not determined. Cathespin D levels were analyzed from a 30 gram sample of muscle removed from the longissimus dorsi. Cathespin D activity has been used as an indicator of protein degradation (Waterlow et al., 1978). Cathespin D levels were decreased in TBA-treated animals when compared to control animals. Since TBA-treated animals gained more weight than control animals, it was concluded that rates of degradation must have been reduced more than the rate of protein synthesis. The net effect would, therefore, be an increase in protein accumulation.

Another hypothesis to account for the effects of TBA is that TBA may interfere with the catabolic activity of glucocorticoids. Mayer and Rosen (1975) suggested that anabolic steroids might compete for the glucocorticoid receptor of muscle and thus reduce the catabolic effects of glucocorticoids. However, Snochowski et al. (1980, 1981) showed that

in rat and porcine muscle there was little evidence to support suggestions of competitive-binding. Although TBA may not compete directly with glucocorticoids. Thomas and Rodway (1983) have shown that TBA can influence circulating corticosterone concentrations. TBA was able to act directly at the adrenal level suppressing corticosterone production by 25%. Thus, TBA may exert a direct action upon the adrenal cortex or by reducing corticosterone releasing factor and/or adrenalcorticotrophic hormone. The net result of these actions would be a reduction in catabolism through decreased secretion of corticosterone.

There is a large amount of evidence suggesting multiple mechanisms of TBA in vivo. It seems reasonable considering the complexities of growth, that many mechanisms may be working in concert to produce the observed effects of TBA. Further investigation may provide more direct evidence for additional modes of action of TBA in stimulating animal growth.

## CHAPTER 2

### MATERIALS AND METHODS

#### General Reagents and Tissue Culture Ware

Dulbecco's Modified Eagle's Medium (DMEM), antibiotic antimycotic mix, Deutsch fetuin, ascorbic acid, fetal bovine serum, and vitamin E were purchased from Gibco (Grand Island Biological Co., Grand Island, N.Y.). Linoleic acid, basement membrane MATRIGEL, selenium and fibronectin were purchased from Collaborative Research, Inc. (Bedford, MA). Gentamicin sulfate was purchased from Irving Scientific (Santa Ana, CA), and pronase was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Dexamethasone, biotin, heparin, bovine serum albumin standard, deoxyribonucleic acid (DNA) and sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO). 3,5-diaminobenzoic acid dihydrochloride (DBA) was purchased from Eastman Kodak Co. (Rochester, N.Y.). Dimethyl sulfoxide was purchased from Fisher Scientific (Fair Lawn, NJ). Centrifuge tubes were obtained from Falcon Co. (Oxnard, CA) and tissue culture plates and cryotubes were purchased from Nunc Intermed (Vangard International Inc., Neptune, NJ).

#### Animals

Young female sprague dawley rats, 60 to 90 grams, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Animals were housed in a University animal care satellite facility, with 4-5 rats per

cage. The animals were allowed food (rat chow, Eagle Milling Co., Tucson AZ) and water ad libitum.

### Hormones

#### Fibroblast Growth Factor.

Fibroblast Growth Factor (FGF) was purchased from Collaborative Research, Inc. (Bedford, MA). FGF is a bovine-derived polypeptide mitogen and greater than 90% pure by SDS-PAGE. FGF was hydrated in sterile deionized distilled water, and aseptically aliquoted into small 1 ml cryotubes and stored at 4° C.

#### Insulin-Like Growth Factor-I.

Thr-59 insulin-like growth factor-I (IGF-I) was purchased from AMGEN (Thousand Oaks, CA). This recombinantly produced product is 99% pure and 145% active as determined by HPLC and radio-immunoassay respectively. IGF-I was dissolved in 0.1 M sterile acetic acid and aseptically aliquoted into 1 ml cryotubes and stored at 4°.

Ovine somatomedin (oSm) was donated by Dr. K. Hosner, Colorado State University.

#### Insulin.

Insulin, purified from bovine pancreas, was purchased from Collaborative Research Inc. (Bedford, MA). The specific activity was  $2.2 \times 10^{-5}$  insulin units per nanogram insulin. Contamination includes 1.16 ppm glucagon, and 16.0 ppm of proinsulin, as determined by radioimmunoassay.

Trenbolone.

Trenbolone (TBOH) was generously provided by William A. Olson, Center For Regulatory Services (Reston, Va.). TBOH was diluted in ethanol and brought up to the necessary concentration in corn oil.

#### Satellite Cell Cultures

Primary satellite cells were harvested according to the procedure described by Allen (1980), as modified from Bischoff (1974). In brief, animals were sacrificed and washed in 70% ethanol. Tissue from the major muscle groups are excised under aseptic conditions in a laminar continuous flow hood. After excessive connective tissue and adipose tissue were trimmed away, the tissue was rinsed in phosphate buffered saline, (PBS) and ground in a tissue grinder. The ground tissue was weighed and transfer into sterile centrifuge tubes (approximately 10 grams per tube). About 30-35 ml of PBS, containing pronase (1 mg/ml) was added, and the tubes were incubated in a 37°C water bath for one hour. The tubes were agitated at fifteen minute intervals to insure adequate exposure to pronase. At the end of the hour, the tissue was subjected to a series of centrifugations designed to isolate satellite cells. The cells were then incubated in DMEM-10% HS for several hours in 144 mm culture plates (during the incubation period fibroblasts attached to the plates while satellite cells did not). After several hours the satellite cells were poured off, spun down, aliquoted into 3 ml cryotubes and frozen in 20% fetal bovine serum and 10% dimethyl sulfoxide.

#### Serum Preparation.

Horse and rat serum were prepared in basically the same manner; blood was collected, allowed to clot at 4° C, spun in the centrifuge at 1400 x g for 20 minutes, and the serum drawn off and frozen at -70° C, in small aliquots.

#### Cell Culture Studies.

All cell culture studies followed the same basic protocol. Twenty-four well culture dishes were chilled then coated with basement membrane MATRIGEL (BMM). BMM was diluted 1 to 10 in Dulbecco's Modified Eagle Medium (DMEM) and stored frozen in small aliquots for convenience. A small amount of BMM (150 ul) was pipeted into each well and then removed, leaving only a thin film. Satellite cells were then added in DMEM-10% HS. Cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% carbon dioxide. After 24 hours, cultures were washed twice with DMEM to insure removal of cellular debris and serum, and the various treatment media were introduced for 72 hours. With the exception of studies using defined media, all treatment media were supplemented with 2% horse serum. Cell cultures were washed two times with DMEM at the end of the culture period. The cells were then exposed to fusion medium for 18-24 hours. Fusion medium, which consisted of DMEM-1.5% HS, linoleic acid (1 ug/ml) and heparin (100 ug/ml), has been shown to depress satellite cell proliferation and induce differentiation resulting in fusion. After removal of fusion medium, the cells were fixed with methanol and stained with gimesa. Mononucleated cells and nuclei in fused cells were counted microscopically and used as an

experimental index of the satellite cell proliferation.

In experiments where two different tissue types were used, TBOH-treated and control rat muscle, the results were normalized back to a 24 hour cell count (cells were plated in a separate dish during initial plating, fixed at 24 hours, stained and evaluated). This procedure takes into account any variation in plating efficiency between the different tissues and allows for unbiased comparison of results.

Two Studies utilized serum free medium. The treatment media consisted of the following constituents: selenium (3.75 ng/ml), linoleic acid (1 ug/ml), dexamethasone ( $10^{-9}$ M), fetuin (500 ug/ml), insulin ( $10^{-9}$ M), fibronectin (50 ng/ml), biotin (500 ng/ml), AB (1 ml/100ml), gentamycin (.200ml/ml), vitamin E (10 ng/ml), ascorbic acid (10 ug/ml), and DMEM and MCDB Medium 104 in a 3:1 ratio. FGF (150 ng/ml) was present in defined medium, unless FGF was a treatment variable. The different hormones, FGF and IGF-I, were included in defined medium at the indicated concentrations. After 72 hours in the defined treatment medium, cells were fixed, stained and evaluated as previously described.

#### TBOH Injections

Preliminary experiments which were conducted, concluded that the concentration of TBOH (80 ug TBOH/100 grams of body weight) used in other experiments (Vernon and Buttery, 1976) would generate an adequate growth response. Rats were injected subcutaneously via the neck skin fold. Treated animals were injected with TBOH, dissolved in 95% ethanol and brought up to the afore mentioned concentration in corn oil. The non TBOH treated animals were injected with the corn oil carrier only.

The animals were anesthetized with ether and injected every morning for 14 consecutive days.

#### DNA Assay

Total amount of DNA present was determined in the selected muscle. Three muscles from the lower leg were selected for evaluation: the gastrocnemius, the peroneus group, and the tibialis anterior. These muscles were selected because they could be excised in toto, from tendon to tendon. It was necessary to obtain the complete muscles to permit accurate determination of total DNA and subsequent comparisons between muscle groups. Rats received treatments fourteen days and on the fifteenth day rats were sacrificed. Muscles were excised from the left hind limb. The muscles were placed in tarred vials, and the wet weight was obtained. The muscles were then frozen at  $-70^{\circ}\text{C}$  until the day of the assay. On the day of the assay, the muscles were removed, one at a time, from the freezer, placed in liquid nitrogen, pulverized to a powder, placed in a tarred test tube and the preparation weight was recorded. Three milliliters of .15 M NaOH was added, and the samples were stored at  $4^{\circ}\text{C}$  for several hours. The tissue samples were then homogenized with a Kinematia Polytron for two 10 second bursts. The final volume was brought up to 10 ml for the peroneus muscle group and tibialis anterior. The final volume for the gastrocnemius muscle was 13 ml, due to its much larger mass. Twenty-five microliters of each sample was pipeted into test tubes. DNA concentrations from 0-30 mg/ul were used to establish a standard curve. Two percent SDS was added to each tube resulting in a final volume of all tubes was 75 microliters.

Seventy-five microliters of 3, 5-diaminobenzoic acid (DBA) was added, tubes were covered with parafilm and incubated at 60°C for one-half hour. At the end of 30 minutes the samples were allowed to cool and 1.5 milliliters of 1 N HCl was added to each tube and aspirated. The samples were then read on a Perkin-Elmer Fluorescence Spectrophotometer. Excitation was set at 405 nm and emission at 520 nm with a slit width of 2.

Total Protein Analysis: Micro-Biuret

Total protein determinations were made using the micro-biuret procedure. The micro-biuret procedure was performed as described by Gornall (1949). Briefly, the biuret reagent was prepared using 30% NaOH, 0.21%  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ . Standard curve concentrations range from 0.025-0.800 mg BSA/ml. All standard curve samples were brought up to a final volume of 200 milliliter. Samples and tissue were prepared as follows: 500 ul of biuret reagent, 800 ul of  $\text{H}_2\text{O}$ , and 200 ul of sample. After fifteen minutes the absorbance at 310 nm was determined in a Gilford 260 Spectrophotometer.

## CHAPTER 3

### RESULTS

#### In Vivo Effects of TBOH

##### Animal Growth Characteristics

In the initial growth study female rats weighing 60 to 90 grams received daily injections of TBOH or carrier. After nineteen days of injections animals receiving the TBOH treatment were significantly larger ( $p < .05$ ) than animals in the control group (fig. 1). At nineteen days the injections concluded and during the following twenty-one day period the growth curves converged. After twenty-eight days, there was no longer a significant difference between the two groups.

The results of seventeen two-week studies, conducted during a two year period, are shown in table 1. TBOH treatment improved animal growth performance in two areas: total weight gained and weight gain per day. The average starting weights among the seventeen studies ranged from 63 to 124 grams. This disparity was a result of animal availability. The difference between the animals receiving TBOH treatments and those not receiving treatments was highly significant ( $p < .0001$ ) for both parameters. In eight studies, body weight gain per gram of feed consumed was also monitored. TBOH treatment groups gained significantly ( $p < .05$ ) more body weight per unit of feed consumed than control groups.

Figure 1. Animal Growth in Response to TBOH-Treatment.

Sixteen animals received daily sub-cutaneous injections, via the neck skin fold, for 19 days. TBOH treated animals received 80 ug of TBOH per 100 grams of body weight. Control animals received an equal volume of the corn oil carrier. Each point represents mean and standard error (SE) of eight animals. Statistical differences were determined using the student's t-test.

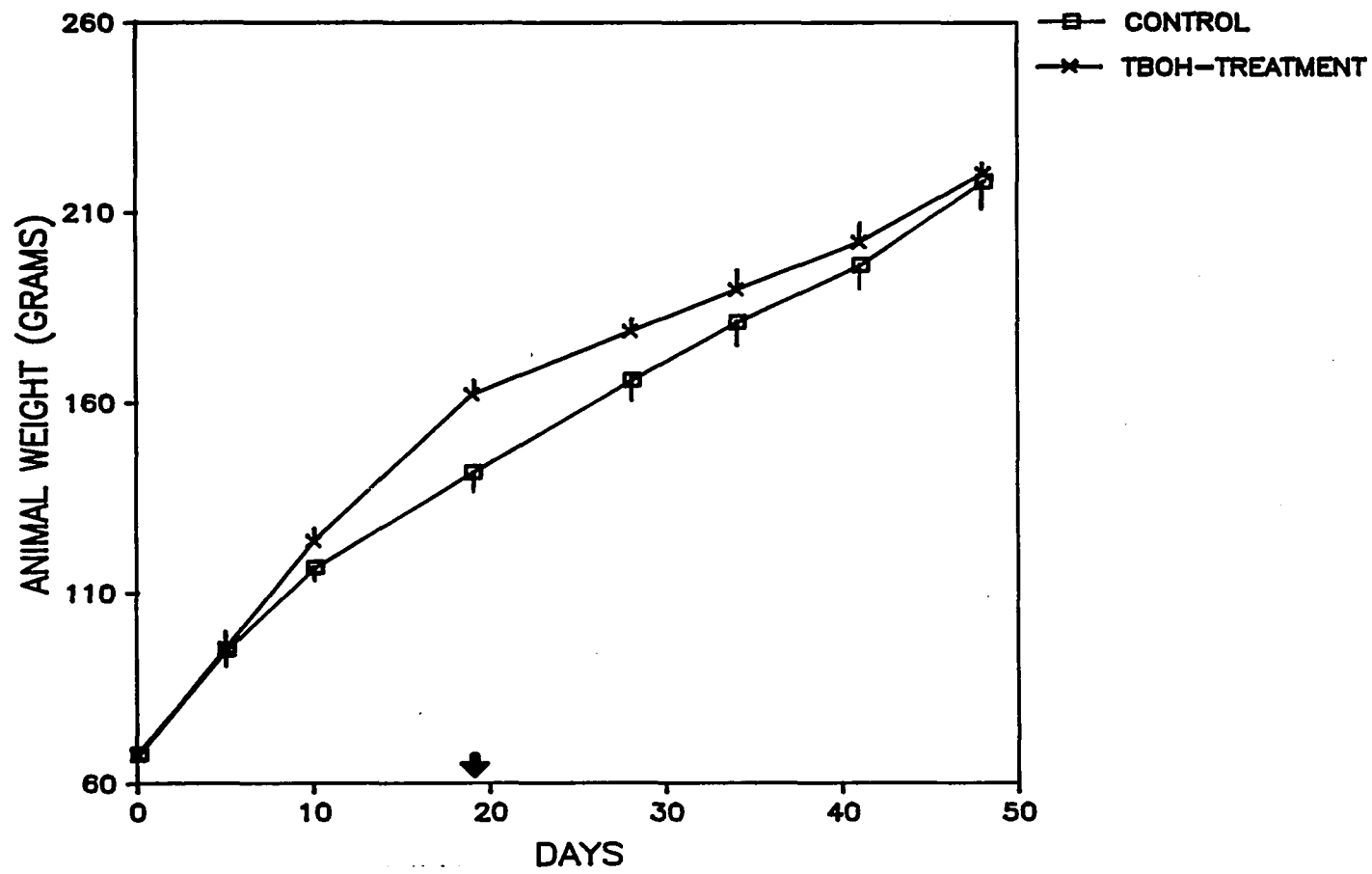


Figure 1. ANIMAL GROWTH IN RESPONSE TO TBOH-TREATMENT

Table 1. Growth characteristics.

Animal growth was measured during seventeen two week injection periods. There were four animals per cage for both treatments. For eight experiments the total feed consumed was also recorded. Weight gain per day, total weight gain, final weight at conclusion of experiment and amount of feed consumed per weight gain were calculated. Statistical differences were determined using the student's t-test.

Table 1. GROWTH CHARACTERISTICS

TREATMENT GROUP	LIVE WEIGHT (gm)	TOTAL WEIGHT GAINED (gm)	WEIGHT GAINED /DAY (gm)	WEIGHT GAINED /GRAMS OF FEED
TBOH	182±21.6 <sup>a</sup> n=72	67.13±15 <sup>a</sup> n=72	4.53±74 <sup>a</sup> n=72	.394±.51 <sup>c</sup> n=8 <sup>b</sup>
CONTROL	166±20.8 n=70	49.99±17 n=70	3.31±.91 n=70	.298±.05 n=8 <sup>b</sup>

all values are mean ± S.E.

compared to control using student's t-test

a =  $p < .0001$

b = the value 8 represents cages of four rats each

c =  $p < .05$

### Muscle Growth and Composition.

Animals from one of the growth trials were used to study changes in muscle composition. The gastrocnemius, peroneus group, and tibialis anterior muscles were removed in toto, and the weight were recorded. Total muscle protein and DNA content were determined for each of these muscles; results are presented in table 2.

Muscle from TBOH-treated rats were not significantly larger than the muscles from the control animals. The total protein values of muscles from the two treatment groups were also not significant. Total DNA content of muscle from TBOH-treated animals, however, was significantly greater than the DNA content of control rat muscles for both tibialis anterior ( $p < .025$ ) and the peroneus group ( $p < .0001$ ). The gastrocnemius from TBOH-treated rats had a slightly higher DNA content, but the difference was not statistically significant. The ratio of DNA to protein was increased in all muscle from TBOH-treated animals compared to controls. Gastrocnemius and tibialis anterior muscle DNA to protein ratios from TBOH-treated rats were significantly greater at  $p < .005$ , when compared to the control. The peroneus group was significantly different at  $p < .0001$ .

The final weight and total weight gained by the four groups of rats used for the DNA and total protein studies are shown in table 3. The final weight attained by the TBOH-treated animals was approximately 10% greater ( $p < .025$ ) than the weight gained by the control group. The total weight gained by TBOH-treated rats during the treatment period was significantly more than the control animals ( $p < .001$ ).

Table 2. Muscle Weight, DNA and Protein Content.

The gastrocnemius, peroneus muscle group, and tibialis anterior muscles were removed in toto from 21 animals, 10 control and 11 TBOH-treated. These muscles were analyzed for DNA and total protein. Statistical differences were determined using the student's t-test.

Table 2. MUSCLE WEIGHT, DNA AND PROTEIN CONTENT

MUSCLE	NET WEIGHT (gm)	TOTAL PROTEIN /MUSCLE (mg)	TOTAL DNA /MUSCLE (mg)	DNA/ PROTEIN $\times 10^{-3}$
<b>GASTROCNEMIUS</b>				
CONTROL n=10	1.05 $\pm$ .06	108.6 $\pm$ 4.6	.568 $\pm$ .05	5.23 $\pm$ .37
TBOH n=10	.94 $\pm$ .18	102.1 $\pm$ 1.3	.596 $\pm$ .06	5.48 $\pm$ .49 <sup>a</sup>
<b>PERONEUS GROUP*</b>				
CONTROL n=10	.171 $\pm$ .02	26.9 $\pm$ 3.0	.085 $\pm$ .009	3.15 $\pm$ .27
TBOH n=11	.161 $\pm$ .04	27.0 $\pm$ 6.0	.100 $\pm$ .01 <sup>b</sup>	3.69 $\pm$ .43 <sup>b</sup>
<b>TIBIALIS ANTERIOR</b>				
CONTROL n=10	.361 $\pm$ .03	37.9 $\pm$ 3.0	.134 $\pm$ .02	3.54 $\pm$ .47
TBOH n=11	.342 $\pm$ .05	37.0 $\pm$ 1.5	.158 $\pm$ .03 <sup>c</sup>	4.27 $\pm$ .21 <sup>a</sup>

All values are mean  $\pm$  S.E.

compared to control using student's t-test

a = p < .005

b = p < .0001

c = p < .025

\* longus, brevis, tertius

Table 3. Animal Weights From Muscle Composition.

These values represent the final live weight and total weight gained for the 21 animals used in the muscle DNA and total protein determinations, recorded in Table 2. Statistical differences were determined using the student's t-test.

TABLE 3. ANIMAL WEIGHTS FROM MUSCLE COMPOSITION STUDY (TABLE 2)

TREATMENT GROUP	FINAL LIVE WEIGHT (gm)	TOTAL WEIGHT GAINED (gm)
TBOH n=11	171.6±8.7 <sup>a</sup>	79.0±6.2 <sup>b</sup>
CONTROL n=10	159.6±13.8	64.3±10.3

All values are mean ± S.E.

compared to control using student's t-test

a =  $p < .025$

b =  $p < .001$

### In Vitro Effects of TBOH

#### Effect of Serum From TBOH-Treated and Control Rats on Satellite Cell Growth

Blood was collected from TBOH-treated and control rats, and serum was prepared. Satellite cells from untreated female rats were cultured for this study. After 24 hours in medium containing 10% serum, treatment media containing rat serum from TBOH-treated or control rats was added in 1, 3, 5, 7, and 10 percent concentrations. The satellite cells responded to both types of serum in a dose dependent manner (fig. 2). Serum collected from TBOH-treated animals appears to have had the ability to shift the growth curve to the left. The maximum response to TBOH serum was observed at 7%. However, the maximum response of the control serum is  $\geq 10\%$ . The satellite cell response to TBOH serum appears to be greater overall and the maximum response is much greater. Two-way analysis of variance revealed a significant ( $p < 0.01$ ) TBOH treatment by serum concentration interaction.

#### Effect of TBOH, Dexamethasone and Growth Factors on Satellite Cells Growth

Steroid Hormone and Insulin-Like Growth Factor-I Interaction. The objective in this study was to examine the interaction of IGF-I with TBOH or dexamethasone. Satellite cells from untreated female rats were cultured; after 24 hours the cells were exposed to defined media containing DEX, TBOH, or ethanol. These treatments were prepared with and without 0.5m/IGF-I (100 ng/ml). One-way analysis of variance

Figure 2. Effects of Serum on Cell Growth.

Satellite cells from untreated female rats were treated with serum collected from control and TBOH-treated rats. Rat serum concentrations ranged from 1, 3, 5, 7, and 10% rat serum. Each point represents the mean and SE of 4 wells. Statistical differences were determined using two-way analysis of variance.

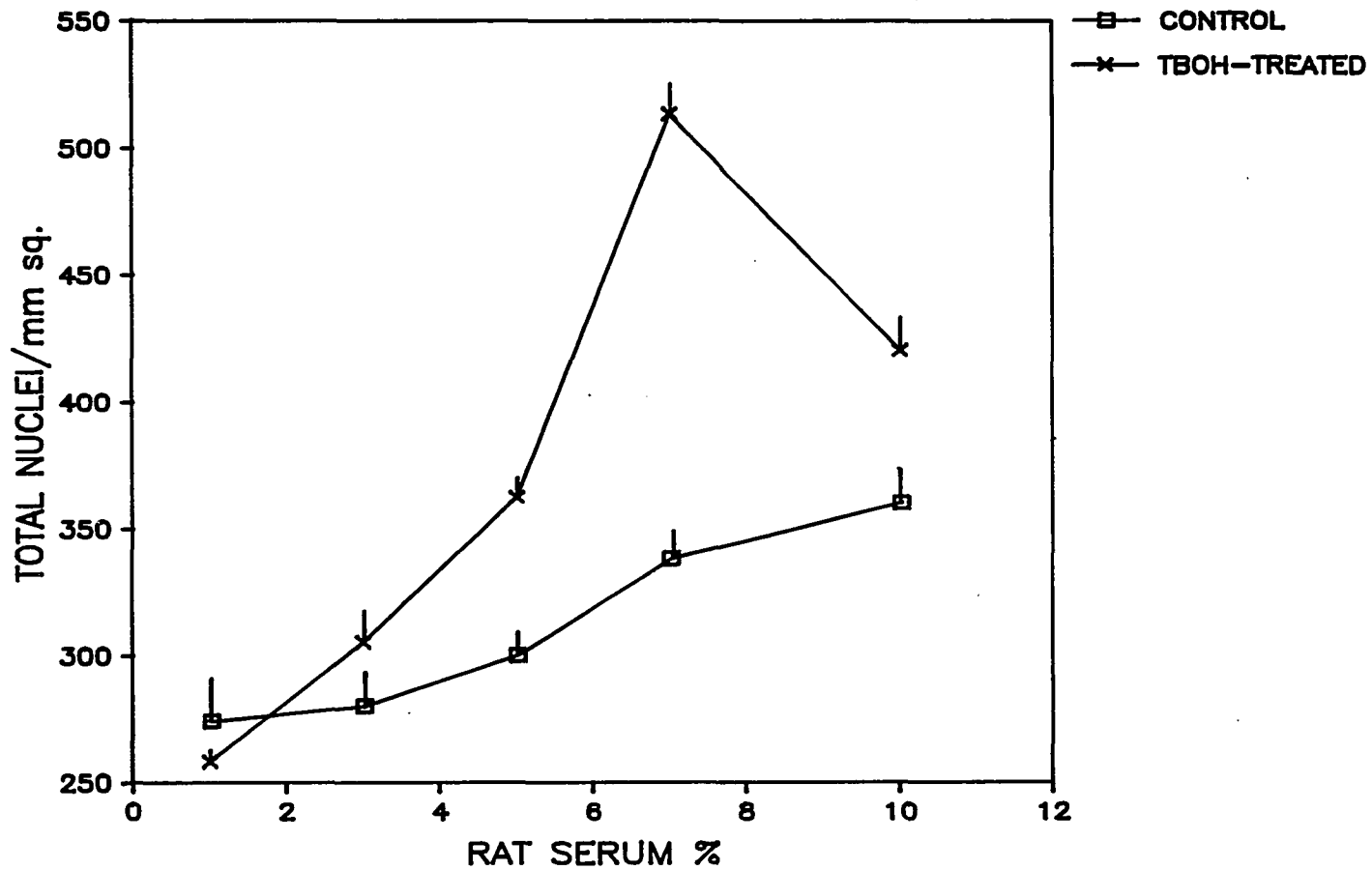


Figure 2. EFFECT OF TREATMENT RAT SERUM ON CELL GROWTH

that total cell density was not affected by the presence of TBOH, in the presence or absence of IGF-I. In the presence of dexamethasone, however, total cell density was significantly increased in the presence and absence of IGF-I (fig. 3). Furthermore, in the absence of dexamethasone (control and TBOH treatments) no significant IGF-I stimulation of cell growth was observed. In the presence of dexamethasone, IGF-I stimulated an increase in cell density. Figure 4 shows the effect of these treatments on myotube nuclei density in culture. Identical results were found with total cell density and myotube nuclei density.

Steroid Hormone and Fibroblast Growth Factor Interaction. This experiment was designed to identify any additive effects that TBOH or dexamethasone might have with FGF. Satellite cells derived from untreated female rat muscle were cultured as in the previous experiment. After 24 hours, the media was replaced with defined media containing either TBOH, DEX or ethanol. Each of the three treatments was prepared with and without 50 ng/ml FGF (fig. 5) One-way analysis of variance demonstrated that in the absence of FGF, no significant differences ( $p < .0001$ ) in total cell density occurred, with either steroid treatment; in the presence of FGF, however, cell density increased significantly ( $p < .0001$ ) in all treatment groups. TBOH and control treatments did not differ, while dexamethasone caused a dramatic and significant ( $p < .0001$ ) increase in cell density when FGF was present.

Myotube nuclei density (fig. 6) was affected in a similar manner as total nuclei density. The only difference was that the increase in

Figure 3. Interaction of Steroids and IGF-I. (Total Nuclei)

Satellite cells from untreated female rats were treated with DEX and TBOH or ethanol with or without IGF-I in defined media. Concentrations of growth factors were: 100 ng oSm/IGF-I per ml, and TBOH and DEX at  $10^{-7}$ M. Each bar represents the mean and SE of 4 wells. Statistical differences were determined using the student's t-test. Treatments identified with different letters differ significantly ( $p < .01$ ).

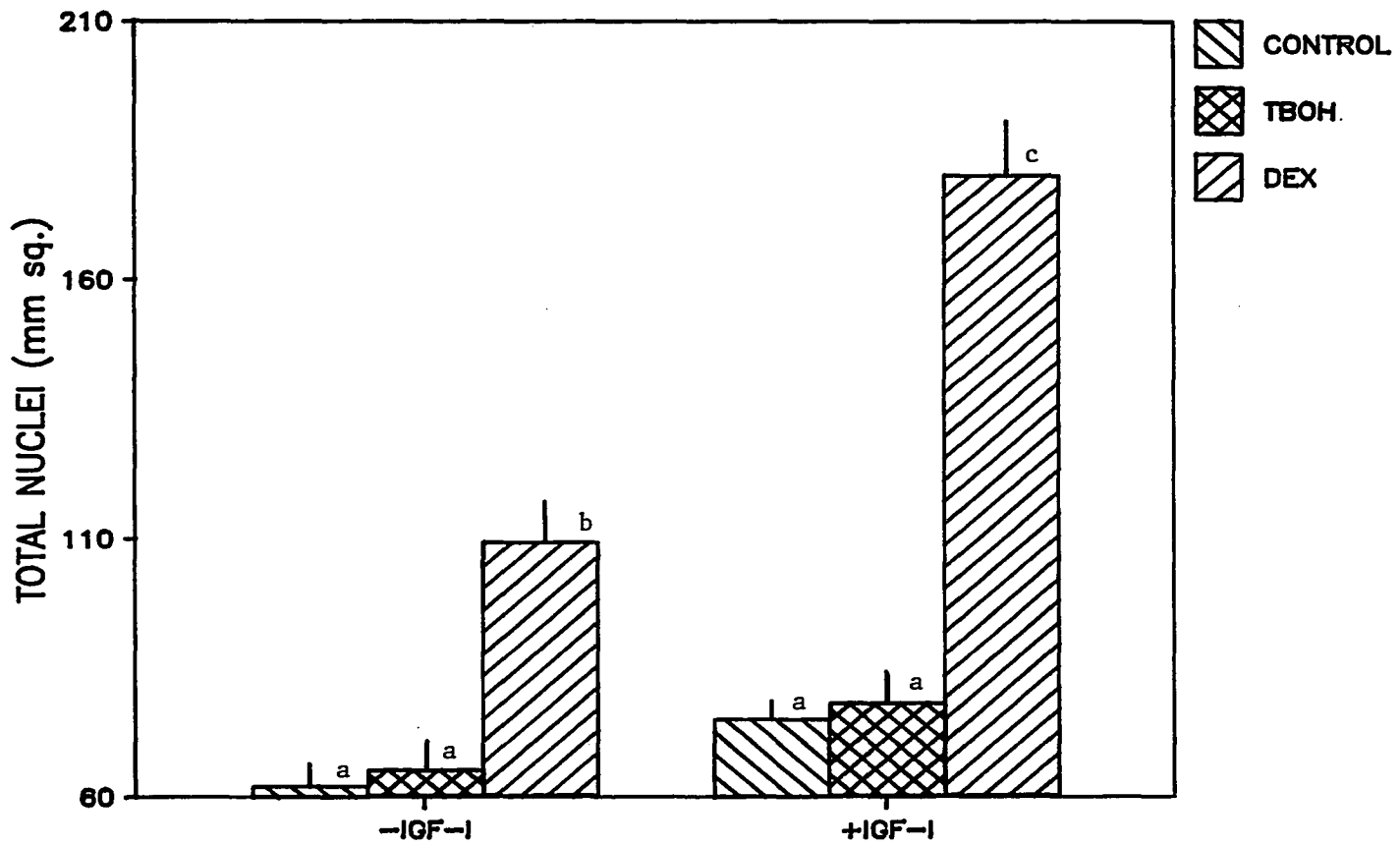


Figure 3. INTERACTION OF STEROIDS AND IGF-I

Figure 4. Interaction of Steroids and IGF-I. (Myotube Nuclei)

Satellite cells from untreated female rats were treated with DEX, TBOH or ethanol, with and without IGF-I in defined media. Concentrations of growth factors were: 100 ng oSm/IGF-I per ml, and TBOH and DEX were 10<sup>-7</sup>M. Each bar represents the mean and SE of 4 wells. Statistical differences were determined using the student's t-test. Treatments identified with different letters differ significantly (p < .01).

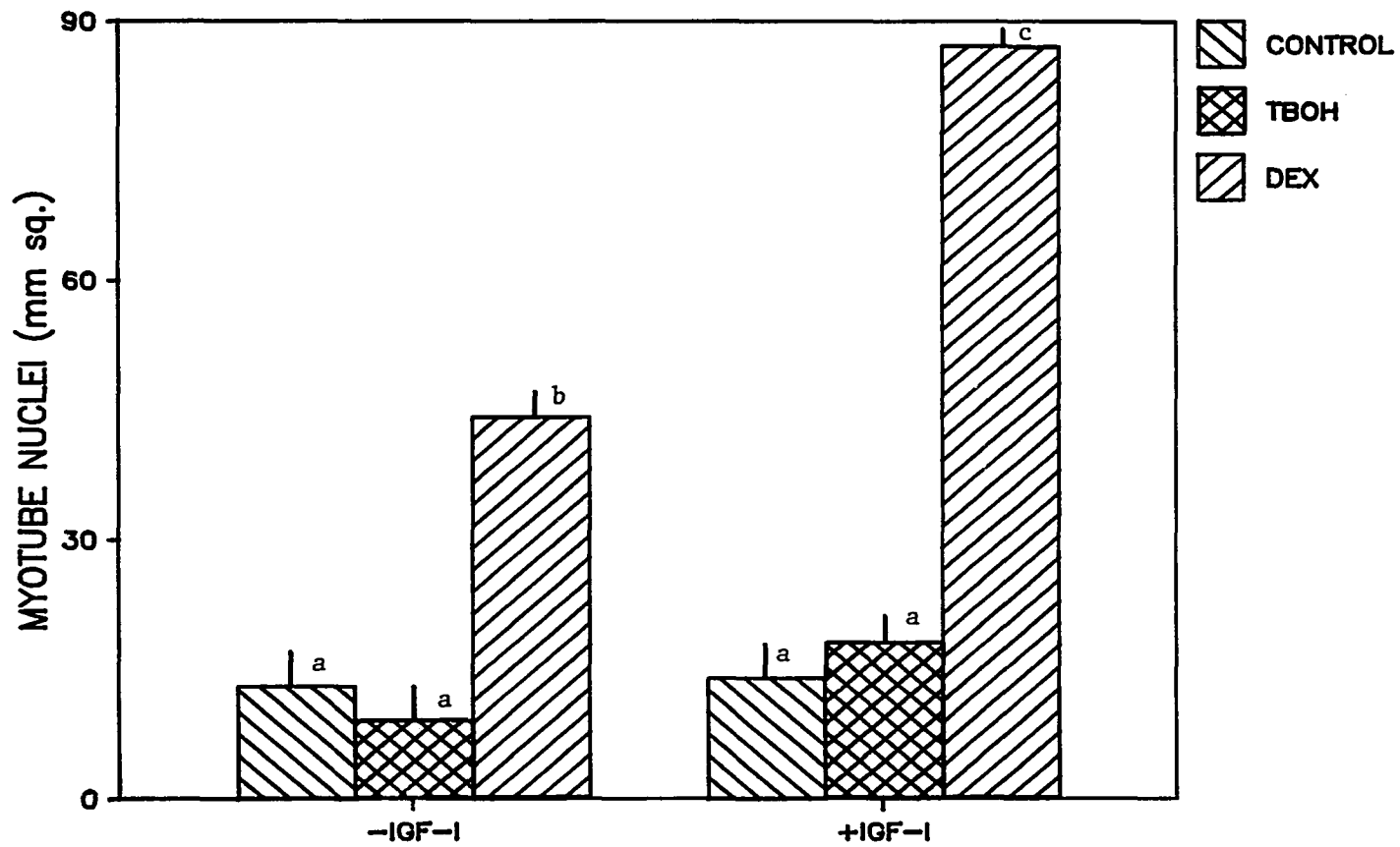


Figure 4. INTERACTION OF STEROID AND IGF-I

Figure 5. Interaction of Steroids and FGF. (Total Nuclei)

Satellite cells from untreated female rats were treated with DEX, TBOH and ethanol with and without FGF in defined media. Concentrations of growth factors were: 50 ng FGF per ml and, DEX and TBOH at  $10^{-7}M$ . Each bar represents the mean and SE of 4 wells. Statistical differences were determined using the student's t-test. Treatments identified with different letters differ significantly ( $p < .01$ ).

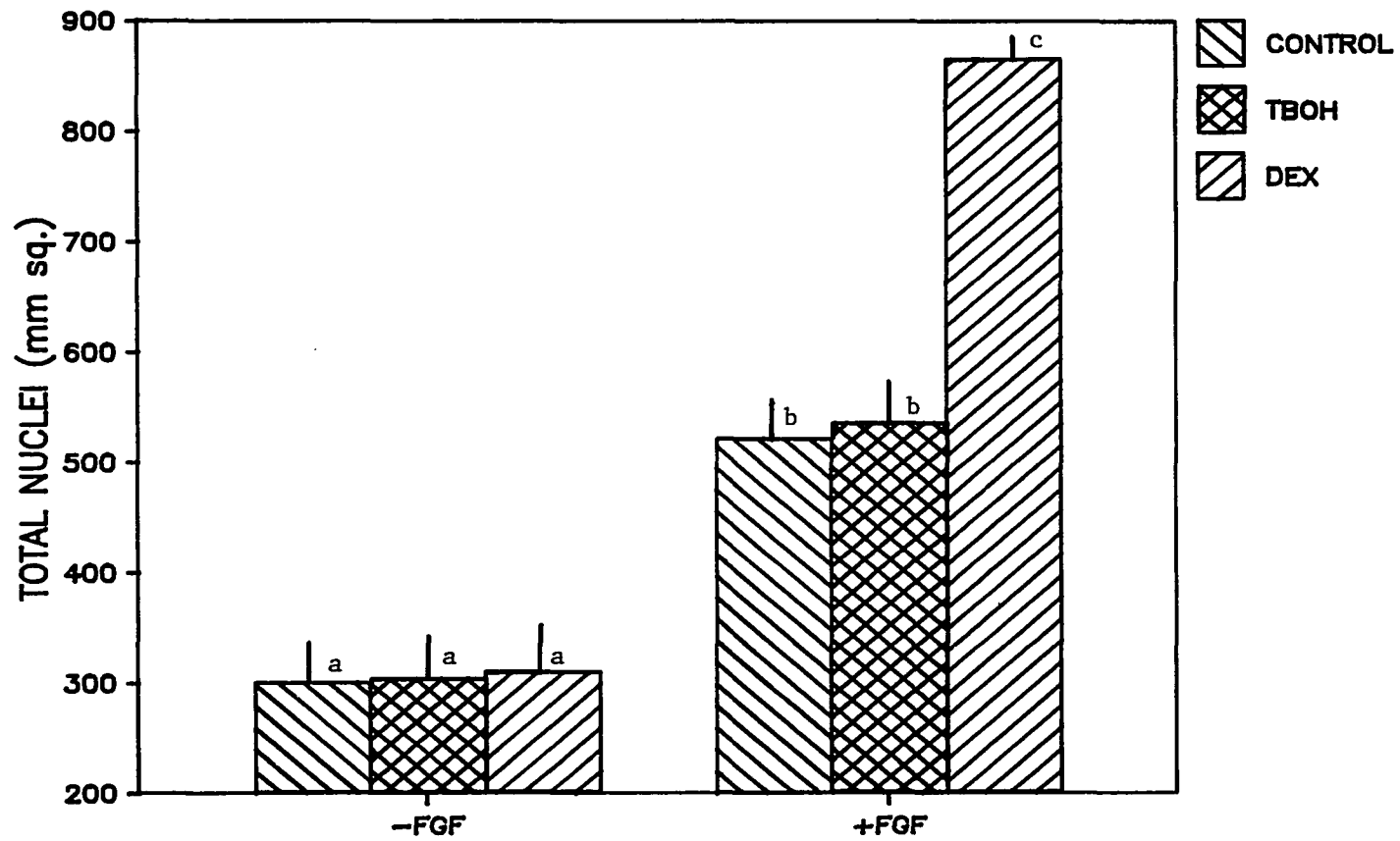


Figure 5. INTERACTION OF STEROIDS AND FGF

Figure 6. Interaction of Steroids and FGF. (Myotube Nuclei)

Satellite cells from untreated female rats were treated with DEX and TBOH with and without FGF in defined media. Concentrations of growth factors were: FGF at 50 ng/ml and DEX and TBOH at  $10^{-7}$ M. Each bar represents the mean and SE of 4 wells. Statistical differences were determined using one-way analysis of variance. Treatments identified with different letters differ significantly ( $p < .01$ ).

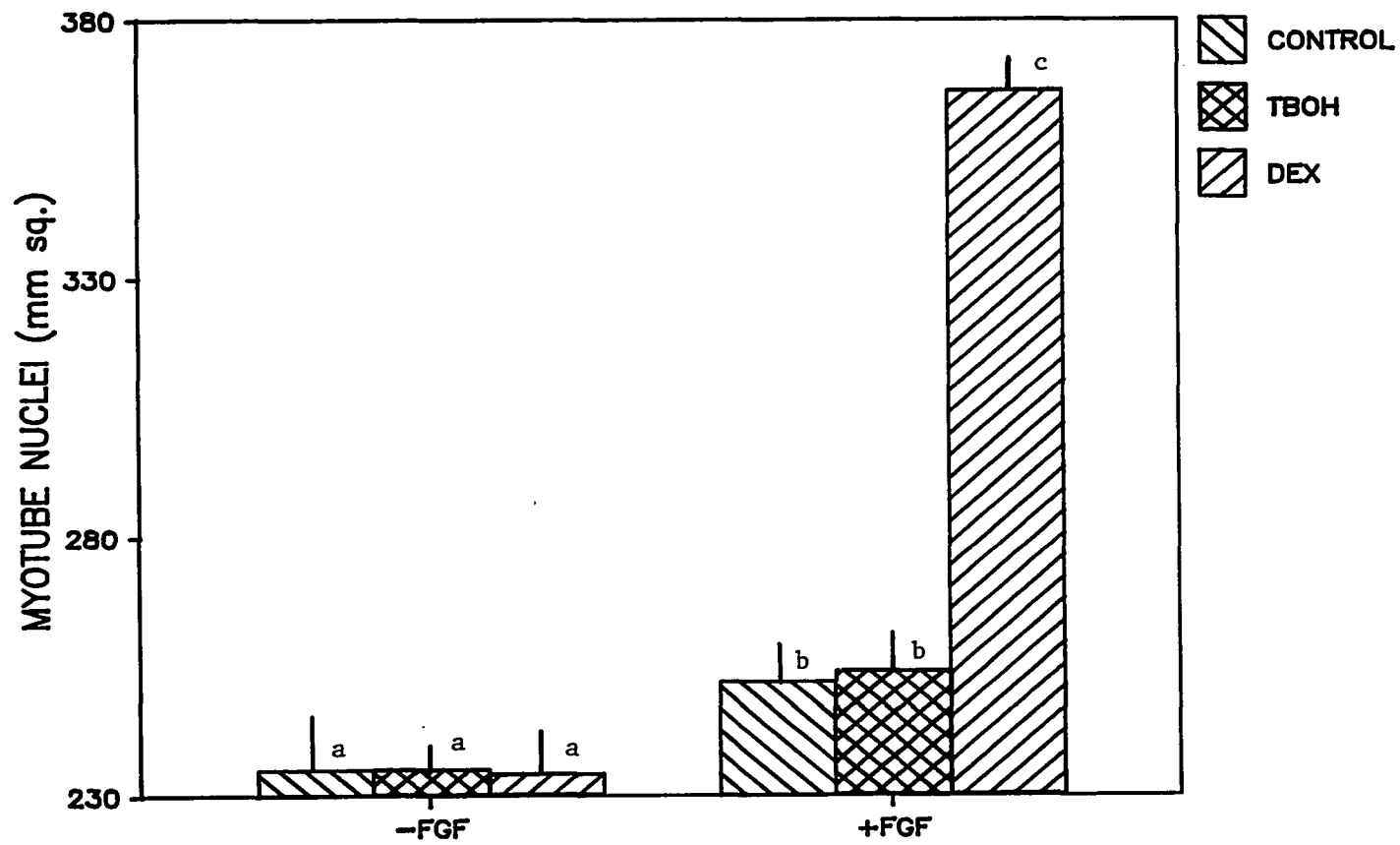


Figure 6. INTERACTION OF STEROIDS AND FGF

myotube nuclei numbers observed in TBOH and control medium in the presence of FGF did not differ statistically from numbers in TBOH and control medium without FGF. A large, statistically significant ( $p < .0001$ ) increase in myotube nuclei density occurred in the presence of dexamethasone and FGF.

Insulin-Like Growth Factor Effect. In this study satellite cells from untreated female rats were used. The treatment media contained five different doses of insulin-like growth factor (IGF-I), two percent horse serum, and TBOH or ethanol carrier. Satellite cells responded to the increasing IGF-I concentrations in a dose-dependent manner (fig. 7), but there was no significant difference in the response observed between cultures that received TBOH in the medium and cultures that only received the ethanol carrier.

Fibroblast Growth Factor Effect. As in the previous experiment, satellite cells from untreated female rats were used. The treatment media contained two percent horse serum, TBOH or ethanol, and increasing concentrations of fibroblast growth factor (FGF). Cultures in both treatment groups responded to the increasing concentrations of FGF in a dose-dependent manner (fig. 8). The FGF dose response curve appears to have been altered by the presence of TBOH in the medium. Maximum response in the presence of TBOH occurred at FGF concentrations less than 50 ng/ml, whereas the peak response in the absence of TBOH was found at 100 ng/ml. Two-way analysis of variance revealed a significant ( $p < 0.01$ ) FGF- by TBOH-treatment interaction.

Figure 7. IGF-I Dose Response Curve.

Satellite cells from untreated rats were exposed to increasing concentrations of IGF-I with or without TBOH in serum containing medium. Concentrations of IGF-I were 0, 1, 5, 10, and 50 ng/ml. TBOH was added in vitro at  $10^{-7}M$ . Both treatments were supplemented with 2% horse serum. Each point represents the mean and SE of 4 wells. Statistical differences were determined using two-way analysis of variance.

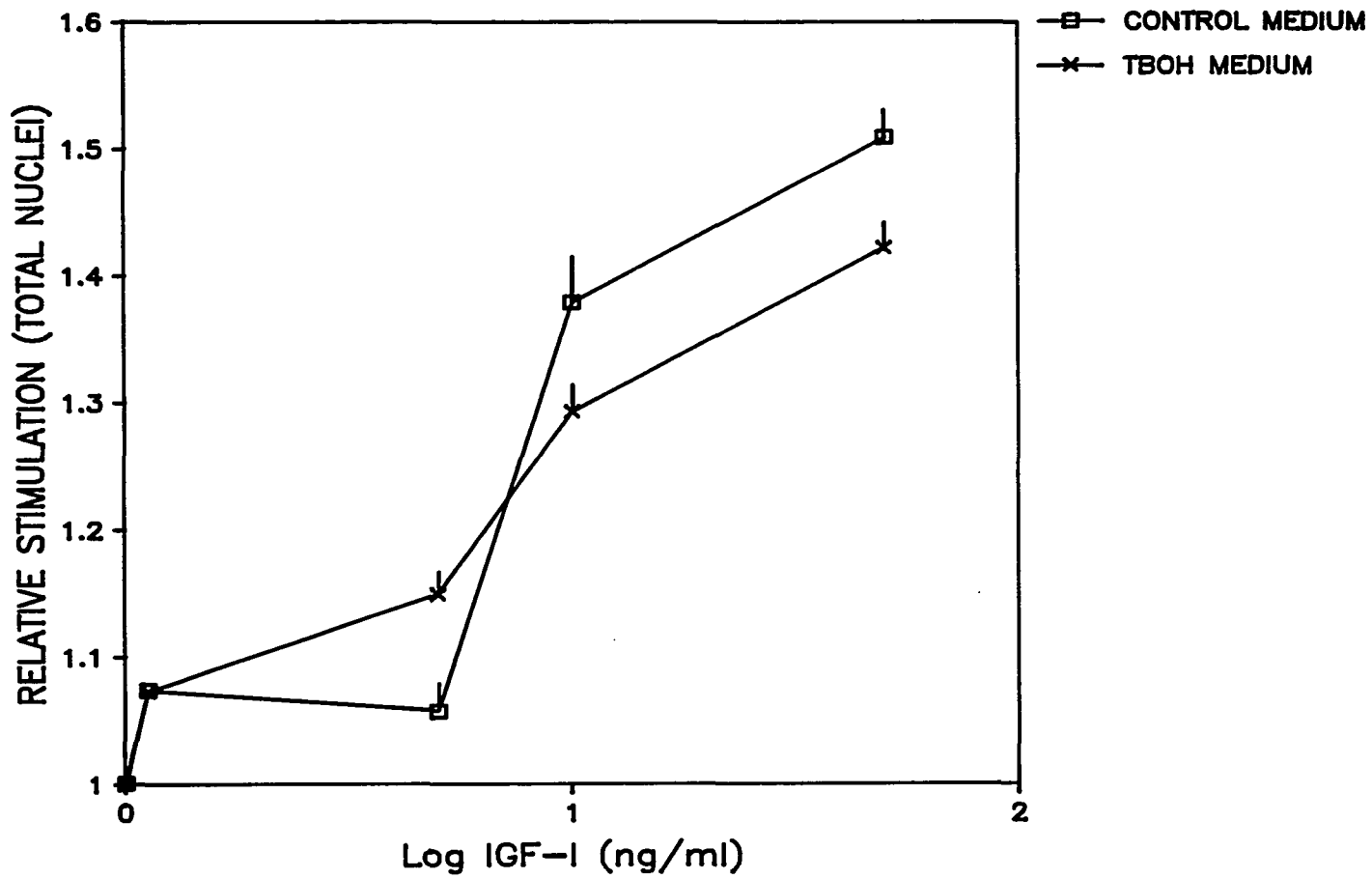


Figure 7. IGF-I DOSE RESPONSE CURVE

Figure 8. FGF Dose Response Curve.

Satellite cells from untreated rats were exposed to increasing concentrations of FGF with or without TBOH in medium containing 2% horse serum. Concentrations of FGF were 0, 50, 100 and 150 ng/ml. TBOH was added in vitro at  $10^{-7}M$ . Each point represents the mean and SE of 4 wells. Statistical differences were determined using two-way analysis of variance.

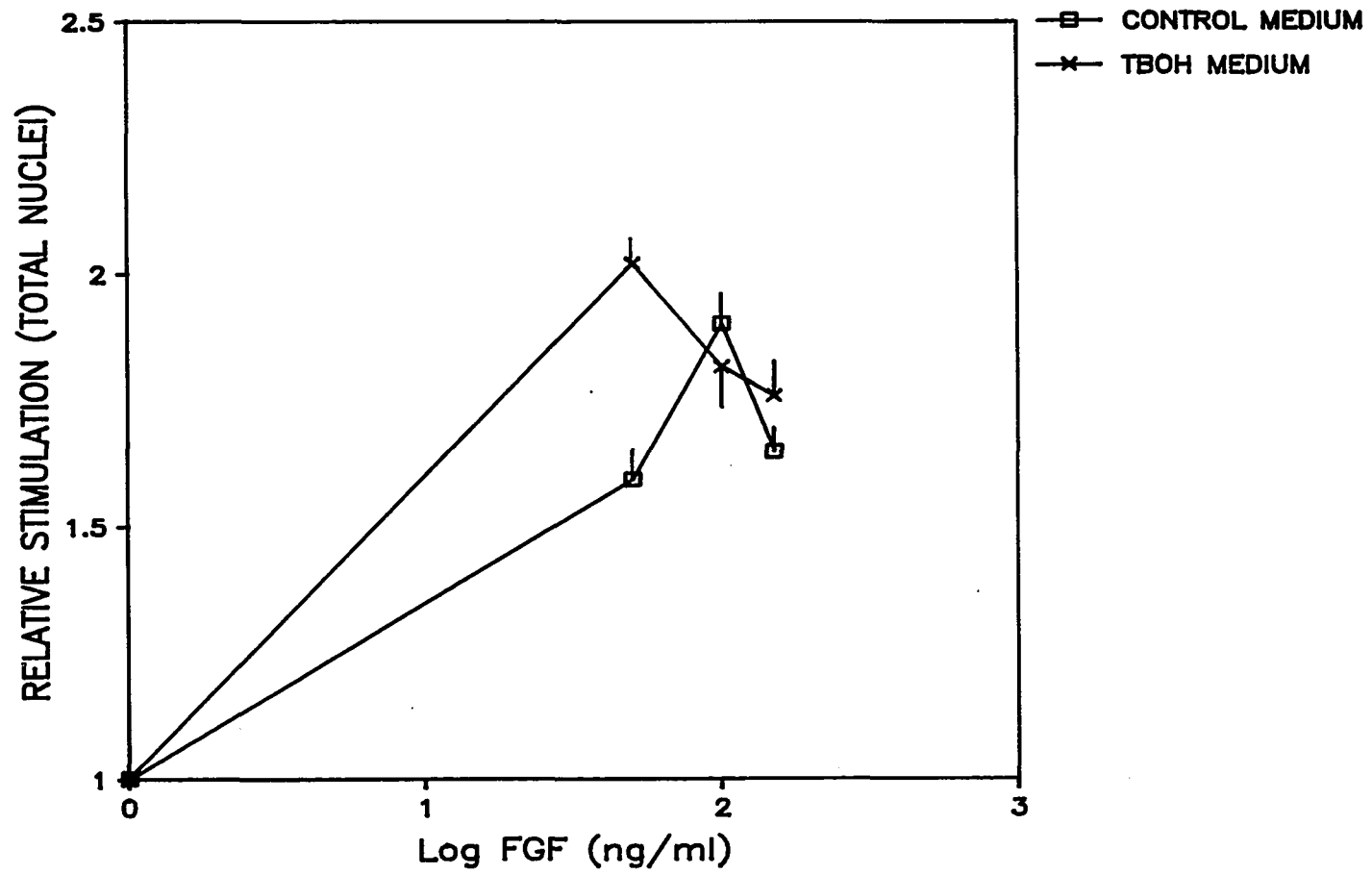


Figure 8. FGF DOSE RESPONSE CURVE

Figure 9. Kinetics of Cell Growth.

Satellite cells from TBOH-treated and control rats were grown in 10% horse serum. Cultures were evaluated at regular intervals 24-144 hours. Each point represents the mean and SE of 4 wells. Statistical differences were determined using two-way analysis of variance.

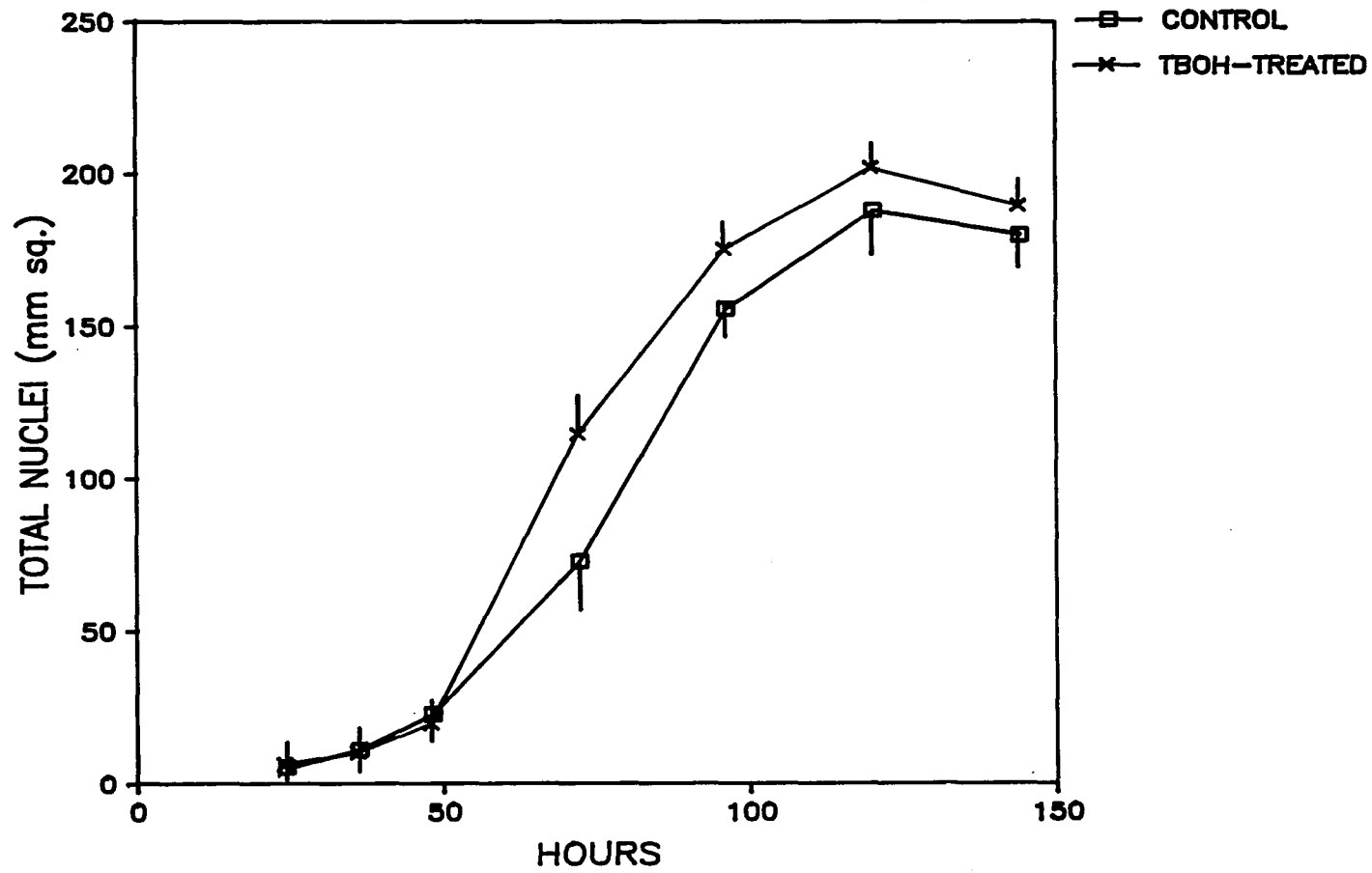


Figure 9. KINETICS OF CELL GROWTH

Effect of TBOH-Treatment in Vivo  
on Properties of Satellite Cells

Growth Kinetics. A preliminary study was performed to determine if cells exposed to TBOH in vivo exhibited altered growth kinetics when grown in vitro (fig. 9). Satellite cells were cultured from TBOH-treated and control rats and were grown in serum-containing medium (DMEM + 10% HS) for six days. No difference in the lag time prior to initiation of proliferation or the rate of proliferation of cells from the two cultures groups were evident.

Insulin-Like Growth Factor Effects. Cells from TBOH-treated and control rat tissue were subjected to various concentrations of IGF-I in the presence of medium containing two percent horse serum. Cells from both tissue sources responded to the increasing concentrations of IGF-I in a dose-dependent manner (fig. 10). The cells which had been treated with TBOH exhibited a greater response to IGF-I at 10 ng/ml than the cells from the control rat tissue. This suggests a shift in the dose-response curve for IGF-I; however, two-way analysis of variance did not detect a significant cell source by IGF-I treatment interaction.

Fibroblast Growth Factor Effect. Cells derived from TBOH-treated and control female rat muscle tissue were cultured and treated with media containing 2% HS and various concentrations of FGF. Cells from TBOH-treated and control rat muscle responded to FGF in a dose-dependent manner (fig. 11). The satellite cells from the TBOH treated tissue exhibited a maximum response at concentrations of FGF equal to or below 50 ng/ml; cells from the control tissue required 100 ng FGF/ml to achieve a maximum response. In addition, the magnitude of the response

Figure 10. In Vivo TBOH-Treatment and IGF-I Effect.

Satellite cells from TBOH-treated and control rats were exposed to increasing concentrations of IGF-I in medium containing 2% horse serum. IGF-I concentrations were 0, 1, 5, 10, and 50 ng/ml. Each point represents the mean and SE of 4 wells. Statistical differences were determined using two-way analysis of variance.

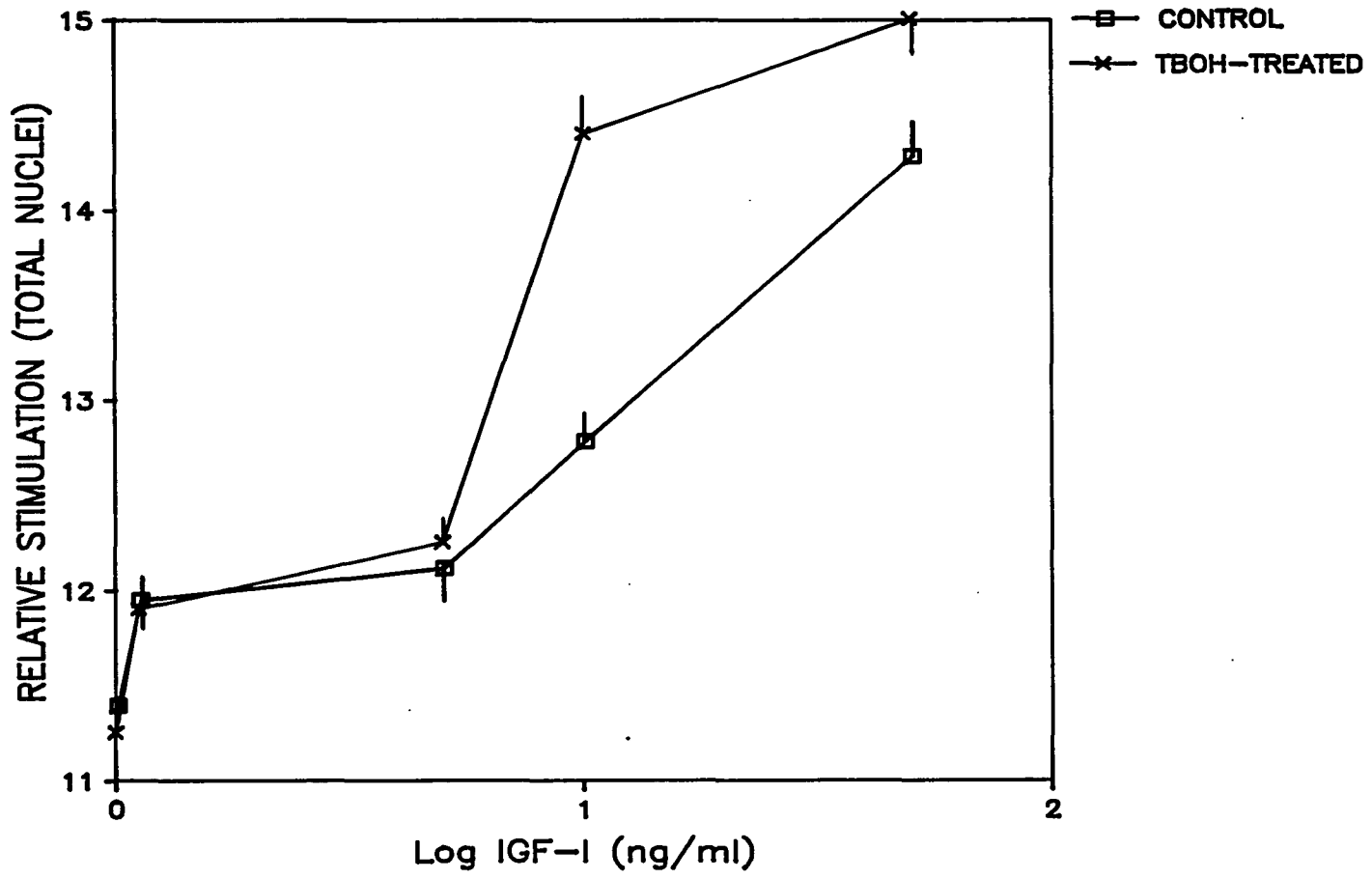


Figure 10. IN VIVO TBOH-TREATMENT AND IGF-I EFFECT

Figure 11. In Vivo TBOH Treatment and FGF Effect.

Satellite cells from TBOH-treated and control rats were exposed to increasing concentrations of FGF in medium containing 2% horse serum. FGF concentrations were 0, 50, 100, and 150 ng/ml. Each point represents the mean and SE of 4 wells. Statistical differences were determined using two-way analysis of variance.

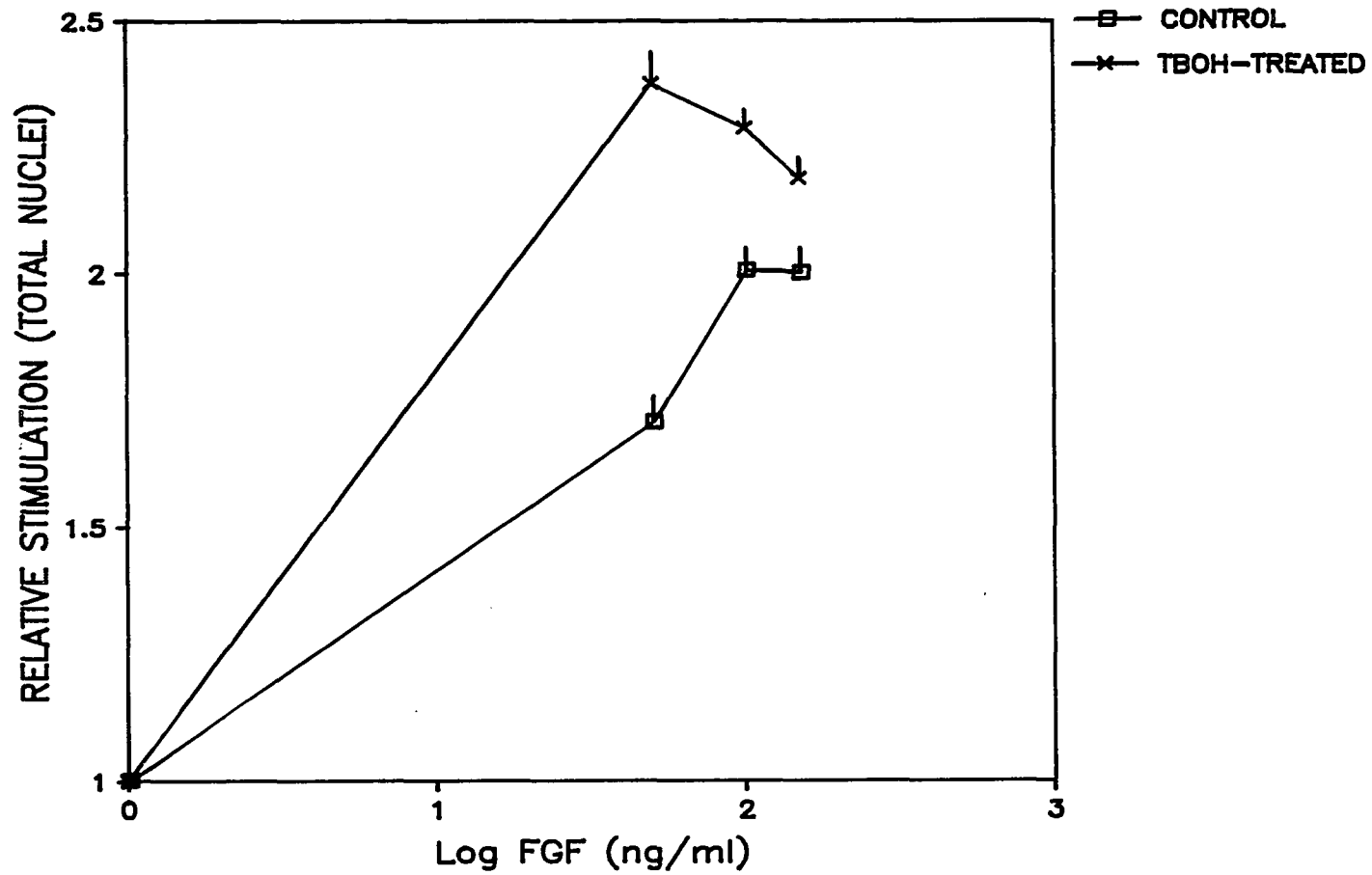


Figure 11. IN VIVO TBOH-TREATMENT AND FGF EFFECT

was greater in cells from TBOH-treated muscle. Two-way analysis of variance showed a significant ( $p < 0.05$ ) cell source tissue by FGF interaction.

In an additional series of experiments, satellite cells from TBOH-treated rats and control rats were cultured in defined medium (containing dexamethasone) and subjected to IGF-I (10 ng/ml) and FGF (50 ng/ml). The relative stimulation of total cell growth by IGF-I and FGF in cultures from the two tissue sources is presented in figure 12. Cells from TBOH-treated tissue were significantly more responsive to IGF-I ( $p < 0.001$ ) and FGF ( $p < 0.001$ ) than cells from the control rat muscle. In figure 13, the increased responsiveness of cells from TBOH-treated rats was also evident when relative stimulation of myotube nuclei concentration by IGF-I and FGF was evaluated, ( $p < 0.001$ ) for each growth factor. The lower relative stimulation of myotube nuclei concentration observed for FGF-treated cells is a result of the proliferation-stimulating and differentiation-inhibiting effect of FGF on satellite cells.

Figure 12. Altered Cell Responsiveness to Growth Factors. (Total Nuclei)  
Satellite cells from TBOH-treated and control rats were exposed to 10 ng IGF-I per ml or 50 ng FGF per ml of in defined medium. Each bar represents the mean and SE of 4 wells. Statistical differences were determined using the students's t-test. Treatments identified with different letters differ significantly ( $p < .01$ ).

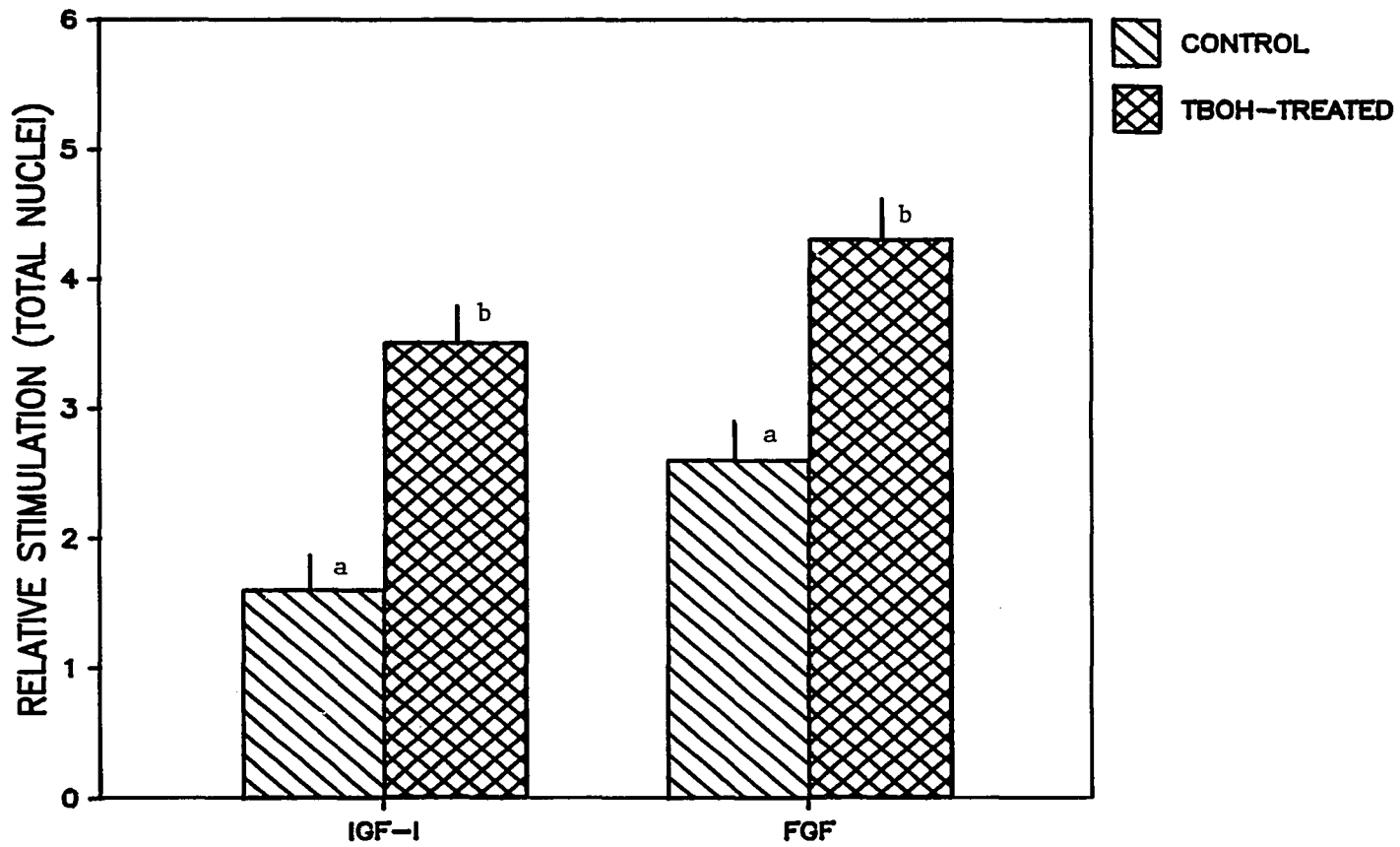


Figure 12. ALTERED CELL RESPONSIVENESS TO GROWTH FACTORS

Figure 13. Altered Cell Responsiveness to Growth Factors.  
(Myotube Nuclei).

Satellite cells from TBOH-treated and control rats were exposed to 10 ng IGF-I per ml or 50 ng FGF per ml in defined medium. Each bar represents the mean and SE of 4 wells. Statistical differences were determined using the student's t-test. Treatments identified with different letters differ significantly ( $p < .01$ ).

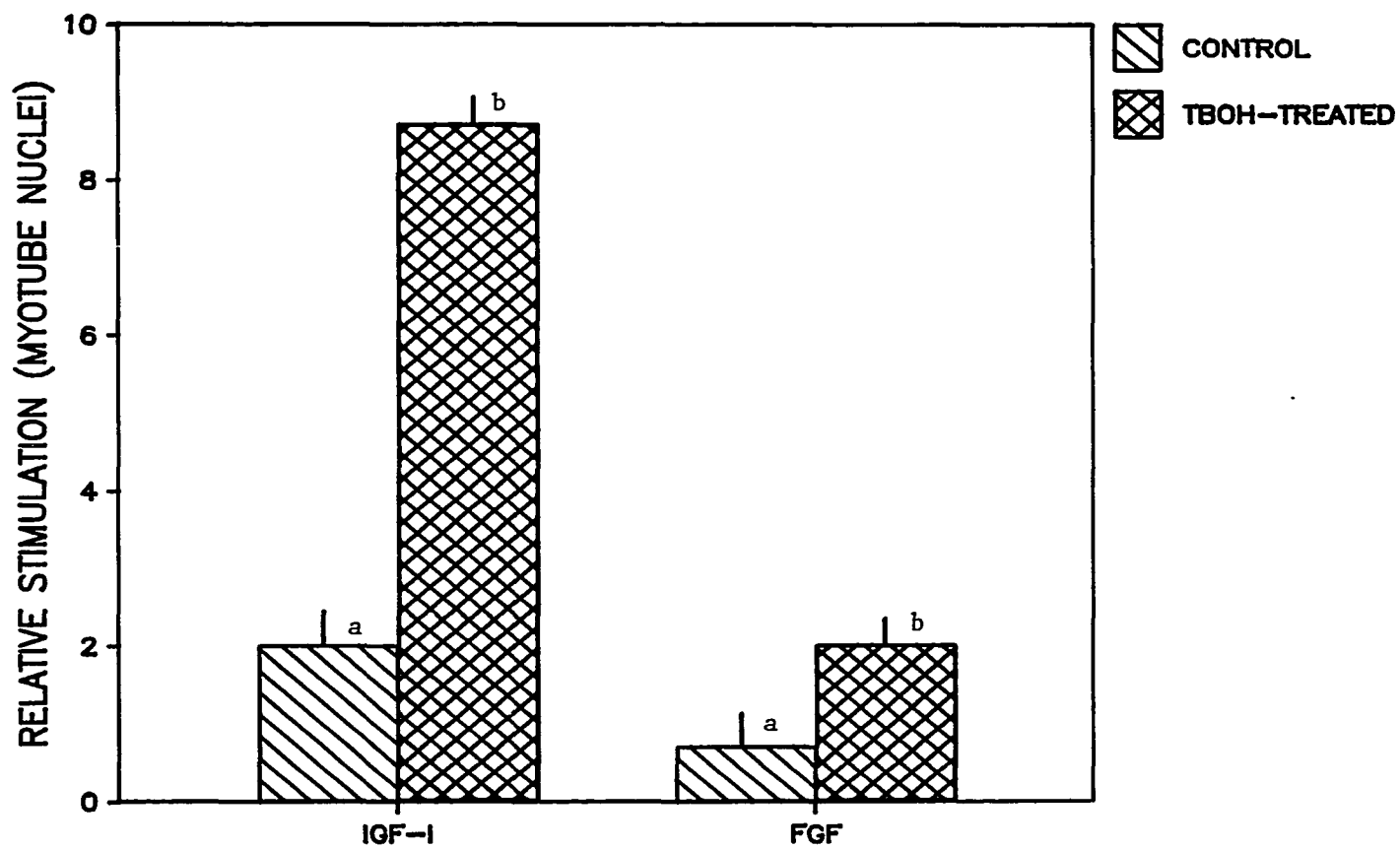


Figure 13. ALTERED CELL RESPONSIVENESS TO GROWTH FACTORS

## CHAPTER 4

### DISCUSSION

Daily injections of female rats with TBOH for a two week duration consistently resulted in an increase in overall growth performance. TBOH increased rate of weight gain and weight gained per unit of feed consumed (table 1). This is in agreement with previous observations (Vernon and Buttery, 1976, 1978; Sinnett-Smith et al. 1983). Clancy et al. (1982) observed an increase in cross-sectional area of longissimus dorsi muscle following exposure of TBA in cattle. The anabolic response elicited by TBA apparently reflects a simple hypertrophy of muscle fibers as there is no change in structure or composition of skeletal muscle (Venable, 1966). The result of TBOH injections appears to be a shift of the growth curve to the left, allowing treated animals to attain a more mature weight sooner (fig. 1.). Increased growth seems to be dependent upon constant exposure to TBOH. When TBOH injections were stopped the growth curves of the TBOH-treated and control animal groups came together. Since both TBOH-treated and control rats were in a rapid growth phase, it seems likely that cessation of TBOH injections resulted in a decrease in growth rate in TBOH-treated rats that allowed the control group to catch-up.

TBA has been reported to reduce muscle protein degradation rate and also to reduce muscle protein synthesis rate slightly (Vernon and Buttery, 1976). These changes should result in a net increase in muscle

mass. Some have speculated that the improved feed efficiency achieved by TBA-treated animals is indirectly due to the decrease in protein turnover (Sinnott-Smith, 1983). The energetic cost of protein turnover are high. Should this energy expenditure be reduced, conceivably, more energy would be available for growth.

From the data presented by many investigators (Winick and Noble, 1966; Moss, 1970) it is evident that DNA accretion in muscle cells is correlated with postnatal muscle growth. The amount of DNA in muscle increases during postnatal growth and the most rapid period of DNA accretion occurs during the most rapid growth phase of the muscle. Since fiber nuclei are mitotically quiescent (Stockdale and Holtzer, 1961) the source of this DNA is the satellite cells (Mauro, 1961; Moss and Leblond, 1970). Di Marco, Baldwin, and Calvert, (1987) indicated that DNA accretion in cattle, throughout the growth phase, may be the most important factor regulating postnatal muscle growth.

Since injections of TBOH were able to increase animal growth performance an increase in muscle DNA content might be expected. To test this hypothesis individual muscles were removed from TBOH-treated and control female rats. The muscles (gastrocnemius, peroneal group, and tibialis anterior) were removed in toto and analyzed for total protein and DNA content (table 2). Rats receiving TBOH had more DNA per muscle and a greater ratio of DNA to protein than the control rats. This would indicate that TBOH, directly or indirectly, was able to influence the proliferation and differentiation of satellite cells, resulting in the addition of nuclei to muscle fiber. Increasing the amount of DNA in muscle should result in a greater capacity to synthesize myofibrillar

proteins and this should result in increased muscle mass. For the three lower leg muscles studied in these experiments, this was not the case; muscles taken from TBOH-treated rats were not larger than control muscle (table 3). There are a couple possible explanations for these apparently conflicting results. The muscles used in the analyses may not be representative of the muscles that experienced TBOH-induced growth. One would assume that muscles experiencing the most significant growth would be major muscle groups in the upper leg and back. The most probable explanation is that satellite cells were stimulated to proliferate and differentiate by the TBOH treatments and subsequently donated their nuclei to the myofibers. However, the use or demand placed upon the muscles selected for the assay was inadequate to induce the addition of myofibrillar protein components. It is conceivable that had the rats been forced to exercise, on a tread-mill for example, that the excess DNA could have facilitated more rapid work-induced hypertrophy. A critical question arising from this study is: does an increase in nuclear content necessarily have to be accompanied by a concomitant increase in muscle mass? Moss (1970) showed that a relationship existed between DNA content and muscle mass in a study comparing muscle of normal and starved chickens. A more appropriate model for comparison to the TBOH study would be that of a weight lifter who has large muscles due to work induced hypertrophy. Muscle fibers in weight lifters would most probably have elevated DNA content, donated from satellite cells. If the weight lifter stopped lifting weights, his muscles, lacking constant stimulus, would atrophy and a higher DNA to

protein ratio should be observed. The weight lifter is in the same position as the TBOH-treated rats higher DNA to protein ratio, although arrived at by different pathways. Should either engage in work induced hypertrophy, the presence of additional myonuclei would facilitate increased ability to produce myofibrillar proteins and subsequent protein accretion.

The hypertrophy of additional muscles, in response to TBOH-treatment, and their DNA and protein content must be examined. In addition body lipid content should also be analyzed to verify that the increased body weight is not strictly due to excess fat deposition.

#### In Vitro Effects of TBOH

Because the most impressive differences between muscles from TBOH-treated and control animals centered around DNA, accretion, the influence of TBOH treatment in vitro and in vivo on satellite cell proliferation was studied. Before discussing specific experiments, it is important to note that total cell number was generally used as an indicator of satellite cell growth in culture. These data would include satellite cells as well as non-muscle cell contaminants. However, crucial observations were always verified by assessing myotube nuclei density, a more accurate indication of satellite cell growth. In all cases myotube nuclei data agreed with total nuclei data.

In vitro, satellite cells from untreated female rat muscle were more responsive to serum collected from TBOH-treated rats than serum collected from control animals. Exposure to serum from TBOH-treated rats caused alterations in the serum dose-response curve. Maximal

response was observed at a lower concentration and the overall serum effect was significantly greater. These results suggest the presence of some mitogenic factor in the TBOH-treated serum. It is unlikely that elevated TBOH concentrations in serum alone is responsible for the observed effect. Several recent studies have shown that androgens and estrogens alone do not stimulate cell proliferation or synthesis in muscle cell culture (Allen et al. 1983; Florini, 1987).

The results of studies using TBOH in defined media on untreated female cells are presented in figures 3, 4, 5, and 6. These results clearly show that TBOH alone in defined media has no direct effect on satellite cells in culture. The increased response of cells exposed to TBOH-treated serum is probably due to a serum borne factor facilitated by the in vivo action of TBOH.

Several studies were conducted in an attempt to identify the factor responsible for the proliferative effects of TBOH-treated serum. Satellite cells from untreated female rats were cultured and then exposed to TBOH and increasing concentrations of IGF-I. IGF-I is a known mitogenic factor for myogenic cells and satellite cells (Florini et al. 1977, 1987; Dodson et al. 1985) in culture. The hypothesis that IGF-I might have a role in the actions mediated by the TBOH-treated serum was based upon evidence that other steroid hormones had been shown to up-regulate IGF-I and insulin receptors (Knutson et al. 1982; Bennett et al. 1984). IGF-I was certainly present in the serum obtained from the rats, and TBOH was probably high in concentration in the TBOH-treated serum. The results, presented in figure 7 show that the addition of TBOH to serum-containing medium did not effect the IGF-I dose-response

curve. These results are in agreement with the studies conducted in the defined medium (fig. 3 and 4) where untreated satellite cells were also exposed to TBOH and IGF-I. TBOH and IGF-I in combination did not stimulate proliferation above control treatments which contained only IGF-I.

Another study was performed on untreated female cells using FGF together with TBOH. The mitogenic effect of FGF on muscle cells is well established (Allen et al. 1984; Gospodarowicz, 1987). FGF has been shown to elicit diverse cellular activities (Gospodarowicz et al., 1986; 1987); the proposed mode of action of FGF is as a competence factor (Gospodarowicz, 1987) which renders cells capable of entering the cell cycle. In this study cell proliferation was stimulated in the presence of TBOH at a lower concentration of FGF than in the absence (fig. 8) of TBOH. The TBOH and FGF treatment appears to have shifted the dose response curve to the left. In light of the evidence, TBOH might be making cells more sensitive to competence factors, such as FGF and therefore making them more sensitive to progression factors, such as IGF-I, that are normally present in serum. This combination would account for increased cell proliferation.

In defined medium TBOH and FGF yielded no interactive responses; FGF stimulated proliferation only in the presence of DEX, a steroid that facilitates the action of IGF-I. In contrast to the experiments containing serum, IGF-I was not present in these experiments. Insulin was the only protein from this family that was added to the medium; insulin can act as an IGF-I under appropriate conditions. A possible

interpretation of this experiment that would put it in agreement with the FGF dose response curve data in figure 8 is that a FGF effect or a FGF-TBOH effect would not manifest itself in proliferation if there was no IGF signal. The only significant stimulation, therefore, occurred when DEX was present to facilitate the generation of an IGF signal by insulin.

#### In Vivo Effects Of TBOH

As indicated previously exposure to TBOH alone, *in vitro*, did not generate a positive TBOH response. But the addition of serum from TBOH-treated rats and FGF in 2% horse serum with TBOH stimulated significant cell proliferation (Fig. 2). These results suggest that TBOH could be making cells more responsive to FGF. Therefore, it seems possible that cells from TBOH-treated rats might respond differently in culture. When cell growth kinetics was monitored, cells from TBOH-treated and control rats behaved in a similar manner in the presence of medium containing 10% horse serum (fig. 9). These results might seem to contradict the *in vitro* experiments since it has been suggested that TBOH might function to make cells more responsive to the IGF component in serum. However, the growth factor concentration in horse serum might be so great as to mask any differences in cell sensitivity to growth factors. To examine this theory, a series of studies were performed to investigate any differences in response to growth factors that may have resulted from TBOH-treatment *in vivo*.

When cells from TBOH-treated tissue and control tissue were exposed to increasing concentrations of IGF-I in serum-containing

medium, cells from both tissue types responded in a dose-dependent manner (fig. 10). However, cells from TBOH-treated tissue appeared to be more responsive to IGF-I at 10 and 50 ng/ml. The maximal response and overall magnitude attained by cells from TBOH-treated tissue was greater than controls. These results suggest that TBOH-treatment in vivo has made satellite cells more sensitive to IGF-I. To insure that the observed effects of IGF-I on TBOH-treated cells were indeed mediated by IGF-I not and by some interaction with components in the media (media contained 2% horse serum), another study was performed in defined medium. The concentration of IGF-I selected was 10 ng/ml which earlier had exhibited the maximal proliferative response (Fig. 10). The results of the defined media study were in agreement with the observation that TBOH-treated tissue was more responsive to IGF-I (fig. 12 and 13). It is important to note that the defined medium formulation used in this experiment contained DEX and FGF.

The effect of FGF on cells from TBOH-treated tissue was also investigated. Increasing concentrations of FGF were added to cultured cells from TBOH-treated and untreated tissues, in the presence of 2% horse serum. Cells from both tissues responded to the FGF treatments in a dose dependent manner. The FGF treatment had a significantly greater stimulatory effect upon cells from TBOH-treated muscle as compared to cells from control muscle. Once again, to ensure that the observed effects were due to FGF and not a serum-borne component, the study was repeated in defined media. FGF was present at 50 ng/ml, the concentration which had been shown to generate the greatest proliferative response in earlier studies (Fig. 11). FGF significantly

increased proliferation of cells from TBOH-treated muscle when compared to control (fig. 12 and 13). Analysis of the data presented in figures 5 and 8 had suggested that FGF required a serum factor to stimulate proliferation. But, a serum factor is not necessary for a FGF mediated response in cells from TBOH-treated muscle, as shown in figures 12 and 13. The most likely explanation of these results is that whatever effect the serum contributed in vitro was manifested in cells in vivo by the actions of TBOH. Thus TBOH-treated cells were already activated by some means in vivo which made them more responsive than control cells to FGF in vitro.

TBOH most likely mediates its response in the whole animal through a series of complex mechanisms. However this study has specifically identified some potential areas of cell growth regulation. The analysis of rat growth and muscle properties provide the interesting observation that DNA accretion was accelerated in response to TBOH treatment. Because this aspect of muscle growth is one of the most important regulatory events in postnatal muscle, the effect of TBOH on satellite cell activity was investigated. Satellite cells were exposed to TBOH in vivo and in vitro and their proliferative activity was examined. The general finding was that satellite cells from TBOH-treated animals were much more responsive to a variety of growth factors that are expected to regulate satellite cell growth in vivo, than satellite cells from untreated animals.

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