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# STEROID-INDUCED GROWTH DELAY AND BONE ABNORMALITIES IN PRETERM INFANTS AND PIGLETS DURING EARLY DEVELOPMENT: THE INTERACTION OF STEROIDS AND THE GH-IGF-I AXIS

By

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# STEROID INTERACTION WITH THE GH-IGF-I AXIS DURING DEVELOPMENT

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## ABSTRACT

Dexamethasone (DEX) treatment in very preterm infants has proven to facilitate earlier weaning from mechanical ventilation and supplemental oxygen, thereby lessening the severity of lung disease incurred by long-term oxygen dependency. However, DEX therapy is not without negative side-effects; studies in preterm infants and piglets have reported DEX-induced impairments in growth and bone mineral metabolism. DEX may act by altering the concentration or activity of specific components of the growth hormone (GH)/insulin-like growth factor (IGF-I) axis which are essential for regulating growth and bone mass. The first study, in preterm infants, characterized how DEX alters the circulating components of the GH-IGF-I axis and suggested potential mechanisms by which DEX delays growth and bone development as both plasma IGF-I and biochemical markers of bone metabolism were reduced during DEX. The objectives of the piglet studies were to delineate the effectiveness of adjunctive GH or GH+IGF-I to counter the detrimental effects of DEX on growth, protein turnover and bone mass. In the first studies, we administered GH, GH+IGF-I or placebo to piglets while they received a two week course of DEX. GH and GH+IGF-I partially attenuated the reductions in growth and bone mass to a similar extent. Only with respect to protein metabolism was an additional benefit observed with combined treatment (GH+IGF-I). A dose-response study revealed the minimal effective GH dose, and demonstrated that bone cell activity and

weight and length gain returned to control levels during a period of rehabilitation in which no DEX or GH were administered. Currently, it is uncertain if DEX-treated infants experience similar metabolic improvements in weight and length growth or bone mineral mass post-DEX treatment or whether the metabolic insults of DEX are sustained. Longer term follow-up of DEX-treated preterm infants is required to fully comprehend the long-term consequences of DEX on growth into childhood. If there are long-term effects on growth and bone development, future studies should focus on whether GH is more effective post-DEX compared to during DEX treatment or whether adjunctive administration of other anabolic agents will counter the negative effects of DEX during development.

## ACKNOWLEDGEMENTS

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I would also like to express my thanks to Sharon Donovan of the University of Illinois, Urbana, who encouraged me to pursue GH and IGF-I research. Your guidance and advice were invaluable and my discussions with you were rewarding. Sharon, thank you also for your warm hospitality during my study visits to Illinois.

I offer my sincerest gratitude to the other members of my supervisory committee, Bosco Paes, Larry Arsenault and Pat Chang, for sharing their expertise and knowledge about the various clinical and experimental research areas of my studies. Your genuine interest in my research was greatly appreciated.

I am grateful for the advice and input of the many friends and colleagues in the Pediatric Nutrition and Metabolism Group, the Neonatal Unit at The Children's Hospital of the Hamilton Health Sciences Corporation and the Central Animal Facility. You provided an enjoyable environment in which to learn and conduct these studies.

I also extend my sincere thanks to the parents who allowed me to study their very special infants in order to help future premature infants.

I also wish to thank my family for their steadfast love and support. Mom, Dad, Mark, Poppy and Peter, you have been the constants in my life.

> To my mother, Pamela, and my father, Wilfred. Thank you mom and dad, for your support of my scientific endeavours throughout my life. Whether it was collecting snowflakes for my grade 2 science experiment, helping build a science fair display board or allowing me to conduct countless experiments in the basement, you were always supportive of your "little scientist".
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> To my brother, Mark. Thank you Mark, for always showing me how to enjoy life's little pleasures and reminding me to have fun.
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I dedicate this thesis to the memory of my maternal grandparents, Edward Uriah "Poppy" and Phyllis Oteoline Spriggs, and to my paternal grandmother, Edith Cavell Ward, who encouraged me to pursue my dreams.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BMC: | bone mineral content |
| BMD: | bone mineral density |
| BPD: | bronchopulmonary dysplasia |
| BUN: | blood urea nitrogen |
| BW: | birth weight |
| DEX: | dexamethasone |
| DXA: | dual energy x-ray absorptiometry |
| ELISA: | enzyme-linked immunoassay |
| GA: | gestational age |
| GH: | growth hormone |
| HPAA: | hypothalamus-pituitary-adrenal axis |
| IGF-I: | insulin-like growth factor-I |
| IGFBP: | insulin-like growth factor binding protein |
| 3-MH: | 3-methylhistidine |
| NTx: | N-telopeptide |
| PMA: | postmenstrual age |
| P-RNIs: | recommended nutrient intakes for premature infants |
| RIA: | radioimmunoassay |

| | |
|---|---|
| SPA: | single photon absorptiometry |
| VLBW: | very low birth weight |
| WLB: | western ligand blot |

## PREFACE

This thesis investigated the interaction between glucocorticoid treatment and the GH-IGF-I axis with the goal of understanding the mechanisms by which exogenous steroids affect bone and protein metabolism and overall growth during development by altering one or more aspects of the GH-IGF-I axis.

I was actively involved in all aspects of this research. This included assisting Dr. Atkinson with writing grant proposals; coordinating blood and urine sample collections and analyses, as well as body composition measurements for the infant study; daily piglet care and sample collection and analyses; and the preparation of each manuscript. I was responsible for writing the drafts of each of the four manuscripts which constitute Chapters 2 through 5. Dr. Atkinson reviewed each draft and made suggestions which not only helped me to focus the chapter, but allowed me to perfect my editorial skills. I was also responsible for writing the rebuttals in response to the comments of reviewers who critiqued our manuscripts. All the studies, Chapters 2 through 5, were primarily designed and conducted by myself and Dr. Stephanie Atkinson. However, Dr. Bosco Paes and Dr. Sharon Donovan made contributions to specific studies and provided constructive criticism of the chapter(s) of which they are co-author(s). Dr. Paes, a neonatalogist, provided insight pertaining to the logistics of studying very sick infants (Chapter 2), actively assisted during the recruitment phase by identifying suitable infants and critiqued Chapter 2 prior to submission for publication. Dr. Sharon Donovan, an experienced

researcher in the field of nutrition and the GH-IGF-I axis, provided technical expertise with respect to the analyses of the circulating and tissue components of the GH-IGF-I axis. I performed all these analyses for both the clinical and piglet studies under Dr. Donovan's supervision at the University of Illinois. Dr. Donovan reviewed Chapters 2, 3 and 5 and provided suggestions to clarify issues. In addition to Dr. Paes and Dr. Donovan, several individuals have been acknowledged at the end of the study chapters and their specific contribution to each study is stated.

Chapter 1, the introduction of this thesis, represents the state of knowledge on the effects of glucocorticoids on the GH-IGF-I axis and bone and protein metabolism at the time when I commenced my graduate studies at McMaster. Thus, I discuss and reference only studies which were published prior to September 1993. Also, Chapter 1 orients the reader to the reasons why glucocorticoids are used in preterm infants and provides an overview of both the short-term and potential long-term consequences of dexamethasone on bone and protein metabolism and growth. Thus, the introduction provides the rationale for the piglet intervention trials. My objectives and hypotheses, as well as the rationale for the outcome measurements and the methods used to assess growth, bone, protein and the GH-IGF-I axis are also included in Chapter 1. The reference list for Chapter 1 is at the end of the thesis.

Chapters 2 through 5 represent the four different studies that were conducted. Each of these chapters is written and referenced in the format set out by the different journals to which the papers have been accepted, submitted or will be submitted for

publication. The respective reference lists are included at the end of each chapter. Chapter 2 details the impact of dexamethasone and development on the circulating components of the GH-IGF-I axis in dexamethasone-treated premature infants. The subsequent chapters (Chapters 3 through 5) are piglet studies in which GH alone or in combination with IGF-I were used to counter the deleterious effects of dexamethasone on protein and bone metabolism and overall growth. These piglet studies provided insight into the mechanisms of DEX action on bone and protein metabolism during development.

The final discussion (Chapter 6) outlines the implications of this research and identifies specific questions which should be answered in future investigations. The reference list for Chapters 1 and 6 is at the end of this chapter. Appendices 1 through 4 contain the study design for each of the 4 studies which comprise this thesis; the treatment groups, sample sizes, as well as the outcomes and the timing of the outcome measurements are explicitly illustrated to assist the reader. Appendices 5 and 6 contain data on organ weights which were not incorporated into manuscripts but provide useful additional information. The figure in Appendix 7 illustrates the time course of $^{15}N$ enrichment in the urine after administration of the $^{15}N$-glycine tracer (Chapter 4).

# Chapter One

# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

## 1.1 Global rationale, objectives and hypotheses

One of the major clinical consequences of being born very prematurely is pulmonary injury resulting in bronchopulmonary dysplasia (BPD) which progresses to chronic lung disease. The etiology of BPD is multifactorial but the main causation is secondary to oxygen toxicity and barotrauma incurred by reliance on mechanical ventilation and supplemental oxygen for extended periods of time as treatment of respiratory failure (Northway 1990). The most effective way to reduce the severity of BPD is to minimize the amount of time an infant requires mechanical ventilation or supplemental oxygen. This can be achieved by treating infants with dexamethasone (DEX), a potent glucocorticoid, which reduces oxygen requirements and ventilator dependency and improves lung compliance (Cummings et al. 1989, Ohlsson et al. 1992a, Harkavy et al. 1989).

While DEX treatment has irrefutable benefits for lung function in the short term, the long term consequences of DEX on growth and more specifically bone and protein metabolism are unknown. Clinical studies have consistently shown dramatic reductions in weight and length gain during DEX treatment (Brownlee et al. 1992, Ohlsson et al. 1992a) and a lower bone mass compared to untreated infants at 33 weeks of gestation (Weiler et al., pilot study). With respect to long term effects, the

detrimental effects on length growth have been shown to be present at 6 months corrected age (Weiler et al., pilot study) and continue to be studied in Dr. Atkinson's laboratory. These negative effects of DEX have become disconcerting since VLBW infants should experience rapid growth and development during early postnatal life.

Based on evidence in steroid-treated animals (Altman et al. 1992), children (Hyams et al. 1988) and adults (Reid et al. 1989), it is hypothesized that DEX partially mediates its negative effects by interfering with the normal function of the components of the GH-IGF-I axis which are essential mediators of growth (detailed in section 1.3.2). Lower circulating GH and IGF-I, alterations in the IGFBP profile and tissue expression of IGFBPs during DEX treatment have all been implicated (Reid et al. 1989, Hyams et al. 1988, Altman et al. 1992) but the influence of DEX on the natural ontogeny of the circulating components of the GH-IGF-I axis has not been studied in VLBW infants. The relative contribution of circulating IGF-I and the IGFBPs on bone mass and overall growth compared to other factors during infancy is unclear. However, infants who are GH deficient experience growth failure from birth (Gluckman et al. 1992) and infants born with Laron Syndrome are shorter at birth compared to control infants (Laron and Pertzelan 1969). These findings have identified that circulating GH and IGF-I are essential mediators of extrauterine growth, and that IGF-I also has a role in fetal growth.

*Thus, our first objective was to characterize the developmental changes in the circulating components of the GH-IGF-I axis in a group of steroid-treated infants and*

*to also assess the changes in specific biochemical markers of bone metabolism, dietary intakes, and growth velocities from the start of DEX treatment through to term corrected age to provide insight into the relationship between the GH-IGF-I axis, growth, nutrition and bone metabolism. Our secondary objective was to compare weight, length and body composition at term corrected age in DEX-treated infants with appropriate reference values from non-steroid treated preterm and term infants.*

To expand our understanding of the mechanisms by which DEX disrupts the GH-IGF-I axis and mediates its negative effects, we designed studies which used the DEX-treated piglet model. With the evidence that both growth hormone and insulin-like growth factor-I have potent anabolic effects on bone and protein metabolism in humans and animals, *our objective was to evaluate the clinical benefits of administering GH alone or in combination with IGF-I to counter the effects of DEX on bone mineral homeostasis and protein metabolism, body composition and overall growth; and, whether the detrimental effects of DEX are sustained after the cessation of DEX therapy among piglets receiving DEX alone.*

**Hypotheses**

In DEX-treated VLBW infants:

i) During DEX treatment, the developmental rise in IGF-I and IGFBP-3 will be suppressed, and the concentrations of the biochemical markers of bone turnover and growth velocities will be reduced compared to pre and post-DEX treatment.

ii) At term corrected age, infants who received DEX will be shorter, lighter and have a lower lean, fat and bone mineral mass when compared to appropriate reference values from non-steroid treated preterm and term born infants.

In DEX-treated piglets:

i) DEX treatment will lower circulating IGF-I and IGFBP-3 and alter liver mRNA expression of the IGFBPs, thereby partially accounting for the reduction in bone and lean mass, and growth delay which accompanies DEX treatment.

ii) Administration of adjunctive GH during DEX will counter the effects of DEX, promoting bone mineral accretion and protein anabolism; moreover, these improvements will be dependent on the dose of GH administered.

iii) The combination of GH and IGF-I administration during DEX treatment will be more anabolic on whole body metabolism as both GH and IGF-I can act independently to stimulate the accretion of bone mineral and enhance the rate of protein synthesis.

iv) After DEX treatment, piglets who received DEX alone will remain shorter and lighter than control or GH-treated piglets and differences in body composition will persist.

## 1.2 Bronchopulmonary dysplasia and dexamethasone treatment in preterm infants

Since VLBW infants cannot produce sufficient quantities of lung surfactant to reduce the surface tension in their lungs and prevent their alveoli from collapsing, mechanical ventilation is required for extended periods of time (e.g. > 3 weeks) (Kumar et al. 1992a). Bronchopulmonary dysplasia (BPD) starts to develop immediately after birth when the infant's immature lungs are exposed to high oxygen concentrations and positive pressure ventilation which generates oxygen free radicals that directly damage the pulmonary endothelium. As well, the presence of leukotrienes, thromboxanes and prostacyclins in tracheal aspirates provides evidence that the inflammatory response is triggered (Rush and Hazinski 1992, Stenmark et al. 1987). In addition, ventilation is often difficult resulting in lung parenchymal injury which destroys bronchiolar architecture, leading to changes in cell morphology, tissue death, infiltration of macrophages and increased lung interstitial elastic tissue (Southall and Samuels 1990).

DEX treatment is commonly prescribed to manage BPD as it has proven to facilitate earlier extubation from mechanical ventilation (Avery et al. 1985, Cummings et al. 1989, Harkavy et al. 1989, Ohlsson et al. 1992a). At the Children's Hospital, Hamilton Health Sciences Corporation, DEX treatment is initiated within the first 3 weeks of postnatal life and the average course of DEX is 42 days. However, the duration of treatment can vary according to the infants clinical pulmonary

improvement. Some infants may require longer courses of DEX before they can be successfully weaned from the ventilator. The exact mechanism by which DEX improves pulmonary compliance is unknown. It is speculated that DEX acts by several mechanisms. DEX may decrease the inflammatory response by stabilizing cell and lysosomal membranes by inhibiting phospholipase $A_2$ and the subsequent cycloxygenase and lipoxygenase pathways (Gerdes et al. 1988, Kumar et al. 1992b), improve lung mechanics by decreasing pulmonary edema (Northway 1990) or stimulate surfactant synthesis by Type II epithelial cells (Delemos et al. 1970).

Clearly, in the short-term, DEX treatment has dramatic effects on weight and length growth and the accretion of bone mineral mass (Brownlee et al. 1992, Weiler et al., pilot study). It is uncertain whether treatment with DEX during early life prevents VLBW infants from attaining their maximal growth potential and peak bone mass. The acquisition of bone mass is of particular concern as these infants may be at a greater risk of developing adult osteoporosis. With advances in medical technology, including improvements in mechanical ventilation and the use of exogenous surfactant and DEX, the survival rate of VLBW infants is steadily increasing (Northway 1990). As a direct consequence of this phenomenon, a larger percentage of infants are developing BPD due to their extreme premature birth (Northway 1990, Parker et al. 1992). Approximately 70% of infants requiring more than 2 weeks of mechanical ventilation develop BPD (Greenough 1990) and most infants will receive DEX in the

management of their BPD. Thus, the medical community is challenged to consider how the deleterious side-effects of steroid treatment can be attenuated.

## 1.3 Steroid effects on the GH-IGF-I axis

### 1.3.1 The GH-IGF-I axis and the regulation of lean, fat and bone mass

Many changes occur in the concentrations of IGF-I and the IGFBPs in non-steroid treated preterm and term born infants during the first months of postnatal life. Premature infants experience a developmental rise in plasma IGF-I and IGFBP-3 from birth to term corrected age (Bennett et al. 1983, Bernardini et al. 1992, Lineham et al. 1986). Accordingly, we speculated that DEX alters these important developmental changes and consequently, impairs growth and development.

Before discussing the effects of exogenous steroid treatment on the GH-IGF-I axis, it is beneficial to examine the normal metabolism of GH and IGF-I, and the IGFBPs with particular interest on the metabolic effects of each component on whole body protein, fat and bone metabolism. Few studies have investigated the GH-IGF-I axis of preterm infants. Thus, this discussion is limited to studies in older children, adults or animals. Observations from in vitro studies have been included to provide greater insight into the mechanisms of GH, IGF-I and IGFBP action at the level of the cell.

In healthy individuals, GH releasing hormone is secreted by the hypothalamus into the portal blood and signals the release of GH from the somatotrophs of the

anterior pituitary. A small quantity of GH is stored in the somatotrophs for immediate release while further GH releasing hormone signals the somatotrophs to produce more GH, accounting for the pulsatile release of GH from the pituitary. At all stages of development, GH is released in a pulsatile manner with the maximal GH secretion occurring during deep sleep, usually between midnight and 4 am (Mendelson 1982, Finkelstein et al. 1972). Even preterm infants have been shown to have a pulsatile release of GH, providing evidence that the GH regulatory mechanisms are functioning at the earliest stages of postnatal life although GH secretion is not as controlled as in later stages of development (Wright et al. 1992). At birth, preterm infants have elevated plasma GH concentrations due to the immaturity of the feedback systems; preterm infants have a lower number of GH receptors than term infants and the somatotrophs are relatively insensitive to somatostatin (Wright et al. 1992). There is some evidence that the pulsatile release of GH is necessary to stimulate growth as a constant infusion of GH in animals is not as effective as a once daily injection at stimulating growth (Isgaard et al. 1983).

As shown in Figure 1, circulating GH and IGF-I levels are regulated via negative feedback inhibition at the hypothalamus or the anterior pituitary. Since exogenous GH or IGF-I increases circulating GH and/or IGF-I, it is possible that endogenous synthesis and release of GH or IGF-I could be suppressed or inhibited. Studies in humans and animals, however, have demonstrated that any abberations in endogenous GH or IGF-I production or release do not persist as circulating GH and IGF-I return to baseline values after GH or IGF-I administration is stopped (Brixen et al. 1990, Ebeling et al. 1993).

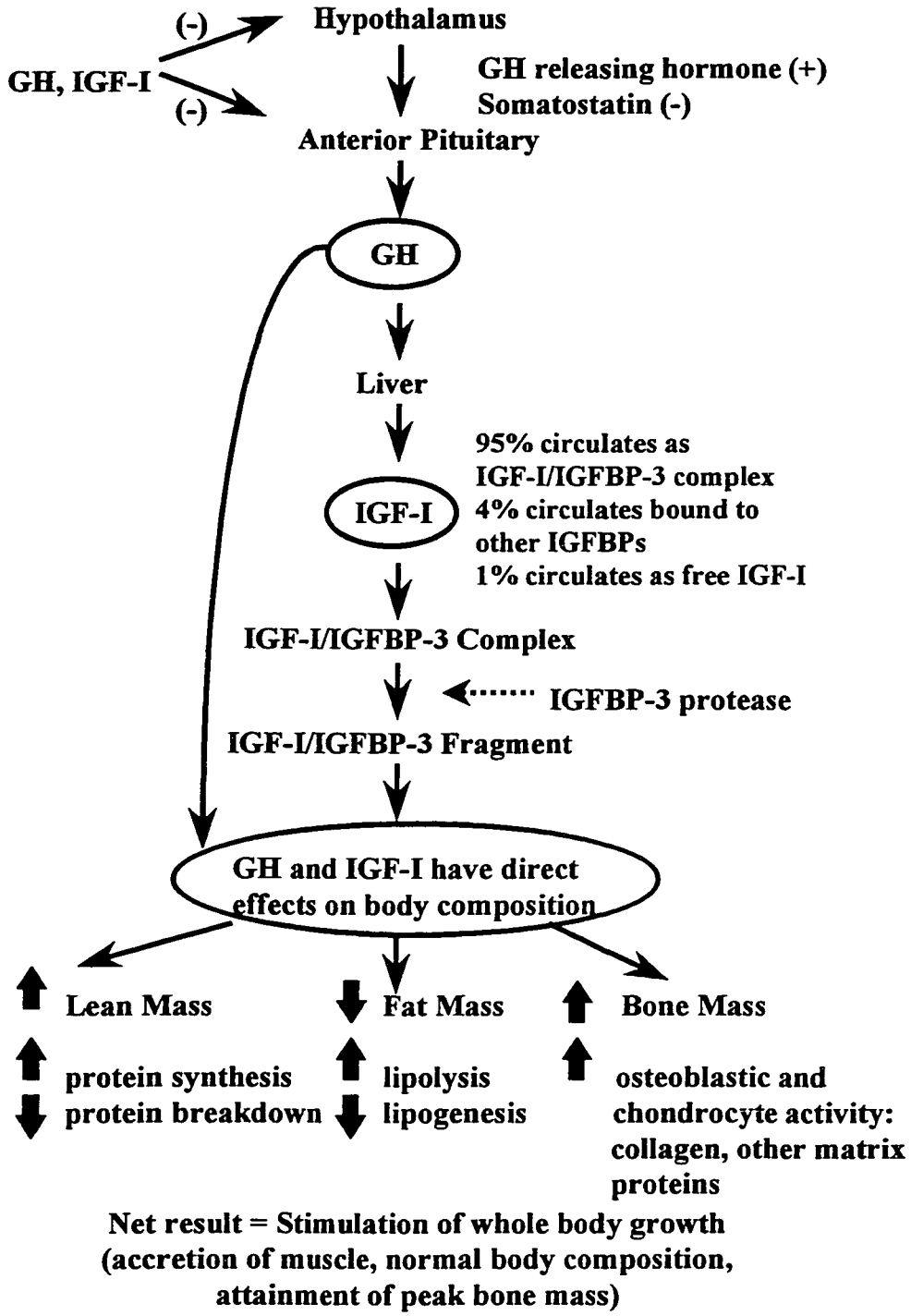



**Figure 1.** The GH-IGF-I axis: regulation of lean, fat and bone mass by GH, IGF-I and the IGFBPs.

In the circulation, GH is bound to a GH-specific binding protein which is similar to the extracelluar component of the GH receptor. GH can mediate direct effects on bone and lean mass by binding to GH receptors on osteoblasts and myoblasts, signalling the production of various transcription factors to promote the growth and differentiation of osteoblasts (Kassem et al. 1993, Morel et al. 1993) or myoblasts (Quinn et al. 1993). Addition of GH to epiphyseal chondrocytes, in vitro, stimulates the proliferation of chondrocytes and the production of matrix proteins (Ohlsson et al. 1992b). GH receptors are also present on adipocytes; GH suppresses the mRNA expression and activity of critical enzymes involved with lipogenesis in pigs (e.g. fatty acid synthase) (Dunshea et al. 1992, Walton and Etherton 1986). GH replacement therapy in GH-deficient children or adults results in higher circulating free fatty acids and glycerol, indicators of higher rates of lipolysis (Gertner 1993, Wabitsch and Heinze 1993). Addition of GH to cultures of preadipocytes stimulates the differentiation of these cells to mature adipocytes and may reduce the number of adipocytes, thereby regulating the deposition of fat mass (Wabitsch and Heinze 1993).

GH can also act indirectly on bone, lean and fat tissue by stimulating the release of IGF-I from the liver, thereby increasing the level of IGF-I which is available to act on target tissues. Osteoblasts and muscle cells express IGF-I receptors to which IGF-I binds (Hock et al. 1988, Beguinot et al. 1985), and like GH, signals the proliferation and differentiation of target cells. Osteoblasts (Hock et al. 1988, Canalis et al. 1980, Canalis et al. 1988) and myoblasts (Allen and Boxhorn 1989,

Quinn et al. 1993) respond to the addition of IGF-I in culture. Dose-dependent increases in DNA synthesis and bone matrix have been observed in cultured rat calvaria cells (Canalis et al. 1980, Hock et al. 1988). IGF-I receptors have not been identified on adipocytes despite the fact that addition of IGF-I has acute effects on human fat cells (Bolinder et al. 1987). It is postulated that IGF-I interacts with the insulin receptor and thereby exerts an insulin-like effect on fat cells, promoting glucose transport into the cell. There is limited information about IGF-I action on fat cell metabolism.

Approximately 99% of IGF-I circulates bound to one of six different IGFBPs (IGFBP-1 through IGFBP-6). The IGFBPs bind IGF-I with a high affinity, controlling IGF-I activity and extending the half-life. IGFBP-3 binds approximately 95% of circulating IGF-I. IGFBP-3 forms a 150 kDa complex with an acid labile subunit. Due to the size of this complex, it has limited permeability across the capillary and extends the half-life of IGF-I from minutes to hours (Guler et al. 1989). The increased half-life may heighten the actions of IGF-I by providing a larger pool of slow-release IGF-I to act on target tissues such as bone or muscle which may enhance the binding of IGF-I to the IGF-I receptor (Conover et al. 1992). Before IGF-I can interact with its receptor on a target tissue, the complex must be cleaved by a circulating IGFBP-3-specific protease. Each of the IGFBPs has a specific protease which is postulated to cleave the IGF-I/IGFBP complex into a fragment which has a higher affinity for IGF-I receptors than does the IGF-I/IGFBP complex.

The specific roles of the IGFBPs other than IGFBP-3 are not as well defined but some functions have been identified. IGFBP-I appears to be sensitive to changes in insulin levels; when circulating insulin is high, the binding of IGF-I by IGFBP-1 may protect against hypoglycemia (Conover et al. 1993). The IGFBPs may also regulate the passage of IGF-I from the intravascular to the extravascular space or exert IGF-independent effects on target cells by binding to IGFBP-specific receptors (integrin receptors) (Ruoslathi and Pierschlaber 1987). All the IGFBPs have an integrin receptor binding sequence (Oh et al. 1993). Thus, the IGFBPs and the corresponding IGFBP-proteases are influential modulators of IGF-I action, which under specific situations such as glucocorticoid treatment, could substantially interfere with tissue growth.

In addition to GH and IGF-I acting as endocrine factors, there is increasing evidence from in vitro studies that locally produced IGF-I and IGFBPs are essential for the local regulation of bone metabolism (Figure 2, Mohan and Baylink 1991, Canalis et al. 1988). Plasma GH and other systemic factors such as parathyroid hormone or vitamin D can stimulate the osteoblast to synthesize and release IGF-I or IGFBP-4 (Scharla et al. 1991, Mohan and Baylink 1991). Osteoblast-derived IGF-I can act as both a paracrine or autocrine factor by promoting the differentiation of osteoprogenitor cells to mature osteoblasts (paracrine action) or acting on neighbouring osteoblasts to further stimulate IGF-I and IGFBP production and release into the bone matrix (autocrine action). Other studies using cultured chick

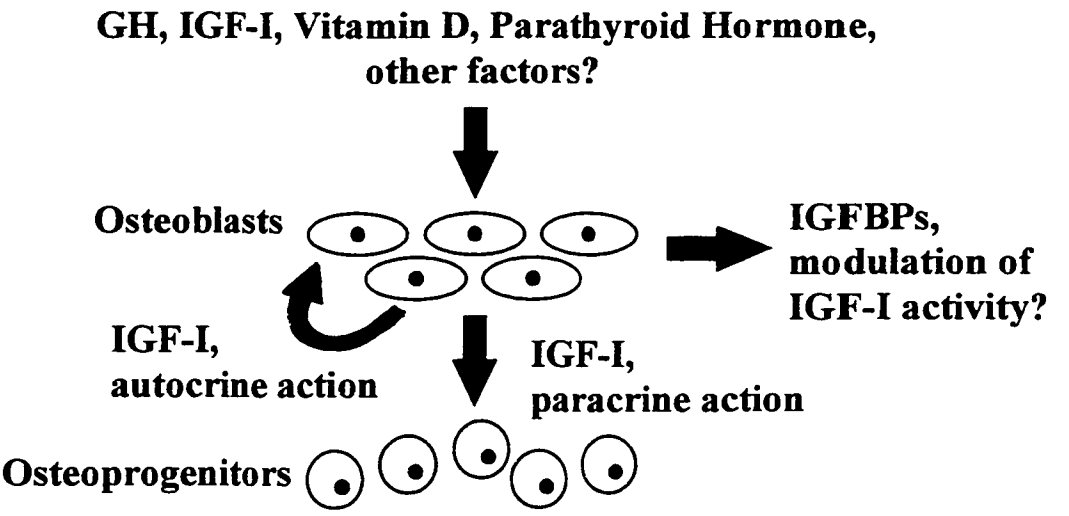



**Figure 2.** Endocrine factors such as GH, IGF-I, vitamin D and parathyroid hormone may stimulate local production of IGF-I. Osteoblasts secrete IGF-I and IGFBPs which can act as paracrine or autocrine regulators; IGF-I can promote the differentiation of osteoprogenitor cells to mature osteoblasts (paracrine action) or can act directly on neighbouring osteoblasts (autocrine action) while the IGFBPs can either enhance or inhibit tissue IGF-I activity.

calvaria cells have demonstrated that bone-derived IGF-I is incorporated into the osteoid matrix and released during bone resorption to further stimulate the proliferation of osteoprogenitor cells or differentiation to osteoblasts (Farley et al. 1987, Mohan and Baylink 1991). In fetal rat calvaria culture, IGFBP-2 suppressed cellular proliferation and collagen synthesis (Feyen et al. 1991) and both IGFBP-4 and IGFBP-5 exerted an inhibitory effect on bone by lowering the affinity of IGF-I for the IGF-I receptor (Mohan et al. 1989, Chevalley et al. 1993). Clearly, these in vitro studies have identified mechanisms by which local production of IGF-I and the IGFBPs links bone formation and resorption.

In vivo, both GH or IGF-I administration favourably alters the profile of the biochemical markers of bone turnover by elevating circulating osteocalcin and type 1 collagen propeptide (Brixen et al. 1990, Ebeling et al. 1993). The stimulation of osteoblast activity is indicative of an increase in bone mass. Exogenous administration of GH is also known to alter lean and fat tissue. In humans, exogenous GH or IGF-I has only been administered for short durations (e.g. 6-7 days) to healthy adult volunteers, and has never been evaluated in healthy infants or children due to the potential side-effects. Exogenous GH stimulates amino acid uptake by muscle (Fryburg et al. 1991), increases the rate of protein synthesis (Tomas et al. 1992a) and in some studies has been shown to suppress whole body protein breakdown (Horber et al. 1991). Thus, the net result of administering exogenous GH is an overall increase in lean mass. The effects of exogenous IGF-I on protein metabolism in healthy

humans has not been studied. Dose-dependent increases in lean mass have been observed in pigs treated with GH and this change in lean mass is accompanied by a reduction in fat mass, due to nutrient partitioning (Thiel et al. 1993, Etherton 1993).

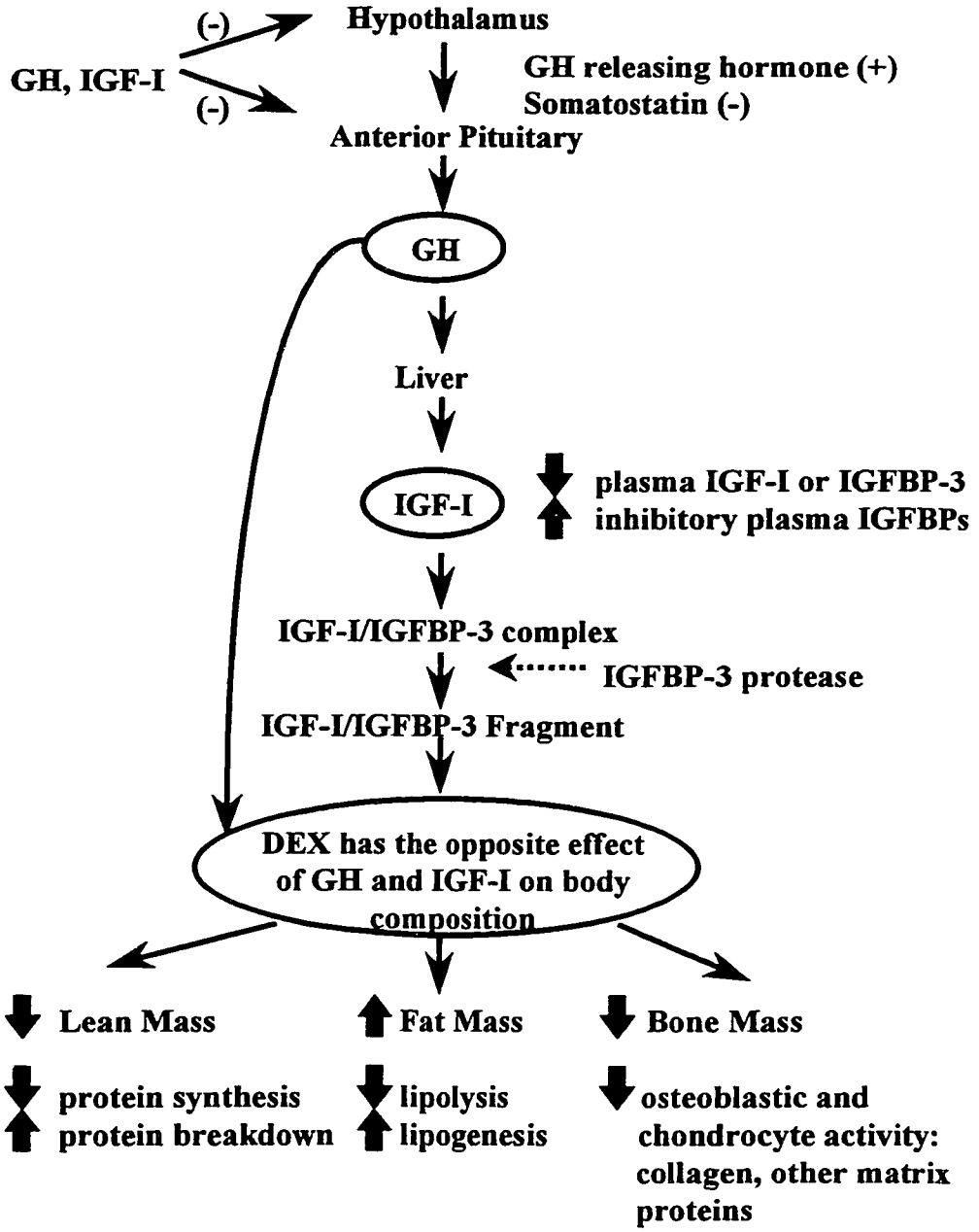
*In summary, it is evident that interference with the normal functioning of one or more components of the GH-IGF-I would have significant consequences on growth. Since the components of the GH-IGF-I axis mediate effects by endocrine, autocrine and paracrine regulation, there are many sites of regulation which could be altered by DEX.*

### 1.3.2 Steroid effects on the GH-IGF-I axis, growth, bone and protein metabolism: in vivo and in vitro evidence

In contrast to the anabolic effects of GH and IGF-I on bone and protein metabolism, steroid treatment inhibits osteoblastic activity, stimulates osteoclastic activity and promotes protein catabolism as illustrated in Figure 3. In developing rats, one month of DEX treatment reduced tibial length, growth plate and cortical bone width, trabecular bone volume, and the bone mineral content of 5 lumbar vertebrae (Altman et al. 1992). Studies in steroid-treated adults have reported reductions in circulating osteocalcin and type 1 procollagen, markers of osteoblastic activity (Reid et al. 1989, Lukert and Raisz 1990). Histomorphometric studies in adults revealed that glucocorticoids reduced the mean wall thickness, trabecular bone volume and lowered mineral apposition rates (Dempster et al. 1983, Lukert and Raisz 1990).

Overall lean mass can be reduced by exogenous steroid treatment (DEX or prednisone) by steroid-mediated increases in protein breakdown, decreases in protein synthesis or both processes simultaneously (Odedra et al. 1983, Horber and Haymond 1990, Bennet and Haymond 1992). Amino acid efflux, particularly by muscle is enhanced in steroid-treated dogs (Mulbacher et al. 1984) and reduced ribosomal activity and lower RNA content is observed in steroid-treated rats (Kelly et al. 1986).

Since both GH and IGF-I are positive modulators of bone development and protein homeostasis, DEX is hypothesized to act by altering specific aspects of the GH-IGF-I axis as illustrated in Figure 3. Although the precise mechanism by which DEX stunts growth and alters normal bone development and protein metabolism is currently not fully understood, DEX may impair release of GH from the anterior pituitary, reduce circulating or tissue levels of IGF-I, or modulate the activity of IGF-I by altering the circulating or tissue-specific IGFBP profile. There is limited evidence to support these hypotheses from human and animals studies as only a few studies have measured circulating components of the GH-IGF-I axis as well as functional measurements such as bone mass or protein turnover (Altman et al. 1992, Tomas et al. 1992a, Horber and Haymond 1990). Moreover, none of these studies have thoroughly investigated the IGFBP profile. The effects of DEX on IGF-I and/or IGFBP production in vitro are largely limited to bone cell culture systems. Addition of DEX to osteoblast cell cultures lowers DNA synthesis and indicates a reduced rate of cellular proliferation (Hughes-Fulford et al. 1992). Using cultured rat calvaria




**DEX effects on tissue regulation of [IGF-I] & IGFBP profile?**
**Net result = Growth delay**
**(lower muscle mass, higher fat mass, osteoporosis)**

**Figure 3.** DEX may interfere with one or more aspects of the GH-IGF-I axis and thereby result in reduced protein deposition, bone abnormalities and overall growth delay.

cells, DEX inhibited osteoblastic activity as indicated by reductions in alkaline phosphatase activity and Type 1 collagen synthesis (Canalis 1983). Since osteoblastic activity is reduced, IGF-I and IGFBP production by the osteoblast is diminished and can thereby interfere with local regulation of bone metabolism by IGF-I and the IGFBPs. Furthermore, studies have consistently demonstrated that chronic exposure to DEX in bone depletes osteoprogenitor reserves in cell culture, providing a mechanism by which long-term steroid treatment reduces bone mass (Kamalia et al. 1992). A study using cultured rat osteoblast-like cells reported that DEX reduced IGFBP-2 in a dose and time-dependent manner (Chen et al. 1991). The clinical significance of the reduction in IGFBP-2 is not understood, but if IGFBP-2 potentiates IGF-I activity within the bone matrix, this may be one mechanism by which DEX suppresses IGF-I activity and results in a lower bone mass. While these in vitro studies assist us in explaining local regulation of bone metabolism, they do not provide specific insight into the functional significance of these changes.

*Both in vivo and in vitro studies provide evidence that steroids affect the components of the GH-IGF-I axis, thereby suggesting mechanisms by which DEX mediates its detrimental effects on whole body bone and protein metabolism. Thus, we proposed to administer exogenous GH with or without IGF-I during treatment with DEX to attenuate DEX-induced effects.*

## 1.4 The efficacy of adjunctive GH, IGF-I or GH+IGF-I therapy during catabolic states in vivo

There are only a few studies in steroid-treated humans and animals which have investigated the benefits of administering GH or IGF-I to counter the detrimental effects of steroids on bone (Table 1) or protein (Table 2) metabolism and overall whole body growth. Of these studies, only one study in rats has considered the advantages of IGF-I alone on protein metabolism during steroid therapy while no study investigated the potential benefits of combined GH+IGF-I therapy on either bone or protein homeostasis. Moreover, none of the studies have detailed the changes in the circulating components of the GH-IGF-I axis by DEX; characterizing the changes in these components during DEX would provide us with a greater understanding of the mechanisms of DEX action on the GH-IGF-I axis in vivo.

### 1.4.1 Growth and bone metabolism

The only GH intervention studies in humans are in children, mostly renal transplant recipients. The largest studies conducted in renal transplant patients which included measurements of height velocity and bone indices were selected for review. Only one study evaluated children who were not renal transplant patients; this study involved children who required chronic steroid treatment for severe asthma and autoimmune colitis (Allen and Goldberg 1992). The four studies in children which are summarized in Table 1 all demonstrated an improvement in height velocity during adjunctive GH therapy and two studies reported that improvements in height velocity

corresponded with advances in bone age. Only one study assessed biochemical markers of bone activity; higher concentrations of plasma type 1 procollagen were correlated with height velocity, emphasizing that GH stimulates osteoblastic activity (Allen and Goldberg 1992).

The observation that some children advanced into puberty during these investigations may have confounded the findings since puberty is a potential stimulus for height and bone growth. Since all but one study included only renal transplant patients who received a variety of other drugs (e.g. cyclosporin) and have other medical complications, other factors may have also confounded the findings. It is also important to acknowledge that all these studies in children are descriptive since no randomized controlled trials have been conducted in steroid-treated children. This is partly due to the controversy surrounding whether children who do not have classic GH deficiency (defined as plasma GH $< 10$ ng/mL after provocative testing) will experience functional benefits such as improved adult height and bone mass. Steroid treatment does not result in classic GH deficiency. Moreover, the safety of GH in children without GH deficiency is actively debated (Lantos et al. 1989, Bischofberger and Dahlstrom 1989). The Canadian Pediatric Society does not recommend GH therapy in non-GH deficient children because of the unproven clinical benefits and potential side-effects although the United States and Europe do permit its use in renal transplant patients or other non-GH deficient states (e.g. autoimmune colitis, rheumatoid arthritis) under close supervision. Randomized controlled trials in non-GH

deficient, steroid-treated children are necessary to determine the efficacy of GH treatment either during or post-steroid treatment. In addition, long-term follow-up will be required to establish whether GH treatment does improve final height and bone mass. Despite the weaknesses of the clinical studies summarized in Table 1, they provide preliminary evidence that GH may improve height velocity and at least partially promote the acceleration of bone age during steroid treatment.

The only animal study was conducted in young, growing mice. Adjunctive GH during DEX fully countered the reduction in weight, plasma IGF-I, and normalized bone structure and mass (Altman et al. 1992) but both the dose of DEX and GH would be considered pharmacological doses in humans. The fact that these mice are young and developing suggests that exogenous GH is effective at stimulating growth during a period of rapid development.

### 1.4.2 Protein metabolism

The only two human studies which examined the effect of GH during steroid treatment were in adults, and both showed improved N retention (Table 2). In fact, N retention, protein synthesis and protein breakdown were completely normalized in healthy adults who received adjunctive GH treatment during a one week course of steroids (Horber and Haymond 1990). Because the study subjects were all healthy, the higher rates of protein breakdown could be attributed only to steroid treatment and were not due to an underlying disease state (Horber and Haymond 1990). In addition, the small descriptive study in steroid-treated adults with chronic lung disease

demonstrated higher rates of protein synthesis after a conservative dose of GH compared to pre-treatment with GH (Bennet and Haymond 1992).

Since there were no studies in children, only the rat study provides insight into the benefits of IGF-I during development (Table 2). Improved growth and N balance was shown to be dependent on the dose of IGF-I administered and was accompanied by elevations in plasma IGF-I (Tomas et al. 1992a). The mechanism of action was an increase in muscle RNA and protein synthesis during DEX treatment. This is the only study which examined dose dependent effects of IGF-I during steroid treatment. The dose-dependent effect of GH on either bone or protein metabolism during DEX has not been examined.

**Table 1: Effect of adjunctive GH on growth and bone metabolism during steroid therapy in vivo[1]**

| Study | Subjects | Steroid dose | GH dose & duration | Outcomes | Findings |
|---|---|---|---|---|---|
| **Van Dop et al.[2] 1992** n=9 | <16 y renal transplant | Prednisone 4-19 mg/m$^2$ Alternate day | 0.3-0.35 mg/kg/wk 3,6 or 7 doses/wk 2 y | Height, bone age | Improved height velocity but not bone age |
| **Fine et al.[2] 1991** n=9 | 11-17 y renal transplant | Prednisone 0.11-0.43 mg/kg/d Daily | 0.1-0.4 mg/kg/wk 3 or 7 doses/wk 0.5-2.5 y | Height, bone age | Improved height velocity but not bone age |
| **Benfield et al.[2] 1993** n=13 | 5-15 y renal transplant | Prednisone 0.3-1.5 mg/kg/d Daily | 0.05 mg/kg/d Daily doses 2 y | Height, bone age | Improved both height velocity & bone age |
| **Allen & Goldberg[2] 1992** n=7 | 9-15 y asthma, colitis renal transplant | Prednisone 0.4-0.7 mg/kg/d Alternate day | 0.3 mg/kg/wk 3 or 7 doses/wk 0.5-1.8 y | Height, plasma IGF, type 1 procollagen (pColl) | Improved outcomes; height velocity correlated with pColl |
| **Altman et al.[3] 1992** n=5/group | 3 wk Healthy, growing mice | DEX 1 mg/kg/d 5 times/wk | 1 mg/kg/d 5 doses/wk 4 wk | Weight, plasma IGF, tibia length, growth plate width, trabecular bone volume,BMC | Improved all outcomes compared to DEX alone & similar to controls |

[1]Prior to September 1993, the potential benefits of IGF-I±GH had not been investigated in vivo.
[2]All studies in children were descriptive. Height velocity and bone age during GH treatment was compared to pre-GH treatment measurements. Steroids were administered orally and GH was administered by subcutaneous (s.c.) injection.
[3]Mice were randomized to placebo, DEX, GH alone or DEX+GH. DEX and GH were administered by s.c. injection.

**Table 2: Effect of adjunctive GH or IGF-I on protein metabolism during steroid therapy[1]**

| Study | Subjects | Steroid dose[4] | GH or IGF-I[5] dose & duration | Outcomes | Findings |
|---|---|---|---|---|---|
| **Horber & Haymond[2] 1990** n=8/group | 18-36 y healthy adults | Prednisone 0.8 mg/kg/d Daily | GH, 0.1 mg/kg/d Daily 7 d | N retention, protein kinetics by leucine tracer | Normalized N retention, protein synthesis & breakdown |
| **Bennet & Haymond[3] 1992** n=4 | 31-71 y asthma, chronic obstructive airways disease | Prednisone 10-27.5 mg/d Daily | GH, 0.0125 mg/kg/d Daily 7 d | Plasma GH & IGF, protein kinetics by leucine tracer | ↑ plasma GH & IGF, ↑ protein synthesis |
| **Tomas et al.[2] 1992** n=6/group | Age?, healthy, growing rats weight=150 g | DEX 0.9 mg/kg/d Continuous infusion | IGF-I, 0.74, 1.85 or 4.63 mg/kg/d Continuous infusion | Weight, plasma IGF, N retention, muscle RNA, protein synthesis | Compared to DEX alone: improved weight & N balance, ↑ plasma IGF-I, ↑ muscle RNA, ↑ protein synthesis, Dose-dependent effects |

[1]Prior to September 1993, the potential benefits of administering the combination of GH+IGF-I during steroid treatment had not been investigated in vivo.
[2]Randomized control trial
[3]Outcomes after GH treatment were compared to pre-GH treatment measurements.
[4]Prednisone was administered orally and DEX was administered by subcutaneously implanted osmotic pump.
[5]GH was administered by subcutaneous injection and IGF-I was administered by a subcutaneously implanted osmotic pump.

### 1.4.3 Non-steroid-induced states in which adjunctive GH, IGF-I or GH+IGF-I have been administered to improve protein metabolism

In addition to steroid treatment, there are several other clinical situations in which GH, IGF-I or GH+IGF-I intervention have been more extensively studied. These include studies in which adults are recovering from surgery (Mjaaland et al. 1993, Ward et al. 1987) or receiving prolonged parenteral nutrition (Ziegler et al. 1992) as well as experimentally-induced diet restriction in healthy volunteers (Kupfer et al. 1993). In randomized double blind controlled trials in patients requiring prolonged total parenteral nutrition due to gastrointestinal or pancreatic disorders (Ziegler et al. 1992) or recovering from surgery (Ward et al. 1987), exogenous GH was shown to improve N balance by increasing both protein synthesis and fat oxidation. As well, skeletal muscle breakdown and the efflux of amino acids from skeletal muscle were lower among GH-treated compared to untreated patients (Mjaaland et al. 1993). These improvements in GH-treated patients were accompanied by elevations in circulating IGF-I during the first days of GH treatment.

Combined treatment of GH+IGF-I resulted in a higher nitrogen retention than treatment with IGF-I alone during dietary restriction in healthy volunteers, providing the only in vivo evidence of an additive effect of IGF-I on protein metabolism during a catabolic state (Kupfer et al. 1993). This study purports that the additional response was due to the induction of IGFBP-3 and the acid labile subunit by GH. As discussed earlier, IGFBP-3 is known to circulate as a ternary complex (M.W. = 150 kD)

comprised of IGFBP-3, IGF-I and the acid labile subunit (Baxter and Martin 1989) accounting for 95% of all IGF-I. IGF-I which is bound to this complex has a significantly longer half-life (12-15 hours) than unbound IGF-I (10-12 minutes) in both humans and piglets (Guler et al. 1989, Walton et al. 1989) and may thereby provide a greater opportunity for IGF-I to interact with target tissues (Kupfer et al. 1993). In addition to this explanation, it is possible that combined treatment may result in additional benefits as both GH and IGF-I have anabolic actions which are independent of each other (Tomas et al. 1992a, Tomas et al. 1992b, Turkalj et al. 1992, Fryburg et al. 1991, Brixen et al. 1990, Ebeling et al. 1993, Richter et al. 1987).

It is unclear whether the responses to GH±IGF-I are different than that which occurs during steroid treatment. Surgery, total parenteral nutrition or experimentally-induced diet restriction causes a suppression in plasma IGF-I and IGFBP-3 which is not a consistent finding during steroid treatment. However, a steroid-induced reduction in plasma IGF-I and IGFBP-3 may not be detected due to the descriptive nature of the majority of the studies (Allen and Goldberg 1992, Bennet and Haymond 1992, Van Dop et al. 1992, Fine et al. 1991, Benfield et al. 1993). The important message from these studies is that exogenous GH, IGF-I or GH+IGF-I can exert their known functions during metabolic insults or disease states in which rates of protein synthesis are reduced and/or rates of protein breakdown are elevated. This finding

suggests that providing additional GH or IGF-I stimulates protein synthesis and lower rates of protein breakdown, the net result being protein deposition.

***Effect of DEX, GH, IGF-I and GH+IGF-I on glucose homeostasis:***

Combined therapy of GH+IGF-I also has the potential to oppose any negative effects of GH or IGF-I on glucose homeostasis. Hyperglycemia can occur with exogenous GH administration (Brixen et al. 1990, Gustafsson 1989) while IGF-I has the opposite effect on blood glucose levels, resulting in hypoglycemia unless the subject is closely monitored (Mauras et al. 1992). Thus, there is some evidence that administering appropriate doses of each factor together will cancel out their negative effects on glucose homeostasis (Kupfer et al. 1993). DEX treatment alone has been shown to cause hyperglycemia in VLBW infants (Harkavy et al. 1989, Ohlsson et al. 1992a).

*In summary, the intervention trials which we have proposed in piglets will make significant contributions to our understanding of the efficacy of GH or GH+IGF-I to stimulate bone formation and promote protein anabolism during steroid treatment. Moreover, these studies will allow us to evaluate the risk of hyperglycemia or hypoglycemia.*

## 1.5 Approaches to study the effects of exogenous steroids during development with or without adjunctive GH or GH+IGF-I therapy

### 1.5.1 Components of the GH-IGF-I axis

Circulating IGF-I and/or IGFBP-3 are positively correlated with the birth weights of preterm and term born infants (Bennett et al. 1983). In children, measurement of the circulating components of the GH-IGF-I can be used to assess GH status in short children (Smith et al. 1993), emphasizing the relationship between growth and the GH-IGF-I axis throughout development. Plasma GH, IGF-I and IGFBP-3 are sensitive to metabolic insults which impair growth (Smith et al. 1993). Thus, assessment of these factors can provide insight into the mechanisms of growth delay.

Steroid treatment and suboptimal nutrition are among the most common metabolic insults experienced by VLBW infants during hospitalization. As detailed above, steroid treatment in animals and older humans is sometimes manifested by alterations in plasma IGF-I and the IGFBP profile but is consistently associated with growth reduction or muscle protein catabolism (section 1.3.2). As well, moderate caloric restriction and/or malnutrition results in higher plasma GH, lower plasma IGF-I and IGFBP-3 and higher plasma IGFBP-2 (Counts et al. 1992). The nutritional state of DEX-treated VLBW infants is compromised as fluid intake is moderately restricted in the management of their lung disease (Tammela et al. 1992) and full oral feeds are not tolerated during the first weeks of life due to the immaturity of the

gastrointestinal tract. Thus, these infants receive total parenteral nutrition for extended periods of time. Circulating IGF-I and IGFBPs can be used as indicators of the nutritional status of DEX-treated preterm infants.

There is currently no information pertaining to the effect of DEX on the circulating IGF-I and the IGFBPs among DEX-treated preterm infants and only minimal data in non-DEX-treated preterm infants born earlier than 28 weeks of gestation (Lineham et al. 1986, Bennett et al. 1983). Although the ontogeny of IGF-I and the IGFBPs has been described in healthier preterm infants, there is a paucity of data in the most immature infants who are at greatest risk of growth failure due to DEX treatment. No studies have thoroughly evaluated/considered the relationship between growth velocities, circulating IGF-I and IGFBPs, and nutritional intake in this population.

Administration of GH to GH deficient children results in improved height velocity, bone maturation and bone mineral density which is accompanied by increases in circulating GH, IGF-I and sometimes IGFBP-3 (Saggese et al. 1993, Smith et al. 1993, Burns et al. 1981). As well, GH administration to GH deficient children resulted in a favourable effect on body composition (e.g. lower fat mass) with GH treatment (Wabitsch and Heinze 1993). Non-GH deficient children who are more than 2 standard deviations less than the mean height for age also experience a rise in circulating IGF-I and improved height velocities after a minimum of 6 months of GH therapy (Genentech Collaborative Study Group 1989, Hopwood et al. 1993,

Allen and Goldberg 1992, Van Dop et al. 1992, Fine et al. 1991, Benfield et al. 1993). Measurement of plasma GH, IGF-I and the IGFBP profile can be used as indicators of the metabolic response to exogenous GH treatment during development (Smith et al. 1993).

Due to the extremely pulsatile release of GH into the circulation of both preterm infants and piglets, a single measurement is likely not representative of the GH concentrations over a 24 hour period and is not a reliable indicator of GH status. Therefore, plasma GH will not be assessed in infant samples because only a small quantity of blood can be sampled from preterm infants. Since a larger amount of plasma can be sampled from the piglets, circulating GH will be evaluated to delineate whether exogenous GH or GH+IGF-I treatment elevates circulating GH. Plasma IGF-I and the plasma IGFBP profile in both infants and piglets will be measured to characterize any developmental changes or DEX-induced alterations, and the response to exogenous GH and IGF-I. The relative quantities of IGFBP-1 through IGFBP-4 will be assessed by western ligand blotting to evaluate the relative changes in each of the IGFBPs. The two predominant IGFBPs in preterm infants during early life, IGFBP-2 and IGFBP-3, will be quantified using specific radioimmunoassay and enzyme-linked immunoassay. Due to a lack of homology between the IGFBP sequences among piglets and humans, we are limited to evaluating the relative changes in IGFBP-1 through IGFBP-4 in piglets by western ligand blot.

There is speculation that tissue production of IGF-I and the IGFBPs in response to endocrine factors can have a critical role in mediating the effects of DEX on bone and protein metabolism and overall growth during postnatal life. Because a suppression in circulating IGF-I and/or IGFBP-3 during steroid treatment is not a consistent finding, it is possible that steroids exert their effect at the target tissues. Studies in human fetal and piglet tissue have demonstrated a tissue-specific expression of IGFBP in skeletal muscle and liver (Funk et al. 1992, Lee et al. 1993). The local availability and action of IGF-I, as discussed in section 1.3.1, can be modulated by both locally produced IGFBPs and systemic stimuli. Thus, in addition to measuring the circulating components of the GH-IGF-I axis, tissue IGFBP mRNA expression in liver, muscle and bone tissue will be evaluated in order to understand tissue-specific regulation by DEX.

*Collectively, measurement of the circulating and tissue components of the GH-IGF-I axis may elucidate some of the mechanisms by which DEX alters bone and protein homeostasis, resulting in growth delay. Any effects of exogenous GH or GH+IGF-I on these components will also be evaluated to determine the mechanisms by which these anabolic agents may counter the effects of DEX.*

### 1.5.2 Growth and body composition

***Anthropometry:*** Anthropometry will be performed at frequent intervals to assess whole body growth in infants and piglets. In infants, weight and length measurements are the least invasive method for monitoring growth during early

postnatal life. Steroid treatment reduces weight and length growth velocities in infants (Ohlsson et al. 1992a, Brownlee et al. 1992) and height velocity in children (Allen and Goldberg 1992). The growth of steroid-treated infants can be compared to the size-at-birth standards which were developed using the birth weights and lengths of a group of preterm and term born reference infants (n=1231) (Blidner et al. 1984). Since the size-at-birth standards are based on cross-sectional data, including birth weight and length of preterm and term born infants from the same geographical region as the infants to be studied, the extrauterine growth of a preterm infant can be compared to the intrauterine growth they should have experienced if they remained in utero. Since it is a well-documented observation that premature infants do not achieve rates of intrauterine growth, a comparison of absolute weight and length between premature infants and term born reference infants is not particularly helpful. It is more useful to calculate a z-score, the standard deviation (SD) of the study group from the reference group, to compare the growth of premature infants to a reference group of term infants. A z score for weight or length is calculated according to the equation:

**z-score = (subject value-mean of reference group value)/SD of reference group**

For example, z-scores of 0, -1 and -2 indicate that an infant is gaining weight or length at the 50th, 16th or 2.5th percentile, respectively, of term born reference infants.

In piglets, a reference growth curve is not required since the weight and length growth of experimental groups can be compared to the placebo control group. Daily weight measurements will indicate whether adjunctive GH or GH+IGF-I improves weight gain. Although length measurements in infants are a measure of long bone growth as well as vertebral growth, the length of a piglet, measured from the tip of the snout along the vertebrae until the base of the tail, reveals only vertebral growth. Long bone growth can be measured after femurs are excised at necropsy.

***Body composition by dual energy x-ray absorptiometry:*** While anthropometry reveals the changes in total weight and length growth, dual energy x-ray absorptiometry (DXA) discerns between lean, fat and bone mass and can be used to monitor changes in each body compartment, providing insight into the quality of growth. Administration of DEX alone or GH alone alters body composition. In humans, the only studies which demonstrated steroid-mediated changes in body composition are in adults. Patients who received steroids to manage kidney disease had a higher fat mass compared to the pre-steroid treatment measurement (Horber et al. 1986). GH administration to growing pigs dramatically alters body composition by promoting a higher percentage of body weight to be deposited as lean tissue compared to non-GH-treated pigs in a dose-dependent manner (Etherton et al. 1993, Thiel et al. 1993). All of the studies in GH-treated pigs evaluated body composition by carcass analysis, the gold standard for quantifying lean, fat and bone mass.

DXA technology was previously validated in Dr. Atkinson's laboratory by comparing the results of whole body composition obtained by DXA with those obtained by carcass analysis (Brunton et al. 1993). In small piglets (1.6 kg), DXA overestimated fat mass and underestimated lean mass but, in heavier piglets (6 kg) the lean, fat and bone mass measurements were similar to those obtained by carcass analysis. Since the weight of most piglets will be close to 6 kg, the results from the piglet studies will be accurate. Assessment of body composition in the infants will need to be interpreted with caution as their body weights will be lower than 6 kg but higher than 1.6 kg. However, DXA can detect changes in all three compartments (bone, lean and fat mass) in prematurely infants who are measured at term, 3, 6, and 12 months corrected age (Brunton et al. 1993) which suggests that DXA can be used to assess preterm infants at term corrected age and at older ages.

The most significant limitation of DXA is the radiation exposure to the infant; a whole body scan exposes an infant to 7 $\mu$Sv. Although this amount of radiation is considered to be safe, performing duplicate scans or additional regional scans at a single visit is unacceptable in infants. Because repeated radiation exposure is not a concern for piglets, additional scans can be performed. The femur can be scanned using a software program which has a higher resolution, enabling smaller quantities of bone to be detected and quantitated.

*In infants, anthropometry and body composition assessment will reveal the impact of premature birth and DEX treatment on extrauterine weight and length*

*growth compared to intrauterine growth rates, underlining the compromised growth of steroid-treated infants. In piglets, the combination of anthropometry and body composition measurements will reveal whether adjunctive GH or GH+IGF-I during DEX treatment improves growth and positively alters body composition.*

### 1.5.3 Bone mineral metabolism

***Biochemical markers of bone turnover:*** Measurement of the biochemical markers of bone turnover, indicators of osteoblastic or osteoclastic activity, provide insight into the mechanisms which regulate bone mass accretion during various stages of development or in response to DEX, GH or IGF-I. Plasma osteocalcin, a marker of osteoblastic activity and urinary N-telopeptide (NTx) of Type 1 collagen, a marker of osteoclastic activity, have been selected for analyses.

Osteocalcin is the most abundant non-collagenous protein in bone and is synthesized and secreted exclusively by the osteoblast during the mineralization process (Gundberg 1993). While the majority of the newly synthesized osteocalcin binds to hydroxyapatite via three carboxylated glutamic acid residues along the osteocalcin peptide, a small proportion of osteocalcin is released into the circulation (Gundberg 1993) and is indicative of bone formation.

NTx is a collagen crosslink at the N-terminal region which stabilizes newly formed collagen fibrils in the extracelluar matrix (Hanson et al. 1993). Lysyl oxidase forms aldehydes on specific lysine and hydroxylysine residues within the collagen fibril; these residues then condense with lysine and hydroxylysine residues on adjacent

collagen fibrils and form a NTx crosslink. During bone resorption, NTx is released from the breakdown of collagen and is an indicator of osteoclastic activity (Hanson et al. 1993).

Frequent, sequential measurements of these markers can be made to assess the impact of development, steroids or anabolic agents on bone metabolism in infants and piglets since very small quantities of blood (25 $\mu$L) and urine (50 $\mu$L) are required. In piglets, it is also possible to monitor the dose-dependent changes during a tapering dose of DEX. Since both osteocalcin and NTx are responsive to acute changes in bone cell metabolism, subtle differences due to steroid (Wolthers et al. 1993), GH (Brixen et al. 1990, Johansen et al. 1990, Markowitz et al. 1989) or IGF-I treatment (Ebeling et al. 1993) can be observed before changes in bone mass can be detected by DXA. In addition, it would be unethical to perform several whole body scans using DXA over a short time frame (e.g. 3 scans over 3-4 months) due to the radiation exposure to the infant. The gold standard for investigating changes in bone metabolism is standard histomorphometry, but obtaining a biopsy sample from this population of infants is too invasive. It is also very difficult to obtain a sample that is representative of the entire bone due to the small amount of tissue sampled. Fortunately, the changes in the biochemical markers of bone do reflect the actual changes in bone cell metabolism and are accepted as indicators of bone changes. Changes in circulating osteocalcin (Delmas et al. 1985) and urinary collagen crosslinks (e.g. pyridinoline) (Delmas et al. 1991) have been shown to correlate with

histomorphometry. The specific relationship between urinary NTx and histomorphometry has not yet been studied.

Steroid treatment has been consistently shown to suppress osteoblastic activity (Lukert and Raisz 1990) and circulating osteocalcin (Wolthers et al. 1993). This effect may be explained by the findings that, in vitro, the glucocorticoid receptor complex binds to the promoter of the osteocalcin gene, inhibiting promoter activity (Morrison and Eisman 1993) and resulting in a reduction in the synthesis of osteocalcin. In contrast to DEX, GH administration to GH deficient children results in higher osteocalcin concentrations in vivo (Markowitz et al. 1989, Johansen et al. 1990). GH is hypothesized to stimulate osteocalcin production via a $1,25(OH)_2D$ mediated mechanism since there is a $1,25(OH)_2D$-responsive element in the promoter region of the osteocalcin gene (Morrison et al. 1989) and GH promotes the conversion of 25(OH)D to $1,25(OH)_2D$ (Goff et al. 1990, Brixen et al. 1992). The effect of IGF-I on the production of osteocalcin or $1,25(OH)_2D$ has not been clearly defined. Because NTx is a relatively new biochemical marker of bone turnover there is no information about the effects of steroids, GH or IGF-I on urinary NTx levels. However, due to the fact that osteoclastic activity is often elevated with steroid treatment (Lukert and Raisz 1990), it is expected that the excretion of other collagen crosslinks would also be higher among steroid-treated compared to non-steroid-treated subjects.

Only one report describes the ontogeny of plasma osteocalcin in preterm infants from 29 weeks gestational age through to term corrected age (Pittard et al.

1992) while there have been no reports of circulating osteocalcin in steroid-treated preterm infants. Also, no studies have monitored changes in NTx during early postnatal life. Establishing the changes in these markers may help to explain why preterm infants do not attain bone mineral at intrauterine rates and thus have a lower bone mass at term corrected age compared to term born infants (Horsman et al. 1989, Steichen et al. 1980). Differences in bone cell metabolism in preterm compared to term infants may compromise the less mature infant's ability to accrue bone during extrauterine life.

*Assessing the changes in plasma osteocalcin and urinary NTx in very premature infants will elucidate the developmental changes in bone cell activity throughout early life and any alterations imposed by DEX. If bone mass is improved in DEX-treated piglets receiving GH or GH+IGF-I, the biochemical data will reveal whether the improvements in bone mass are due to higher rates of osteoblastic activity, lower rates of osteoclastic activity or both.*

### 1.5.4 Protein metabolism

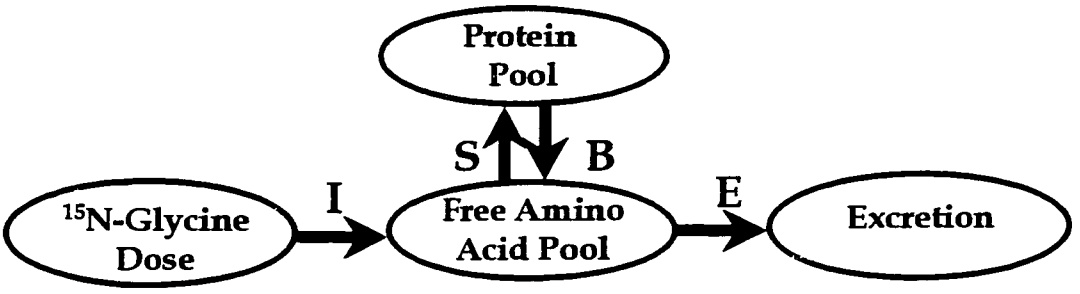
***Stable isotope methodology***

***$^{15}$N-glycine tracer:*** Stable isotope methodology has been used to assess changes in the rates of whole body protein synthesis and breakdown in response to steroid, GH or IGF-I therapy (Beaufrere et al. 1989, Tomas et al. 1992b, Horber and Haymond 1990, Kupfer et al. 1993). We specifically chose to administer $^{15}$N-glycine to our piglets because it is extensively involved in protein metabolism (Mathews et al.

1981), is relatively inexpensive compared to other tracers, can be administered as a single oral dose, and has been frequently used in preterm infants to examine changes in protein kinetics due to differences in nutrient intake (Heine et al. 1983) or in children (Richter et al. 1987) and pigs (Tomas et al. 1992b) to assess the protein kinetic response to GH treatment. Also, this methodology could be used to assess protein kinetics in VLBW infants in whom obtaining multiple blood samples over a short period of time would be too invasive since serial urine samples rather than multiple blood samples are required for the calculations. Administering $^{15}N$ is safe to both infants and pigs since the chemical activity of stable isotopes and the metabolism of isotopes are similar. Only the atomic mass differs between isotopes; $^{15}N$ has an additional neutron compared to $^{14}N$ (Wolfe 1992). It is the difference in the mass of the N isotopes and the differences in the natural abundance of the N isotopes ($^{14}N$=99.63% abundance versus $^{15}N$=0.37% abundance) which allows us to detect how rapidly $^{15}N$ is excreted from the body.

***The single pool model:*** The single pool model (Figure 4), developed by Waterlow et al. (1978), will be used to determine the rates of protein synthesis and protein breakdown. The single pool model is concerned with the overall process of whole body protein turnover rather than focusing on several different metabolic pools of N (Wolfe 1992). Thus, after ingestion of the tracer, $^{15}N$-glycine enters a free amino acid pool where it is either incorporated into protein or oxidized and excreted in the form of urea or ammonia. Total urinary $^{15}N$, including $^{15}N$ which is

incorporated into urea and ammonia, is quantified and used to determine N flux (Equation 3). This model is based on the assumptions that $^{15}N$ is not recycled due to protein degradation and that the rate of protein synthesis and protein breakdown are constant during the study period.




$$Q = S + E = B + I$$

**Q, nitrogen flux; I, nitrogen intake; S, protein synthesis**
**B, protein breakdown, E, nitrogen excretion**

**Figure 4.** The single pool model (based on the concept of Waterlow et al. 1978).

***Determining the enrichment of $^{15}N$ in urine samples:*** The isotope ratio mass spectrometer expresses the enrichment of $^{15}N$ in a urine sample as delta per mL ($\delta$). Delta per mL is defined in equation 1:

**Equation 1:** $\delta^{15}N$ **per mL** $= [(r_{sa}-r_r)/r_r)]*1000$

**$r_{sa}$ and $r_r$ are the ratio of $^{15}N^{14}N$:$^{14}N^{14}N$ in the sample and reference gas, respectively.**

The first step in calculating the rates of protein synthesis and protein breakdown requires the conversion of $\delta$ per mL values to atom percent excess (APE). The APE of a sample is the atom percent enrichment of the sample in excess of the reference gas. All samples, including baseline, atmosphere and post-dose urine samples will be converted from $\delta$ to APE.

**Equation 2:** $\mathbf{APE = [(\delta)(r_r)/((\delta)(r_r)+1000)] * 100}$

**$r_r$ is the ratio of $^{15}N^{14}N$:$^{14}N^{14}N$ of the reference gas**

To correct for the baseline $^{15}N$ enrichment in the food source and in the atmosphere, the APE for the baseline and atmosphere samples are subtracted from the APE of each of the post-dose samples. In order to quantify the amount of $^{15}N$ excreted at each time point, the APE will be multiplied by the total urine volume at each specific collection time. The quantity of $^{15}N$ excreted is expressed as a function of the original dose of $^{15}N$. To calculate the cumulative $^{15}N$ excretion over 48 hours, the quantity of $^{15}N$ excreted at each time point is summed. After plotting the cumulative excretion of $^{15}N$, the time at which a constant value is obtained is determined and the cumulative excretion of $^{15}N$ at this time

point is used to calculate N flux, represented by Q (calculation is shown in the methods section of Chapter 4). The rate of protein synthesis and protein breakdown will be calculated using the equation shown in Figure 4 as both the N intake (1.92 g/kg/d) and total urinary N excretion are known. Total urinary N, including both urea and ammonia N, will be measured using the Kjeldahl method (Bradstreet 1965) which does not distinguish between different N isotopes.

***Biochemical markers of protein metabolism:*** Blood urea nitrogen (BUN) and urinary 3-methylhistidine (3-MH) can be measured to assess protein metabolism in steroid-treated preterm infants. Due to the precarious state of VLBW infants, it is usually more practical to measure these biochemical markers rather than perform a stable isotope tracer study (e.g. $^{15}$N-glycine) for several reasons: i) VLBW infants have frequent routine blood chemistry so a single blood sample can be obtained for BUN analysis; ii) obtaining a spot urine for 3-MH analysis is fairly non-invasive; iii) a $^{15}$N-glycine tracer study requires frequent handling of the infant to ensure a complete 48 h urine collection when only minimal handling is acceptable; and iv) measuring the enrichment of $^{15}$N in multiple urine samples is more time-consuming and expensive than analysing a single blood (BUN) or urine (3-MH) sample.

Both BUN and 3-MH are sensitive to acute changes in protein metabolism. Within the first days of steroid treatment, elevations in BUN and 3-MH are observed in preterm infants (Brownlee et al. 1992) and rodents (Odedra et al. 1983). An elevation in BUN is indicative of excess nitrogen in the body; this surplus of N can be due to excess N in

the diet, decreased uptake of amino acids followed by a lower rate of protein synthesis, or due to increased proteolysis. A rise in circulating plasma amino acids accompanies DEX treatment in preterm infants (Williams and Jones 1992). Thus, changes in BUN do not identify whether protein synthesis, breakdown or both processes are altered by DEX. In contrast, urinary 3-MH is a specific indicator of protein breakdown (Young and Munro 1978). 3-MH is formed by a post-translational modification of actin and myosin proteins and is almost exclusively found in muscle (Kayali et al. 1987). During muscle protein breakdown, 3-MH is released from muscle and is not further metabolized. Thus, an elevation in 3-MH excretion is indicative of increased muscle protein breakdown.

*In the proposed piglet study, assessing protein homeostasis by both methods, stable isotope methodology and the biochemical markers, will verify the agreement between the two approaches for assessing changes in whole body protein metabolism due to DEX, GH or GH+IGF-I. Measurement of urinary 3-MH will specifically reveal how DEX and adjunctive GH and GH+IGF-I during DEX alters muscle protein metabolism.*

## 1.6 The piglet as a model for very low birth weight infants

Term born piglets are used extensively to understand the regulation of bone and protein metabolism in response to nutrition and drug treatments in premature infants (Weiler et al. 1993, Burrin et al. 1992). Because potential confounding variables which cannot be controlled in the clinical setting can be eliminated and tissue samples can be

collected, piglet studies allow us to fully test our hypotheses and gain a further understanding of the mechanisms mediating growth in the postnatal period.

During early life, both piglets and infants have a similar quality of diet (Atkinson et al. 1993) and digestive physiology (Moughan et al. 1992). As well, the absorption and utilization of nutrients are comparable (Moughan and Rowan 1989, Moughan et al. 1992) and both share a similar body composition (Brunton et al. 1993). A major difference between infants and piglets born at term, which makes term born piglets a particularly appropriate model for VLBW infants, is the degree of maturity at birth. Piglets born at term have undergone substantially less late stage fetal development than human infants born at term and are therefore estimated to be at a comparable stage of development as infants born at 27 weeks of gestation (Book and Bustad 1974). However, because piglets have a substantially higher rate of weight gain and maturation than human infants postnatally (Miller and Ullrey 1987), studying a piglet from birth to 3 weeks of age is postulated to parallel the developmental changes experienced by infants during the first 3 months of extrauterine life (Moughan et al. 1992).

Both piglets and VLBW infants have a comparable metabolic response to DEX, manifested by alterations in the biochemical markers of bone and protein turnover, a suppression of bone and lean mass deposition and an overall delay in growth (Weiler et al. 1993, Weiler et al., pilot study). Thus, the DEX-treated piglet is an established animal model for steroid-treated VLBW infants. Furthermore, growing, non-steroid treated pigs (Goff et al. 1990, Tomas et al. 1992b) who are treated with GH experience

similar metabolic changes with respect to bone and protein homeostasis as children and adults who receive exogenous GH (Allen and Goldberg 1992, Brixen et al. 1992, Brixen et al. 1990). These findings emphasize that the piglet is an appropriate model for investigating potential adjunctive therapies in humans. Another important similarity between pigs and VLBW infants is that the ontogeny of the circulating components of the GH-IGF-I axis is comparable during early postnatal life; both experience a postnatal rise in IGF-I and IGFBP-3 (Bernardini et al. 1992, Lee et al. 1991, Lineham et al. 1986) and have tissue specific expression of the IGFBPs during intrauterine life (Lee et al. 1993, Funk et al. 1992). There is also an increase in GH receptors in hepatic tissue of both piglets and humans during early life (Daughaday et al. 1987, Breier et al. 1989).

*In summary, the DEX-treated piglet is an appropriate animal model in which to investigate the impact of DEX on the GH-IGF-I axis; and to evaluate the effectiveness of adjunctive GH alone or GH+IGF-I to counter the potent effects of DEX on whole body bone and protein metabolism during early postnatal development.*

# Chapter Two

# BONE METABOLISM AND THE GH-IGF-I AXIS IN DEXAMETHASONE-TREATED PRETERM INFANTS*

* This chapter represents a manuscript which was submitted to Early Human Development, July 1998.

# Bone metabolism and the GH-IGF-I axis in dexamethasone-treated preterm infants

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**Summary**

Aim: To characterize the ontogeny of circulating IGF-I, the IGF binding proteins (IGFBPs) and biochemical markers of bone turnover in dexamethasone (DEX)-treated preterm infants with chronic lung disease.

Methods: Plasma and urine samples from 17 infants were obtained prior to DEX, after 7 days of DEX and at the completion of DEX to assess plasma IGF-I, IGFBPs, osteocalcin and urinary N-telopeptide. Nutrient intakes and growth were monitored from birth until term corrected age at which time body composition was evaluated by dual energy x-ray absorptiometry.

Results: Although nutrient intakes did not differ during or after DEX, weight gain (115 vs. 174 g/wk) and length gain (0.7 vs. 1.0 cm/wk) were higher after DEX treatment. Plasma IGF-I, IGFBP-3 and osteocalcin increased over time. N-telopeptide was the only biochemical parameter which appeared to be suppressed during DEX (1342 nM bone collagen equivalents/mM creatinine vs. 2486 (pre-DEX) and 2292 (post-DEX)).

Conclusion: Changes in the circulating components of the GH-IGF-I axis paralleled the changes reported in non-steroid-treated infants and provide limited insight into the mechanisms by which DEX alters growth and bone turnover.

*Keywords:* bone, bronchopulmonary dysplasia, dexamethasone, GH-IGF-I axis, premature infants

**Introduction**

Infants born very prematurely with a birth weight less than 1200 grams exhibit growth rates and bone mass accretion which are substantially lower than intrauterine growth. Administration of dexamethasone (DEX), which is an accepted therapy for bronchopulmonary dysplasia (BPD) [14,22,28], imposes an additional impediment to growth and skeletal development in this population. DEX treatment is associated with reduced weight and length growth during treatment, with a delay in length growth still apparent at 6 months corrected age [36]. Radial bone mineral content (BMC) at term corrected age is also reduced in DEX-treated infants compared to infants of similar birth weight and gestational age [36].

There is speculation that interference with the GH-IGF-I axis is responsible for the growth delay and altered bone mineral status in DEX-treated very low birth weight (VLBW) infants; however, the mechanism of steroid action remains to be determined. Studies in older children [1,32,39] and animals [2,9,35] provide evidence that steroids can alter the circulating components of the GH-IGF-I axis and, thereby, mediate the negative effects of steroids on growth and/or bone mineral homeostasis. A functional GH-IGF-I axis is essential for tissue growth, particularly the acquisition of bone during development. Children who suffer from abnormalities of the GH-IGF-I axis (e.g. GH deficiency, Laron Syndrome) experience significant delays in weight, height and bone growth, providing direct evidence that interference with the GH-IGF-I axis can have deleterious effects on normal human development [23,25]. The extent

to which the growth of the fetus or the prematurely born infant is regulated by the GH-IGF-I axis is currently not fully understood. However, the developmental changes in the tissue expression of IGF-I receptors and IGF binding proteins (IGFBPs), as well as circulating concentrations of IGF-I and the IGFBPs, suggest a potential role in intrauterine development [4,18].

The ontogeny of circulating GH, IGF-I and the IGFBPs in prematurely born infants has been described [6,19,26,29,30]. Most studies have reported increases in circulating IGF-I [19,26,29,30] and IGFBP-3 [26,29,30] and a decrease in circulating IGFBP-2 with increasing postnatal age [19,26,29]. There are no published reports defining how DEX might alter the natural ontogeny of the GH-IGF-I axis in VLBW infants, nor the relationship of these proteins to changes in somatic growth and bone metabolism during early development at known nutrient intakes.

The objective of this study was to characterize the changes in the concentrations of the circulating components of the GH-IGF-I axis, specific biochemical markers of bone metabolism, dietary intakes, and growth velocities during and after the completion of DEX treatment. Measurements of bone mineral mass at term corrected age in steroid-treated VLBW infants were compared with appropriate reference values from non-steroid treated preterm and term born infants.

## Subjects and methods

### *Subjects*

Infants admitted consecutively to the Neonatal Intensive Care Unit at the Children's Hospital, Hamilton Health Sciences Corporation, were recruited, after obtaining informed written consent, according to the following entry criteria: appropriate size for gestational age without any malformations known to affect growth; birth weight < 1200 g; and a prescribed course of DEX for greater than 7 days since this is the minimum time required to suppress the hypothalamus-pituitary-adrenal (HPA) axis in preterm infants [31,38]. Gestational ages were determined by menstrual history corroborated by the results of an ultrasound and were expressed in completed weeks. The study was approved by the Research Advisory Group (Ethics Board) at McMaster University.

At the start of the study all infants were receiving or had received oral feeds, consisting of either expressed breast milk (EBM) or SMA Preemie formula (Wyeth-Ayerst, Toronto, ON, Canada), initially in combination with total parenteral nutrition. Human milk fortifier (Enfamil, Mead Johnson, Belleville, ON, Canada) was added to EBM to provide supplemental nutrients as needed. Infants fed fortified or unfortified EBM also received a calcium (0.7-1.32 mmol Ca/d) and phosphorus (0.13-1.0 mmol P/d) supplement and 0.5-1.0 mL/d of Poly-Vi-Sol (Mead-Johnson, Belleville, ON, Canada) which provided 200-400 IU of vitamin D/d. Intake of EBM with or without fortifier, formula, total parenteral nutrition and/or various mineral or vitamin

supplements were recorded daily by the nursing staff. Infants receiving EBM did not successfully suckle at the breast until $\geq$ 34 weeks postmenstrual age (PMA) and all infants were hospitalized until they were $\geq$ 37 weeks PMA. Average daily nutrient intakes during DEX and from the end of DEX until discharge or term corrected age, which ever came first, were calculated using the nutrient composition information of the commercial products provided by the respective manufacturers. The nutrient composition of EBM was based on published reports [3].

The starting dose of DEX (sodium phosphate salt, Hexadrol, Organon, Toronto, ON), either 0.5 mg/kg/d (n=5) or 0.3 mg/kg/d (n=12), was at the discretion of the attending physician. Infants who were prescribed a starting DEX dose of 0.5 mg/kg/d received this dose for the first 48-72 hours; the dose was then reduced to 0.3 mg/kg/d and subsequently tapered in all infants by 10% every 72 hours [14]. The duration of DEX treatment depended on the individual infant's clinical pulmonary improvement.

*Clinical and laboratory monitoring*

Weight was measured to the nearest gram by the nursing staff using an electronic scale (Olympic Smart Scale, Olympic Medical, Seattle, WA) every 48 hours. Recumbent length was measured to the nearest mm by WW and a research nurse every 1-1.5 weeks using a polyacrylic board (Ellard Scientific, UT). Weight and length growth were compared to the size-at-birth growth standards [10] generated

from growth data of infants born in the Hamilton area. In addition, z-scores were calculated using the size-at-birth growth standards [10] to assess weight and length at term corrected age.

Whole blood (0.6 mL), providing 0.35 mL of plasma, was collected by heel prick during routine clinical sampling into 3-4 EDTA-coated glass capillary tubes (200 $\mu$L capacity), centrifuged at 10 000 g for 20 minutes and stored at -70°C until analyses were performed. Blood was sampled at three different times during the study: pre-DEX: immediately prior to receiving the first dose of DEX; during DEX: 9-12 days after the start of the course of DEX; and post-DEX: 10 days after the final dose of DEX since it was reported that suppression of the HPA axis in preterm infants does not persist past 10 days [38]. A spot urine sample was obtained at the time of each blood sampling and was stored at -70°C. In order to collect clean samples, female infants were placed on a sterile plastic weigh dish while male infants had a 6 mL sterile plastic tube placed below the penis until they voided.

Plasma IGF-I was separated from the IGFBPs by acid-ethanol extraction and measured by ELISA (Diagnostic Systems Laboratories, Webster, TX). The intra-assay and interassay CVs were 6.9% and 5.3%, respectively. For IGFBP-2 and IGFBP-3 analyses, plasma was diluted 1:50 and quantitated by competitive RIA (IGFBP-2) or ELISA (IGFBP-3) (Diagnostic Systems Laboratories). Intra-assay and interassay CVs were $< 10\%$ and $< 12\%$, respectively, for both assays. In addition, the plasma IGFBP profile was characterized by SDS-PAGE and western ligand blotting,

providing information about the relative amounts of circulating IGFBP-1 through IGFBP-4 [17]. Subjects were randomly selected for IGFBP analysis at all three sampling times and 3 $\mu$L of plasma was diluted 1:10 before analysis. Plasma osteocalcin was measured by competitive RIA and the intra-assay and interassay CV's were 6.8% and 9.2%, respectively (Diagnostic Systems Laboratories). Urinary NTx of Type I procollagen was measured using a competitive inhibition ELISA (Osteomark, OSTEX, Seattle, WA). The intra-assay CV was $<12\%$. NTx measurements were expressed as a function of creatinine excretion (Procedure No. 555; Sigma Diagnostics, St. Louis, MO). Intra-assay and interassay CVs for creatinine were 9.5% and 12%, respectively.

Whole body bone mineral content (BMC), lean and fat mass were assessed at term corrected age using dual energy x-ray absorptiometry (DXA) (Hologic QDR1000W®, Waltham, MA) according to our laboratory's established protocol [11]. Body composition was not assessed prior to this age as current limitations of DXA technology are such that the quantity of BMC prior to term corrected age is too small to be measured reliably [11]; moreover, the safety and logistics of transporting infants for diagnostic tests are complicated when infants are medically unstable.

BMC was also expressed as a function of weight and length to determine whether the deposition of bone mineral was proportionate to body size when compared to preterm infants [36] and term born infants (FASEB J 1994;8:A1604). Z-scores were calculated to compare the absolute BMC, lean and fat mass, as well as

the ratio of BMC/weight and BMC/length of study infants with these two different reference groups.

*Statistical analyses*

Linear regression analysis was performed to determine the rates of weight and length gain (Microsoft Excel, Microsoft Corporation, Redmond, WA). Unpaired t-tests were used to determine whether the infant characteristics between males and females were different. Paired t-tests were used to determine if weight and length growth velocities differed during DEX versus post-DEX treatment. To assess differences among the three sampling times, IGFBP-1, IGFBP-3, osteocalcin and NTx data were analyzed using a one-way repeated measures analysis of variance since the data were distributed normally. The Friedman repeated measures on ranks was used to assess IGF-I, IGFBP-2 and the age at each sampling time. All post-hoc analyses were performed using the Student-Newman-Keuls test. Statistical analyses were done using SigmaStat software (Jandel Scientific, San Rafael, CA). Data are expressed as mean$\pm$SD and differences were considered significant if $p<0.05$.

**Results**

Seventeen out of 19 parents consented to the study and the study cohort comprised 9 male and 8 female infants, including one set of twins. Approximately half (9/17) of the infants were delivered by cesarian section and all infants received

surfactant therapy during the first 48 hours of life. Birth weight, gestational age, exposure to antenatal steroids, the duration of DEX treatment and the cumulative dose of DEX were similar between sexes. Therefore, the data for males and females were combined and are shown with other infant characteristics in Table 1.

Fourteen out of the 17 infants received EBM as their first oral feed and of these 14 infants, 10 received EBM until term corrected age. The other 4 infants received EBM until a PMA of 31, 33, 36 and 36 weeks, respectively. 3/17 infants were fed formula from birth. There were no differences in calcium, phosphorus, energy, protein or fluid volume intakes during DEX treatment (mean number of days=41$\pm$17) compared to after DEX treatment until discharge or term corrected age (mean number of days=26$\pm$16) (Table 2). Fluid volume intake was at the lower limit of the current recommended nutrient intakes for preterm infants (P-RNIs) because of moderate fluid restriction in the management of chronic lung disease [34]. Intakes of calcium, phosphorus, energy and protein were below the P-RNIs both during DEX and post-DEX treatment (Table 2) [27].

Weight and length growth of each infant were divided into two distinct periods: during DEX treatment (Figure 1A, 2A); and, from the end of DEX treatment through to term corrected age (Figure 1B, 2B). During DEX, the mean rate of weight gain (115$\pm$34 g/wk) was lower ($p<0.002$) than the rate of weight gain after DEX treatment until term corrected age (174$\pm$52 g/wk). Further analysis revealed that the weight gain during week 1 (55$\pm$54 g/wk, n=17) and week 2 (67$\pm$51 g/wk, n=17) of

DEX treatment were significantly lower than week 3 (129±40 g/wk, n=17) and week 4 (130±51 g/wk, n=16) while the weight gain during the first 3 weeks post-DEX treatment were similar (week 1: 213±68 g/wk, n=17; week 2: 222±67 g/wk, n=15; and week 3: 195±80 g/wk, n=11). The rate of length gain during DEX was significantly lower (0.7±0.2 cm/wk versus 1.0±0.2 cm/wk, $p<0.003$). From a qualitative perspective, 13 infants were growing below the 5th percentile for weight during DEX treatment and 12 infants were growing below the 5th percentile for weight after the completion of DEX through to term corrected age. Two infants were growing at or above the 50th percentile for weight post-DEX treatment. Length growth of all infants during DEX was below the 5th percentile. After DEX treatment, only 1 infant crossed the 5th percentile. The z-scores for weight and length at term corrected age were -1.45±1.08 and -2.73±1.12, respectively.

The PMA (mean±SD) of the infants at each blood and urine collection were significantly different ($p<0.001$) (Table 3). The concentration of plasma IGF-I pre-DEX did not differ from the concentration during DEX but plasma IGF-I was significantly higher ($p<0.001$) post-DEX compared to both the previous sampling times (Table 3). Plasma IGFBP-2 concentration was similar at all sampling times (Table 3). The concentration of plasma IGFBP-3 during DEX treatment was significantly higher ($p<0.001$) than the pre-DEX concentration and remained higher ($p<0.001$) post-DEX treatment (Table 3). Densitometric analysis of a representative western ligand blot demonstrated similar findings to those obtained by RIA (IGFBP-2)

or ELISA (IGFBP-3) and also provided information about IGFBP-1 and IGFBP-4 (Figure 3). IGFBP-3 was higher ($p=0.043$) in infants during DEX ($202\pm76$ arbitrary densitometric units) and post-DEX ($221\pm68$) compared to pre-DEX values ($110\pm34$). In this same blot, IGFBP-2 (pre-DEX $221\pm29$; during DEX $197\pm36$; post-DEX $230\pm58$) and IGFBP-4 (pre-DEX $51\pm12$; during DEX $58\pm11$; post-DEX $56\pm13$) were unchanged among sampling times. IGFBP-1 was higher ($p=0.029$) post-DEX ($96\pm27$) compared to pre-DEX ($45\pm20$) and during DEX ($61\pm18$) values.

The pre-DEX and during DEX concentrations of osteocalcin were not different, but the post-DEX osteocalcin concentration was significantly higher ($p=0.001$) than both the earlier sampling times (Table 3). During DEX, urinary NTx was significantly lower ($p<0.05$) than baseline values and by the end of DEX treatment NTx was higher ($p<0.05$) than during DEX (Table 3).

There were no differences between males and females for any body composition measurement. In addition, the duration of DEX treatment or cumulative dose of DEX was not related to BMC, fat or lean mass at term corrected age. When compared to a group of preterm infants who did not receive any DEX [36], or a group of term infants (FASEB J 1994;8:A1604), the infants treated with DEX had a lower bone mineral content and lean mass (Table 4). However, when BMC was expressed as a function of weight or length, the ratios were similar to preterm but not term born comparison infants (Table 4).

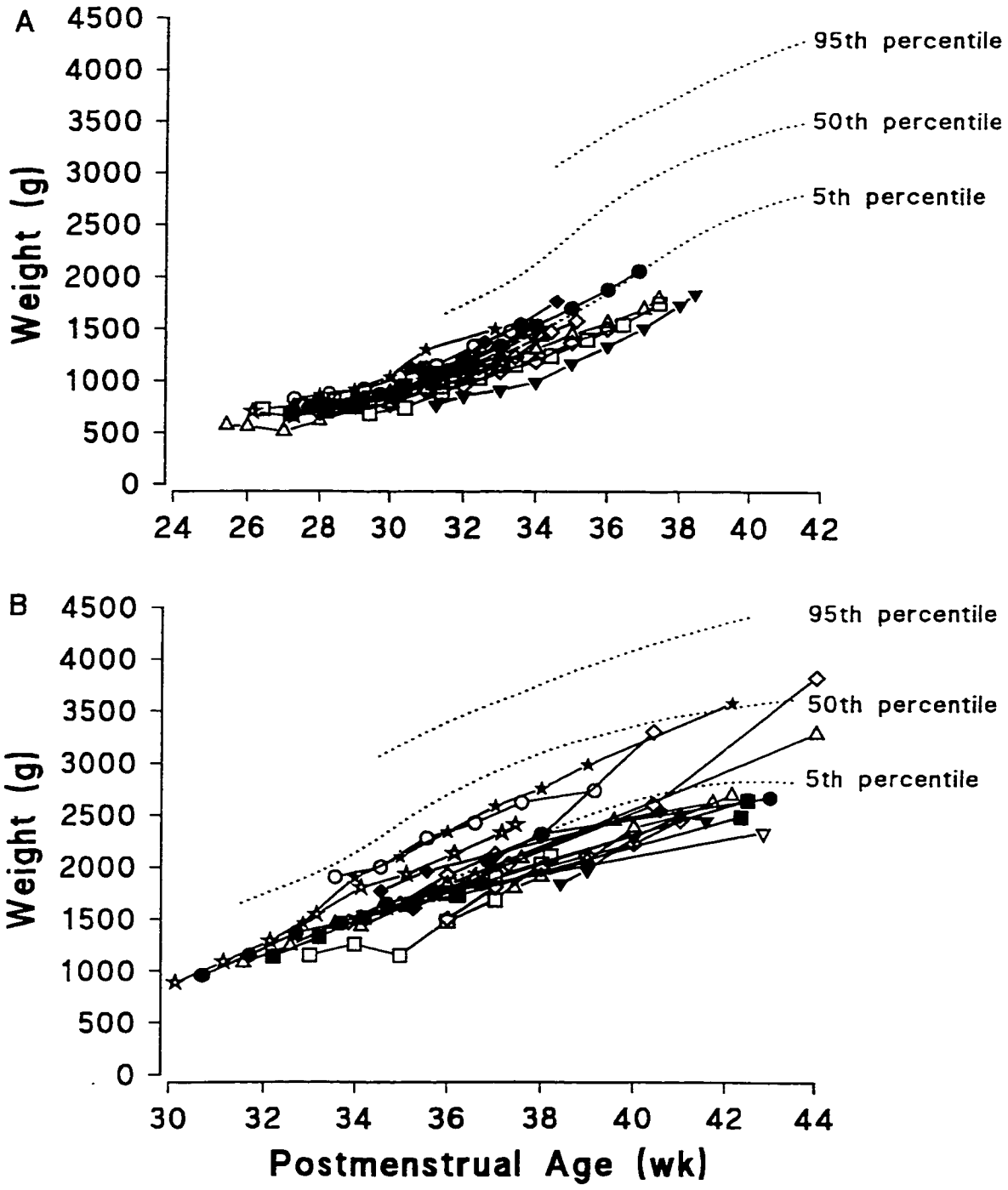


**Figure 1.** Weight growth A) during DEX treatment and B) from the end of DEX treatment until term corrected age for each infant is plotted using the size-at-birth growth curves [10] which were generated based on a cohort of preterm infants born in the same geographical region as the infants in this study (Hamilton, Ontario, Canada).

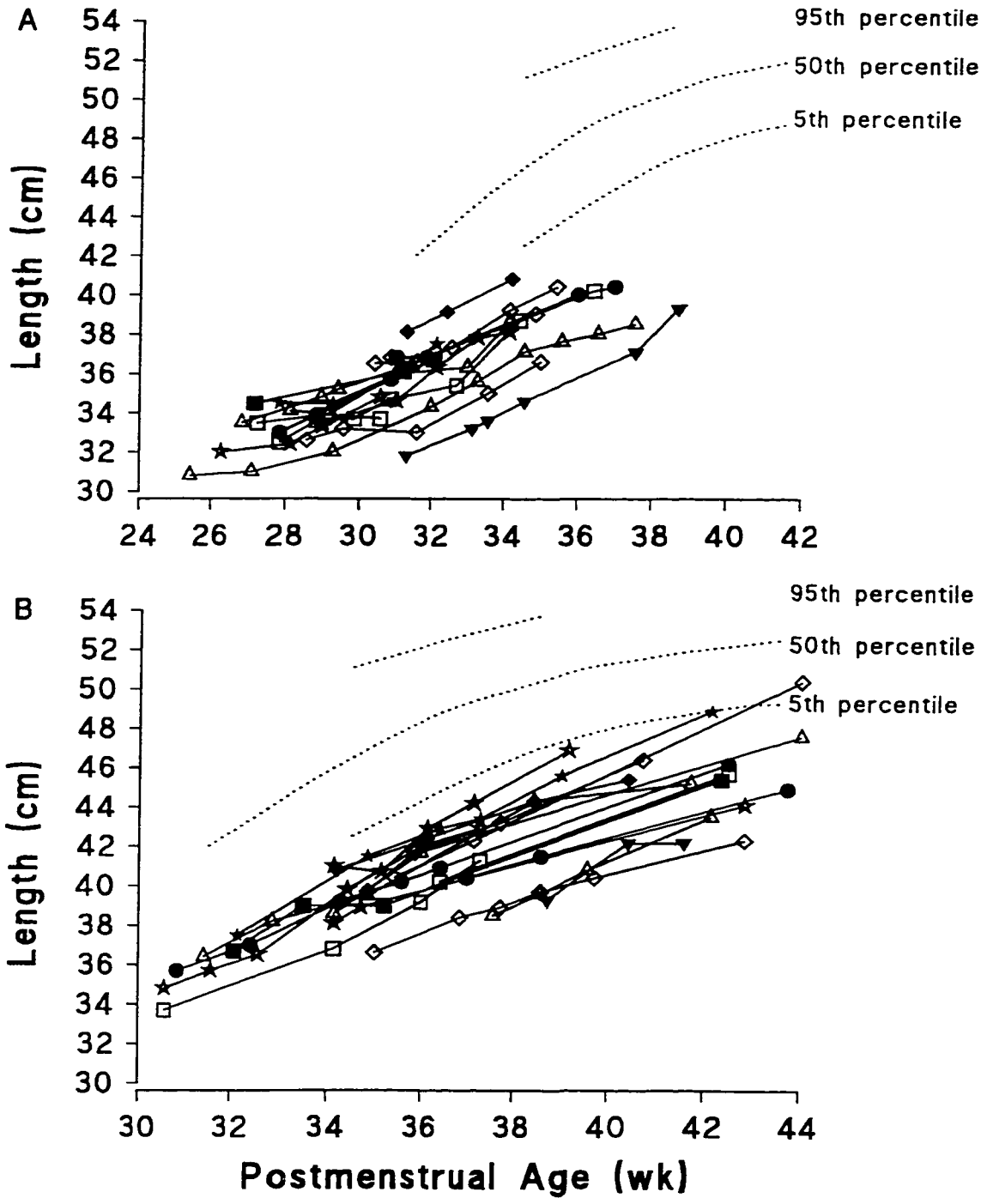


**Figure 2.** Length growth A) during DEX treatment and B) from the end of DEX treatment until term corrected age for each infant is plotted using the size-at-birth growth curves [10].

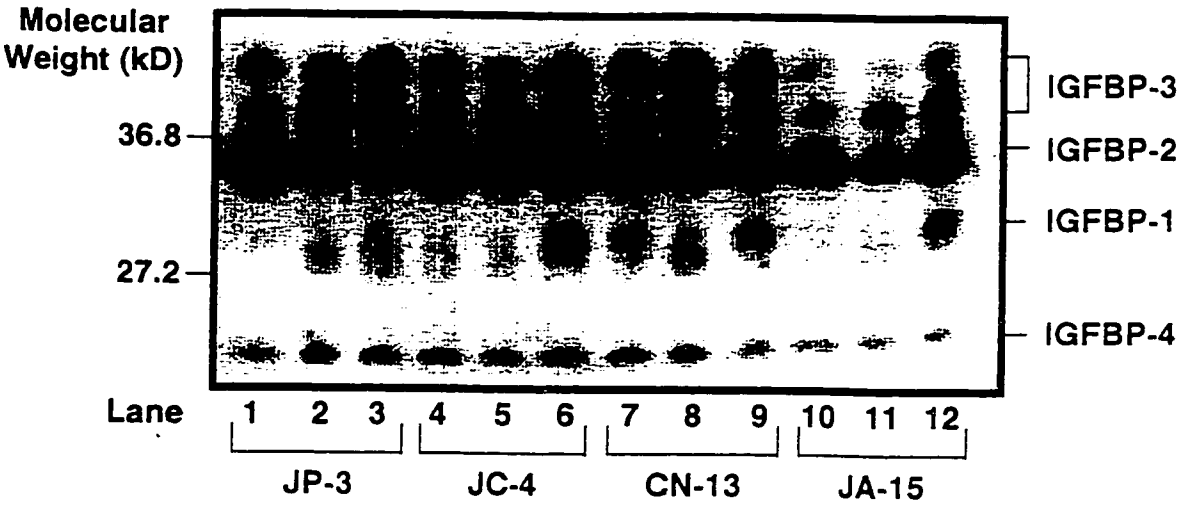


**Figure 3.** An autoradiograph of a representative western ligand blot showing the circulating IGFBP profile of four infants (2 males (M-1, M-2), 2 females (F-1, F-2)) at each of the sampling times. The mean PMA at each sampling time point: pre-DEX 28.0±1.8 wk; during DEX 29.8±2.0 wk; and post-DEX 36.4±2.3 wk. Lanes 1,4,7,10: pre-DEX samples. Lanes 2,5,8,11: during DEX samples. Lanes 3,6,9,12: post-DEX samples.

Table 1 - Clinical characteristics of study infants (n=17).

| | |
|---|---|
| Gestational Age (wk) | 25.4 ± 1.3 |
| Birth weight (g) | 782 ± 185 |
| Apgar Scores: 1 minute | 4.9 ± 2.8 |
| 5 minutes | 7.2 ± 2.1 |
| Antenatal steroids (# of infants) | 10 |
| Number of days ventilated (d) | 57.8 ± 16.8 |
| Number of days oxygen (d)* | 80.9 ± 22.3 |
| Postnatal age at first day of DEX (d) | 19.5 ± 8.4 |
| Duration of DEX (d) | 41.0 ± 16.5 |
| Cumulative DEX (mg/kg/d) | 6.1 ± 2.3 |
| Severity of BPD** (n): | |
| Mild | 1 |
| Moderate | 6 |
| Severe | 10 |

Values are expressed as mean ± SD.
*Number of days of oxygen includes time on ventilation.
**The severity of BPD is defined as: Mild = oxygen therapy at 28 days postnatal age but discontinued prior to 36 wk PMA; Moderate = ventilated at 28 days postnatal age but ventilation and oxygen discontinued by 36 wk PMA; and Severe = oxygen or ventilation at > 36 wk PMA based on a modified clinical classification according to the definitions established by Shennan et al. 1988 [33].

Table 2 - Average daily nutrient intakes during DEX treatment and post-DEX treatment until term corrected age.

| | During DEX treatment | Post-DEX treatment* | P-RNI [27] |
|---|---|---|---|
| Fluid Volume (mL/kg/d) | 130 ± 21† | 140 ± 11 | 120 - 200 |
| Calcium (mM/kg/d) | 2.3 ± 0.5 | 2.6 ± 0.6 | 4.0 - 6.0 |
| Phosphorus (mM/kg/d) | 1.5 ± 0.5 | 1.8 ± 0.4 | 2.5 - 3.8 |
| Energy (kcal/kg/d) | 95 ± 11 | 100 ± 12 | 105 - 135 |
| Protein (g/kg/d) | 2.8 ± 0.2 | 2.8 ± 0.2 | 3.5 - 4.0 |

Values are expressed as mean ± SD, except for the P-RNIs which are expressed as a lower and upper limit.

*Post-DEX treatment represents the time from discontinuation of DEX until term corrected age.

†Represents a combination of oral and intravenous nutrition.

Table 3 - Plasma IGF-I, IGFBP-2, IGFBP-3, osteocalcin and urinary NTx in relation to DEX treatment.

| | Pre-DEX | During DEX | Post-DEX |
|---|---|---|---|
| Postmenstrual Age (wk)* | 28.0 ± 1.8[a] | 29.8 ± 2.0[b] | 36.4 ± 2.3[c] |
| IGF-I (nM)* | 1.9 ± 0.9[a] | 2.1 ± 1.5[a] | 7.3 ± 4.6[b] |
| IGFBP-2 (nM) | 48.9 ± 22.8 | 38.8 ± 12.8 | 43.7 ± 22.2 |
| IGFBP-3 (nM)* | 17.9 ± 5.1[a] | 31.8 ± 8.0[b] | 31.8 ± 7.2[b] |
| Osteocalcin (nM)* | 2.7 ± 1.4[a] | 2.4 ± 1.9[a] | 5.0 ± 2.2[b] |
| NTx/Creatinine (nM BCE/mM)* | 2486 ± 613[a] | 1342 ± 873[b] | 2292 ± 1322[a] |

Values are expressed as mean ± SD.
BCE, bone collagen equivalents
*Sampling time was significantly different ($p < 0.05$); means were tested by Student-Newman Keuls test with significant differences represented by different superscripts within a row.

Table 4 - Body composition at term corrected age.

| | DEX-treated infants | Preterm reference infants[1] | z-scores: relative to preterm infants | Term reference infants[2] | z-scores: relative to term infants |
|---|---|---|---|---|---|
| BMC (g) | 51 ± 11 | 60 ± 12 | -0.85 ± 0.99 | 74 ± 13 | -1.78 ± 0.86 |
| BMC/Weight (g/kg) | 18.2 ± 2.0 | 18.1 ± 1.5 | 0.03 ± 1.31 | 21.5 ± 1.9 | -1.75 ± 1.03 |
| BMC/Length (g/cm) | 1.1 ± 0.2 | 1.2 ± 0.2 | -0.43 ± 1.04 | 1.5 ± 0.2 | -1.48 ± 0.87 |
| Lean Mass (kg) | 2.2 ± 0.3 | 2.6 ± 0.3 | -1.17 ± 0.84 | 3.0 ± 0.3 | -2.30 ± 0.84 |
| Fat Mass (kg) | 0.7 ± 0.3 | 0.8 ± 0.3 | -0.19 ± 0.86 | 0.6 ± 0.2 | 0.58 ± 1.16 |

Values are expressed as mean ± SD.
[1]Preterm infants who did not receive steroids, n=8 [36]
[2]Term reference infants, n=46 (FASEB J 1994;8:A1604).

**Discussion**

Treatment with DEX, an immature GH-IGF-I axis and inadequate nutrition are all potential contributors to the growth failure of very preterm infants during the first few months of extrauterine life. These three factors may also be interrelated; in older children and adults, the GH-IGF axis is sensitive to both steroid treatment [1,32,39] and nutritional status [13,16]. Extremely premature infants may also be unable to fully respond to endogenous hormonal stimuli, representing an additional impediment to growth. To our knowledge, this is the largest study to examine nutrient intakes, biochemical measurements and functional outcomes in a group of infants born $\leq$ 28 weeks of gestation who were treated with DEX.

The improvement in linear growth post-DEX treatment which was associated with higher plasma osteocalcin concentrations was likely due to deposition of new collagen and essential matrix proteins in long bones. Although steroid treatment reduces osteoblastic activity in piglets [37] and children [20], we did not observe a reduction in circulating osteocalcin during DEX treatment. This may be due to low osteocalcin concentrations during the first weeks of life, indicating that bone formation is relatively inactive. Both radiological and histomorphometrical analyses have suggested that higher rates of bone resorption and greater numbers of osteoclasts, in addition to low osteoblastic activity, may account for the low bone mass often associated with premature birth [7,8]. The dramatically lower z-score for BMC in this population of DEX treated infants at term corrected age compared to

both non-DEX treated preterm infants [36] and term born infants (FASEB J 1994;8:A1604) at term corrected age may be due to low osteoblastic activity and a lower rate of bone turnover during early life while infants are receiving DEX. Indeed, the suppression of urinary NTx during DEX, reported in this study and one other [36], is indicative of a lower rate of bone turnover. Our observation that the lower BMC is proportional to body weight but not axial length when compared to non-steroid treated preterm infants provides evidence that DEX interferes with mineral accretion in the axial skeleton. The rise in both plasma osteocalcin and urinary NTx by 36 wk PMA suggests that the bone cells are more metabolically active compared to earlier sampling times among DEX-treated infants. Since these biochemical markers reflect acute changes in bone metabolism, we speculate that the month between the final sampling time and the DXA measurement at term corrected age did not provide a sufficient amount of time for infants to attain a bone mass which was comparable to non-steroid treated preterm and term born comparison infants.

The deposition of lean mass was also compromised in the study infants, as the z-scores for lean mass were lower in DEX-treated infants compared to both non-steroid treated preterm infants and term born comparison infants. Although the rate of weight and length gain improved during the post-DEX period compared to during DEX, we do not know whether body composition was altered. Higher circulating IGF-I and IGFBP-3 post-DEX treatment coincided with the improved weight and length gain and the rise in circulating osteocalcin. During DEX, the differences in

weekly weight gain corresponded with the dose of DEX as previously reported [22]. In addition, during the first two weeks of DEX, most infants received parenteral nutrition alone or in combination with oral feeds which may have contributed to their lower rates of weight growth.

Overall, we observed similar trends to previous reports with respect to a rise with age in plasma levels of IGF-I [19,26,29,30] and IGFBP-3 in some [26,29,30] but not all studies [19]. The latter discrepency among studies may be due to the small numbers of infants evaluated who were below 30 weeks PMA or that cord rather than circulating blood samples were analyzed. Clearly, our study showed a dramatic increase in IGFBP-3 between 28 (pre-DEX) and 29 (during DEX) weeks PMA, providing evidence that studies which sampled infants older than 29 weeks PMA might have missed this change. Nonetheless, we cannot exclude the possibility that DEX treatment may have influenced circulating IGF-I and/or IGFBP-3. The fact that the increase in IGFBP-3 by 29 weeks (during DEX) was not accompanied by an increase in IGF-I, as would be expected, may indicate that the developmental rise in IGF-I was marginally suppressed by DEX. It would appear that DEX did not alter circulating IGFBP-3 as the levels remained elevated post-DEX treatment. IGFBP-2 concentration was unchanged with age and the result is similar to previous observations [19,29].

It is possible that the low levels of IGF-I and IGFBP-3 during early life, noted in this study and others [19,26,29], are due to a small number of GH receptors [15]

or the immaturity of hormonal feedback mechanisms regulating GH secretion [40] in premature infants. In fact, there is no concrete evidence that GH is a major regulator of fetal growth and development [21] which implies that very premature infants may be unresponsive to GH during the earliest stages of extrauterine life. To our knowledge, there is currently no information about the responsiveness of VLBW infants to exogenous GH during early life.

Nutritional status is the third factor that can affect circulating IGF-I and the IGFBP profile [24]. Plasma IGF-I and IGFBP-3 concentrations are reduced with food restriction while plasma IGFBP-2 concentration is increased [13,16]. The increase in circulating concentrations of IGF-I and IGFBP-3 post-DEX cannot be attributed to differences in nutrient intakes because these were consistent throughout the study. The daily nutrient intakes overall were lower than the current P-RNIs. However, current P-RNIs may not be appropriate for VLBW infants with BPD because they are mostly based on data from term or premature infants who are more mature and clinically stable. In the medical management of BPD, nutrient intake may be limited by a moderately restricted fluid regimen [34]. Moreover, the residual lung disease post-discharge may limit the quantity of fluid an infant can consume. Provision of supplemental nutrients to infants with BPD results in a higher rate of length gain, whole body lean mass and radial BMC at 3 months corrected age compared to infants receiving standard infant formula, indicating that VLBW infants can respond to a

short-term nutritional intervention [12]. The effect of an enriched diet on circulating IGF-I or the IGFBP profile has not been investigated.

A limitation of this study is the fact that it was not ethically feasible to obtain a true control group since randomized controlled trials [14,22,28] have proven that DEX reduces oxygen requirements and ventilator dependency and significantly improves pulmonary resistance and lung compliance of preterm infants with chronic lung disease [5]. We attempted to recruit infants who would form a comparison group, but were unable to match infants not treated with DEX to those in the DEX group who were of a similar gestational age and weight at birth. Furthermore, it is important to acknowledge that even a comparison group would not constitute a control group in the truest sense, since infants not treated with DEX would have milder disease than DEX-treated infants.

Clearly, infants treated with DEX do not achieve intrauterine growth rates during postnatal life as the z-scores for weight and length at term corrected age were -1.45 and -2.73, respectively, indicating disproportionate length for weight. Since the ontogeny of the circulating IGFBPs does not appear to be altered by DEX and circulating IGF-I may be only marginally altered by DEX, we postulate that assessment of blood levels provides little information into the mechanisms by which DEX reduces growth rates and radial bone mass [36]. In order to elucidate the specific mechanisms of DEX action on bone and lean tissue growth, more invasive studies, using an animal model, are required.

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# Chapter Three

# DEXAMETHASONE-INDUCED ABNORMALITIES IN GROWTH AND BONE METABOLISM IN PIGLETS ARE PARTIALLY ATTENUATED BY GROWTH HORMONE WITH NO SYNERGISTIC EFFECT OF INSULIN-LIKE GROWTH FACTOR-I*

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# Dexamethasone-induced abnormalities in growth and bone metabolism in piglets are partially attenuated by growth hormone with no synergistic effect of insulin-like growth factor-I

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**Abstract.**

Dexamethasone (DEX) therapy improves pulmonary compliance in premature infants with chronic lung disease; however, normal growth and bone development are impaired. Since DEX may mediate its effects by altering the GH-IGF-I axis, we investigated whether adjunctive therapy with GH or GH+IGF-I during DEX could attenuate these DEX-induced effects. Piglets were randomized to placebo, oral tapered DEX (0.5 mg/kg/d, 0.3 mg/kg/d, and 0.2 mg/kg/d over 14 days), DEX+GH (0.1 mg/kg/d) or DEX+GH+IGF-I (0.1 mg/kg/d). Final whole body weight and length were improved with GH or GH+IGF-I compared to the DEX alone group. Plasma GH and IGF-I were not influenced by DEX, but infusion of IGF-I resulted in higher ($p<0.05$) plasma IGF-I compared to all other groups at d 15. DEX reduced ($p<0.05$) circulating IGFBP-2 and IGFBP-3 and liver IGFBP-2 and IGFBP-4 mRNA expression compared to controls. Treatment with DEX alone resulted in lower ($p<0.05$) plasma osteocalcin, urinary N-telopeptide and whole body and femur BMD compared to controls while piglets receiving adjunctive GH or GH+IGF-I were similar to controls. Given adjunctively, GH alone appears to partially counter the abnormalities in growth and bone metabolism associated with DEX therapy; however, this improvement cannot be attributed to higher circulating IGF-I since combined therapy did not further improve growth or bone homeostasis compared to DEX+GH treatment. Growth hormone therapy has the potential to stimulate growth in infants exposed to steroid treatment.

**Abbreviations.**

BMC, bone mineral content

BMD, bone mineral density

DEX, dexamethasone

DXA, dual energy x-ray absorptiometry

IGFBPs, insulin-like growth factor binding proteins

SPA, single photon absorptiometry

VLBW, very low birth weight

**Key Words.**

bone mineral, GH-IGF-I axis, glucocorticoids, piglet, very low birth weight infant

**Introduction.**

Dexamethasone (DEX), a potent glucocorticoid, is commonly prescribed to improve pulmonary compliance in very low birth weight (VLBW) infants with chronic lung disease. The improvement in pulmonary function due to DEX treatment facilitates earlier extubation (1,2,3), and thereby alleviates some of the side-effects of long-term mechanical ventilation. However, it has been reported that overall growth is reduced during DEX (1,2,4), and studies in our laboratory indicate that bone metabolism is directly impaired (5,6). In VLBW infants, bones are not well mineralized at birth since mineral accretion in utero occurs primarily in the third

trimester of pregnancy. The negative effects of DEX may thus compromise the infant's ability to achieve rapid somatic and bone growth during critical stages of development in early extrauterine life. Since prolonged DEX therapy is common practice for chronic lung disease of prematurity, it is important to consider potential adjunctive therapies which can prevent or at least ameliorate DEX-induced growth delay and abnormalities in bone metabolism.

Growth hormone (GH) and insulin-like growth factor-I (IGF-I) are critical mediators of tissue growth via endocrine and autocrine regulation, respectively. More specifically, GH and IGF-I are potent stimuli of growing long bones; both are essential for the complex regulation of bone modelling and turnover (7). Bone tissue expresses all the components of the GH-IGF-I system (reviewed in 8). Therefore, the steroid-induced bone abnormalities which have been extensively described in animals (9,10) and in selected human populations (11,12,13,14) may be attributed to alterations in circulating and/or bone tissue concentrations or activity of one or more of these components including GH, IGF-I and/or modification of the insulin-like growth factor binding protein (IGFBP) profile (reviewed in 15).

The metabolic response of piglets to DEX is similar to that of VLBW infants in that both overall growth and bone homeostasis are significantly impaired (5,6). The ontogeny of the GH-IGF-I axis is similar among piglets and human infants; both experience a rise in circulating IGF-I with increasing gestational age and the IGFBP profile is also similar in early postnatal life (16,17,18,19). Growth hormone receptors

are present in small numbers in porcine liver at birth and treatment with exogenous GH was shown to increase hepatic GH receptor numbers (20). Furthermore, treatment of healthy, developing pigs with GH stimulated bone formation (21). Thus, piglets are an appropriate animal model in which to study the interactions between DEX, somatic growth, bone homeostasis and the GH-IGF-I system with application to the VLBW infant.

The objective of this study was to determine if adjunctive administration of GH alone or combined with IGF-I during DEX treatment could attenuate DEX-induced abnormalities in growth and bone homeostasis in the developing piglet. Growth hormone and IGF-I were administered in combination to understand whether an additive effect, as has been reported in non-DEX treated adults (22) or rodents (23,24) in catabolic states, occurs in piglets treated with DEX.

**Methods.**

Animals. Four to five day old male Yorkshire piglets (n=32) were removed from the sow at the Arkell Swine Research Station (Guelph, ON), transported to the Central Animal Facility at McMaster University and housed singly in steel metabolic cages maintained at 28°C using heat lamps. Piglets were adapted to a liquid diet (5) over the first 3 days and then fed 400 mL/kg/d, divided over 4 feeds per day, throughout the study. Formula intake and urine output were monitored for each 24-hour period.

Piglets were allowed to exercise and socialize for 1 hour outside their cages in a fenced pen twice daily.

Experimental Design. On day 1 of the study (postnatal age = 7-8 d), piglets were randomized to one of the four treatment groups: placebo control, DEX alone, DEX+GH or DEX+GH+IGF. DEX (sodium phosphate salt, Hexadrol, Organon, Toronto, ON) was administered twice daily (0900 h and 1700 h) as a tapered dose by orogastric gavage technique (#8 french tube). For the first five days (d 1 through d 5) DEX was administered at a dose of 0.5 mg/kg body weight/d. During the following five days (d 6 through d 10) the DEX dose administered was 0.3 mg/kg body weight/d and for the last 4 days (d 11 through d 14) the DEX dose was 0.2 mg/kg body weight/d. This DEX protocol mimics the tapering doses used in the management of VLBW infants in the Neonatal Intensive Care Unit at The Children's Hospital, Hamilton Health Sciences Corporation. Recombinant porcine GH (rpGH, Monsanto Co., St. Louis, MO) was administered from d 1 through d 14 at a dose of 100 $\mu$g/kg/d by intramuscular (i.m.) injection once daily at 1700 h. Recombinant human IGF-I at a dose of 100 $\mu$g/kg/d (rhIGF-I, Genentech Inc., South San Francisco, CA) was continuously infused using an Alzet™ osmotic pump (Model 2ML2, Alza Corp., Palo Alto, CA). Osmotic pumps were surgically implanted subcutaneously behind the left scapula using aseptic technique while piglets were anesthetized with isoflurane (AErrane, Anaquest, Mississauga, ON). Placebo controls received vehicle to control

for administration of DEX (sterile water by orogastric technique), rpGH (sterile water, i.m.) and rhIGF-I (sterile saline by an Alzet™ osmotic pump).

Weight was measured each morning using an electronic scale (Sartorius, Gottingen, Germany) with an animal weighing program. Snout to rump length was measured by the same person (WEW) using a plastic measuring tape each time piglets were anesthetized for blood sampling. Fasting blood samples were obtained by blind stab technique from the external jugular vein between 0800 h and 0900 h at baseline (d 0) and after each change in DEX dose (d 6, d 11) and by cardiac puncture at necropsy (d 15). Blood was collected in heparinized tubes, immediately centrifuged for 20 minutes at 3000 x g at 4°C and stored at -70°C. Twenty-four hour and spot urine samples were obtained after each change in DEX dose using a metabolic tray or a urine collection bag, respectively, and were immediately frozen and stored at -20°C. After obtaining the blood sample on d 15, piglets were killed by lethal cardiac injection (euthanol overdose) while anesthetized with isoflurane. This protocol was approved by the Animal Ethics Committee at McMaster University and was conducted in accordance with the Guide for the Care and Use of Experimental Animals (25). After 7 days of DEX treatment, one piglet died from sepsis due to an ulceration of the cecum.

Blood and urine analyses. Plasma GH was measured by $^{125}$I-RIA using anti-porcine antibody (provided by Dr. D. N. Marple, Auburn University, Auburn, AL). To

precipitate the bound complex, a second antibody (goat anti-guinea pig serum) and polyethylene glycol were added. All samples were assayed in triplicate. The interassay and intra-assay CVs were $<10\%$. Plasma IGF-I and the IGFBP profile were measured in Dr. Donovan's laboratory using established protocols (26). In brief, 0.5 mL of plasma was acid chromatographed to separate IGF-I from the IGFBPs; the eluent containing free IGF-I was lyophilized, resolubilized and quantified by RIA using polyclonal rabbit IGF-I antibody generated by Drs. Underwood and Van Wyk, University of North Carolina at Chapel Hill and distributed by the National Hormone and Pituitary Program (Bethesda, MD). Samples were diluted 1:20 and were measured in duplicate at three different volumes (25 $\mu$L, 50 $\mu$L and 100 $\mu$L). The intra-assay CV was 6.7%. Samples were analyzed in three consecutive assays; the interassay CV was 9.0% using the same batch of antibody and $^{125}$I-IGF-I. Plasma IGFBP-2, IGFBP-3 and IGFBP-4 were characterized by SDS-PAGE and Western ligand blotting (26). Four or five piglets from each treatment group were randomly selected for IGFBP analysis at all four sampling times. Briefly, 3 $\mu$L of plasma (diluted 1:10) was separated on a 12.5% SDS-PAGE gel. Proteins were electroblotted to nitrocellulose, which was incubated with $^{125}$I-IGF-I. After washing, the blots were exposed to film (Kodak X-Omat AR film, Rochester, NY) for 4-7 days at -70°C. The relative pixel intensities of the bands on the audioradiographs were determined using the FotoAnalyst II Imager System and Collage software (Fotodyne, New Berlin, WI). Plasma osteocalcin was measured using a commercially available competitive RIA

(INCstar Corp., Stillwater, MN) (27). All plasma samples were analyzed in duplicate and some in triplicate for calculation of the intra-assay CV (CV=4.4%). The interassay CV was 6.1%. Ionized plasma Ca was measured in duplicate using an ionized Ca analyzer (ICa1; Radiometer, Copenhagen). Urinary N-telopeptide of Type I procollagen was measured using a commercially available competitive inhibition ELISA (Osteomark, OSTEX, Seattle, WA) (27). Triplicate analysis of two piglet samples at three different concentrations demonstrated the linearity of the assay with correlation coefficients of 0.99, similar to the correlation coefficients reported in human samples by Ostex. The intra-assay and interassay CVs were 2.8% and 5.7%, respectively. N-telopeptide measurements were expressed as a function of creatinine excretion which was measured by a colorimetric assay (Procedure No. 555, Sigma Diagnostics, St. Louis, MO). Interassay and intra-assay CVs for creatinine were 2.5% and 3.4%, respectively. Urinary Ca was measured by atomic absorption spectrophotometry (intra-assay CV = 2.2%; Model 703; Perkin-Elmer, Norwalk, CT) and fractional Ca excretion was calculated as a function of creatinine excretion. Plasma glucose was measured using a commercial colorimetric assay (Procedure No. 510, Sigma Diagnostics). The interassay and intra-assay CVs were 4.5% and 2.6%, respectively.

Liver IGFBP-2 and IGFBP-4 mRNA expression by Northern Analysis. Total cellular RNA was isolated from liver samples as previously described by Zhao et al. (28).

The liver samples from the same piglets which were randomly selected from each treatment group for IGFBP profile analysis were measured (n=4 liver samples/treatment group). RNA samples (15 $\mu$g) were size fractioned on a 1.2% agarose-formaldehyde gel, capillary transferred to a nitrocellulose membrane and then hybridized with rat cDNA for IGFBP-2 (29) and IGFBP-4 (30). The relative pixel intensities were determined by the method used for the Western ligand blots.

Bone mineral density analyses. Immediately prior to necropsy, piglets were lightly anesthetized with isoflurane and whole body and femur bone mineral density (BMD) were measured using dual-energy x-ray absorptiometry (DXA, Hologic QDR1000W$^{R}$, Hologic Inc., Waltham, MA) as previously described (31). Whole body BMD was measured using the pediatric whole body software program (V5.63P, Hologic Inc.). Piglets were placed on the scan field in a spread eagle position. For measurement of femur BMD, the piglet remained in the spread eagle position with the left leg extended for clearer definition of the femur using the right hip software program (V4.47P, Hologic Inc.). Placement CVs for the whole body and femur scans were 1.2% and 3.6%, respectively. The mean$\pm$SD of triplicate CVs for manual isolation of the femur in six piglets was 4.1$\pm$1.8%.

The left femur was also excised at necropsy, cleaned of all soft tissue and then BMD, bone mineral content (BMC) and bone width (BW) were measured at the 1/4, 1/3 and 1/2 site distal to the proximal femur using single photon absorptiometry

(SPA) (278A, Norland, Fort Atkinson, WI) as previously described (5). In order to mimic soft tissue, excised femurs were submerged in a water bath during scans. The intra-assay CVs for BMC and BW were 2.4% and 1.4% (interassay CV for BMC=1.3% and BW=0.4%).

Statistical analysis. Bone density data and Northern analyses were analyzed using one-way analysis of variance (ANOVA) and a repeated measures ANOVA was used for all other outcomes. The Student-Newman-Keuls test was used for comparison of multiple means at each time point in which significant differences due to treatment were observed. Differences were considered significant when $p<0.05$. All data were expressed as mean$\pm$SD and all analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

**Results.**

Postnatal age (control 7.0$\pm$0.7 d; DEX 7.0$\pm$0.7 d; DEX+GH 7.0$\pm$0.7 d; and DEX+GH+IGF-I 7.1$\pm$0.8 d), weight and length (Figure 1A,1B) were not significantly different among groups at randomization to treatment. Treatment with a tapered dose of DEX alone over a two week course significantly reduced absolute weight and length growth (Figure 1A,1B). By d 6, DEX alone treated piglets were shorter ($p<0.05$) than control piglets and by d 11 through d 15, all DEX-treated piglets were significantly lighter and shorter than controls despite adjunctive

administration of GH or GH+IGF-I. Treatment with GH alone or combined with IGF-I partially attenuated the DEX-induced growth failure at d 15; piglets receiving DEX+GH or DEX+GH+IGF-I were significantly heavier and longer than DEX alone piglets at the end of the study. The mean 24 h formula intake over the study period (control 366±25 mL/kg/d; DEX 392±6 mL/kg/d; DEX+GH 390±7 mL/kg/d; DEX+GH+IGF-I 391±9 mL/kg/d) was not significantly different among treatment groups while the mean urinary excretion volume was higher in all DEX-treated groups compared to controls (control 149±25 mL/kg/d; DEX 182±15 mL/kg/d; DEX+GH 182±24 mL/kg/d; DEX+GH+IGF-I 181±29 mL/kg/d, $p=0.002$).

There were no significant differences in mean fasting plasma GH concentrations among treatment groups or among sampling times during the study (Control 0.3-0.7 nmol/L; DEX 0.4-0.7 nmol/L; DEX+GH 0.2-0.5 nmol/L; DEX+GH+IGF-I 0.2-0.4 nmol/L, values represent the range over the 14 days). A limitation of this finding is that it represents a single measurement whereas GH is released in a pulsatile manner. A pilot study using piglets of a similar weight and age to this study demonstrated that plasma GH peaked 2 hours post-injection (unpublished observations). Plasma IGF-I concentration did not change significantly with DEX alone or DEX+GH treatment compared to control piglets throughout the study (Figure 2). IGF-I was successfully delivered to the piglets as indicated by the significantly higher ($p<0.05$) IGF-I at d 15 in the GH+IGF-I group compared to all

other groups. DEX-induced reduction ($p<0.05$) of plasma IGFBP-2 predominated at d 6, d 11 and d 15, and was not altered by either GH or combined GH+IGF-I intervention (Figure 3A). The percent change in circulating IGFBP-3 from baseline was lower ($p<0.05$) in all groups treated with DEX compared to control group at d 11 (Figure 3B); these differences were not observed at necropsy. DEX, GH or GH+IGF-I administration did not alter the percent change in plasma IGFBP-4 from baseline at any sampling time compared to controls (Figure 3C). Hepatic expression for both IGFBP-2 and IGFBP-4 mRNA was reduced in all DEX-treated piglets irrespective of adjunctive GH or GH+IGF-I treatment (Table 1). In addition, hepatic IGFBP-4 mRNA expression in piglets receiving DEX+GH+IGF-I was lower ($p<0.05$) than the DEX alone and DEX+GH treated groups.

Plasma osteocalcin was significantly reduced in DEX alone treated piglets at d 6 and d 11 compared to control, but was not significantly different at d 15 (Figure 4A). Both GH and GH+IGF-I treatment attenuated the DEX-induced reduction in osteocalcin at all sampling times. Urinary N-telopeptide excretion was reduced in DEX alone treated piglets compared to controls at d 6 (Figure 4B). Ionized plasma calcium and urinary calcium excretion were not significantly altered by any treatment (data not shown). There were no significant differences in morning, fasting plasma glucose among treatment groups or among treatment days at any time during the study (Control 4.8-5.7 mmol/L; DEX 4.9-6.3 mmol/L; DEX+GH 5.0-6.2 mmol/L; DEX+GH+IGF-I 5.2-5.9 mmol/L, values represent the range over the 14 days)

indicating that the doses of GH and IGF-I were safe with respect to glucose homeostasis.

Whole body and femur BMD as assessed by DXA were reduced ($p<0.05$) in DEX alone treated piglets while mean values for the DEX+GH and DEX+GH+IGF-I groups were intermediary between the control and DEX alone treated piglets (Table 2). Similarly, the BMD of femurs from DEX piglets, measured by SPA, were significantly reduced ($p<0.05$) compared to controls at the 1/2, 1/3 and 1/4 site distal to the proximal femur. Similar alterations were observed in femur BMC at these same three sites (data not shown). Femur length was shorter ($p<0.001$) in all DEX-treated piglets compared to the control group; adjunctive GH or combined therapy did not prevent the stunting of this long bone (Control $8.5\pm0.5$ cm; DEX $6.9\pm0.3$ cm; DEX+GH $7.3\pm0.4$ cm; DEX+GH+IGF-I $7.4\pm0.4$ cm).

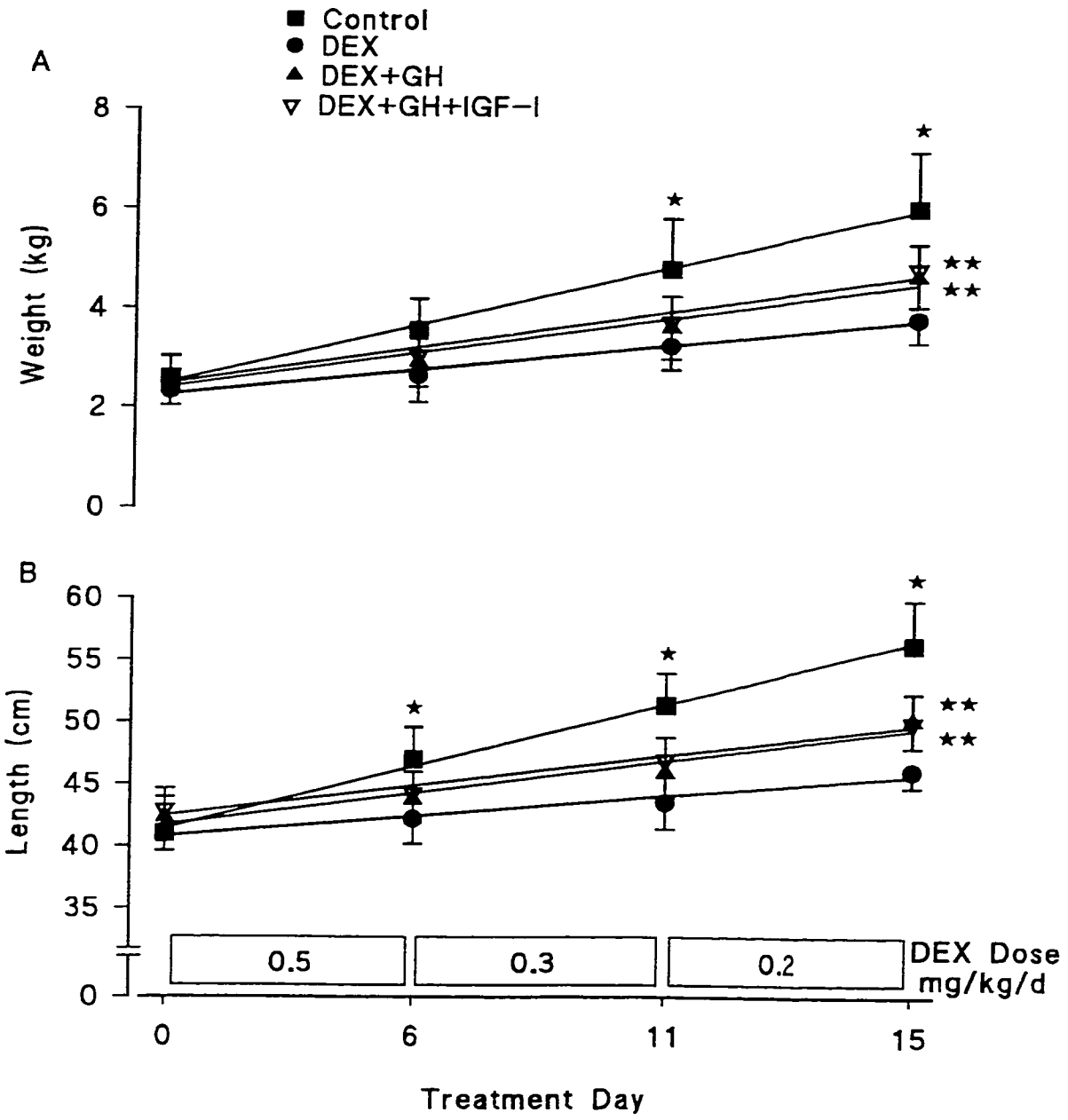


**Figure 1.** Anthropometry. (A) Weight and (B) length growth for Control, DEX, DEX+GH and DEX+GH+IGF-I groups. Values are expressed as mean ± SD. ★ Control vs. all DEX groups, $p<0.05$; ★★ DEX+GH, DEX+GH+IGF-I vs. DEX, $p<0.05$.

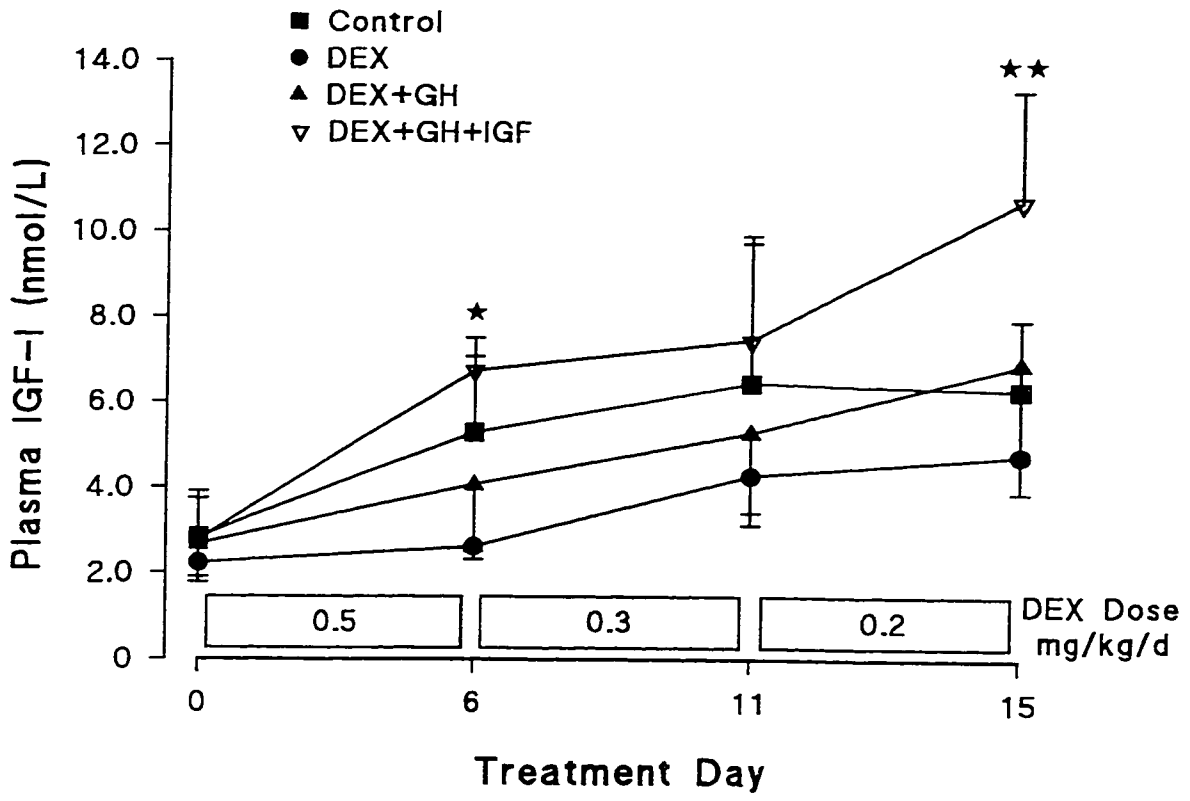


**Figure 2.** Plasma IGF-I at baseline and after each change in DEX dose. Values are mean ± SD. ★ DEX+GH+IGF-I vs. DEX, $p<0.05$; ★★ DEX+GH+IGF-I vs. all groups, $p<0.05$.

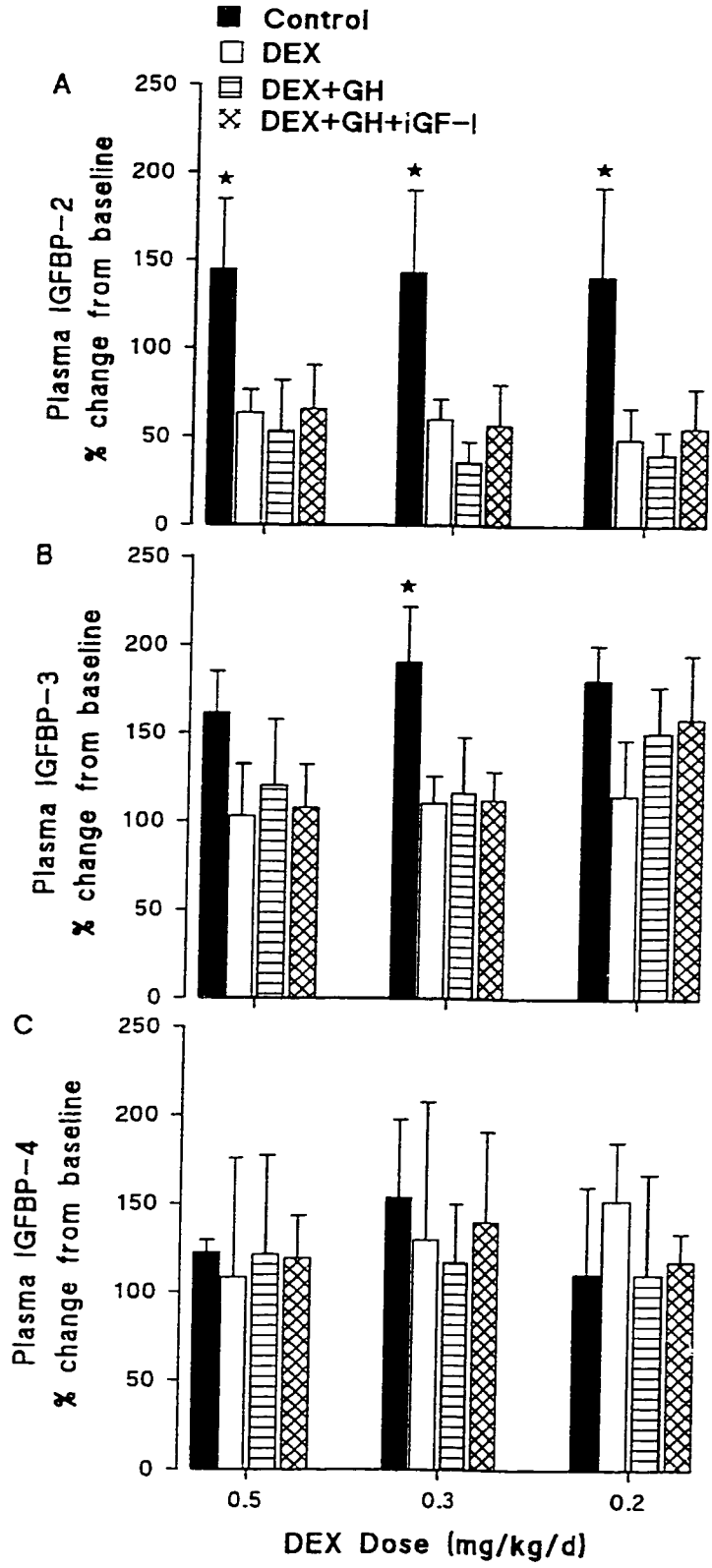


**Figure 3.** The plasma IGFBP profile. The percent change from baseline for (A) IGFBP-2, (B) IGFBP-3 and (C) IGFBP-4 after each change in DEX dose. Bars represent mean ± SD of four or five piglets. ★ Control vs. all DEX groups, $p < 0.05$.

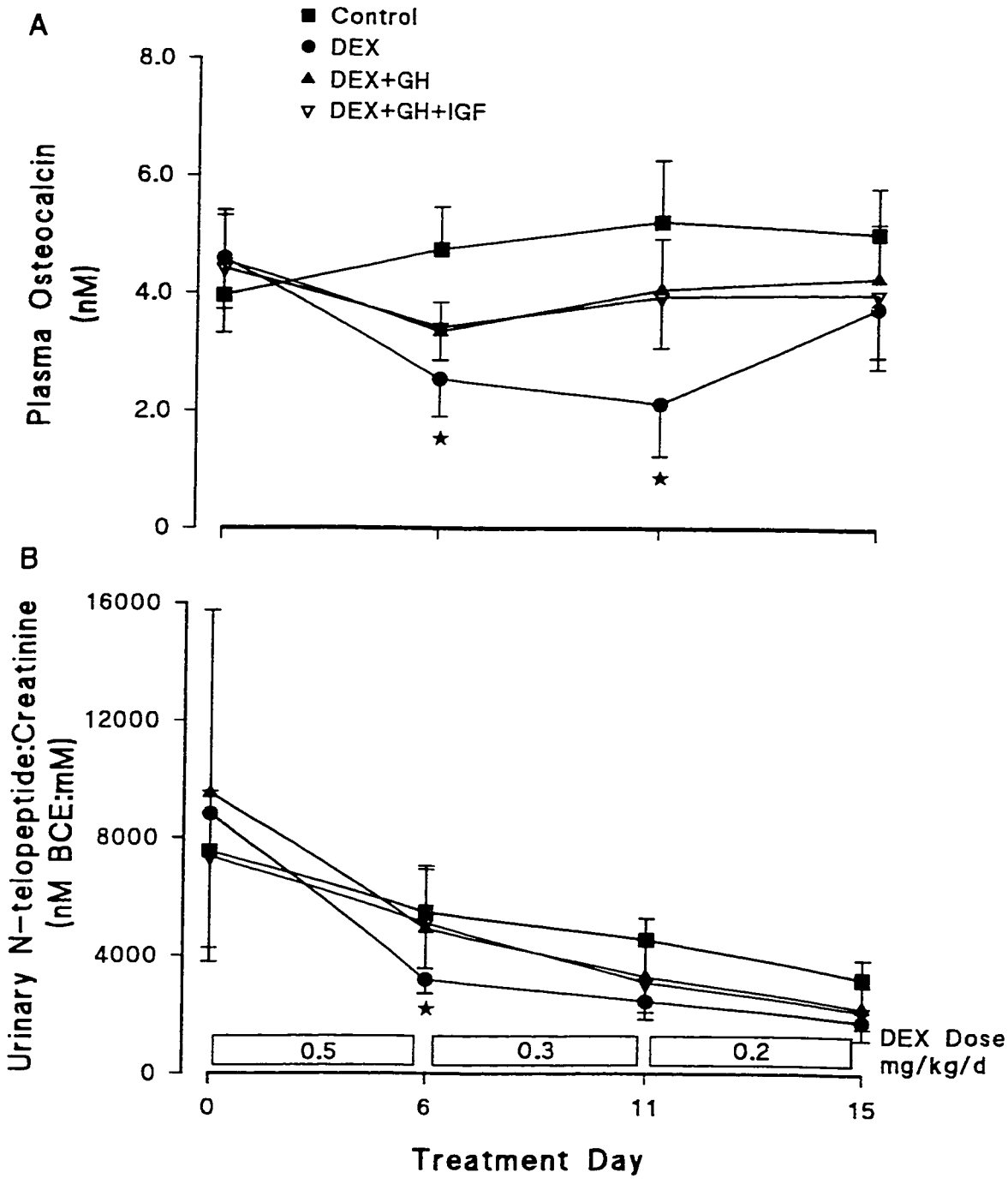


**Figure 4.** Plasma osteocalcin (A) and urinary N-telopeptide (B) concentrations at (d 0) and after each change in DEX dose. ★ DEX vs. Control, $p<0.05$.

**Table 1. Liver IGFBP-2 and IGFBP-4 mRNA expression***

| Arbitrary Units of Pixel Intensity | Control | DEX | DEX+GH | DEX+GH+IGF-I |
|---|---|---|---|---|
| IGFBP-2† | $2.05 \pm 0.66^{a}$ | $0.51 \pm 0.12^{b}$ | $0.65 \pm 0.42^{b}$ | $0.33 \pm 0.15^{b}$ |
| IGFBP-4† | $1.22 \pm 0.37^{a}$ | $0.49 \pm 0.16^{b}$ | $0.33 \pm 0.07^{b}$ | $0.07 \pm 0.04^{c}$ |

Values are expressed as mean $\pm$ SD.

* IGFBP-2 and IGFBP-4 mRNA data were corrected by $\beta$-actin mRNA

† Treatment was significant ($p < 0.05$); means were tested by Student-Newman Keuls Test with significant differences represented by different superscripts within a row.

**Table 2. Whole body and femur bone mineral density**

| BMD ($g/cm^2$) | Control | DEX | DEX+GH | DEX+GH+IGF-I |
|---|---|---|---|---|
| Whole Body* | $0.240 \pm 0.018^a$ | $0.210 \pm 0.012^b$ | $0.219 \pm 0.021$ | $0.224 \pm 0.018$ |
| Whole Femur* | $0.443 \pm 0.054^a$ | $0.374 \pm 0.029^b$ | $0.414 \pm 0.053$ | $0.412 \pm 0.047$ |
| Femur 1/2 Site* | $0.520 \pm 0.044^a$ | $0.392 \pm 0.032^b$ | $0.466 \pm 0.038$ | $0.477 \pm 0.097$ |
| Femur 1/3 Site* | $0.518 \pm 0.057^a$ | $0.414 \pm 0.040^b$ | $0.476 \pm 0.060$ | $0.480 \pm 0.085$ |
| Femur 1/4 Site* | $0.439 \pm 0.068^a$ | $0.294 \pm 0.029^b$ | $0.376 \pm 0.099$ | $0.342 \pm 0.070$ |

Values are expressed as mean $\pm$ SD, BMD, bone mineral density.

* Treatment was significant ($p < 0.05$); means were tested by Student-Newman Keuls test with significant differences represented by different superscripts within a row.

**Discussion.**

A tapered dose of DEX, as used clinically in VLBW infants to treat chronic lung disease, caused similar abnormalities in somatic and bone growth in rapidly growing piglets as have been previously reported by our laboratory using a constant high dose of DEX (5). The fact that even a tapering dose of DEX mediates these effects emphasizes the potentially deleterious effect of DEX during development. Of interest is our finding that the reduction in growth velocity and plasma osteocalcin is dose-dependent, persisting only during the two highest doses of DEX; urinary N-telopeptide differed only at the highest DEX dose. Since osteocalcin and N-telopeptide are indicators of acute changes in osteoblastic and osteoclastic activity, respectively, it appears that bone cell activity was returning to normal by the end of the study; however, a longer term study would be needed to determine whether bone mass (BMD) normalizes during a low dose of DEX or post-steroid treatment. In our study, GH prevented the dramatic reduction in osteocalcin and N-telopeptide during the highest DEX dose that was observed in the DEX alone group. Thus, it appears that DEX inhibited both osteoblast and osteoclast activity but GH had a positive, anabolic effect on bone. It is important to note that while GH improved somatic growth only during the lowest dose of DEX, GH was able to partially attenuate the suppression of bone cell activity at the higher doses of steroids, providing evidence that GH can exert a direct effect on developing bone.

The dose-dependent response to DEX may also provide an explanation for the partial attenuation of the DEX-mediated reductions in somatic growth and bone mineral metabolism with adjunctive GH±IGF-I therapy. For example, adjunctive GH therapy in children receiving long-term steroid treatment (> 3 years) improved linear growth only when the steroid dose was below a threshold amount (32). The results of the present study also suggest that GH±IGF-I therapy showed a functional benefit, e.g. improved somatic growth, only during the lowest dose of DEX. Thus, it appears to be important to consider the DEX dose being administered prior to initiating GH administration. Although no randomized controlled trials have evaluated the effectiveness of long-term adjunctive GH therapy in children receiving prolonged courses of steroids, descriptive studies have demonstrated positive responses to GH treatment (11,13). Improvements in weight and height velocities, and higher circulating C-terminal type 1 procollagen concentrations and bone formation rates with GH treatment have been reported in children who received prednisone (>6 months) to manage chronic diseases (e.g. asthma, lupus, autoimmune colitis) or after kidney transplantation (11,13).

All measures of BMD (whole body, whole femur, and at the 1/2, 1/3, 1/4 sites of the femur) in animals receiving adjunctive GH±IGF-I were intermediate between control and DEX-treated groups, indicating that the piglet's bones were responsive to these therapies, which is in agreement with the osteocalcin and N-telopeptide data. In addition, since the reduction in BMD at all three sites was

diminushed with GH±IGF-I, it appears that both cortical and trabecular bone were responsive to these hormones. However, the fact that BMD was not significantly higher than DEX alone treated piglets indicates that only a partial attenuation of bone mineral mass was attained by GH, which is evidence of the potent effects of steroid drugs on developing bone. Alternatively, piglets may not be fully responsive to GH during the first three weeks of postnatal life although it has been shown that hepatic GH receptor number is increased in 19 day old piglets treated with GH treatment for 12 days (20). A study in older (3 months old), non-steroid treated pigs demonstrated a higher rate of bone formation in pigs treated with exogenous GH compared to control animals suggesting that GH will increase BMD in the absence of steroids (21).

Our observations are supported by in vitro and in vivo studies which have demonstrated the anabolic effects of exogenous GH. In cultured bone cells, GH interacts with functional GH receptors (33) and promotes the proliferation and differentiation of human osteoblast-like cells (34). In vivo, GH is a direct stimulus of osteoblastic activity in healthy adults (35) leading to higher rates of bone matrix production. Due to the action of GH on both protein and bone metabolism, deposition of lean (36) and bone mass (35) are observed. In addition, the bones of DEX-treated mice (9) responded to exogenous GH as demonstrated by histomorphometrical analyses; DEX-mediated reductions in cortical bone width, epiphyseal growth plate width and trabecular bone volume were not observed in mice receiving adjunctive GH therapy.

There are conflicting reports about the effects of steroids on circulating GH, IGF-I or the IGFBP profile depending on the species studied and the stage of development (9,11,12,13,37). In the current study, circulating GH and IGF-I concentrations were not altered by DEX. The plasma GH concentration was likely unchanged due to the fact that blood was sampled 15 hours post-dose. Treatment with DEX reduced circulating IGFBP-2 and IGFBP-3, which were not altered by exogenous GH±IGF-I. IGF-I bioactivity may be altered in DEX-treated piglets due to changes in circulating IGFBP-2 and IGFBP-3 levels. We speculate that the relative quantification of IGFBP-4 by Western ligand blotting was not sensitive enough to detect a reduction in circulating IGFBP-4 as liver IGFBP-4 mRNA expression was significantly lower at necropsy. Our observation that the reductions in liver IGFBP-2 and IGFBP-4 mRNA expression and plasma IGFBP-2 and IGFBP-3 were not related to differences in growth and bone metabolism among DEX-treated groups, suggests that circulating IGF-I and/or the IGFBPs are not the integral component(s) responsible for mediating the actions of DEX. A plausible explanation is that DEX acts directly at the bone by altering autocrine and/or paracrine regulation of tissue IGF-I and/or GH receptors. Studies in normal human bone cell cultures have demonstrated that DEX reduces IGFBP-3 and IGFBP-5 mRNA expression in a dose-dependent manner (38,39). Since there is evidence that IGFBP-3 and IGFBP-5 potentiate IGF-I action (8), this alteration in the IGFBP profile provides a mechanism whereby DEX may limit the amount of IGF available for maintenance of bone remodelling (38).

There was no added benefit of combining IGF-I with GH for any outcome measured in this study; thereby it is likely that GH alone was the stimulus for the attenuation of the DEX-induced reduction in growth and bone mineral mass. This finding is in contrast to several other studies in humans (22) and rats (23,24) in which combined therapy (GH+IGF-I) provided additional benefits on deposition of lean mass and growth recovery after a period of malnutrition than GH alone. It is possible that the GH-IGF-I axis is affected differently in the steroid-induced catabolic state. In studies in pigs (40) and rats (10) adjunctive treatment with IGF-I alone did not overcome the catabolic effects of DEX, although the dose of DEX used in the piglet study was 10 times that used in the present study.

A legitimate concern about extrapolating these study results to VLBW infants treated with DEX is that a side-effect of both DEX (2,3) and GH (35,37) treatment in humans is hyperglycemia. However, blood glucose concentrations were not elevated in piglets receiving DEX or DEX+GH treatment. It is possible that the glucose regulatory system of the piglets may be more mature than that of VLBW infants, as insulin is sometimes prescribed to VLBW infants to counter the hyperglycemia which can accompany DEX therapy. Although administering GH+IGF-I versus GH alone conferred no additional benefit with respect to growth and bone outcomes, combined treatment has been shown to counteract hyperglycemia and prevent protein catabolism in animals (23,24) and humans (37). Thus, the safety of GH administration with respect to glucose homeostasis must be addressed in VLBW infants.

The DEX-induced alterations in growth and bone observed in the piglets were equally or more dramatic than that observed in premature infants (6). By extrapolation, any reversal of these abnormalities that might be achieved with GH are likely to be of clinical significance in human infants. The timing of an adjunctive therapy is also an important consideration as it appears that the clinical benefits of adjunctive GH are most apparent during the lowest dose of DEX in piglets. Thus, the findings in both piglets and infants receiving DEX suggest that adminstering GH post-steroid treatment, rather than during DEX treatment, may be more effective at facilitating 'catch-up' growth.

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# Chapter Four

# GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I THERAPY PROMOTE PROTEIN DEPOSITION AND GROWTH IN DEXAMETHASONE-TREATED PIGLETS

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# Growth hormone and insulin-like growth factor-I therapy promote protein deposition and growth in dexamethasone-treated piglets

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## ABSTRACT

**Background:** Dexamethasone treatment facilitates the weaning of premature infants from mechanical ventilation but impairs protein homeostasis, lean tissue deposition and growth. We investigated whether dexamethasone mediates these effects by reducing protein synthesis or elevating protein breakdown, and whether adjuvant growth hormone±insulin-like growth factor-I therapy can attenuate such effects.

**Methods:** Piglets (n=24) were randomized to placebo, a tapered course of dexamethasone (0.5, 0.3, 0.2 mg/kg/d for 5, 5 and 4 d each, respectively), dexamethasone+growth hormone (0.1 mg/kg/d) or dexamethasone+growth hormone+insulin-like growth factor-I (0.1 mg/kg/d) for 14 days. On day 13, $^{15}$N-glycine was administered as a single, oral dose and urine was collected at timed intervals over the subsequent 48 hours.

**Results:** Total urinary N and cumulative $^{15}$N excretion were higher in all dexamethasone groups compared to controls. Protein synthesis was suppressed while protein breakdown was unaltered by dexamethasone. Adjunctive growth hormone±insulin-like growth factor-I therapy enhanced protein synthesis, but only combined therapy improved net protein gain compared to DEX alone. Higher circulating insulin-like growth factor-I may have mediated the greater net protein gain. Blood urea nitrogen was elevated in all dexamethasone-treated groups at d 6 and 11 but was normalized by d 15 with adjunctive GH±IGF-I. From a functional perspective, both adjunctive growth hormone and GH+insulin-like growth factor-I

partially attenuated the dexamethasone-induced reduction in weight and length gain but not whole body lean and fat mass.

**Conclusion:** Adjunctive growth hormone±insulin-like growth factor-I therapy partially reverses the dexamethasone-induced reduction in protein synthesis, resulting in improved growth when given concurrently with a low tapering dose of dexamethasone.

**Keywords:** Dexamethasone, GH-IGF-I Axis, $^{15}$N-Glycine, Piglets, Preterm Infants, Protein Metabolism

## INTRODUCTION

Administration of dexamethasone (DEX) to very low birth weight (VLBW) infants to manage chronic lung disease during early neonatal life is associated with significant elevations in urinary 3-methylhistidine (3-MH), plasma amino acids and blood urea nitrogen (BUN) (1,2,3). Together, these biochemical findings are manifested functionally by a lower rate of weight growth (1,4,5). In adult humans (6,7), glucocorticoid treatment resulted in a negative nitrogen balance due to increased amino acid oxidation. In rats, glucocorticoids act directly at the muscle by activating branched-chain $\alpha$-keto acid dehydrogenase and thereby accelerating amino acid oxidation (8).

In contrast to the catabolic effects of DEX therapy, growth hormone (GH) therapy has anabolic effects on whole body protein metabolism by stimulating amino acid uptake and protein synthesis in the muscle (9) as well as promoting the accretion of lean tissue at the expense of fat tissue (10). Studies in healthy pigs destined for market have consistently shown that treatment with porcine GH increases lean mass which is accompanied by a corresponding decrease in fat mass compared to placebo controls (10). In healthy humans, administration of exogenous IGF-I lowered whole body protein breakdown as well as plasma triglycerides and free fatty acids (11).

During catabolic states such as malnutrition (12), prolonged parenteral feedings (13), diet-restriction (14) or glucocorticoid therapy (7) in animals or adult humans, adjunctive GH therapy has been shown to promote weight gain, stimulate protein synthesis and improve nitrogen balance by enhancing N retention. The anabolic action of exogenous insulin-like growth factor-I (IGF-I) is controversial as some studies have reported improvements in protein metabolism during catabolic states (15,16) while other studies reported that IGF-I alone does not promote recovery from malnutrition (12) or attenuate steroid-induced effects (17). However, there is substantial evidence that the combination of GH and IGF-I is the most effective promoter of lean mass deposition compared to the administration of either treatment alone in both animals (18,19) and humans (20).

The DEX-treated piglet has proven to be an appropriate animal model for VLBW infants who receive DEX as both experience severe wasting in response to

DEX treatment (4,21,22). We chose to administer $^{15}$N-glycine since this tracer has been widely used to study protein metabolism in pigs and pediatric populations to demonstrate differences in protein kinetics due to differing dietary intakes (23,24) or the response to GH treatment (25,26). Moreover, since we may want to confirm our findings in VLBW infants, $^{15}$N-glycine is a favourable choice of tracer since the enrichment of $^{15}$N is measured in urine which is less invasive than obtaining multiple blood samples.

Our objectives were to elucidate whether the previously documented DEX-induced reductions in lean mass and overall growth rates can be attributed to reduced protein synthesis, higher protein breakdown or a combination of both; and, to determine whether adjunctive administration of anabolic agents such as GH or the combination of GH+IGF-I can counter the effects of DEX. This study provides insight into the potential benefit of administering GH alone or GH+IGF-I to infants during prolonged DEX treatment.

## MATERIALS AND METHODS

Animals and Care: As previously reported (21), male Yorkshire piglets (n=24) were removed from the sow at the Arkell Swine Research Station (Guelph, ON) and transported to the Central Animal Facility at McMaster University. Piglets were housed singly in steel metabolic cages which were maintained at 28°C using heat lamps. Piglets received 400 mL of liquid diet per kg body weight each day, divided

over 4 feeding times, which provided adequate nutrient intakes to facilitate growth at the rate of sow fed piglets (27). Twice daily, all piglets were removed from their cages and allowed to exercise and socialize together in a fenced pen for 1 hour. We report the findings from 22 piglets; one piglet died from sepsis due to an ulcerated cecum after 7 days of DEX alone treatment while several samples from one control piglet were contaminated at the time of the urine collection due to illness.

Drug Administration: Piglets were randomized to receive placebo, DEX alone, DEX+GH or DEX+GH+IGF. The drug dosing protocols have been reported previously (21). Briefly, DEX (sodium phosphate salt, Hexadrol, Organon, Toronto, ON) was administered twice daily as an oral, tapering dose: 5 days at 0.5 mg/kg/d followed by 5 days at 0.3 mg/kg/d and then 0.2 mg/kg/d during the final 4 days. From d 1 through 14, recombinant porcine GH (rpGH, generously provided by Monsanto Co., St. Louis, MO.) was administered at a dose of 100 $\mu$g/kg/d by intramuscular injection once daily at 1700 h. Recombinant human IGF-I (rhIGF-I, generously provided by Genentech Inc., South San Francisco, CA) was continuously infused at a dose of 100 $\mu$g/kg body weight using an Alzet™ osmotic pump (Model 2ML2, Alza Corp., Palo Alto, CA). Placebo controls received vehicle to control for administration of DEX (sterile water by orogastric technique), rpGH (sterile water, i.m.) and rhIGF-I (sterile saline by an Alzet™ osmotic pump).

Anthropometry: Daily weights were obtained between 8 and 9 am using an electronic scale (Sartorius, Gottingen, Germany). Snout to rump length was measured by the same person (WEW) using a plastic measuring tape each time piglets were lightly anesthetized with gas inhalant (isoflurane, AErrane, Anaquest, Mississauga, ON) for blood sampling.

Body Composition: Prior to necropsy, piglets were lightly anesthetized and whole body lean and fat mass were quantified by dual-energy x-ray absorptiometry (DXA) (Hologic QDR1000W®, Hologic Inc., Waltham, MA) using the infant whole body software program (V5.63P, Hologic Inc.) as previously described by our laboratory (28). The percent coefficient of variation for lean mass and fat mass were 0.8% and 5.8%, respectively.

Blood and Urine Collections and Analyses: Fasting blood samples were obtained by blind stab technique from the external jugular vein between 0800 h and 0900 h at baseline (d 0) and after each change in DEX dose (d 6, d 11) and by cardiac puncture at necropsy (d 15). Blood was collected in heparinized tubes, immediately centrifuged for 20 minutes at 3000 x g at 4°C and stored at -70°C. Twenty-four hour urine collections for 3-MH analyses were performed after each change in DEX dose. On d 15, piglets were killed by a lethal cardiac injection of euthanol while heavily anesthetized with gas inhalant. BUN was measured by a colorimetric assay using a

commercial kit (Urea nitrogen, Procedure 640A, Sigma Chemical Company, St. Louis, MI). Urea was hydrolyzed by urease to ammonia and subsequently quantified. The interassay CV was 3.4%. High pressure liquid chromatography was used to measure urinary 3-MH. Duplicate urine samples (950 $\mu$L) were acid precipitated (50 $\mu$L of 70% perchloric acid), vortexed and centrifuged for 1 minute. Using 75 $\mu$L of the supernatant, fluorescamine derivatives were prepared and sample elution was performed using a gradient of 10 mM sodium phosphate buffer and 25-40% acetonitrile (29). As previously reported, plasma GH and IGF-I were measured by established protocols (21). In brief, plasma GH was quantified in triplicate by a porcine specific $^{125}$I-RIA (porcine antibody provided by Dr. D. N. Marple, Auburn University, Auburn, AL) with interassay and intra-assay CVs $<10\%$. Prior to quantifying IGF-I by $^{125}$I-RIA using polyclonal rabbit IGF-I antibody (generated by Drs. Underwood and Van Wyk at the University of North Caroline at Chapel Hill and distributed by the National Hormone and Pituitary Program, Bethesda, MD), 0.5 mL of plasma was acid chromatographed to separate IGF-I from the IGF binding proteins. The mean intra-assay CV was 6.7%. The interassay CV for 3 consecutive assays using the same batch of antibody was 9.0%.

Administration of $^{15}$N-Glycine and 48 Hour Urine Collection: At 9 am on d 13, each piglet received 20 mg/kg body weight of $^{15}$N-Glycine (chemical purity of 98%+, Cambridge Isotope Laboratories, Andover, Mass.) as a single, oral dose prior to the

first feeding of the day. $^{15}$N-Glycine was reconstituted in 3 mL of sterile water and administered using the same oral gavage technique used to deliver DEX. A baseline urine sample was obtained between 8 and 9 am, immediately prior to dosing, to correct for $^{15}$N present in the diet. Post-dosing, urine samples were collected at frequent intervals (2, 3.5, 5.5, 7.5, 11.5, 14, 22.5, 28, 34.5 and 48 h after $^{15}$N-glycine dose). At each collection time, the total urine volume excreted was recorded and an aliquot ($<$100 mL) was immediately frozen at -70°C. Fecal collection bags were used throughout the 48 hour urine collection to prevent fecal contamination of the urine samples. After administering the $^{15}$N-glycine tracer, a sterile urine bag was placed on the piglet each time they were removed from their cage for exercise or feeding to prevent an incomplete urine collection. Piglets were fed outside their cage to prevent contamination with formula.

Total urinary nitrogen: The N content of urine and formula samples was measured using a standard Kjeldahl technique (30). The interassay and intra-assay CVs were $<$10% and $<$8.5%, respectively.

Enrichment of urinary $^{15}$N: Enrichment of $^{15}$N in urine samples was measured using isotope ratio mass spectrometry (IRMS; VG SIRA 10 Series II, Cheshire, UK). Urine samples (5 mL) were lyophilized and 12-20 mg of lyophilized sample was placed in 6 mm glass tubes with an excess of cupric oxide. The tubes were sealed under vacuum

and baked at 550°C for 2 hours and allowed to cool. Immediately prior to measurement on the IRMS, the sealed tubes were frozen in liquid nitrogen and then cracked to release $N_2$ gas into the spectrometer. The ratio of $^{15}N^{14}N$:$^{14}N^{14}N$ was measured and used in the final protein kinetic calculations.

Determination of protein kinetics: The single pool model (31) was used to determine the rates of protein synthesis and breakdown using total $^{15}N$ as the end product. The cumulative excretion of $^{15}N$ over the 48 hour period was calculated and it was determined that a constant value occurred 48 hours post-dosing (Figure 1). Thus, this is the value that was used in the calculation of nitrogen flux denoted by Q:

$$Q \text{ (mg N/d)} = E_m * d/e_m$$

where $E_m$=excretion of total nitrogen; d=dose of $^{15}N$ administered; and $e_m$=cumulative excretion of total $^{15}N$. Total nitrogen excretion included only urinary N as fecal N excretion was not measured and may have resulted in an underestimation of the rates of protein synthesis and protein breakdown. The rates of protein synthesis and protein breakdown were then determined using the following equation:

$$Q = S + E = B + I$$

where S = protein synthesis; E = N excretion; B = protein breakdown; and I = N intake. A factor of 6.25 was used to convert from N to protein.

Statistics: Statistical analyses were performed using SigmaStat software for DOS (Jandel Scientific, San Rafael, CA). One-way ANOVA was used for body composition and protein kinetic data since measurements were only obtained at d 15. One-way ANOVA was also used to compare weight and length gain among groups. Repeated measures ANOVA was used for analysis of BUN and 3-MH data at multiple sampling times (baseline, d 6, d 11 and d 15). The Student Newman-Keuls test was used for comparison of multiple means at each time point in which significant differences due to treatment were observed. Results were considered significantly different if $p<0.05$. All data were expressed as mean$\pm$SD.

**Ethical Considerations**

All animal handling and procedures were conducted in accordance with the Guide for the Care and Use of Experimental Animals (32). Ethical approval for the study was obtained from the Animal Review Board of McMaster University.

**RESULTS**

At the start of the study, all piglets were between 6 and 8 days postnatal age and the mean weight and length were similar between treatment groups. Daily formula intakes during the tracer study were identical among treatment groups (400 mL/kg/d) since formula which was not consumed within one hour of feeding was administered by feeding tube to ensure that all piglets received the same quantity of

nutrients per kg body weight. The mean analyzed nitrogen content of samples taken from 7 different preparations of formula was 7.0±0.3 g N/L of formula, providing each piglet with 2.8 g N/kg/d or 17.5 g protein/kg/d. The change in body weight and axial length over the 15 day study was significantly lower ($p<0.05$) among all DEX-treated animals compared to controls; however, piglets who received adjunctive treatment with GH alone or combined with IGF-I had a higher ($p<0.05$) weight and length gain than the piglets who received DEX alone (Table 1).

The 1.8-2.3 fold reduction in weight gain was represented by a lower ($p<0.05$) whole body lean mass among all DEX treated groups irrespective of whether adjunctive GH or GH+IGF-I were administered (Table 1). Also, the fat mass was lower ($p<0.05$) in the piglets receiving DEX alone compared to controls; piglets receiving GH or GH+IGF-I were intermediary between the DEX alone group and control group (Table 1). Whole body lean and fat mass were also expressed as a percentage of body weight since there were differences in body weight and axial length by the end of the study when body composition was assessed. The percent lean mass and the percent fat mass did not differ among treatment groups indicating that both lean and fat tissue were deposited proportionately to total body weight.

The cumulative excretion of $^{15}N$ reached a constant value for all treatment groups 48 hours following the administration of $^{15}N$-glycine (Figure 1). At this time, all DEX-treated groups had excreted significantly more ($p<0.05$) of the $^{15}N$ label than the control group. Similarly, total nitrogen excretion, a mixture of $^{14}N$ and $^{15}N$,

was also significantly higher ($p<0.05$) among all DEX-treated groups (DEX alone, $0.77\pm0.06$ g N/kg/d; DEX+GH, $0.67\pm0.15$ g N/kg/d; DEX+GH+IGF-I, $0.58\pm0.12$ g N/kg/d) compared to the control group ($0.23\pm0.03$ g N/kg/d). The rate of protein synthesis was suppressed ($p<0.05$) in the DEX alone treated piglets compared to the control group while adjunctive administration of either GH or GH+IGF-I prevented this reduction in the rate of protein synthesis (Figure 2A). Protein breakdown was similar among all groups during the study (Figure 2B). The rate of net protein gain, the difference between the rate of protein synthesis and protein breakdown, was significantly higher ($p<0.05$) in piglets receiving adjunctive GH+IGF-I during DEX compared to the DEX alone treated piglets (Figure 2C). However, the rate of net protein gain among all DEX-treated groups was lower ($p<0.05$) than the rate of net protein gain of control piglets.

BUN was significantly elevated ($p<0.05$) after 5 days of receiving the highest DEX dose and remained higher at d 11 compared to control piglets (Figure 3A). Adjunctive GH or GH+IGF-I treatment did not lower BUN levels until day 15, at which time only the BUN levels of piglets receiving DEX alone remained significantly higher ($p<0.05$) than controls. 3-MH did not differ significantly among treatment groups at any time but there was a non-significant trend ($p=0.094$) which indicated that 3-MH in the DEX alone treated group was elevated after 5 days of the highest DEX dose (Figure 3B).

Plasma GH and IGF-I concentrations have been previously reported (21). In brief, plasma GH was not altered by DEX, GH or GH+IGF-I treatment while plasma IGF-I in piglets receiving exogenous IGF-I was higher ($p<0.05$) than the DEX alone group (6.72 vs. 2.61 nM) at d 6 and higher ($p<0.05$) than all other groups at d 15 (control 6.29 nM; DEX 4.76 nM; DEX+GH 6.88 nM; DEX+GH+IGF-I 10.72 nM).

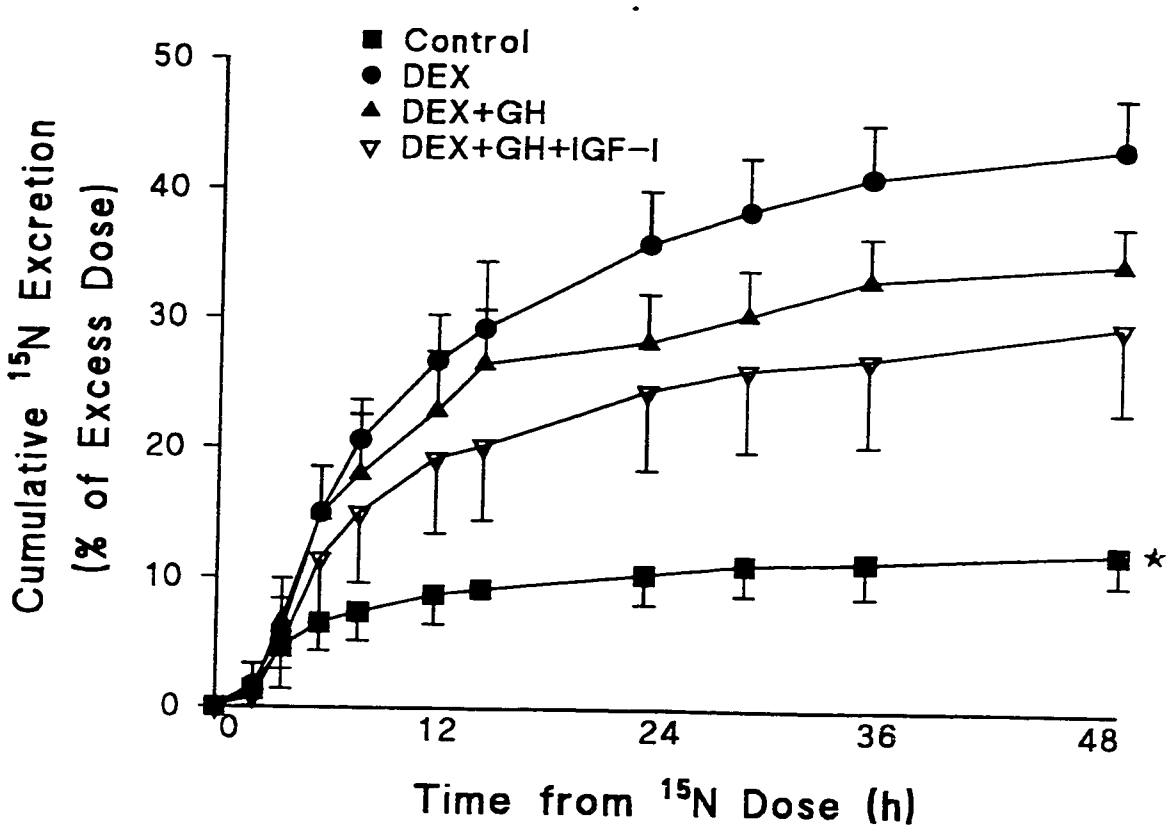


**Figure 1** - Cumulative excretion of $^{15}$N over the 48 h urine collection period. A plateau was established 48 hours after the administration of the $^{15}$N-glycine dose. Values are expressed as mean$\pm$SD. * Control vs. all DEX-treated groups, $p<0.05$.

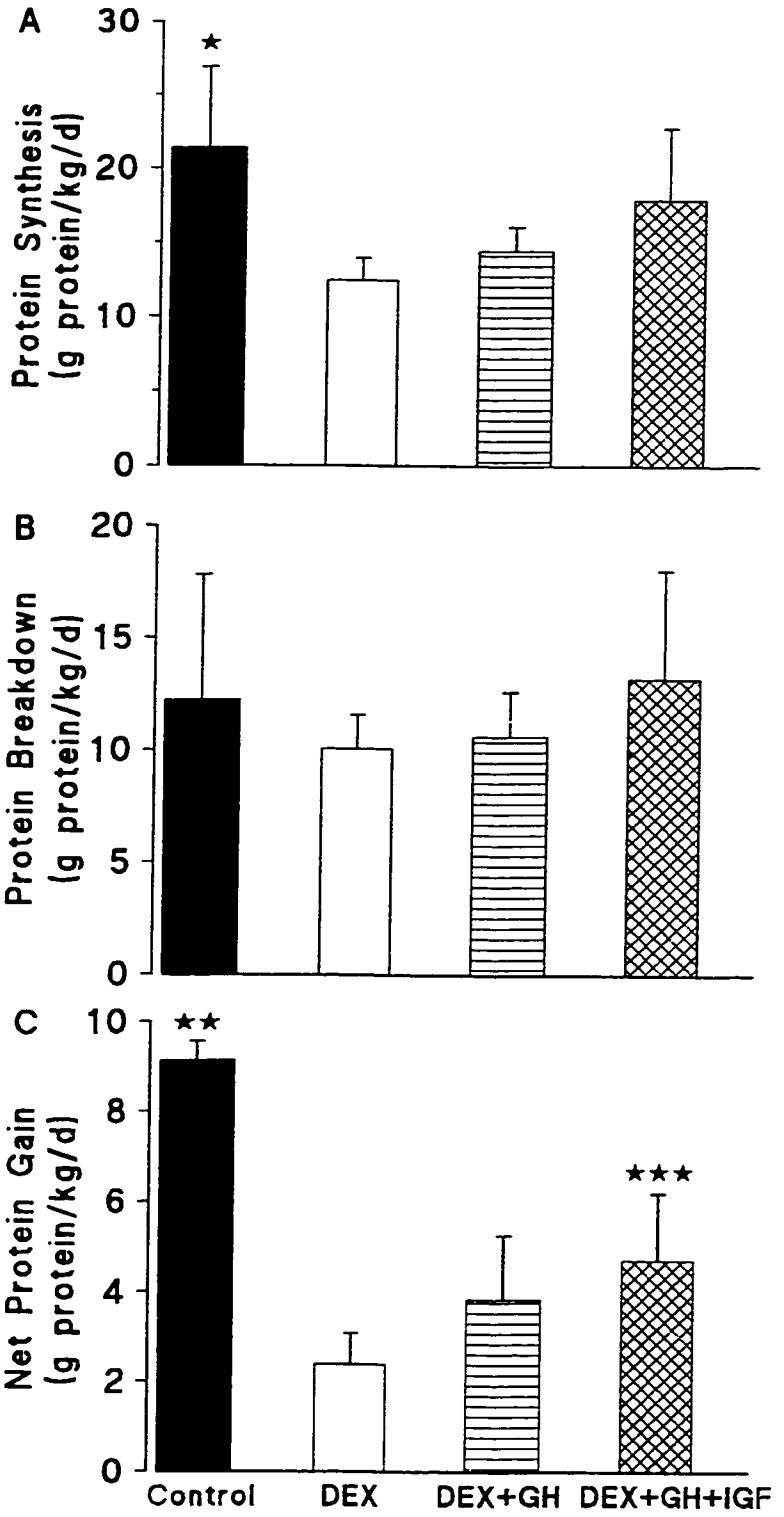


**Figure 2** - Protein kinetics: rates of A) protein synthesis, B) protein breakdown and C) net protein gain for control, DEX, DEX+GH, and DEX+GH+IGF-I groups. Values are expressed as mean±SD. * Control vs. DEX alone, $p<0.05$; ** Control vs. all DEX-treated groups, $p<0.05$; *** DEX+GH+IGF-I vs. DEX alone, $p<0.05$.

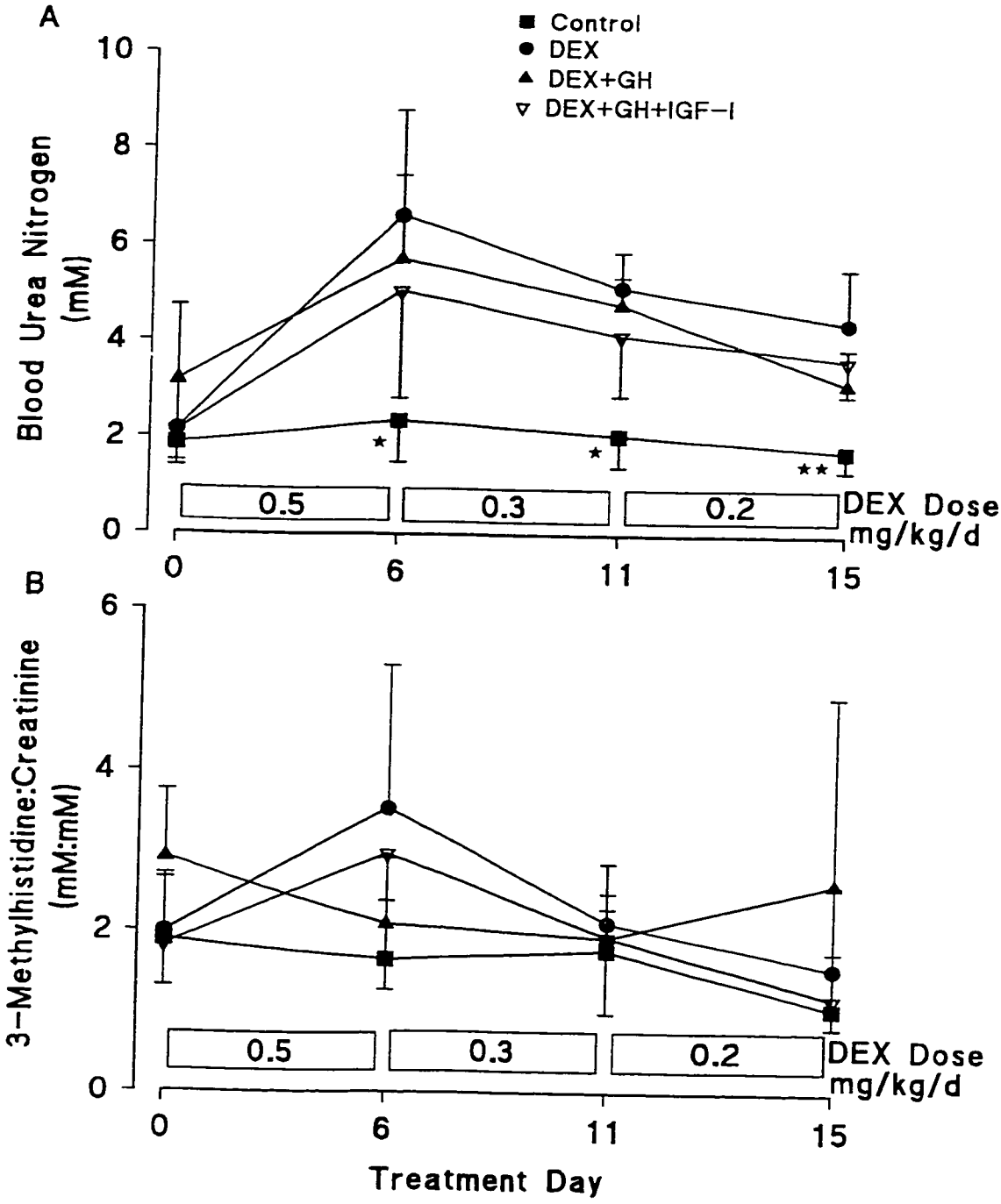


**Figure 3** - A) Blood urea nitrogen and B) urinary 3-methylhistidine:creatinine at baseline and after each change in DEX dose. Values are expressed as mean±SD. * Control vs. all DEX-treated groups, $p<0.05$; ** Control vs. DEX alone, $p<0.05$.

Table 1 - Weight and length gain during the study and body composition at the end of the 15 day study period

| | Control | DEX | DEX+GH | DEX+GH+IGF-I |
|---|---|---|---|---|
| Weight Gain (kg/15 days)* | $3.5\pm0.5^{a}$ | $1.5\pm0.2^{b}$ | $2.0\pm0.3^{c}$ | $2.1\pm0.2^{c}$ |
| Length Gain (cm/15 days)* | $15.0\pm0.1^{a}$ | $4.3\pm1.5^{b}$ | $7.4\pm1.7^{c}$ | $7.4\pm2.4^{c}$ |
| Lean Mass (kg)* | $5.7\pm1.1^{a}$ | $3.6\pm0.5^{b}$ | $3.9\pm0.6^{b}$ | $4.3\pm0.8^{b}$ |
| Lean Mass (% of body weight) | $91.3\pm1.1$ | $91.7\pm0.9$ | $91.2\pm1.1$ | $93.3\pm2.7$ |
| Fat Mass (kg)* | $0.46\pm0.14^{a}$ | $0.26\pm0.04^{b}$ | $0.30\pm0.07$ | $0.31\pm0.08$ |
| Fat Mass (% of body weight) | $7.0\pm1.0$ | $6.7\pm0.9$ | $7.1\pm1.1$ | $6.5\pm0.9$ |

Values are expressed as mean$\pm$SD.

* Treatment was significant ($p<0.05$); means were tested by Student-Newman Keuls test with significant differences represented by different superscripts within a row.

**Discussion**

Using the DEX-treated piglet model we have established that whole body protein synthesis is specifically suppressed even during a low tapering dose of DEX. This finding was somewhat unexpected as we had hypothesized that protein catabolism would be enhanced with DEX treatment based on findings in glucocorticoid-treated infants (33), adult humans (7,20) and piglets (17). One explanation to account for these disparate findings is that protein breakdown may be increased only during the earliest phase of steroid treatment. For example, in developing rats (34,35) and very preterm infants (2,3), 3-MH is elevated over controls only during the early phases of steroid treatment, returning to control or baseline levels before the end of the steroid protocol. Thus, there appears to be an adaptive response to the stress of extended steroid treatment. This transient stimulation of protein breakdown by DEX has been observed in VLBW infants using a leucine tracer (33). Infants who received a tapering dose of DEX had a significantly higher rate of protein breakdown at d 4 compared to d 19 of DEX treatment (33).

Based on the current study, we speculate that protein catabolism was significantly higher after 5 days of the highest DEX dose based on the biochemical data (urinary 3-MH and BUN). This speculation awaits verification which could be obtained by measuring daily changes in 3-MH or by conducting a tracer study five days into the tapered DEX dosing protocol. The accompanying elevation of BUN does not discern between alterations in protein synthesis, protein breakdown or both. Our

observation that protein synthesis was suppressed with DEX has not been observed in DEX-treated VLBW infants (33) or piglets (17) but has been consistently observed using rodent models in which rates of protein synthesis were suppressed after five days of steroid treatment and remained low throughout treatment (35,36). VLBW infants have additional stresses that could potentially trigger a catabolic state; this includes marginal-nutritional adequacy due to prolonged parenteral feedings or feeding intolerances, effects of other medications, or the underlying lung disease. Such factors would not be present in our piglets. In the study by Hellstern et al. (17), piglets had received only 3 days of DEX treatment at the start of the leucine tracer study; it is possible that protein synthesis was not altered until after this point in time. While our study did not explore the precise mechanisms by which DEX alters protein homeostasis, prior studies in DEX-treated animals have provided insight into our findings. In rat muscle tissue, a lower RNA content, reduced ribosomal activity and a smaller quantity of protein in several different muscles compared to untreated rats were associated with both lower rates of muscle and whole body protein synthesis (36). In dogs, DEX caused a marked efflux of free amino acids from the muscle, particularly glutamine, which led to a negative nitrogen balance (37).

The improved net protein gain among piglets receiving GH+IGF-I treatment compared to DEX alone treated piglets suggests that, over a longer study period, this group would have experienced a functional improvement in lean mass and weight gain compared to GH alone treated piglets; it is possible that the study duration allowed

insufficient time to delineate differences between the GH and GH+IGF-I groups as both demonstrated improved growth and lowered BUN levels only during the last 4 days of the study (during 0.2 mg/kg/d dose). Despite these improvements, neither adjunctive therapy fully reversed the effect of DEX on overall somatic growth. The specific mechanisms by which GH or GH+IGF-I partially attenuated the actions of DEX are unknown; but we and others hypothesize that GH and IGF-I acted via their normal pathways, independently of DEX-stimulated pathways (7). Perhaps the additional action of IGF-I is mediated by insulin-like biological actions which promote protein anabolism as insulin itself acts as a growth factor (38).

The piglets in this study had nutrient intakes which were similar to the recommended nutrient intakes for growing swine (60 g protein/d; 850 kcal energy/d; 2.8 g Ca/d; 1.8 g P/d) (39); however, nutrient requirements may be increased when administering anabolic agents during stages of rapid development. It remains uncertain whether the provision of supplemental nutrients would result in additional improvements in weight (e.g. lean mass) and length growth (e.g. axial skeleton) in steroid-treated piglets although non-steroid treated piglets receiving GH have higher rates of growth than non-GH treated piglets without providing a higher quantity of nutrients. The dramatic elevation in BUN by d 6 and 11 and the higher urinary N and cumulative $^{15}N$ by d 15 revealed that DEX treatment, regardless of adjunctive hormonal therapies, inhibited normal utilization of N and suggests that the provision of supplementary nutrients in conjunction with GH or the combination of GH+IGF-I

would likely not improve the metabolic state. Clearly, DEX has an overwhelming suppressive effect on protein turnover which can only be marginally improved with concurrent adjunctive GH or GH+IGF-I.

Despite the fact that DEX can increase fat mass while GH is known to promote the conversion of fat tissue to lean tissue, body composition was not altered by DEX, GH or GH+IGF-I treatment. Perhaps the low quantity of body fat in piglets at this age limits detection of small differences by DXA technology (28).

The next step is to investigate the extent to which DEX alone, DEX+GH or DEX+GH+IGF-I treated piglets recover from DEX treatment. If there is a sustained insult to the accretion of lean mass and overall somatic growth, as has been observed for linear growth in infants treated with DEX (4), potential interventions which can assist the rehabilitation from prolonged DEX therapy.

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# Chapter Five

# IMPROVEMENTS IN GROWTH, BONE STATUS AND BODY COMPOSITION WITH ADJUNCTIVE GROWTH HORMONE DURING DEXAMETHASONE THERAPY IN PIGLETS ARE DOSE-DEPENDENT*

* This chapter represents a manuscript which has been prepared according to the format of the Journal of Bone and Mineral Research.

# Improvements in growth, bone status and body composition with adjunctive growth hormone during dexamethasone therapy in piglets are dose-dependent

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**Abstract**

Previous investigation demonstrated that adjunctive GH (0.1 mg/kg/d) during DEX therapy partially improves steroid-induced abnormalities in growth and bone metabolism (1). We subsequently investigated if these improvements with adjunctive GH are dose-dependent and if piglets further recover during a period after DEX±GH are discontinued. Piglets (n=24) were randomized to placebo; a tapered dose of DEX alone (0.5, 0.3 and 0.2 mg/kg/d over 14 d); DEX+low dose GH (0.05 mg/kg/d); or DEX+high dose GH (0.15 mg/kg/d) for 14 d followed by a 7 d recovery period. During DEX, the DEX+high dose GH group gained more ($p<0.05$) weight than DEX alone piglets and were similar to controls. Length was more severely affected as piglets receiving DEX+high dose GH had a lower ($p<0.05$) length gain than controls but experienced improved ($p<0.05$) length gain compared to treatment with DEX alone. In addition, the femurs of all DEX treated groups were shorter than controls. DEX+high dose GH had higher ($p<0.05$) plasma IGF-I at d 15 compared to DEX alone. High dose GH prevented the reduction ($p<0.05$) in osteocalcin at d 6 and 11, and N-telopeptide at d 6 compared to DEX±low dose GH. GH had no effect on IGFBP profile. GH at both doses prevented the DEX-induced reduction ($p<0.05$) in bone mineral density (BMD). After the 7 d recovery period (d 21), the DEX alone group had a lower ($p<0.05$) BMD compared to all groups. As well, controls had a greater ($p<0.05$) lean mass than DEX±low dose GH groups while the weight and length gain post-DEX were similar among groups. Plasma IGF-I and the IGFBP

profile as well as plasma osteocalcin and urinary N-telopeptide did not differ among groups post-DEX (d 21). Therefore, GH at 0.15 mg/kg/d promotes normalization of somatic and bone growth during DEX treatment to a greater degree than 0.05 mg/kg/d but does not fully counter the detrimental effects of DEX.

**Keywords:** bone, dexamethasone, dose-response, growth hormone, piglets

## Introduction

Treatment with exogenous steroids during development delays linear growth, and reduces the rate of weight gain and the accretion of whole body bone mass in both growing humans (2-6) and animals (1,7-10). These negative effects of steroids are observed even at very low doses of steroids. For example, in preterm infants, who receive dexamethasone (DEX) to improve pulmonary compliance, reductions in bone cell activity and growth occurred during the lowest dose of a tapering course of DEX, and continued to be clinically manifested by a lower rate of length growth and radial bone mineral content at 6 months beyond steroid treatment (2). In addition, children who were randomized to a low dose of prednisolone to manage their asthma, experienced a significant reduction in lower leg growth after 2 weeks of treatment with no "catch-up" growth in the two weeks after steroid treatment was stopped (3). These negative effects are particularly distressing because steroid treatment disrupts

growth at stages where infants or children should be achieving rapid rates of weight and length gain and may have long-lasting effects.

We have specifically shown that steroid treatment, in vivo, reduces circulating IGF-I, IGFBP-2 and IGFBP-3, and alters tissue mRNA expression of IGFBPs (1), providing evidence that steroids mediate delays in growth and bone development via both endocrine and autocrine/paracrine mechanisms. Descriptive studies in children are mostly limited to kidney transplant recipients who receive prolonged, low dose steroid treatment. In these patients, adjunctive GH therapy has been reported to improve growth rates and promote bone maturation (5,11-13) but none of these studies have examined whether improvements in growth velocities and bone development are dependent on the dose of GH administered during steroid treatment. This is likely due to the uncertainty of the safety of GH therapy and the controversy surrounding the efficacy of GH treatment at improving adult height and peak bone mass (14). Thus, it is prudent to establish the safety of GH therapy and the optimal dose of GH which counters growth delay and bone abnormalities inflicted by steroid treatment using an animal model.

Since the DEX-treated piglet model has been shown to respond to both DEX and GH treatment, the piglet is a suitable animal in which to conduct a GH dose-response study. The dose-response of GH on growth rates and body composition have been most thoroughly investigated in pigs due to the financial implications of increasing growth rates, improving feed efficiency and promoting the deposition of

lean mass at the expense of fat tissue. In older, (> 30 kg, age= < 2 months) non-steroid-treated pigs, administration of GH improves weight gain and promotes the gain of bone and lean mass in a dose-dependent manner from 30 μg/kg/d through to 200 μg/kg/d (15)

We previously reported that administration of adjunctive GH (100 μg/kg/d) during DEX treatment partially attenuated the DEX-induced growth delay and reduction in bone mass using our DEX-treated piglet model (1). In this follow-up study, our objectives were to determine whether GH administration attenuated DEX-induced effects on growth and bone mass in a dose-dependent manner, and, whether the metabolic insults incurred by DEX treatment are sustained after the cessation of treatment. Whether steroid treatment during early postnatal life or childhood compromises the attainment of final adult height or body composition, particularly bone mass, has not been described in steroid-treated humans or animals. Thus, this study also provides insight into the recovery from DEX treatment.

## Materials and Methods

### Piglet Care and Study Design.

Littermate, male Yorkshire piglets (4-5 d old, 6 different litters, Arkell Swine Research Station, Guelph, ON) were removed from the sow, brought to McMaster University and weaned onto a specially designed liquid diet (7) over the subsequent three days. After adaptation to the diet, 400 mL of liquid diet per kg body weight was

provided at four different feeding times. In order to perform 24 h urine collections and monitor total formula intakes and urine volumes, piglets were housed individually in metabolic cages which were maintained at 28°C. Since piglets were housed individually, they were allowed to socialize with their littermates and exercise for 2 hours each day in a fenced pen.

Piglets were randomized to receive placebo, a tapering dose of DEX which mimics the protocol used at our institution to manage chronic lung disease in VLBW infants (0.5 mg/kg/d on d 1 thru d 5; 0.3 mg/kg/d on d 6 thru d 10; and 0.2 mg/kg/d on d 10 thru d 14), DEX+low dose GH (0.05 mg/kg/d) or DEX+high dose GH (0.15 mg/kg/d). DEX (sodium phosphate salt, Hexadrol, Organon, Toronto, ON) was administered orally twice daily (0900 h and 1700 h) and GH was administered i.m. once a day (0900 h). To control for the stress of adminstering DEX and/or GH, controls piglets received sterile water by orogastric technique and piglets not randomized to GH treatment received sterile water by i.m. injection.

Anthropometry

Daily morning weights were obtained using an electronic scale (Sartorius, Gottingen, Germany) with an animal weighing program which takes the average of 10 consecutive measurements. The quantity of formula offered and drug doses were adjusted daily according to changes in body weight. Snout to rump length was measured by the same person (WEW) using a plastic measuring tape each time piglets

were anesthetized with isoflurane (AErrane, Anaquest, Mississauga, ON) for blood sampling and at necropsy. In order to evaluate the growth of a developing long bone, the left femur was excised at necropsy, cleaned of soft tissue and the length was measured.

Blood and Urine Analyses

Fasting morning blood samples were obtained 2 hours post-GH administration and 24 hour urine samples were collected as detailed in a prior study (1). The details pertaining to the plasma IGF-I and the IGFBP profile analyses have also been previously reported (1,16). Samples were analyzed in 4 consecutive assays using the same batch of antibody and $^{125}$I-IGF-I (intra-assay CV=3.7%, interassay CV=11.3%). Four piglets from each treatment group were randomly selected to characterize the IGFBP profile by SDS PAGE and western ligand blotting during DEX±GH and during the recovery period. Plasma osteocalcin and urinary N-telopeptide were assessed by a competitive RIA (INCstar Corp., Stillwater, MN) and competitive inhibition ELISA (Osteomark, OSTEX, Seattle, WA), respectively, using commercially available kits. N-telopeptide measurements were expressed as a function of creatinine excretion which was measured by a colorimetric assay (Procedure No. 555, Sigma Diagnostics, St. Louis, MO). Interassay and intra-assay CVs for creatinine were 2.5% and 3.4%, respectively. Urinary Ca was quantified using atomic absorption spectrophotometry (Model 703; Perkin-Elmer, Norwalk, CT).

All samples were measured in triplicate and the intra-assay CV was 6.3%. Fractional Ca excretion was calculated as a function of creatinine excretion. Ionized plasma Ca was measured in duplicate using an ionized Ca analyzer (ICa1; Radiometer, Copenhagen). Plasma glucose was measured using a commercial colorimetric assay (Procedure No. 510, Sigma Diagnostics). The interassay and intra-assay CVs were 6.1% and 4.7%, respectively.

Body Composition Analyses

Whole body bone mineral content and lean and fat mass were assessed by dual energy x-ray absorptiometry (Hologic QDR1000W$^{R.}$ Hologic Inc., Waltham, MA) using the pediatric whole body software program (V5.63P, Hologic Inc.) at d 15 and d 21 while piglets were anesthetized with isoflurane gas (17). After the DXA scans on d 21, piglets were killed by a lethal cardiac injection of euthanol while deeply anesthetized with gas inhalant.

All piglet care and procedures were conducted according to the Guide for the Care and Use of Experimental Animals (18) and approved by the Animal Research Ethics Board at McMaster University.

Statistical Analyses

Outcomes were analyzed using either a one-way analysis of variance (ANOVA) or a repeated measures ANOVA; the Student-Newman-Keuls test was used

for comparison of multiple means at each time point in which significant differences due to treatment were observed. Differences were considered significant if the p value was less than 0.05. All data were expressed as mean$\pm$SD. Statistical analyses were performed using SigmaStat Software (Jandel Scientific, San Rafael, CA).

## Results

### *During DEX*

The mean postnatal age of piglets in each group was 7.3$\pm$1.0 d. The mean initial weight (control 2.4 kg; DEX 2.5 kg; DEX+low dose GH 2.5 kg; DEX+high dose GH 2.3 kg) and length (control 42.3 cm; DEX 42.7 cm; DEX+low dose GH 41.7 cm; DEX+high dose GH 42.3 cm) were not significantly different among groups. The mean 24 h formula intake (control 390$\pm$9; DEX 400$\pm$0; DEX+low dose GH 400$\pm$0; DEX+high dose GH=400$\pm$0 mL/kg/d) and urine volume (control 143$\pm$19 DEX 155$\pm$24; DEX+low dose GH 147$\pm$27; DEX+high dose GH 153$\pm$17 mL/kg/d) did not differ among treatment groups on any day during DEX treatment.

After 14 days of DEX administration, weight gain was reduced ($p<0.05$) in piglets receiving DEX alone or DEX+low dose GH (Table 1). Adjunctive high dose GH prevented the reduction in weight gain by DEX and improved ($p<0.05$) weight gain compared to the DEX alone group. Length growth was more dramatically affected than weight as all DEX treated piglets experienced a significantly lower ($p<0.05$) rate of linear growth than controls; but, piglets receiving DEX+high dose

GH had a higher ($p<0.05$) length gain than DEX alone but not DEX+low dose GH treated piglets (Table 1).

Both low and high dose GH attenuated the DEX-induced reduction ($p<0.05$) in bone mineral content and density (Table 1). Only high dose GH countered the DEX-induced reduction in absolute lean mass while the percent lean mass was not different among groups (Table 1). Fat mass, expressed either as an absolute weight or a percentage of body weight, did not differ among different treatment groups (Table 1).

Plasma IGF-I among DEX treated piglets was not different from controls at baseline, d 6 or d 11 but by d 15, piglets receiving DEX+high dose GH had higher ($p<0.05$) circulating IGF-I compared to DEX alone treated piglets (Figure 1). Adjunctive high or low dose GH did not counter the DEX-induced reduction in plasma IGFBP-2 at d 6, 11 or 15 (Figure 2). IGFBP-3 was lower among all DEX-treated groups compared to controls at d 11 (Figure 2).

Adjunctive high but not low dose GH prevented the DEX-induced reduction of plasma osteocalcin at d 6 and d 11 and urinary NTx at d 6. By d 15, both plasma osteocalcin and urinary NTX concentrations of all DEX groups were similar to the control group (Figure 3). Ionized plasma Ca and urinary Ca did not differ among treatments at any sampling time during DEX treatment (data not shown). Plasma glucose was also unchanged by DEX or GH at either dose (values were between 4.7-5.8 mmol/L).

*Post-DEX treatment*

The weight and length gain over the 7 day recovery period were similar among all groups (Table 2). By d 21, absolute weight (control 8.2±2.3 kg; DEX 6.5±1.0 kg, DEX+low dose GH 6.7±1.3 kg; DEX+high dose GH 7.3±1.2 kg) and length (control 63.0±5.2 cm; DEX 57.1±2.8; DEX+low dose GH 56.5±4.1 cm; DEX+high dose GH 60.9±1.6 cm) were not significantly different among groups. However, control piglets had longer ($p<0.05$) femurs compared to all the DEX-treated groups irrespective of whether piglets had previously received adjunctive GH therapy during DEX.

Despite the similarities in absolute weight and length among groups, piglets who had received DEX alone still had a lower BMC and BMD compared to controls. Furthermore, the reduction in lean mass which was observed at the end of DEX treatment among DEX±low dose GH groups persisted at the end of the recovery period. No differences in the percent lean or fat mass, or absolute fat mass were observed post-DEX.

Circulating IGF-I (Figure 1), IGFBPs (Figure 2) and osteocalcin (Figure 3) as well as urinary NTx (Figure 3) were similar among groups 7 d post-DEX treatment. Ionized plasma Ca and urinary Ca remained unchanged from during DEX values (data not shown). Plasma glucose did not differ among treatment groups (values ranged from 5.0-6.4 mmol/L).

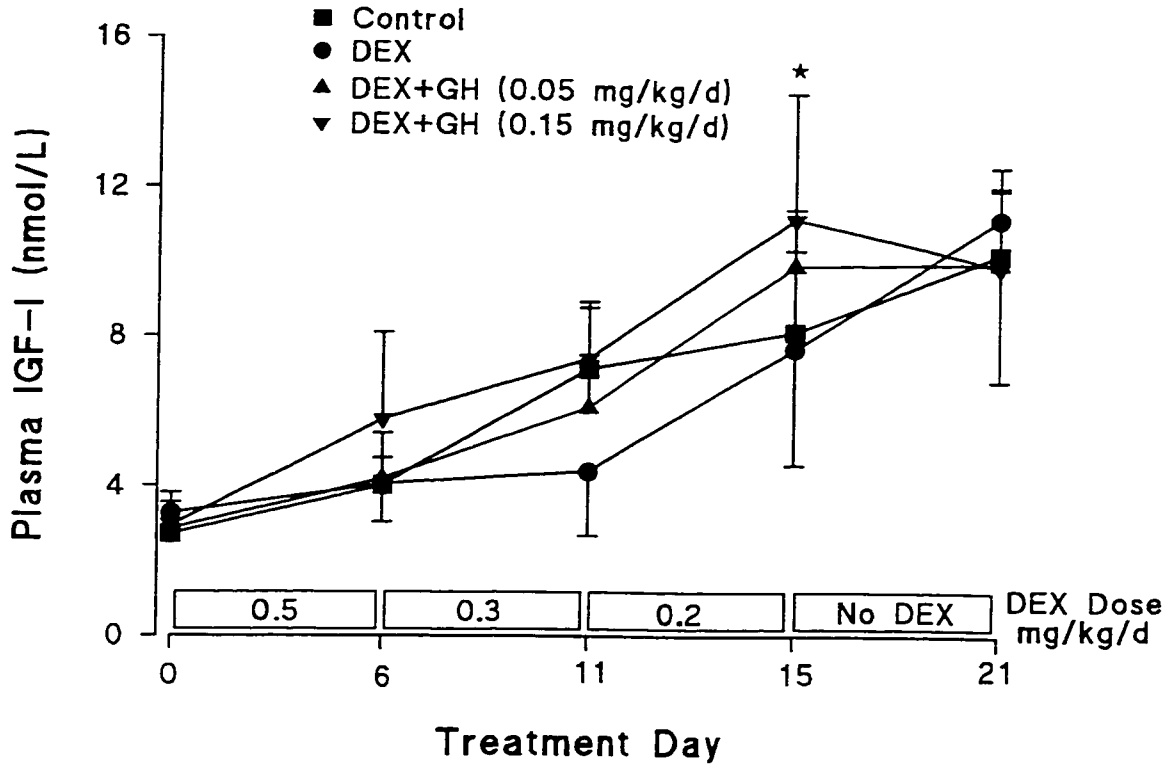


**Figure 1** - Plasma IGF-I at baseline, after each change in DEX dose and after a 7 day recovery period. Values are mean ± SD. * DEX+high dose GH vs. DEX, $p<0.05$.

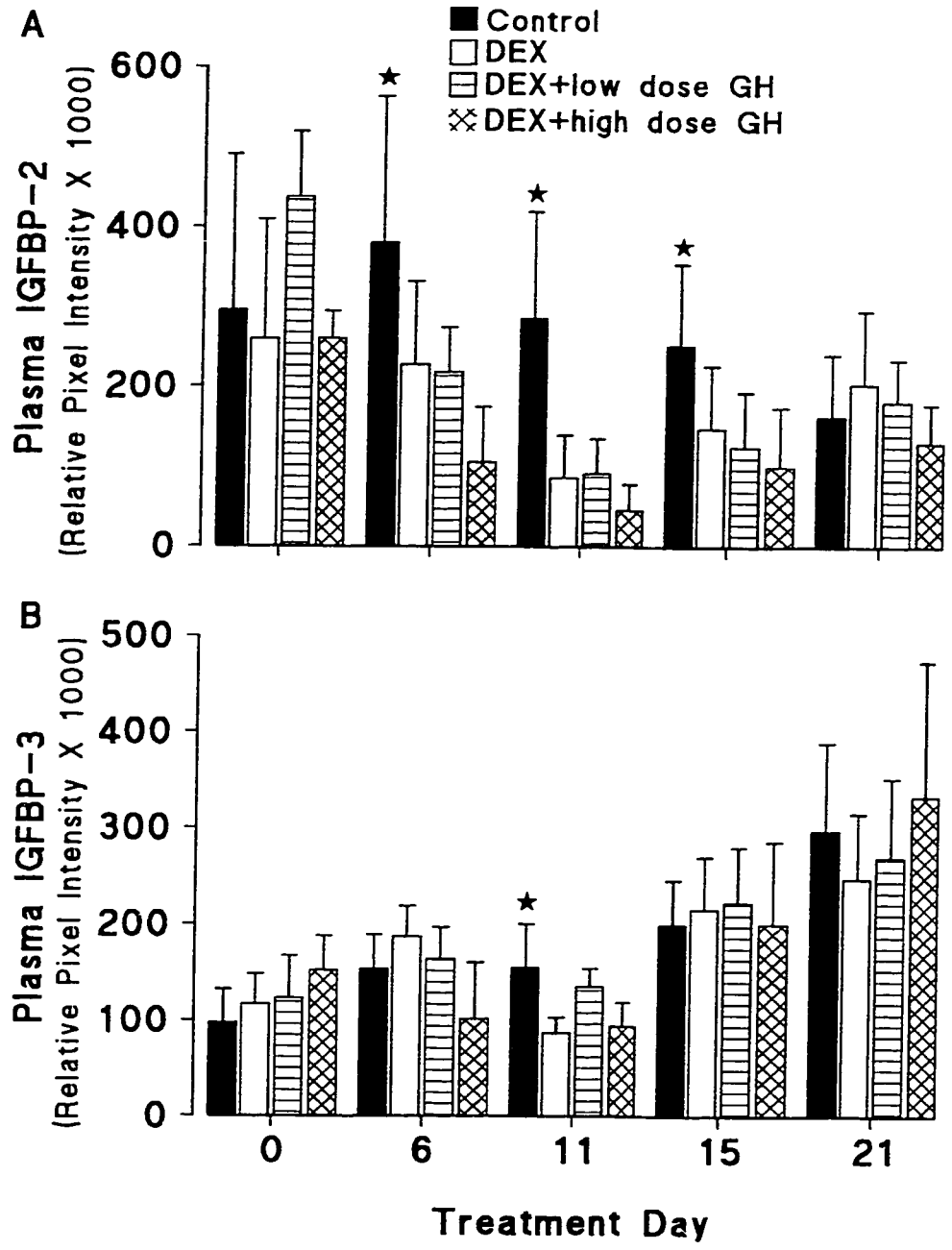


**Figure 2** - The plasma IGFBP profile. The relative pixel intensity of IGFBP-2 (A) and IGFBP-3 (B) at baseline, after each change in DEX dose and after a 7 d recovery period. Bars represent mean ± SD of four piglets. * Control vs. all DEX groups, $p<0.05$.

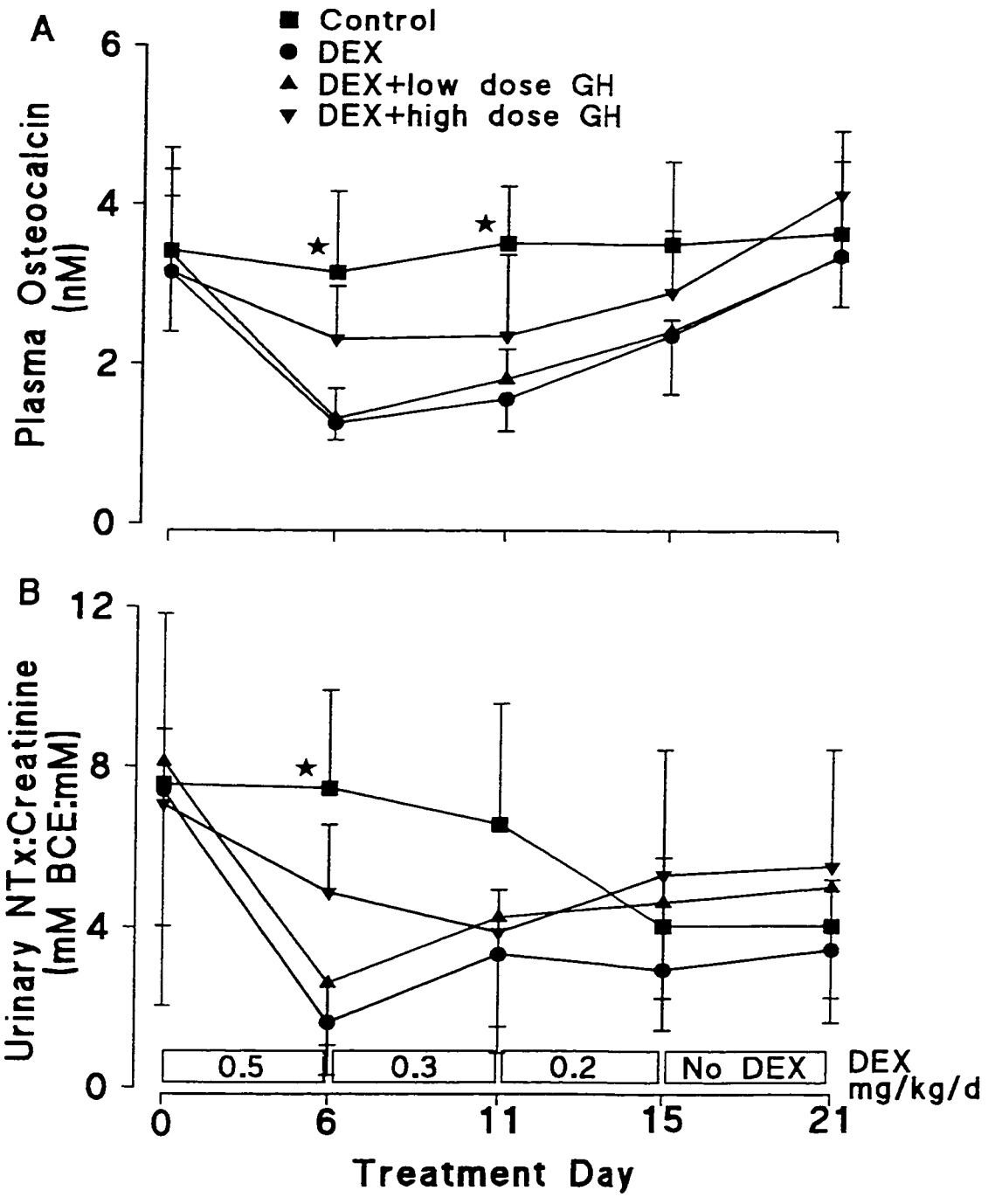


**Figure 3** - Plasma osteocalcin (A) and urinary N-telopeptide (B) concentrations at baseline, after each change in DEX dose and after a 7 d recovery period.
* DEX±low dose GH vs. Control, $p<0.05$.

Table I - Whole body weight and length gain during DEX treatment and body composition after 14 d of DEX treatment

| | Control | DEX | DEX+low dose GH | DEX+high dose GH |
|---|---|---|---|---|
| Weight Gain (kg/15 days)* | $3.6\pm1.1^{a}$ | $1.9\pm0.4^{b,c}$ | $2.3\pm0.5^{b}$ | $2.9\pm0.5^{d}$ |
| Length Gain (cm/15 days)* | $13.7\pm1.6^{a}$ | $7.1\pm2.3^{b,c}$ | $8.8\pm1.7^{b}$ | $9.8\pm1.7^{b,d}$ |
| Bone Mineral Content (g)* | $118\pm29^{a}$ | $78\pm11^{b}$ | $89\pm23$ | $91\pm11$ |
| Bone Mineral Density ($mg/cm^2$)* | $254\pm16^{a}$ | $221\pm10^{b}$ | $234\pm20$ | $241\pm10$ |
| Lean Mass (kg)* | $5.8\pm1.6^{a}$ | $4.0\pm0.5^{b}$ | $4.4\pm0.9^{b}$ | $4.7\pm0.5$ |
| Lean Mass (% of body weight) | $90\pm1.0$ | $90\pm1.4$ | $89\pm1.4$ | $90\pm0.6$ |
| Fat Mass (kg) | $0.52\pm0.19$ | $0.41\pm0.09$ | $0.47\pm0.14$ | $0.43\pm0.37$ |
| Fat Mass (% of body weight) | $7.9\pm1.1$ | $9.1\pm1.4$ | $9.2\pm1.4$ | $8.8\pm0.7$ |

Values are expressed as mean$\pm$SD.

* Treatment was significant ($p<0.05$); means were tested by Student-Newman Keuls test with significant differences represented by a vs. b and c vs. d within a row.

Table II - Whole body weight and length gain, femur length and body composition 7 days post-DEX treatment

| | Control | DEX | DEX+low dose GH | DEX+high dose GH |
|---|---|---|---|---|
| Weight Gain (kg/7 days) | 2.2±0.7 | 2.0±0.4 | 2.0±0.4 | 2.2±0.3 |
| Length Gain (cm/7 days) | 7.0±2.0 | 7.3±2.5 | 6.1±2.2 | 8.9±2.2 |
| Femur Length (cm)* | $9.1\pm0.6^{a}$ | $8.3\pm0.3^{b}$ | $8.4\pm0.6^{b}$ | $8.6\pm0.3^{b}$ |
| Bone Mineral Content (g)* | $159\pm44^{a}$ | $112\pm18^{b}$ | 117±30 | 127±21 |
| Bone Mineral Density ($mg/cm^2$)* | $285\pm25^{a}$ | $247\pm10^{b}$ | 252±20 | 261±20 |
| Lean Mass (kg)* | $7.8\pm1.6^{a}$ | $5.8\pm0.6^{b}$ | $6.1\pm0.9^{b}$ | 6.5±0.7 |
| Lean Mass (% of body weight) | 91±1.7 | 89±1.6 | 89±1.6 | 90±1.1 |
| Fat Mass (kg) | 0.68±0.26 | 0.61±0.14 | 0.64±0.20 | 0.59±0.12 |
| Fat Mass (% of body weight) | 7.6±1.2 | 9.2±1.5 | 9.0±1.5 | 8.1±0.9 |

Values are expressed as mean±SD.

*Treatment was significant ($p<0.05$); means were tested by Student-Newman Keuls test with significant differences represented by different superscripts within a row.

**Discussion**

The findings of this study emphasize the potent effect of DEX during development and provide evidence that administering adjunctive anabolic agents (e.g. GH) during DEX is not an appropriate intervention to counter the negative side-effects of DEX, especially related to bone. We have shown that even when adjunctive GH is administered at a dose considered to be high for clinical use in children, the DEX-induced suppression of weight and length gain or BMD was not fully attenuated. While growth rates were improved with high dose GH, osteocalcin, NTx, BMD and lean mass were not improved compared to DEX alone treatment.

Of particular concern was the observation that the stunting effect of DEX on femur length was sustained 7 days post-DEX although the snout to rump length, a measure of vertebral growth, was similar among all treatment groups. Previously, we reported that the femurs of piglets receiving DEX±GH (0.1 mg GH/kg/d) were shorter than controls after a tapered two week course of DEX (identical to the DEX regimen used in this study) and that GH administered simultaneously did not attenuate the stunting effect of DEX (1). In contrast to our findings in piglets, exogenous administration of GH to steroid-treated children improves height velocity. Some of the discrepancy may be due to differences in the frequency of steroid dosing. Approximately half of these children received alternate day versus daily steroid administration (5,6,11-13). Alternate day steroid therapy has been shown to have

fewer negative side-effects on growth and bone development (19) and may thereby allow GH to exert a more anabolic effect.

It is also possible that administration of GH to other animal species receiving DEX, and more specifically developing humans, would have greater functional benefits as the GH-IGF-I axis in neonatal pigs (birth to 7 days postnatal age) is less responsive to exogenous GH than in older pigs (20). Administration of 1 mg GH/kg/d, divided over 3 doses each day for 1 week, elevated circulating IGF-I, improved weight gain and resulted in a higher rate of protein synthesis in liver as well as other target tissues (20). The dose administered in this study was approximately 7 times the highest dose of GH we administered and is 20 times higher than the dose commonly used in steroid-treated children. The anabolic response to this clinically unrealistic dose of GH, as noted by the authors, was much less than would be observed in more mature pigs. Thus, the response of piglets to exogenous GH is submaximal. The fact that GH was administered over 3 doses each day as opposed to the once daily injection used by us may have further potentiated the growth response by elevating circulating GH, and thus plasma IGF-I for a longer period of time. Indeed, the frequency of GH administration is an important consideration to ensure a maximal stimulation of growth. Several studies have reported that GH-deficient children receiving GH replacement experience higher height velocities when switched from a 3 times per week to a daily dosing of GH (21,22).

Extrapolation of the GH doses necessary to stimulate growth in piglets to preterm infants may not be appropriate or safe. Administering a dose higher than 0.15 μg/kg/d is not feasible for the clinical setting as children can experience negative side-effects on carbohydrate metabolism at lower GH doses (e.g. 0.05 mg/kg/d) (23). Potential serious side-effects include insulin resistance at the peripheral tissues which can result in diabetes and impaired production of insulin by the pancreas (24). Due to the fact that the piglet is not as responsive to exogenous GH as more developed pigs, it will be essential to assess improvements in older children who receive steroids or even in a small clinical trial in steroid-treated preterm infants. Analogous to the neonatal piglet, preterm infants may also have an immature GH-IGF-I axis compared to later stages of development, and may not be fully responsive to exogenous GH. In addition, the added insult of steroid treatment could further compromise a positive, anabolic response to GH. In a group of steroid-treated preterm infants, the natural developmental rise in the circulating components of the GH-IGF-I axis (IGF-I, IGFBP-3) and the biochemical markers of bone turnover were marginally affected by DEX (25). Thus, further investigation is required to determine how DEX may alter the response of preterm infants to exogenous GH.

Combining the findings of this study with our previous study (1), in which GH at a dose of 0.1 mg/kg/d partially attenuated the DEX-induced reductions in somatic growth and promoted normalization of bone metabolism, we estimate that the optimum dose of GH is between 0.05 and 0.1 mg/kg/d. Most outcomes were

similarly improved regardless of whether a dose of 0.1 or 0.15 mg/kg/d was administered.

Our observation that weight and length gain were similar among all groups 7 days post-DEX was encouraging. These findings suggest that by minimizing the initial dose of DEX, and perhaps even the duration of DEX, would assist a recovery from DEX. The long-term consequences of shorter femurs, lower absolute lean mass and BMD requires further investigation using our piglet model. We speculate that over a longer recovery period, femur length and BMD would normalize to control values since the biochemical bone marker data revealed that bone cell activity was not different from controls post-DEX. Unlike the piglets, preterm infants have many other sequelae (neurological disorders, residual lung disease) which could confound their rehabilitation (26). Long-term follow-up studies of steroid-treated infants, requiring a large sample size and amount of funding, are urgently needed to clarify the extent to which these infants attain their genetic growth potential.

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# Chapter Six

# DISCUSSION AND FUTURE INVESTIGATIONS

# DISCUSSION AND FUTURE INVESTIGATIONS

## Summary of findings

Together, the studies in this thesis delineated some of the mechanisms underlying DEX-induced growth delay, bone abnormalities and altered body composition with particular insights into the interaction of DEX and the GH-IGF-I axis during development. The descriptive study in steroid-treated infants (Chapter 2) is the first to characterize the changes in the circulating components of the GH-IGF-I axis, revealing that the natural developmental rise in IGF-I and bone cell activity (NTx) were suppressed during DEX and may have contributed to the delay or restriction in weight, length and bone mass compared to term born reference infants. Despite the fact that growth failure is a common finding in DEX-treated infants (Weiler et al. 1997, Ohlsson et al. 1992a, Brownlee et al. 1992) and a functioning GH-IGF-I axis is known to be essential for growth, this is the only comprehensive study to date in which the circulating components of the GH-IGF-I axis along with nutritional status, biochemical measures of bone metabolism, body composition and growth data have been evaluated.

The piglet studies provided evidence for a positive influence of exogenous GH on weight and length growth and bone cell activity in a dose-dependent manner, and that the combination of GH+IGF-I resulted in an additive improvement in net protein

gain. From these studies, potential mechanisms of DEX action were identified and provided an understanding of how the negative side-effects of DEX could be countered. To provide a global perspective of the effects of DEX and the ability of adjunctive GH alone or with combined GH+IGF-I therapy to counter these effects, the findings from the piglet studies are summarized in Figure 1.

The studies which comprise this thesis have not directly identified the interaction of DEX, GH and IGF-I at the level of the cell. It remains uncertain whether GH and IGF-I acted by directly inhibiting DEX action (acting via similar pathways), opposing the effects of DEX (independent of DEX pathways) or by a combination of both. We attempted to evaluate local regulation of tissue IGF-I in several target tissues (e.g. liver and bone) by assessing IGFBP mRNA expression. In liver, the fact that a reduction in IGFBP-2 and IGFBP-4 mRNA expression was observed after the lowest dose of DEX indicates that there was a sustained insult even during the lowest dose of DEX. Although bone expression of mRNA of the IGFBPs was below the level of detection using our methods, the findings of in vitro studies from the literature provide further insight into potential regulation as detailed in the discussion section of Chapter 3. In bone cell culture, IGFBP-3 and IGFBP-5 mRNA expression are reduced with the addition of DEX, and may thereby lower the availability of DEX and prevent IGF-I from acting as an autocrine and paracrine stimulator of bone growth (Chevalley et al. 1996, Okazaki et al. 1994). Clearly, as discussed in Chapter 1 (Figure 2), local regulation of IGF-I activity by IGFBPs present in bone is an integral part of bone growth. ***Future investigation is required to***

***improve the sensitivity of this analysis so that DEX-induced alterations in bone IGFBP mRNA expression can be evaluated in order to understand local regulation of bone tissue by DEX. In addition, quantifying IGF-I and the IGFBPs in bone is required to provide more mechanistic insight into the ability of GH and IGF-I to counter the effects of DEX in bone.***

We speculate that the measurement of other growth factors (e.g. transforming growth factor $\beta$) which are abundant in the bone matrix and the consideration of potential interactions with GH or IGF-I may help to delineate the mechanisms by which the negative effects of DEX on bone cell metabolism may be attenuated. ***Thus, isolation and quantification of other growth factors which are essential for normal bone homeostasis will be essential to attain a comprehensive understanding of DEX-induced impairments in bone cell regulation by growth factors, in vivo.***

To further our understanding of the mechanisms of glucocorticoid action on bone, it would be helpful to assess the proliferation and differentiation of osteoprogenitors to osteoblasts in response to in vivo DEX, DEX+GH or DEX+GH+IGF-I treatment. As discussed in Chapter 1, osteoprogenitor cell number is reduced with prolonged exposure to DEX, thereby suggesting a mechanism which accounts for the loss of bone mass in steroid-induced osteoporosis. ***Whether GH or IGF-I, potent mitogens in bone, can counter the effect of DEX on osteoprogenitor cells, in vivo, requires investigation.***

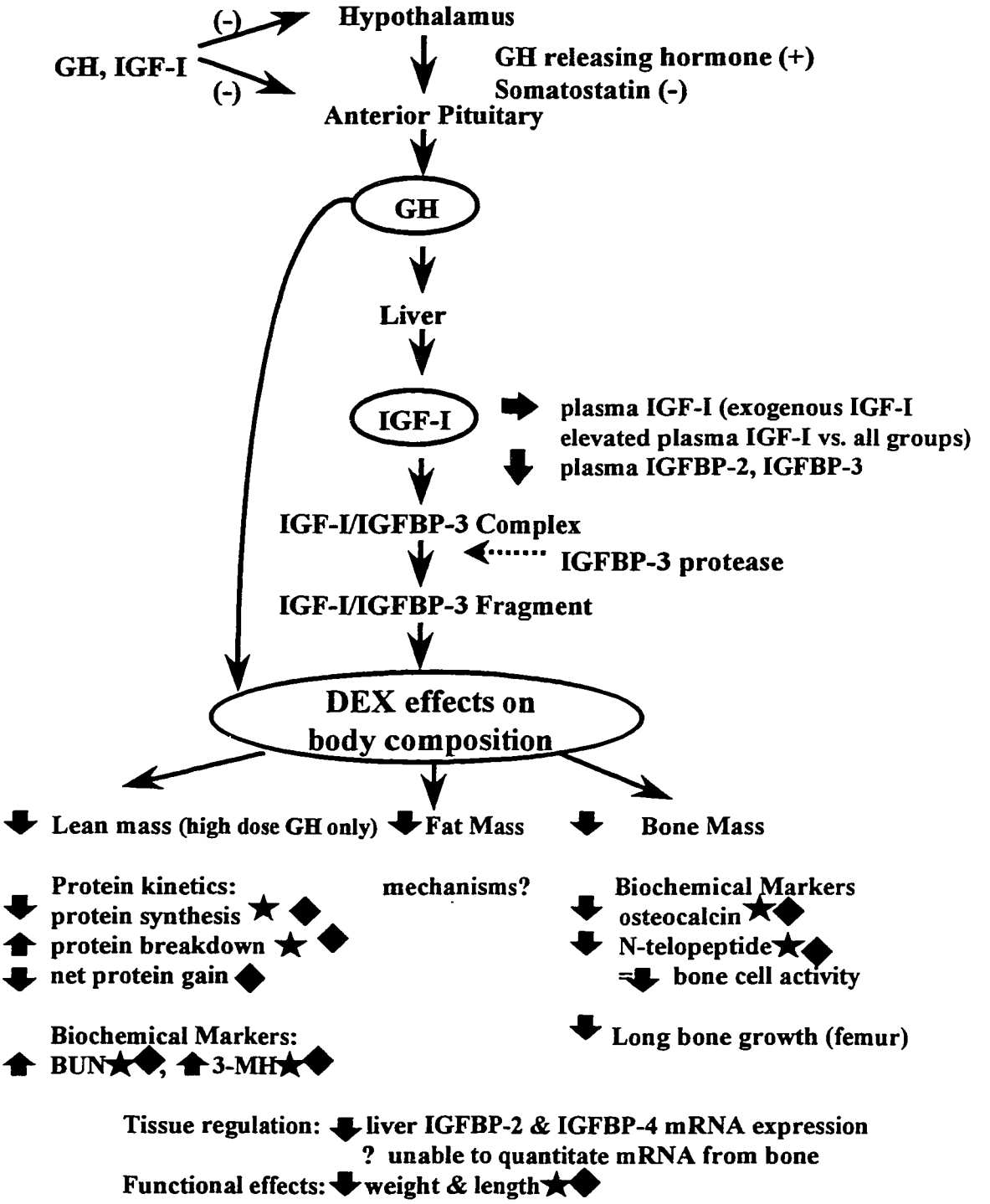


**Figure 1.** A summary of the mechanisms by which DEX delays growth and impairs bone development based on outcomes from the three piglet studies. Stars indicate that adjunctive GH, at a dose of 0.1 or 0.15 mg/kg/d, partially or fully attenuated the DEX-induced effect. Diamonds indicate that adjunctive GH+IGF-I partially or fully attenuated the DEX-induced effect.

**New information about the effects of adjunctive GH, IGF-I or GH+IGF-I on bone or protein metabolism during steroid treatment from studies published after 1993**

With the wide-spread use of steroids to manage a myriad of diseases which afflict infants, children and adults, there is substantial interest in the effectiveness of administering adjunctive anabolic agents such as GH and IGF-I to attenuate the negative side-effects of steroids. Consequently, several studies have been published since we designed the studies in this thesis and are worthy of consideration when interpreting the combined findings from Chapters 3 through 5. The studies summarized in Table 1 and 2 are representative of different species at various stages of development and different aspects of metabolism.

*Bone metabolism (Table 1)*: The findings of the study in children who received GH (Sanchez et al. 1995) are in agreement with earlier studies (Fine et al. 1991, Van Dop et al. 1992, Allen and Goldberg 1992) in which growth rates were higher post-GH administration. A strength of the study by Sanchez et al. 1995, is that a bone biopsy was obtained and, with a relatively small sample size, significant increases in the osteoid perimeter (deposition of collagen) and bone formation rate were observed. In contrast, exogenous administration of IGF-I to rats did not have any anabolic effect on bone as assessed by both biochemical markers and histomorphometry (Binz et al. 1994). The latter is similar to our study (Chapter 3), in which no additional benefit of administering IGF-I in combination with GH with respect to bone outcomes. Together with our piglet experiments, these studies suggest that exogenous administration of

IGF-I does not stimulate the deposition of bone in developing, steroid-treated animals who have normal plasma IGF-I concentrations.

*Protein Metabolism (Table 2)*: IGF-I alone treatment in piglets (Hellstern et al. 1996) or healthy adult males (Oehir et al. 1996) was also shown to be ineffective at attenuating the increase in protein breakdown due to steroid treatment. In contrast to treatment with IGF-I alone, adjunctive GH alone was effective during steroid treatment; the rate of protein breakdown was maintained at pre-treatment rates. Similar to our findings in piglets (Chapter 4), administration of GH+IGF-I to healthy adult males (Berneis et al. 1997) had an additive effect on net protein gain, resulting in a higher protein gain than GH alone treatment. Based on the results of these studies, and our own, it appears that IGF-I is most effective when administered with GH. The fact that we did not observe an additive effect with respect to bone outcome, as examined in the discussion section of Chapter 3, may be due to the duration of IGF-I administration as well as the steroid dose. To date, this is the only study which has assessed changes in bone metabolism with adjunctive GH+IGF-I during steroid treatment.

***Based on these findings by other investigators, several areas of future research using the DEX-treated piglet model have been identified. For instance, it would be helpful to elucidate how DEX alters the structure and biomechanical properties of long bones. Determining bone quality (e.g. structure and biomechanical properties) is likely as important as measuring bone quantity (e.g. BMC). With respect to protein metabolism, the question of whether the higher rate of net protein gain observed with GH+IGF-I therapy in our piglets or in humans***

*(Berneis et al. 1997) translates into functional gains such as greater muscle mass and strength should be determined. Furthermore, whether a higher muscle mass is accompanied by a higher bone mineral mass during development requires further study.*

**Table 1:** **Studies since 1993 which have administered adjunctive GH or IGF-I during steroid treatment to stimulate bone development.**

| Study | Subjects | Steroid dose | Dose & duration: GH or IGF-I | Outcomes | Findings |
|---|---|---|---|---|---|
| **Sanchez et al.[1] 1995** n=7 | mean age=12±3 y lupus, asthma, connective tissue disease | Prednisone Variable doses mean dose = 0.24±0.05 mg/kg/d Alternate day or daily | GH: 0.375 mg/kg/wk 3 doses/wk 1 y | Height, Plasma: IGF-I, osteocalcin, PTH, $1,25(OH)_2D_3$, Bone histomorphometry | Improved height, ↑ plasma IGF-I but not osteocalcin, PTH or $1,25(OH)_2D_3$ ↑ osteoid perimeter & bone formation rate |
| **Binz et al.[2] 1994** n=8/group | growing rats 3 months old 311 g | DEX Approximately 0.12 mg/kg/d Daily | IGF-I: Approximately 0.1 mg/kg/d Continuous infusion 21 d | Weight gain, Plasma: IGF-I, osteocalcin, calcitriol Bone histomorphometry | Improved weight gain, ↑ plasma IGF-I but osteocalcin & calcitriol remained lower than controls No change in trabecular bone density, osteoblast & osteoclast cell numbers with IGF-I |

[1]A descriptive study. Height velocity, biochemical measurements and bone histomorphometry during GH treatment were compared to pre-GH treatment measurements. Steroids were administered orally and GH was administered by s.c. injection.

[2]DEX was provided in drinking water. An osmotic mini-pump was used to deliver IGF-I.

**Table 2:** **Studies since 1993 which have administered adjunctive GH, IGF-I or GH+IGF-I during steroid treatment to improve protein metabolism.**

| Study | Subjects | Steroid dose | Dose & duration: GH, IGF-I or GH+IGF-I | Outcomes | Findings |
|---|---|---|---|---|---|
| **Hellstern et al.[1] 1996** n=5/group | growing pigs age? 10-15.3 kg | DEX 5 mg/kg/d Daily | IGF-I: 0.025 mg/kg/h for 6 h each day 4 d | Protein kinetics using $^{13}$C-leucine tracer | DEX-induced protein catabolism was not attenuated |
| **Oehri et al.[2]** 1996 n=8/group | healthy males 20-30 y | Methylprednisolone 0.5 mg/kg/d Daily | IGF-I: 0.08 mg/kg/d over 2 doses/d GH: 0.30 IU/kg/d over 2 doses/d 7 days | Protein kinetics using $^{13}$C-leucine tracer | GH alone: maintained protein catabolism at pre-DEX values IGF-I alone: did not counter DEX-induced ↑ in protein catabolism |
| **Berneis et al.[3] 1997** n=8/group | healthy males mean age = 24.5±1.2 y | Methylprednisolone 0.5 mg/kg/d Daily | IGF-I: 0.08 mg/kg/d over 2 doses/d GH: 0.30 IU/kg/d over 2 doses/d 7 days | Protein kinetics using $^{13}$C-leucine tracer | GH alone: maintained protein catabolism at pre-DEX values Additive effect with GH+IGF: ↑ net protein gain |

[1]Crossover trial with weekly intervals between each DEX or DEX+IGF-I treatment. DEX and IGF-I were delivered i.v.
[2]Baseline protein kinetic measurements were obtained in all subjects prior to randomization to one of three treatments: methylprednisolone (placebo), methylprednisolone+GH or methylprednisolone+IGF-I. GH and IGF-I were administered s.c. Methylprednisolone was administered orally.
[3]Baseline protein kinetic measurements were obtained in all subjects prior to randomization to one of three treatments: methylprednisolone (placebo), methylprednisolone+GH alone or methylprednisolone+GH+IGF-I. GH and IGF-I were administered s.c. Methylprednisolone was administered orally.

**New information about DEX-treated preterm infants: catch-up growth, GH therapy and nutrition**

Piglets recovered from DEX therapy within 7 days of the cessation of DEX treatment regardless of whether they received adjunctive low or high dose GH. It is presently unclear whether infants will experience a similar recovery. ***Thus, it will be important to determine whether or not steroid-treated preterm infants undergo catch-up growth or whether their genetic growth potential and attainment of peak bone mass are compromised.*** Recovery of the steroid-treated preterm infant is complicated by the fact that in addition to DEX treatment, residual lung disease, suboptimal nutrition and premature birth may also contribute to delays in growth and bone mass accretion (Figure 2). As well, neurological impairments may reduce physical activity and result in a lower muscle mass. Several review papers have identified that the attainment of peak bone mass is critical to prevent osteoporosis and that the acquisition of bone is a complex interaction of nutrition, hormonal stimuli and physical activity (and muscle mass) (Carrascosa et al. 1995, Ribot et al. 1995, Burr 1997). ***It will be essential to determine the extent to which preterm infants catch-up to term born infants during early childhood before deciding whether to administer GH.***

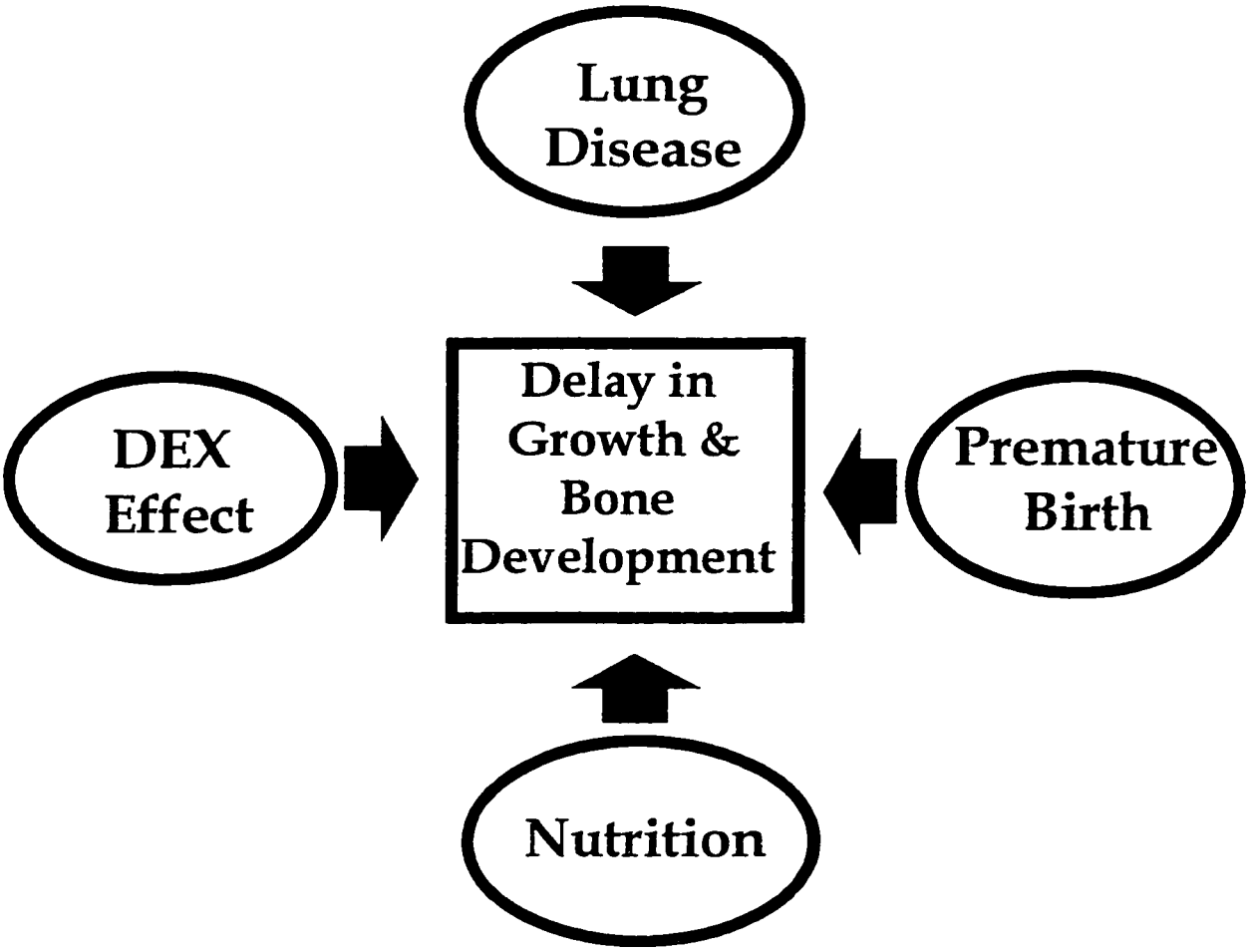


**Figure 2.** Other factors which may contribute to the delay in growth and bone development in DEX-treated preterm infants: residual lung disease, suboptimal nutrition and premature birth.

One approach to assist with the recovery from DEX may be to lessen the insult incurred by steroid treatment. For instance, a consistent finding in our piglet studies was that the reduction in weight and length growth velocity, the suppression of the biochemical bone markers and the elevation in biochemical markers of protein metabolism were dependent on the DEX dose, being most affected after the highest dose of DEX and improving or returning to control levels after the lowest dose of DEX. Similarly, we (Chapter 2) and others (Cummings et al. 1985, Brownlee et al. 1992) have observed that weight and length gain of infants who received a 42 day tapering course of DEX improved as they were weaned off DEX. These findings in piglets and infants emphasize the benefits of minimizing the amount of time the highest doses of steroids are administered.

It is also evident from studies in preterm infants and children treated with steroids that it is important to minimize the frequency of steroids; infants (Gilmour et al. 1995) and children (Ansell et al. 1974) who received pulse or alternate day steroid treatment did not experience the dramatic suppression of weight gain observed with daily steroid administration. The route of steroid administration is another consideration. Inhaled steroids may not result in the severe side-effects of oral or systemic steroids, however, inhaled steroids may not be as therapeutically effective compared to oral or systemically delivered steroids. The lack of therapeutic effectiveness may be due to inadequate delivery of the steroid via a nebulizer

(Dimitriou et al. 1997). Ensuring proper delivery of an inhaled steroid is essential before the side-effects are compared to those that result from systemic administration.

Our observation that steroid-treated preterm infants had nutrient intakes below the lower limit of the P-RNIs (Chapter 2, Table 2) suggests that providing additional nutrition prior to term corrected age may be beneficial for growth and bone development, allowing them to achieve growth rates which are closer to intrauterine growth rates and a bone mass similar to term born infants (Figure 2). Infants who received enriched formula between 37 weeks GA and 3 months corrected age experienced the highest weight and length gain between 37 weeks GA and 1 month corrected age (Brunton et al. 1998). This observation provides evidence that the hormonal stimuli which are required to efficiently utilize nutrients for the deposition of protein and bone mineral are present in infants by 37 weeks gestational age. More importantly, this study identified a critical time during early postnatal life in which catch-up growth could be promoted.

Our findings in DEX-treated infants (Chapter 2) also indicate that hormonal stimuli are present during early life. Although the natural rise in circulating IGF-I may have been suppressed during DEX, circulating IGF-I, IGFBP-2 and IGFBP-3 appeared to undergo similar changes as observed in older, non-steroid treated infants (Bennett et al. 1983, Guidice et al. 1995, Radetti et al. 1997, Rajaram et al. 1995)

after the cessation of DEX treatment (approximately 36 weeks GA). ***To ensure that inadequate nutrition is not a factor which further compromises growth, additional investigation is required to determine the appropriate RNIs for DEX-treated VLBW infants.***

If steroid-treated infants do not achieve acceptable rates of growth, as the current literature indicates (O'Shea et al. 1993, Jones et al. 1995, Furman et al. 1995, Weiler et al. 1997), it will be essential to assess the effectiveness of adjunctive GH therapy in developing humans. Studies which have followed steroid-treated preterm infants during early life (e.g. until 20 months to 3 years of age) consistently report that approximately 30% of infants born less than 1500 g are growing below the 3rd percentile for weight and height (O'Shea et al. 1993, Jones et al. 1995, Furman et al. 1995). ***However, it will be important to consider whether adjunctive GH treatment during steroid treatment is effective in preterm infants.***

As previously discussed, steroid-treated animals and humans are not GH deficient, as defined biochemically by measuring plasma GH concentration in response to provocative stimuli. Consequently, when reviewing the evidence pertaining to the effectiveness of GH therapy in children, it is important to assess studies in which GH has been administered to non-GH deficient children. The two recent randomized controlled trials in non-GH deficient children (Zadik et al. 1994, Fine et al. 1994) reported improvements in growth rates and bone age compared to untreated infants. No randomized controlled trials in preterm infants or children have

been conducted during steroid treatment. Thus, the mechanism of stunting may be different and, accordingly, the responsiveness to GH is uncertain.

If GH treatment is deemed appropriate, another consideration will be when to administer GH. If catch-up growth is variable between subjects, it may be prudent to evaluate weight, height and bone mass in early childhood (e.g. 5 years of age) and subsequently make the decision of whether to administer GH. GH may be even more effective at stimulating growth after DEX therapy has been stopped (Rivkees et al. 1994). The study by Zadik et al. (1994) determined that younger subjects attained greater height after four years of GH therapy than subjects who were older at the start of GH therapy. It is speculated that this is due to the fact that younger children (approximately 4-9 years old) had more time in which to deposit collagen and allow mineralization to occur than children who progressed into puberty during treatment. Whether preterm infants are responsive to GH therapy during early life, at a time when they are receiving DEX, is not known. In light of the knowledge that newborn piglets do not fully respond to exogenous GH (Chapter 5, Wester et al. 1998), we speculate that human infants might also have an attenuated metabolic response to GH. The fact that the natural developmental changes in the circulating components of the GH-IGF-I axis occur in preterm infants may not necessarily indicate that infants can fully respond to exogenous GH as similar changes are observed in piglets during early postnatal life (Lee et al. 1991, Lee et al. 1993, Ward et al. 1998). Premature birth itself may be a factor which limits the metabolic response to GH and IGF-I. There is

still considerable debate and conflicting evidence pertaining to the role of GH and IGF-I in fetal as well as extrauterine growth. ***Future studies are required to elucidate the responsiveness of preterm infants to exogenous GH during the first few months of life.***

The potential serious side-effects associated with GH treatment must be carefully weighed against the benefits of treatment (Allen et al. 1994). Concern about the long-term safety of GH treatment in children or preterm infants who do not have classic GH deficiency is a well-founded but unanswerable at this point in time. ***Certainly, frequent monitoring will be required to evaluate if adjunctive GH therapy during steroid treatment is safe in children or preterm infants.***

**More effective anabolic agents to stimulate growth and bone development?**

Alendronate, a bisphosphonate, is being investigated to understand its potential for preventing bone loss and/or promoting bone formation to treat osteoporosis in adults (Margolis et al. 1996). Bisphosphonates have the advantage of being non-hormonal treatments with no side-effects on carbohydrate metabolism; however, there is some concern about the long-term effects of alendronate on bone structure in rapidly developing bone. In adults receiving alendronate, however, there is no evidence of impaired bone structure by biopsy samples out to 3 years (Bone et al. 1997, Chavassieux et al. 1997). Investigation of the efficacy of anabolic agents other

than GH and IGF-I may prove more useful in countering the effects of DEX on bone development (Margolis et al. 1996).

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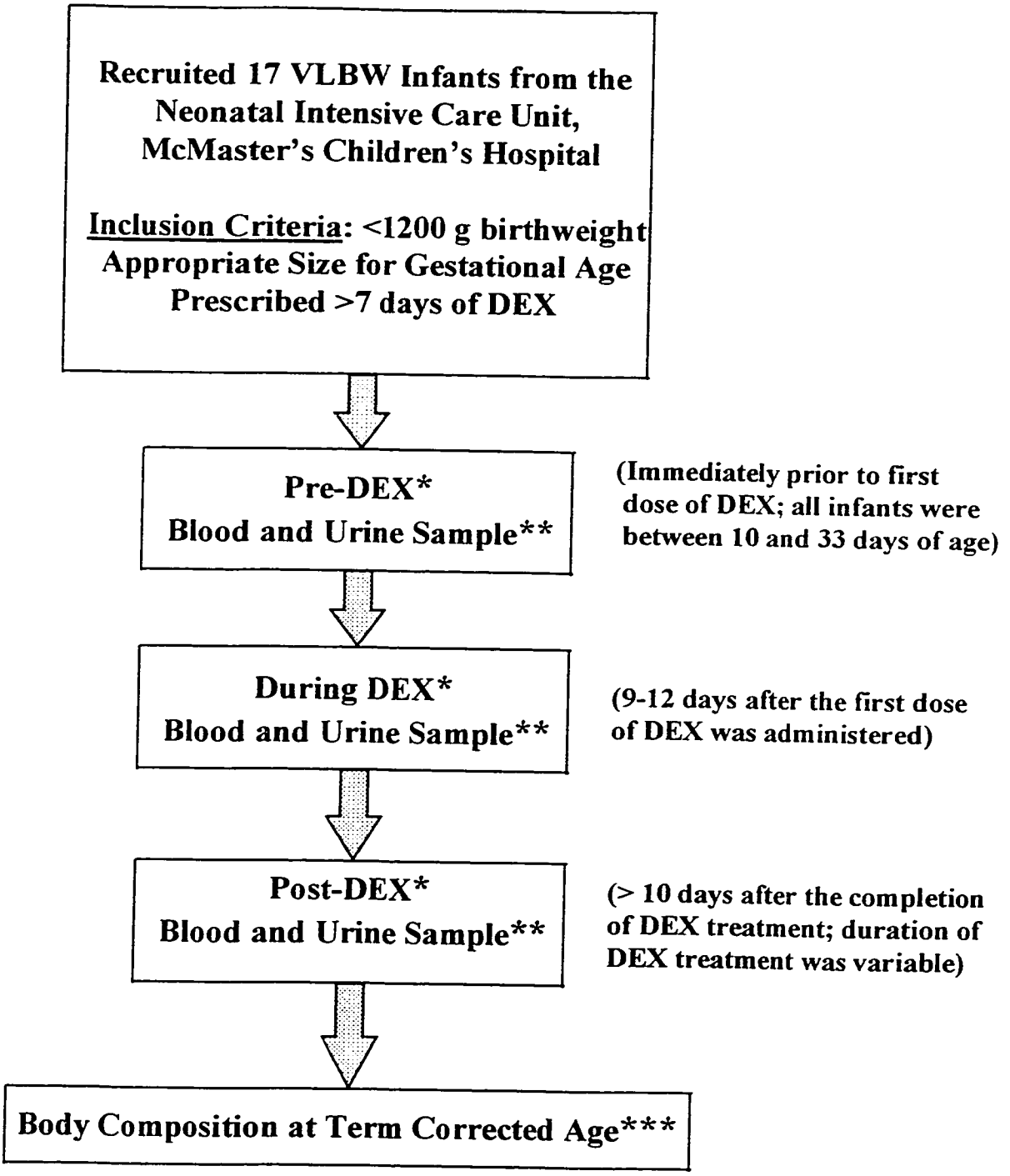



*Daily weights and nutrient intakes
Length every 1-2 weeks

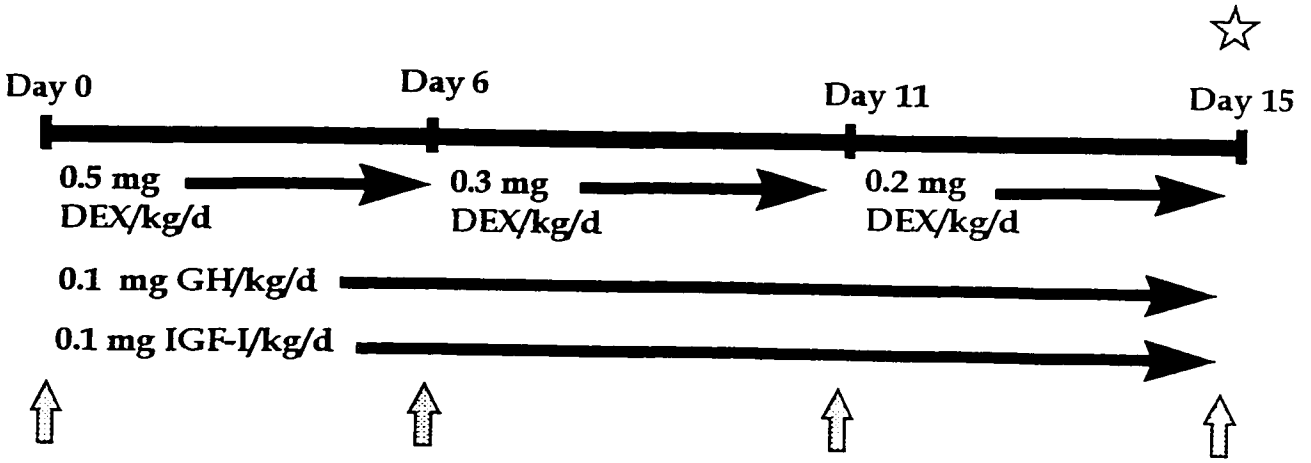
**Blood sample: IGF-I, IGFBP profile, osteocalcin
Urine sample: N-telopeptide

***Weight and Length measured at term visit

## Appendix II

3 day adaptation to diet and environment
Randomization to one of four groups (n=8/group)

1. Control
2. DEX
3. DEX+GH
4. DEX+GH+IGF-I



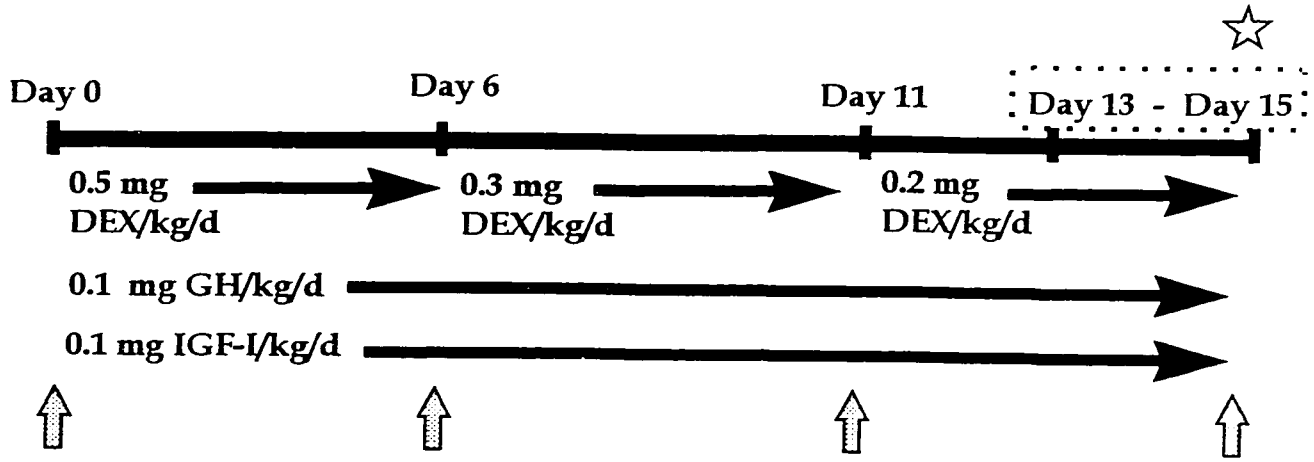

⇧ Weight, Length, 24 h formula intake,
24 h urine volume
Blood : GH, IGF-I, IGFBP profile,
Osteocalcin, Calcium, Glucose
Urine: N-telopeptide, Calcium

☆ Whole body bone scan
Femur scan
Liver IGFBP mRNA expression
Femur length
Organ weights

## Appendix III

**3 day adaptation to diet and environment**
**Randomization to one of four groups (n=6/group)**

1. Control
2. DEX
3. DEX+GH
4. DEX+GH+IGF-I




⇧ **Weight, Length, 24 h formula intake, 24 h urine volume**
**Blood : GH, IGF-I, Urea Nitrogen**
**Urine: 3-Methylhistidine**

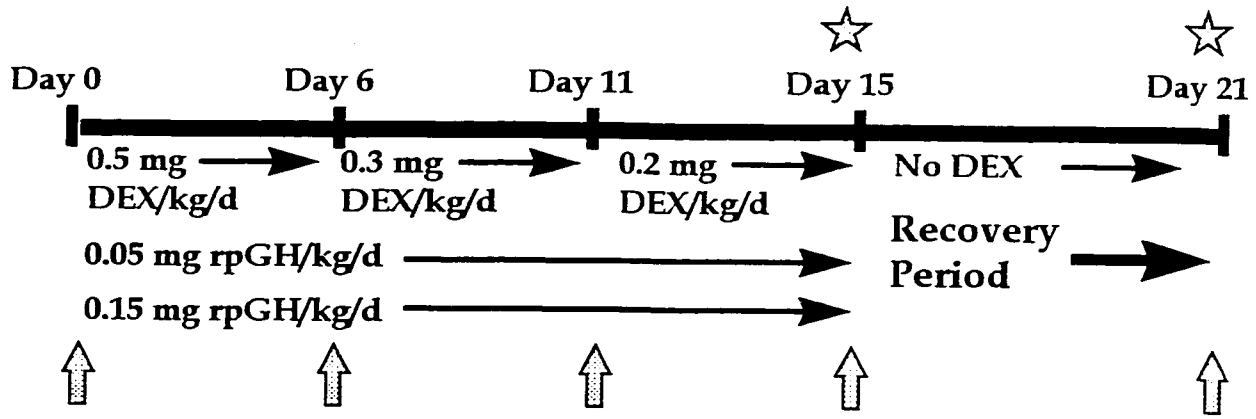
**$^{15}$N-Glycine Dose at 9 am on Day 13**
***48 hour urine collection from Day 13 - Day 15***
**Total urinary nitrogen**
**Enrichment of $^{15}$N in urine**

☆ **Body Composition: Lean and Fat Mass**

## Appendix IV

3 day adaptation to diet and environment
Randomization to one of four groups (n=6/group)

1. Control
2. DEX alone
3. DEX+low dose GH (0.05 mg/kg/d)
4. DEX+high dose GH (0.15 mg/kg/d)




⇧ Weight, Length, 24 h formula intake,
24 h urine volume
Blood : GH, IGF-I, IGFBP profile,
Osteocalcin, Calcium, Glucose
Urine: N-telopeptide, Calcium

☆ Whole body composition
(bone, lean and fat mass)

At day 21 only: Femur length,
Organ weights

## Appendix V

**Liver, kidney, heart and spleen weights after 15 days of DEX, GH and IGF-I treatment**

| Treatment | Liver† g/kg bw | Kidney*,† g/kg bw | Heart g/kg bw | Spleen g/kg bw |
|---|---|---|---|---|
| Control | 29.2±2.3[a] | 3.2±0.4[a] | 7.3±0.6 | 2.7±0.7 |
| DEX | 38.1±6.3[b] | 3.5±0.3 | 7.5±1.1 | 2.3±0.5 |
| DEX+GH | 41.3±5.4[b] | 3.8±0.4[b] | 7.2±0.6 | 2.7±0.9 |
| DEX+GH+IGF-I | 38.3±6.0[b] | 3.8±0.5[b] | 7.1±0.6 | 2.8±1.0 |

Values are expressed as mean±SD.

* Left kidney weights are shown. There was no difference between the weight of the right and left kidney.

† Treatment was significant ($p<0.05$); means were tested by Student-Newman Keuls Test with significant differences represented by different superscripts within a column.

## Appendix VI

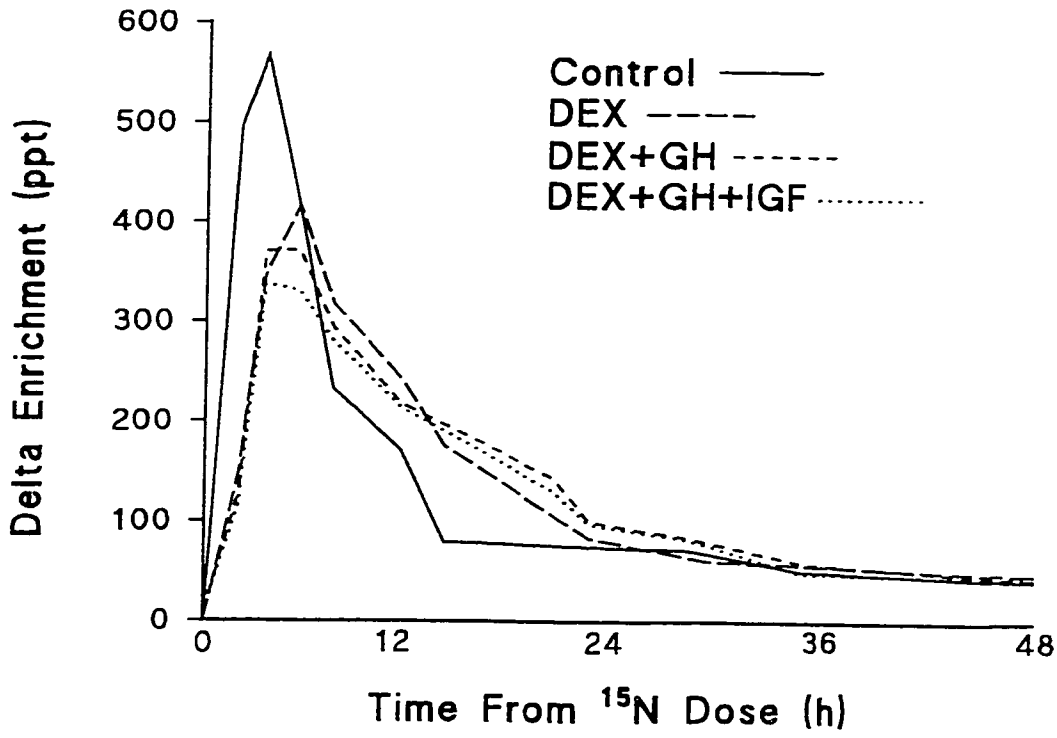
**Liver, kidney, heart and spleen weights after 15 days of DEX and GH treatment followed by 7 days of no treatment**

| Treatment | Liver g/kg bw | Kidney * g/kg bw | Heart g/kg bw | Spleen g/kg bw |
|---|---|---|---|---|
| Control | 32.7±3.4 | 3.4±0.7 | 7.1±1.1 | 2.7±0.9 |
| DEX | 29.3±2.2 | 3.6±0.2 | 7.2±0.8 | 2.3±1.0 |
| DEX+low dose GH | 28.1±4.8 | 3.3±0.2 | 7.2±0.9 | 2.9±1.1 |
| DEX+high dose GH | 28.1±2.9 | 3.3±0.6 | 6.8±0.5 | 2.3±0.3 |

Values are expressed as mean±SD.

* Left kidney weights are shown. There was no difference between the weight of the right and left kidney.

## Appendix VII




The mean delta enrichment of $^{15}N$ in piglet urine over the 48 h urine collection for each treatment group. The excretion of $^{15}N$ peaked between 3 and 6 hours after administration of the $^{15}N$-glycine tracer.