University of Alberta

Effect of growth hormone and therapeutic ultrasound on mandible and mandibular condyle

by

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DEDICATION

To My Parents, Mohammed Emdad Khan and Mahfuza Begum, for their unconditional and relentless support

ABSTRACT

Abstract: Previous studies have shown growth hormone and therapeutic low intensity pulsed ultrasound can enhance mandibular growth separately. The aim of this study is to evaluate the concomitant effect of both of these applications on mandibular growth in rat. Methods: 24 male Sprague Dawley rats were divided into four groups, 6 in each. Groups 1, 2, 3, and 4 were designated as untreated control, recombinant rat growth hormone, Low Intensity Pulsed Ultrasound, and combination of both groups respectively. After 21 days of daily treatment on mandibular condylar, mandibles from euthanized rats are dissected, and scanned by Micro-Computed Tomography to measure the mandibular bone volume, bone surface area, and condylar bone mineral density. Also Real-time Polymerase Chain Reaction was performed on the extracted livers' C-fos, C-jun, and IGF-1 genes expressions. Results: Groups 2, 3 and 4 showed significant ($p<0.05$) growth stimulation when compared to the untreated control group. However, there was no statistical significant difference between groups 2, 3 and 4 with regard to bone volume or surface area. Conversely, condylar bone mineral density for group 4 was significantly reduced than groups 1, 2, and 3. Rats' weights were not significantly different among the treatment groups after the treatment was performed. Additionally, gene expression study showed that the expression of C-jun, in harvested livers for Group 4 was less than that of Group 2 showing fewer side effects. Conclusion: When growth hormone was applied to rats' mandible together with therapeutic ultrasound, preferential increase in bone volume, and surface area occurred with the expense of condylar bone mineral density and with less potential side effects.

Keywords: Growth Hormone, Low Intensity Pulsed Ultrasound, Mandibular growth, Micro-computed Tomography

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LIST OF ABBREVIATIONS

TMJ – Temporomandibular Joints

N – Newton (SI Unit of Force)

N/mm^2 – Newton per square millimetres

FAS – Fetal Alcohol Syndrome

ACEI - Angiotensin Converting Enzyme Inhibitors

HFM - Hemifacial Microsomia

TCS - Treacher Collins Syndrome

NS - Nager Syndrome

PRS - Pierre-Robin Sequence

BMC – Bifid Mandibular Condyle

TMC - Trifid Mandibular Condyle

TCOF1 - Treacher Collins-Franceschetti syndrome 1

SOX9 - SRY (Sex determining region Y)-box 9

CT - Computed Tomography

ROI – Region of Interest

VOI – Volume of Interest

PET - Positron Emission Tomography

FDG - 18-F-2-fluoro-2-deoxyglucose

cm - centimetre

GH - Growth Hormone

GHR – Growth Hormone Receptor

VEGF – Vascular Endothelial Growth Factor

mRNA –messenger Ribonucleic Acid

IGF1 - Insulin like growth factor 1

JAK2 - Janus tyrosine kinase 2

MAPK - Mitogen Activated Protein Kinase

C-fos - FBJ murine osteosarcoma viral oncogene homolog

C-jun- jun proto-oncogene

JunB - jun B proto-oncogene

Spi- Serine Protease Inhibitor

SRE – Serum Response Element

PTH – Parathyroid Hormone

HIFUS - High intensity focused ultrasound

LIPUS - Low-intensity pulsed ultrasound

°C – Degree celcius

mW/ cm^2 – milliwatts per square meter

MHz – Megahertz

KHz – Kilohertz

μ – Micron; μg/mcg – Microgram

nmol - Nanomole

OC – Osteocalcin

BSP - Bone Sialoprotein

Egr-1 - Early Growth Response 1

Cbfa-1 / Runx2 - Core Binding Factor Alpha 1 / Runt-Related Transcription

Factor 2

Alk-3 - Aurora-like Kinase

ALP – Alkaline Phosphatase

OP- Osteopontin

ON- Osteonectin

TGF-β1- Transforming Growth Factor Beta 1

BMP-7 - Bone Morphogenetic Protein

MicroCT - Micro-Computed Tomography

SD – Sprague-Dawley

MBV – Mandibular Bone Volume

MBCV – Mandibular Condylar Bone Volume

MBSA – Mandibular Bone Surface Area

MCBSA – Mandibular Condylar Bone Surface Area

MBMD – Mandibular Bone Mineral Density

MCBMD – Mandibular Condylar Bone Mineral Density

Chapter 1: Introduction

1.1 Mandibular Growth and related diseases with diagnosis and treatment

1.1.1 Background:

Vertebrates went through early evolution in the cephalization process. This makes the jaws specialized for the purpose of collecting food and processing them inside their mouth with a view to prepare them for the lower gastrointestinal tract environment for digestion. Clear-cut distinctions in fossil records of chordates as well as mammals have suggested the evolution of the temporomandibular joint (TMJ) as unique event throughout the life history in our planet [1] Notable earlier studies have pursued the neuromuscular reflects control for the detection of phylogenetic relationships between muscles [2], and human embryologic study of TMJ [3]. Remarkable conclusions has been drawn after anatomical and histological studies by Griffin et al are including but not limited to the lateral and posterior condylar movement on the jaw, irregularity in the thickness of the mandibular meniscus, normal, osteoarthritic (medio-lateral or antero-posterior), and excessive progressive remodeling (due to abnormal condylar position) [4].

Van Eriden [5] suggests from the values of elastic constants obtained by several group of researchers' that the cortical bone of the mandible is anisotropic and the latter is stiffer in longitudinal than in the radial and tangential directions. The author also depicted that the strength of the mandible is also larger in longitudinal than in transverse directions [5]. Moreover, when mastication and static incisor or molar biting happens the mandible is bent in the sagittal plane, which is the result of the vertical components of muscle forces and of the reaction forces at the

condyles and the chewing or bite forces. In addition to this, the magnitudes of sagittal bending moments and shear forces have been suggested to be dependent on the points of application, and consequently on moment arm lengths, of muscle and bite forces [5].

To understand whether neovascularization or endochondral mechanisms are more coherently involved with mandibular growth has drawn a number of research outcomes. Mandibular condyle is one of the major sites of growth regulation for the mandible. Sox 9 differentiation in the proliferative state regulates collagen X synthesis and transforms chondrobrast primarily to cartilage matrix and later on to hypertrophic chondrocyte cells by dint of maturation i.e. increasing the size of the cells [6]. Hypertrophic chondrocytes in turn induced new blood vessel formation by a) secreting the type X collagen to precede endochondral ossification and b) expressing Vascular Endothelial Growth Factor (VEGF). Later on, newly formed blood vessels mesenchymal osteoprogenitor cells invades and further differentiate into osteoblasts and osteocytes to render endochondral ossification and to regulate condylar growth (adapted from cascade of condylar growth [6]. Other authors reported that the onset of increase in bone mass occurs during childhood and puberty via endochondral bone formation [7]. At 20–30 yr of age, gradual increase in bone mass is seen until peak bone mass occurs with the decline in females after menopause.

1.1.2 Abnormal Mandibular Growth:

Although rarely observed compared to the other anatomical growth abnormalities the difficulties of curative approaches draw the researchers' attention to the craniofacial diseases. Not less than 30% women who abuse alcohol happen to deliver an infant affected by fetal alcohol syndrome (FAS) which may feature craniofacial dysmorphology. FAS can also happen due to moderate consumption of 2 drinks per day. Growth restriction may also happen indirectly due to exposure to antineoplastics (1^{st} trimester) anticovulsant (toxic epoxide radicals cause teratogenecity, might be associated with this anomaly) [8, 9]. These abnormalities include but not limited to Hemifacial Microsomia, Treacher Collins Syndrome, Nager Syndrome, and Pierre-Robin Sequence [10]. Other rare anomalies may include bifid (BMC) or trifid mandibular condyle (TMC) [11, 12] and tumor which can be benign or malignant [13]. Regardless of the type of anomalies majority of the above mentioned cases manifested by the altered growth in the mandible especially condylar cartilage [10].

1.1.2.1 Hemifacial Microsomia (HFM):

This congenital disease is manifested by unilateral condyle and underdevelopment of ramus, temporomandibular joint, ear and masticator, being primarily a syndrome of branchial arch [10, 14]. Literatures show different terms of HFM namely otomandibular dysostosis, lateral facial dysplasia, first and second branchial arch syndrome, unilateral intrauterine facial necrosis, unilateral craniofacial microsomia, and oculoauriculovertebral dysplasia [15, 16]. HFM has

a frequency of one in 5000/6000 life birth children (HFM; Online Mendelian Inheritance in Man database of Johns Hopkins University: http://www.ncbi.nlm.nih.gov/sites). However, there is debate about the frequency among authors.

1.1.2.1.1 Etiology:

Disturbance in odontogenic process has been claimed to [17, 18]cause underdevelopment of dental tissue which also has been supported by the fact that patients with HFM suffers from hypodentia due to disturbance in neural crest cell development since the presence and interaction between neural crest ectoderm and mesoderm is a requirement for normal odontogenesis [19]. Patients with Facial, auricular, and vertebral anomalies consistent with Goldenhar syndrome, was found to have growth hormone deficiency [20, 21], but it was unclear to both of the research groups whether this was an association by chance or a rare finding in the syndrome. The most comprehensive and one of the most commonly used classification for HFM [OMENS; (O = orbital distortion; M = mandibular hypoplasia; E = ear anomaly; N = nerve involvement; and S = soft-tissue deficiency)] has been suggested by Vento et. al. [22] and supported by others [23]. It includes the orbit, mandible, ear abnormalities, as well as soft tissue, facial nerve and extracranial problems together with assignment of normal grade usually subscribed with a zero (0) following the letter [23, 24]. Numerical values are assigned pertaining to various type of severity [22, 23]. Several authors have also classified the disease ranging from slightly hypoplastic mandibular condyle to

aplasia or severe hypoplasia or Kaban type III [10, 25]. Multicenter, risk factor evaluation study has reported pseudoephedrine use in the first trimester of pregnancy double the occurrence of hemifacial microsomia in the newborn. Multiple pseudoephedrine products happened to be reported as having similar risk profile as of the single once [9].

1.1.2.2 Treacher Collins Syndrome (TCS):

Also known as Franceschetti-Zwahlen-Klein syndrome which is a craniofacial development disorder representing anti mongoloid slant of the eyes coloboma of the lid, micrognathia, microtia and other deformity of the ears, hypoplastic zygomatic arches, and macrostomia [26] with a prevalence of conductive hearing loss and cleft palate. It has also been described to feature downward-slanting palpebral fissures, hypoplasia of the zygomatic complex, hypoplasia of the mandible, conductive deafness, any degree of microtia, and atresia of the external ear canal [OMIM #154500] & [27].

1.1.2.2.1 Etiology:

Chromosomal abnormalities specifically TCOF1 gene mutation was suggested to cause this autosomal dominant disorder. Defective encoding of a nucleolar protein by TCOF1 gene resulting in impaired maturation of the ribosomes in the neuroepithelial and neural crest cells which finally renders deficiency in the number neural crest cell migrating into the branchial arch [10, 26-28]. Prenatal study has revealed hypoplastic zygomatic bone, unusually short rami and corpus

along with excessive vascularization all of them manifested completely by fifteen weeks [29]. Strikingly similar pathological feature of mandibulofacial dysostosis has been reported by Lungarotti et al due to vitamin A toxicity in pregnant mother hypersensitive to the latter [30].

1.1.2.3 Pierre Robin Syndrome (PRS):

Pierre Robin, a Parisian stomatologist in 1923 described breathing problems in patients with glossoptosis and associated micrognathia and later on with cleft palate in 1934. Although this well-known eponym has had several name changes, this condition is often described as being Pierre Robin sequence, syndrome, complex or anomaly [31].

1.1.2.3.1 Etiology:

Role of a gene encoding transcription factor namely SOX9 has been suggested to the development of PRS, the former being known to be crucial for differentiating chondrocyte and chondrogenesis [32]. It also regulates collage expression during cartilage and endochondral bone formation [33, 34] which in turn is evident by fact that PRS patient having different collagen types with mutation in gene coding [35, 36]

1.1.2.4 Nager Syndrome or Acrofacial dysostosis:

In the literature it has been generally mentioned as additional but relatively rarer than the previously mentioned diseases where size and the shape of the mandible

is affected along with condylar growth [10, 37]. They are associated with condylar ankylosis and Turner syndrome with short mandibular body. Posterior rotation of mandible is also observed [38-41]. It has also been mentioned as a challenging disorder where a multidisciplinary team involving neonatology, otolaryngology, anesthesia, obstetrics, audiology, plastic surgery, hand surgery, and genetics is best suited to care [42].

1.1.2.4.1 Etiology:

Although 10% inheritance rate were described for Nager syndrome, the mode still remains unclear [42] since severe sporadic and autosomal dominant as well as recessive cases have been reported [43, 44]. None of the facts including karyotype duplications, translocations, and deletions [42, 45, 46] have been noted to be causative by the previously mentioned authors [42]. Clinical and pathological difference play an important role in differentiating the Nager syndrome from TCS in the prenatal stage since the latter has less severe micrognathia or retrognathism as compared to the former [47]. Also TCS is accompanied by retarded mental activity however Nager syndrome is not [42]. Paladini et al. has reported that micrognathia, forearm shortening, and malformed ears in conjunction with a normal karyotype are highly diagnostic for Nager Syndrome which has been found by Ultrasound [48].

1.1.3 Diagnosis of Temporomandibular Joint (TMJ) Condylar Abnormalities:

Proper selection of imaging technique to assess facial asymmetry is obvious. With this end in view, clinical differential diagnosis of the condyle or ramus cited in the literature includes both developmental and neoplastic conditions such as condylar hyperplasia, osteochondroma, chondroblastoma, osteoma, osteoblastoma, and chondrosarcoma [13]. Although an enlarged condyle and a longer mandibular neck on the affected side will be shown by Panoramic radiography and computed tomography (CT); they cannot provide information regarding the activity of the abnormality which can be obtained by a comparison of follow-up images for the assessment of the tumor growth [13]. Same authors has also mentioned that in case of immediate detection, skeletal scintigraphy using technetium-99m methylene diphosphate used along with single photon emission CT, or positron emission tomography (PET) using a radiolabeled glucose analog, 18-F-2-fluoro-2-deoxyglucose (FDG), as a tracer, alone or combined with CT (PET/CT), might indicate increased cellular activity on the affected side [13]. To determine the presence of active process in a growth center scintigraphy, single photon emission CT, and PET are useful [49]. Hence, Venturin et al. argues for combination of morphologic and physiologic imaging modalities that offers improved analysis of benign and malignant lesions with a cautionary remark on the fact of FDG uptake by the brain, liver, spleen, bowel, and tonsils which are sites regarded for high glucose metabolism [13] which in turn may precipitate inflammation and infection, and the assessment of abnormal activity becomes cumbersome [50]. Besides, FDG-PET has also gained researchers interest recently still not proven to

be self-sufficient to replace biopsy especially for diagnosing the cartilaginous tumor of bone [51, 52].

1.1.4 Available treatment options for mandibular diseases

1.1.4.1 Tracheostomy:

Clinical management rely on surgery and devoted mostly to manage the airway. Special positioning for the instrument is required for the neonates along with craniofacial reconstruction as suggested by [53]. Conversely, some authors have also emphasize the use of tracheotomy as a cascade joined with mandibular distraction [54].

1.1.4.2 Mandibular Distraction Osteogenesis:

Mandibular distraction osteogenesis seems to revolutionize the treatment of airway obstruction and craniofacial deformities resulting from mandibular deficiency and eventually taking over the tracheotomy in newborn infants suffering from the same [55]. This technique and the device was first developed through Kirschner wire bows to repair complex fraction and non union of bones by a self-trained internist, surgeon, obstetrician, pediatrician, and orthopedist ultimately an unparalled genius of his time Gavril Abramovich Ilizarov as obtained from the quotation of Vladimir Golykhovski, M.D. [56]. Notable development has paved this technique towards the manipulation to treat airway obstruction resulting from mandibular abnormalities [57-61] along with obstructive sleep apnea [55].

Intraoral and external approach combined with three dimensional CT scan evaluations results in subsequent decision making about the location of osteotomies to be performed which relies mainly on the vertical height of the

ascending ramus, the anterior projection of the body, the formation of the temporomandibular joints, and the exact location of the lingual nerves within their bony canals [61]. These authors have also mentioned some requirements in their published literature are given below:

Preoperative CT scans are used to plan whether the osteotomy will be proximal or distal to the angle of the jaw

With both intraoral and external approach the periosteum of the mandible must be exposed approximately 2 cm on either side of the angle

The osteotomy should always be proximal or distal to the angle, but never through it to preserves the mandibular angle as an important

Contour of the facial skeleton

If the external approach is used, care should be taken to identify the internal notch of the mandible so that the osteotomy is not inadvertently carried superiorly into the ascending ramus

Marking from the alveolar surface to the inferior edge of the mandible has also been suggested [61].

1.1.5 Complications with surgical techniques:

Significant morbidity and mortality has been reported in tracheotomy by authors. In addition to this major complications has been observed through Bacterial colonization from cultured tracheal secretions, crusted airways, tracheobronchitis, pneumonia and tracheobronchial bleeding in the form of bloody secretions tracheo-arterial erosion with massive bleeding, tracheo-oesophageal fistula and

the most common tracheal stenosis [62]. Prolonged intubation before the tracheotomy has been criticized by authors to precipitate tracheal damage [63]. Some author have also pinpointed the swallowing and aspiration problem occurred after tracheotomy [64]

Limitation of distraction device may nullify the prosperity of the Mandibular Distraction Osteogenesis [65]. Although, it has also been suggested to use preoperative computerized tomography scanning and three dimensional stereolithographic model formation in vector planning for aberrant mandibular anatomy [65] however, availability of those cutting-edge diagnostic and analytic procedures and equipments cannot be warranted in clinical perspective. Permanent dentition loss, malformation, or cyst formation on the second molars and premolars due to the placement of the osteotomy was reported however, limited positioning of the mandibular angle and also possibilities of significant blunting of important facial landmark may also restrict placement posterior to the tooth buds as suggested by them [65]. Also, the authors admitted the incident of sacrificing tooth bud as a compromise in order to achieve the optimal vector of distraction for airway improvement [65]. Other complications as cited by the previous author may include TMJ abnormalities, ankylosis, and subsequent mandibular growth disruption resulting malocclusion [66-68].

Based on the reports and literatures published to address the mandibular retrognathism, it is obvious to address some key points like, changing the modality of current treatment option, introduction of therapeutic agents as treatment choice in addition to surgery. To the best of our understanding, no

pharmaceutical agent until now has been introduced into the therapy of such diseases. Potent growth factors has been used to alleviate the surgical intervention however, the number of study and the extent of information are limited. Also, different studies finding cannot be correlated with each other. Having all those paucity in this particular research field it is obvious to explore potential therapeutic agents to induce intact bone growth as well as to heal the fractured bone with minimum requirement of cumbersome and lengthy surgical bed operations. It is also important to explore the basic science of mandibular growth in general population to aid further research.

1.2 Growth Hormone; its physiological role in bone and other tissues

1.2.1 Introduction

Human chromosome 17q24.2 of 46.83 kilobases contains genetic locus that encodes GH [69, 70], is a part of a family of five structural genes located on the long arm of human chromosome 17 and oriented in the transcriptional 5' to 3' direction the order of which being 5' hGH-N, hCS-L, hCS-A, hGH-V, and hCS-B 3' [71] which is produced from somatotrophs of common primordial cell of the mature pituitary [72, 73]. GH mRNA rises from 16 to 27 week of gestation in pituitary [74]. Meanwhile, GH itself reaches to the climax in between 25 to 30 weeks' of gestation and until term remains constant as suggested in the early 70's [75]. At the postnatal stage, metabolic homeostasis and maintenance of normoglycemia necessitates GH for proper linear growth [73]. In puberty, mediated by sex steroids, GH secretion increases in terms of rate of production and the actual mass however, the biological half-life does not increase and GH secretion peak gets back to the pre-pubertal amount at the advent of adult age [76-78]. GH production rate has been shown to decrease by 14% along with a 6% decrease in half-life for every 10 years increase in age in healthy individuals [79]. Change in sex-steroids activity may also contribute to this [73].

1.2.2 Age related physiological role of GH

This well-known anterior pituitary hormone serves many established functions in the body regarding growth, metabolism and lot of others [80]. Studies show

beneficial metabolic effects associated with GH replacement [81, 82]. Stimulation of amino acid uptake and that of protein synthesis are two known functions of GH [83, 84]. On the other hand, high protein meals and administration of basic (arginine and ornithine) and aromatic (tryptophan) aminoacids, stimulate GH secretion in normal subjects[73, 85].

Not only in the neonatal or juvenile age but also in the later elderly age GH has shown to be beneficial for bone growth by either its direct action on the skeletal tissue or mediated through the local production of insulin like growth factor 1 (IGF1), a single chain 70 amino acid containing polypeptide [7]. Conversely, Meta analytic and other studies have not concluded the muscle strength increase by human growth hormone [86, 87]. Limited number of studies of the cardiac and pulmonary impact of GH did not find any rigid conclusion on whether or not irregularities of GH are associated with any pathological effect on the former because of contradictory findings [88, 89]. However, GH therapy has shown some beneficial effects for treating the cardiopulmonary disorder associated with GH anomaly [90, 91].

1.2.3 Important patho-physiological aspects of GH

GH secretion is reduced in obese patients [92]. Some author reported 50% decrease in daily GH secretion happens due to 1.5 kg/m^2 increase in body mass index (BMI) [93] while others did not report any significance [94]. Again, researchers did not find hyperglycemia in case of the obese patients to be a cause of inhibition of spontaneous and stimulated GH secretion [95-97]. GH is long been

known to be lipolytic which in turn reduce GH secretion acting at the pituitary and the hypothalamus [98].

1.2.4 GH and other related hormones in endocrine system

In cultured pituitary cells, thyroid hormone was shown to be activator of the GH and to regulate the activity of glucocorticoid hormone on GH mRNA [99]. It is supported by the action of GH on growth plate as well [100]. Direct or indirect thymus epithelial cells has also been suggested by some authors [101] while other dictates for only indirect i.e. IGF-1 mediated proliferation[102]. Regardless the ambiguous findings of the different mechanistic pathways GH has been confirmed by researchers to improve thymic functions along with thymocyte proliferation and migration, which potentiate the fact that the former might well be suited as a treatment adjuvant in peripheral T cell deficiency as well as decrease in thymocyte leading to immunodeficiency [103, 104].

1.2.5 A glimpse of molecular biology of GH

Specific genes' activation is required in response to the GH treatment for the signal transduction pathway leading from the cell-surface GH receptor to the nucleus among which IGF-1 is highly conserved [105, 106] and the latter was shown to be induced by GH long before [107]. Meanwhile, GH receptor proteins was mentioned to share limited amino acid sequence similarity in the extra-cytoplasmic domains along with lacking intracellular domain regions to be

suggested for G-protein coupling mechanism although belonging to the family of cytokine/hematopoietic receptor family [108-110].

Figure 1.1: Mechanism of Gene Expression by GH in Cellular Level

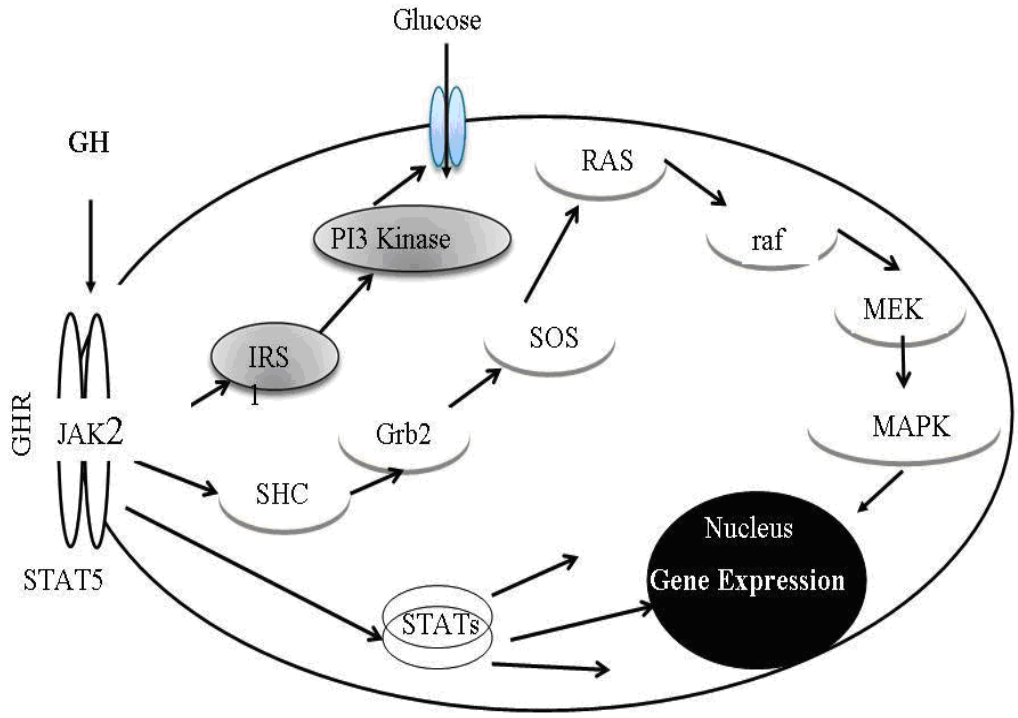

Figure 1.1: Mechanism of Gene Expression by GH in Cellular Level. Dimerization of receptor followed by the induction of Janus tyrosine kinase 2 (JAK2), an intracellular tyrosine kinase stimulated by GH as well as prolactin [111-114] and other members of the cytokine/hematopoietin family and hence it is not so special for GH only [115, 116]. Later on, pathway leading to mitogen activated protein kinase (MAPK) activation through several cascades of intermediate adapter proteins' expression finally leads to the expression of genes in the nucleus [117].

GH causes rapid stimulation (from 15 to 120 minutes) of IGF-I, c-fos, c-jun, JunB, serine protease inhibitors, Spi 2.1 and 2.2 [118-123] . Among them c-fos activation has been shown to mediate through serum response element (SRE) by a region element appear on the former abbreviated as SIE [124]. Similarly single region was detected within the IGF-I locus where rapid alterations by GH in chromatin structure happens.[105].

1.2.6 Effect of GH on skeletal growth

Despite of decades of research on growth hormone, generally accepted adult population studies for GH is scarce since the study methodology employs hGH-deficient adults mostly, and from where extrapolation to healthy subject not seems to be logical [84]. Sport discipline has been suggested to be a remarkable exception [125] although might still lack acceptance due to the bias of the physical conditions as well as different ways of GH intervention by the sportsmen (for example, by doping and abusing) happen to be remarkably different from general population throughout the world. Earlier researchers relate the GH induction and its impact on cartilage. GH and somatomedin was described to regulate the growth of epiphyseal cartilages of long bones, of spheno-occipital synchondrosis of the cranial base, of the cartilage of the nasal septum, of lateral cartilaginous masses of the ethmoid, of cartilage between body and greater wings of the sphenoid (all stemming from the primary cartilaginous skeleton of the organism) condylar, coronoid and angular cartilages of the mandible, of the cartilage of the midpalatal suture, and of the cartilage in some cranial sutures (all

of secondary formation during phylogenesis and ontogenesis). GH is termed as general extrinsic factor which has a marked dissimilarity with orthopedic devices since these devices can alter the direction but not the amount of growth [126].

Later on, classic Somatomedin hypothesis suggested the action of GH on bone relies on the IGF-1 production in liver [107, 127]. Conversely, earlier researchers did not agree with the Somatomedin hypothesis when they observed the stimulation of longitudinal bone growth after local injection of GH into rat tibia [128].

Bone resorption and bone formation conjointly regulate bone remodeling. Both of the processes are regulated by GH. For the formation of bone, GH either alone or through IGF1 controls the process, which is well known to be "dual effector theory" as evidenced from different findings of the researchers [128-130]. Conversely, the mechanism of resorption by GH is not known [131].

Figure 1.2: Regulation of Skeletal Growth by GH.

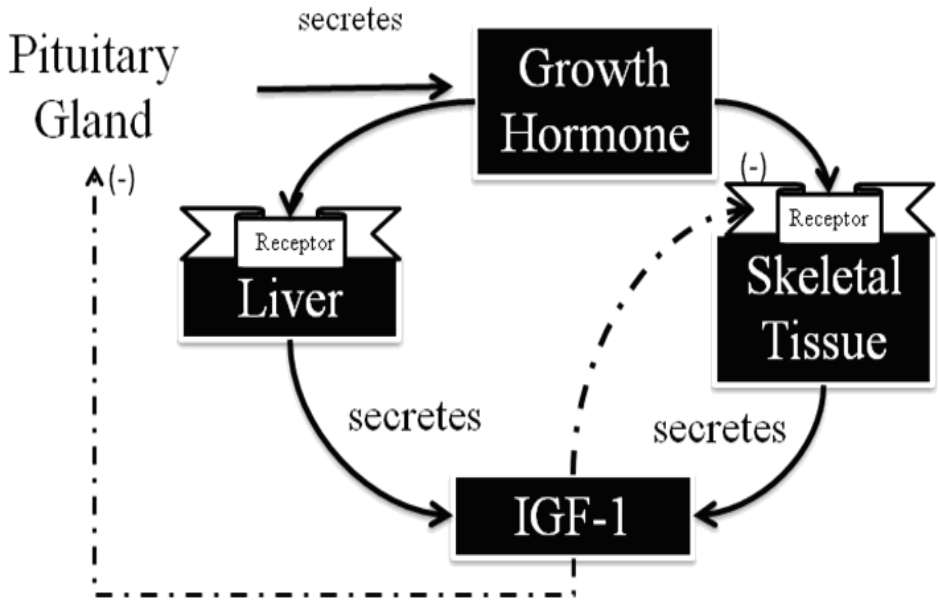

Figure 1.2: Regulation of Skeletal Growth by GH. Contrasting the Somatomedin theory, GH has been suggested to stimulate skeletal growth directly by binding to its specific receptor on the growth site after being secreted by the anterior pituitary gland. Excessive GH in turn secretes Insulin Like Growth Factor -1, the latter, however, prevents further binding of GH with its receptor in skeletal tissue and hence presents feedback mechanism. This mechanism however does not eliminate the importance of liver derived IGF-1 feedback as well (adapted from Leung, K et al, 1996, and Ohlsson, C et al, 1998).

Histomorphometric analysis showed 2% skeletal mass increase because of osteogenesis after treatment with GH for 3 months in adult mongrel dogs [132]. GH was found to act on the resting cell zone but not to differentiated chondrocyte in the growth plate by binding to the specific site present on the former [133].

Again, capacity of GH and IGF1 was assured by such technique to stimulate prechondrocyte by reducing the cell cycle time of the latter and indeed GH induced cell cycle time reduction was 50% less than IGF1 confirming the direct action of growth hormone in single or multiple steps on the growth of chondrocyte in terms of cell volume, height, and matrix production per unit time [134]. In addition to this, renal 25-hydroxyvitamin D-1 alpha-hydroxylase activity is stimulated by GH, which in turn enhances calcium and phosphate absorption in the intestine. It also increases the maximal renal tubular reabsorption of phosphate. On the other hand, excessive GH induced osteoarthritis and other serious metabolic side effects have not dictated for its safe utilization in clinical premises [131]. Having all these, a biphasic model for GH action on skeletal tissue was suggested by Ohlsson, C. et al (1998) which dictates that bone formation induction by GH follows the resorption and a transition point was determined [7] when bone formation supersedes the resorption [131]. It is very unlikely that those authors include biomedical or biophysical interventions in their comment about effectiveness of combination therapy in conjunction with GH. Those were merely combination with other potential therapeutic agent varied in chemical entity.

On the contrary, accelerated bone resorption and decreased bone formation cause postmenopausal and age-related bone loss [135]. Decrease in growth factors, [136] with or without GH [137, 138] and IGF-1 levels [139-141] decrease bone formation. The physiology of bone loss in aging women and men may be attributed to the fact of deficiency of gonadal sex steroid on the skeleton.

Commencing with estrogen deficiency in early rapid postmenopausal bone loss and proceeding with hyperparathyroidism and vitamin D deficiency later in life for age related bone loss in woman [135]. Apart from that, estrogen deficiency also plays a dominant role in the physiology of bone loss in aging men [135]. Impairment of the growth hormone/insulin-like growth factor axis with different other factors comes afterwards but its importance in the neonatal development until juvenile growth has remarkably induced the researchers to focus on its therapeutic mechanisms.

1.2.7 Understanding the importance of GH on mandibular growth

For mandibular growth the importance of GH was reported in early 80's. At a dose of 2.5 nmol/litre GH enhanced the overall growth of cartilage explant and stimulated the differentiation of its cells which resulted in a substantial increase in the number of chondroblasts and young hypertrophic chondrocytes. Additionally, GH also stimulated new bone formation adjacent to mineralized hypertrophic chondrocytes supporting that GH stimulation of cartilage growth which in turn is followed by endochondral ossification leading to mandibular growth [142]. It's in vitro effect it is not as yet clear whether the effect of growth hormone is indeed a direct one or is mediated via the local production of IGF-I. Some recent findings dictate for the therapeutic effect of GH utilization to induce mandibular growth. Topical GH application stimulates new bone formation significantly, as GH was supplied with osteotomy [143]. GHR polymorphisms P561T and C422F were shown to be associated with mandibular ramus height in Japanese population

[144] however, some deny the P561T to be useful for the mandibular growth [145]. Another study confirms the synergistic effect of GH and melatonin to enhance new bone formation during early-stage healing process [146]. Stimulation of mitotic activity and delaying the maturation of cartilaginous cell in mandibular condyle has also been observed by the researchers [147] which gives us a hint about how might the GH happens to induce the bone growth in the secondary cartilaginous region.

Earlier research suggested for positive correlation of GH and skeletal growth including that of mandible. From intact bone to induced injury studies in animal skeleton has proven the potential of this well known therapeutic approach. GH alone may induce variety of side effects systemically, however proper administration is probably the key fact to avoid such incidences. In addition to this, combination treatment of growth hormone with other potent bone growth inducer might allow us to decrease the dose into a level such that it does not induce side effects. Hence, it will remain a precious topic of research for searching appropriate growth inducer to be used with GH. Appropriate doses of both of the treatment approach might also be studied and optimized to ensure optimum benefit for the growth. This can be achieved by performing further research on appropriate animal model for dose titration and on appropriate cell culture model to understand the molecular biology of both the treatment so as to understand any potential untoward, as well as stress effects.

1.3 Therapeutic Ultrasound: Its implication in biology and therapeutics

1.3.1 Background

It is almost going to be a century since the therapeutic applications of ultrasound was first recognized to produce lasting changes in biological systems apart from its use as an imaging technique [148].

1.3.1.1 Definition and Classification

Ultrasound means sound whose frequency is too high to be perceived by the human ear, i.e. above 20000 Hz. Up to several GHz of ultrasound frequencies even present on the top of the scale. These waves are in brief, traveling vibrations. Therapeutic ultrasound can be divided into two classes according to Gail ter Haar (1999):

Low intensity (0.125–3 W/cm^2) – to stimulate normal physiological responses to injury, or to accelerate some processes such as the transport of drugs across the skin

High intensities (≥5 W/cm^2) – to selectively destroy tissue in a controlled fashion

Alternatively, it can be classified based on terms of applications i.e. whether the coupling of sound directly to the tissue or via a coupling medium [149]. This author had presumed the thermal and non-thermal mechanism of Ultrasound action [149]. Thermal mechanism were based on the fact of attenuation of ultrasound beam energy while passing the tissue and hence, theoretically the

intensity is inversely proportional to the distance to be covered which had been expressed as

$I_{(x)} = I_0 e^{-\mu x}$ where;

$I_{(x)}$ = intensity at a distance x from a reference point in the tissue

I_0 = intensity in the beam at a reference point in the tissue

x = distance

μ = intensity attenuation co-efficient

For non-thermal mechanism micro-massage and diffusion of ion resulted by local radiation effect were proposed [150]. But the precise mechanism still remains unknown.

1.3.1.1.1 High intensity focused ultrasound (HIFUS)

HIFUS has been a subject of interest for medical research for half a century. Selective tissue necrosis is manifested through HIFU in a very well defined volume, at a variable distance from the transducer, through heating or cavitation [151]. In addition to this, being the provider of non-ionizing radiation, the thermal and mechanical effects of acoustic radiation (i.e., sound waves) that delivers to target tissues repeatedly one or more times in multiple sessions, also perform necrosis[152]. Near-instantaneous coagulative necrosis and cell death results in through this thermal effect which is achieved by heating tissues to 60°C or higher [153] with more destruction occurs within the focal plane compared to tissues outside the target area [152]. However, it was also claimed that due to the variability of cell death versus temperature increase from cell lines obtained from

different species it is difficult to assign a fix value for the purpose. Eventually, there is no complete reference for the human tissues that would show temperature versus cell death in a predictable and reproducible manner [153].

1.3.1.1.2 Low-intensity pulsed ultrasound (LIPUS)

LIPUS has gained researchers interests later than its counterpart. A good number of studies published in the literature trying to utilize it as a treatment modality in different tissues of human or animal body. Difference in tissue response imparts problem to understand the underlying mechanism of action. However, suggestions have been made regarding the absorption of ultrasonic energy proportionally to the density of the tissue which the former is passing through. So difference in effects on different hard tissues is also expected [154]. The most important aspect of this intervention might be attributed for the skeletal tissue whether it is fracture healing or growing an incomplete bone. Pulsed, low-intensity ultrasound energy, similar to that used for diagnostic purposes, can have beneficial effects on bone tissue. Some of the published reports support the fact of healing [155] as well as accelerating bone maturation [156] whereas some others decline [157]. A gradient of mechanical strain is stimulated when LIPUS is absorbed in different extent in tissues in the healing callus that stimulates periosteal bone formation [158, 159] which informs us how LIPUS may influence the inflammatory and soft callus formation phases of fracture healing [160].

1.3.2 LIPUS parameters

Several different low intensities and frequencies have been utilized with a view to optimize LIPUS exposure as well as treatment. Earlier researchers have used different intensities and obtained results that have guided the later ones to decide the optimum parameters to be used to serve their purposes. Table below shows different values of LIPUS intensities along with the results obtained.

Table 1: Effects Different LIPUS Intensities Used by Earlier Researchers

LIPUS Intensity	Result
11.8mW/ cm^2	Improved radiographic fracture healing and increased bone density in rat femora [161]
30 mW/cm^2	significantly improved interface defect filling and subchondral bone regeneration [162]
50 and 100 mW/cm^2	Increased maximum torque and a slightly decreased torsional stiffness using 50 mW/cm^2 compared to 100 mW/cm2 [163] and Untreated controls had significantly lower maximum torque and torsion stiffness compared to 50 mW/cm^2 treated femora, but not compared to femora treated with 100 mW/cm^2 [163]
120, 390, and 1490 mW/cm^2	Alkaline phosphatase expression positively correlates [164].

In addition to the intensity, the frequency of the US signal has also been investigated in stimulating bone osteogenesis. Earlier, researchers showed using

higher intensity (500 mW/cm^2) pulsed US that 78.6% of 1.5MHz and 56.2% of 3.0MHz treated rat fibulae fractures had more advanced radiographic and histological healing than untreated controls [165]. There was also no significant difference in maximum torque in rat femora [166] with LIPUS (30 mW/cm^2) treatment using a frequency of 0.5MHz compared to 1.5MHz. On the contrary, significantly greater bone mineral density was observed with 1.5MHz 1 kHz repetition rate, 200 μs pulse duration, 30 mW/cm2 spatial intensity [167]. A carrier frequency of 1.5MHz has been more commonly used to stimulate osteogenesis experimentally and clinically.

1.3.3 Important findings related to LIPUS - in the light of biology and therapeutics

There are a number of studies were published having considerable similarity existing in the intensity of the pulsed ultrasound and the outcomes were both significant and not. In some of the cases, there is no mention of the intensities as well. 3 MHz and 1 MHz frequencies were used in the reports that were evaluated in systematic review by Robertson V et al [168]. Among them difference in intensities were observed. 20 minutes daily application of LIPUS characterizing 200-microsecond burst of 1.5-MHz sine waves repeated at 1.0 kHz was used to assess the rate of fibula osteotomy healing in rabbit non-invasively; incident intensity being 30 mW/cm^2 and by these specific frequency and intensity LIPUS happened to result in better profile of maximum strength although not significant [169]. Similar non significant result under similar condition was obtained in the

case of longitudinal bone growth of rats including bone mineral density [157]. On the other hand, LIPUS treated recovery from fracture was found significant on patient with similar parameter of incident intensity, and frequency compared to control group in a computed tomography evaluation [170] and also in rabbit when BMD was accounted for [156]. Reasons for these apparently contradictory findings might be attributed but not limited to appropriate cell recruitment, and appropriate time of gene expression [171] i.e. the sequential steps of the biological system to undergo healing in response to LIPUS. Three overlapping phases has been described to represent the complexity of the fracture healing formation such as:

Inflammatory phase - where hematoma forms from ruptured blood vessel and invades the clot,

Reparative phase - where healing overlaps the end stage of inflammation i.e. invasion of pluripotential mesenchymal stem cells which in turn differentiate into fibroblast, chondroblast, and osteoblast resulting in soft fracture callus to be and finally woven bone. This phase is also accompanied by angiogenesis and mineralization.

Remodeling - which is the slowest stage and overlaps significantly with the reparative phase resulting in slow progression from woven bone to mature lamellar bone and also require higher strength of stimulus in maintenance stage than the bone formation itself [171, 172].

Same authors have also pointed the primary factors contributing to the occurrence of delayed union and non-union namely the severity of the fracture, the location

of the fracture, the nature of the blood supply to the bone, the extent of soft tissue damage and its interposition, bone loss, air contact and contamination, and whether a tumor is involved and the former can also be increased by the treatment itself via inadequate reduction, poor stabilization or fixation, distraction, damage to the blood supply, or postoperative infection. The author also convicted the systemic factors such as smoking, alcoholism, age, and diabetes for severely interrupting the healing process [171].

Two interesting case reports have been published regarding two soccer defenders having right clavicle bone non-union and right mid-foot injury. A 9 mm diastase of Magnetic Resonance imaging fragments in the right clavicle of the first one was reduced to 5 mm after 6 week of LIPUS treatment whereas the fifth metatarsal fracture of the second player after 3 months of the same treatment consolidated the fracture and the reporters claimed both the players performing the highest level during that time [173]. Meanwhile, disagreement in researchers is also known [174]. Furthermore, researchers have suggested LIPUS treatment to commence within 6 months of the most recent operation since the LIPUS is effective, non-invasive, and riskless and hence the treatment might be considered as the first treatment choice for cases of postoperative delayed union or nonunion [175].

Apart from in vivo, study in bone marrow derived stromal cell clone ST2 cells responded to LIPUS in a way that of up regulation in expression of IGF-1, Osteocalcin and Bone Sialoprotein; three important markers for bone growth [176]. A single 20-min dose of LIPUS was also found to stimulate expression of

the immediate-early response genes c-fos and COX-2 and elevate mRNA levels for the bone matrix proteins ALP and OC indicating that the LIPUS has a direct effect on bone formation [177]. Other researchers have also found similar expression pattern together with Egr-1, TSC-22 and bone differentiation marker gene like Osteonectin (ON), and Osteopontin (OP) in 3 hr [178] in real-time polymerase chain reaction (RT-PCR). Apart from these, LIPUS was suggested to stimulate herniated disc resorption in vitro [179], and greater but delayed stimulation of Cbfa-1/Runx2, IGF-receptor, Alk-3, alkaline phosphatase (ALP), osteopontin, TGF-beta1 and BMP-7 in bone marrow stromal cell [180]. These expressions also supported by others in mouse osteoblast cell where expression was significantly higher in ALP, the marker of early osteoblast differentiation [181]. Some researchers were interested in evaluating stress related parameters for example Vickers hardness [182], bone strength index (BSI) [166], failure load and ultimate strength [183]. Earlier, LIPUS has induced a stimulatory biologic effect (intracellular activity, cytokine release and the bone healing process) in vitro studies using cell cultures and research on experimental fractures in animal models [150, 184, 185] and also altered the time course or the sequence of gene expression of several genes, like aggrecan (a proteoglycan), involved in enchondral osteogenesis [163]. LIPUS significantly affects the cellular stress signaling in a pathway where calcium release is involved [186] and also increases total collagen content [187]. Moreover, stimulation of proliferation and differentiation of the fibroblasts, chondroblasts and osteoblasts by increasing fluid

flow and nutrient delivery as well as waste product draining has been reported to happen through the acoustic pressure waves at the fracture site [171, 185].

Another potential approach through which LIPUS induce osteogenesis has been suggested to be therapeutic angiogenesis [188], a treatment or preventive modality to subside hypovascularity and to induce tissue regeneration and healing through neovascularization and neocellularization by utilizing angiogenic factors like IL-8, bFGF (basic Fibroblast Growth Factor), and VEGF where traditionally surgical methods have been employed [189]. But growth in mandibular cartilage has been widely recognized to be favored by endochondral ossification where pressure adaptation towards the direction of the growth is more important rather than intra-membrenous.

LIPUS is combination with other growth inducers show different pattern of induction of bone growth. Study with bone morphogenic protein 2 (BMP 2) did not show any synergistic benefit in growth and separately BMP 2 was quicker in stimulation the responses of bone growth inducing markers than LIPUS although not greater [180]. Conversely, LIPUS along with anterior mandibular jumping appliances enhances mandibular growth in growing baboons [190].

Important findings of growth modifications in baboon monkeys were obtained when jumping appliance were accompanied with that [190]. This finding gives us a progressive idea in the field of skeletal growth research since higher animal shows significant change in the mandibular length and growth activities. Earlier it was studied on rabbit in the same manner and similar result was obtained in the light of endochondral bone growth and mandibular ramus height [191]. Their

histological studies were also in coherence with the quantitative result obtained from mandibular parameters. In both the cases Ultrasound was applied for 20 minutes/day for four weeks to the left of the mandible in each rabbit. The US was applied using a 200-microsecond burst of 1.5 MHz sine waves repeating at 1 kHz that delivered an incident intensity of 30 mW/cm^2 and so the reproducibility of the result was remarkable. In between these two the same laboratory group published a significant decrease in resorption area in the premolar root as well as the number of resorption lacunae which tentatively gives an idea of induction of growth since the ossification supposed to dominate as the resorption gets down-regulated [192]. Now if this finding is similarly applicable to the bones in the other part of the craniofacial system is a must needed research topic. As earlier it was mentioned that the implementation of the growth induction in the particular area of the mandibular system might be supportive to either induce growth or repair malformation, research in growth induction in particular area of the jaw is important. It is well known that mandibular condyle is one of the major growth centers governing of the overall mandibular i.e. craniofacial growth. Although the other part of the mandible has also been described as to control the growth but research in the condyle is by far the most among them. Eventually, it brings to our mind to apply the therapeutic low intensity ultrasound locally to the mandibular condyle so as to evaluation the local effect of the former and its impact on whole hemimandibular growth. However, since previously mentioned published literatures present the fact that growth induction happens very slowly which might poke the issue of patient adherence to the technique. With this end in view, we

understand the necessity to potentiate the growth induction by potent stimulant along with LIPUS.

1.4 Hypotheses and objectives:

1.4.1 Hypotheses:

Based on the literature reviews we are proposing the following hypothesis:

Hypothesis 1: Daily injection of GH and US into the posterior attachment of rat mandibular condyle will enhance whole hemimandibular growth

Hypothesis 2: Daily injection of GH and / or LIPUS into the mandibular condyle will enhance condylar growth

Hypothesis 3: Daily local injection of GH into the posterior attachment of rat mandibular condyle will have no systemic side effects on the body weight, and GH markers

1.4.2 Objectives:

Objective 1: To determine the effect of growth hormone and/or local application of LIPUS on whole hemimandibular bone volume and bone surface area in male Sprague Dawley rat.

Objective 2: To examine the effect of growth hormone and/or local application of LIPUS on mandibular condylar bone volume, bone surface area, and bone mineral density in male Sprague Dawley rat.

Objective 3: To determine whether the treatment with growth hormone and/or local application of LIPUS will cause systemic side effects.

1.5 Rat as an Animal model:

Importance of laboratory animal in research and pre-clinical study is paramount. Mandibular disorders have been assessed on different animal populations. Craniofacial malformations such as retinoic acid syndrome and Treacher Collin syndrome has been shown to induce to the animals resembling the human like features [193] and microscopic study has suggested the coherence of human mandibular disostosis with rat [194]. Appropriate animal model for the hemifacial microsomia has not yet been suggested. Not surprisingly, it is always difficult to extrapolate results from any animals to the human being but this fact does not outweigh their necessity. Babbon monkeys [190], rabbit [191], fetal lamb [195], transgenic mice [195, 196], dog implant [143, 197] and rat [194] have been previously used. Among them expression of hemifacial microsomia gene (hfm) expression in mouse makes it an exceptional choice for HFM study [196]. In contrast to all of those our current research is not related to induce any malformations and subsequently treat them according with treatment of choice. Our research can be categorized as basic understanding of normal mandibular growth induction. The combination treatment we used, to the best of our knowledge has never been utilized before on any animal. So, going to a conclusion of exact animal model for our cause is not possible with current understanding about the normal growth.

Rats are everywhere available, widely used animals for laboratory experiments. They are easier to handle than other animals and require much less space and care from technical personnel. Higher mammals like baboon monkeys have

resemblance with human jaw; however, being a higher mammal, protocol compliance with this animal in North America is not easy. This makes higher mammals not suitable for our purpose. Also, permission to perform study in higher numbers of such animal is restricted too and hence attainment of reliable, reproducible, and even valid data can be questionable. Eventually, rats have a superior advantage of complying with the animal protocols since those are easily attainable in Universities and other research institutions throughout the world. Wild type rats (not transgenic) are also somewhat less expensive than other animals. Above all, their size is another remarkable advantage to be worked with MicroCT. All the extracted mandibles can easily be kept on the gantry of the MicroCT machine and analyzed. Rat might be regarded as better options than its smaller counterpart mouse because of the ease of techniques we are using in this study to apply on the mandibular condyle. Locating the injection site is somewhat easier than mouse in this perspective providing the use of higher gauge needles (>22). Small LIPUS transducer (square shape with 1 cm^2 area) is suggested for this study.

Nonetheless, there are some remarkable drawbacks for rat being an animal model of this study. One major aspect of craniofacial study might be attributed to the positions of teeth in the mandibular bed. For rodents, (including rats) the incisors protrude all the way inside the cortical bone of the either sides of the mandible which is a remarkable difference between themselves and human being. Also, prominent angular process in the ramus is not present in human mandible which is also an obvious difference. Choosing higher mammal might solve the issue but, as

described earlier, acquiring necessary protocol compliance is not easy with mammal cohort. Apart from the scope of this study, applying surgical technique may be difficult for rat because of its small mandibular size.

1.6 Micro-Computed Tomography (MicroCT) Image Process and Analysis

Primeval articles reported in literature on using computerized technology in X-ray published in 1970s [198, 199]. Since then advancement of science bestows us with facilities to quantify cortical and trabecular bone peripherally as of recent [200]. Micro computed tomography (MicroCT) is a non-invasive technique where both ex-vivo and more importantly in-vivo scanning can be performed with small laboratory animals like rat and mouse followed by three dimensional image reconstruction (Micro Photonics Inc. Allentown, PA). Apart from simple mapping of internal anatomy particular molecular events has been made possible to detect and assess due to the availability of different probe [201]. Although widely used in orthopaedic study analysis of research issues in dentistry is also not uncommon and increasing day by day. For human dental regions, computed tomography is regarded as an essential noninvasive and nondestructive tool [202]. Recently Micro-CT imaging study has also been used to study coronary microvessels' formation in an animal model [203]. Sub-retinal implant assessment has also been reported [204].

MicroCT is clearly an important tool in in-vitro studies with bone tissues. Extracted bones from animals are subjected to be fixed by using 10% formalin followed by storing in -80 degree to avoid sample degeneration. Different sizes of gantries allow different shape of bones to be placed on them. Larger gantries have obvious advantage for the smaller bone that multiple samples can be run according to the capacity of the software to batch up the sample. It is important to bear in mind that the samples should be kept sessile inside the machine and more

importantly during the time of scanning. Since a gantry movement is controlled by software it is important to make sure that the positioning of the sample does not change otherwise reproducible, image and data will not be available for analysis.

Obvious prerequisite for the in-vivo study is anesthesia of the animal. Proper onset of anesthesia is necessary since the movement of animal can seriously jeopardize the acquiring of images. Movement in animal body especially on the region of interest gives fuzzy appearance and the whole time and effort of the scanning render useless. In addition, correction for animal breathing is required to obtain optimum quality images and subsequently reproducible data to analyze which is important for the in vivo study design (not for our purpose). Well-equipped ventilation support for the animals undergoing different stages of anesthesia is also important. Also this equipment should be installed in close proximity to the MicroCT scanner for the shortest most distances to cover from the anesthesia bed to the CT scanner. Different set up gantries for animal setting are available to give room to rats and mice.

Symmetry of human body is a unique matter. The left and right division of organs often helps research having an internal control achieved. If we perform intervention on one side, the other might work as internal control depending on the treatment pursued. MicroCT images for the mandibular bone in both ex-vivo, in-vivo cases provide opportunity to select specific region of interest around both the left, and the right hemimandible and quantification of bone parameters can be compared as ratio or percentage. However, the need of traditional control cannot

be outweighed by any means since without proper evidence we cannot just ignore the possibility of effect on right side due to the treatment on left side and vice versa. For small animals like mice and rats the left and right sides of mandibles are separable and can be scanned separately however that increase the scanning time and labor and subsequently additional cost. To avoid this we may setup the whole mandible and mark one side by using a skellite, a calcified material, used to distinguish between the left and right side.

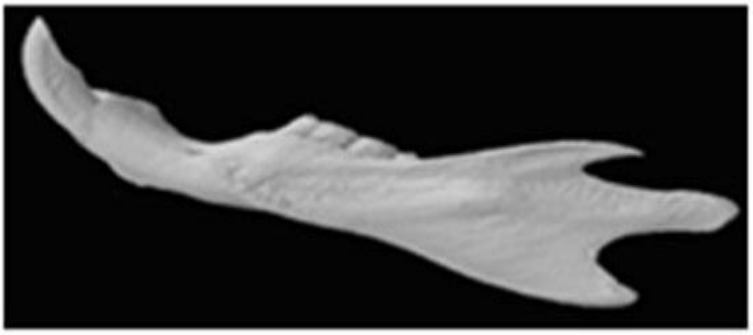

Figure 1.3 a: MicroCT image of Left Hemimandible. Extracted mandibles were scanned in Skyscan 1076 MicroCT imager post-treatment using 35 μ resolution with an Aluminum filter, frame average of 3.0. After reconstruction by NRecon Software the raw 2D images were obtained. Then, by cTAN software those 2D images were processed for 3D model and cTVOL software was used to obtain the model.

Use of MicroCT is not surprisingly very common in mandibular studies [205, 206]. Different bone parameters pertaining to different segment of the mandible ie ramus, condyle, alveolar bone [207], and with glenoid fossa [208] has been evaluated by the researchers. Different resolution offers different sort of detail appearance however, for the measurement purpose our study used both 18 and 35

μ showing no difference in the measurement. We have used 35 μ scan to measure the global bone volume (Figure 1.3 a) while used 18 μ for specific condylar measurement (Figure 1.3 b) as it seems to be a better advice to go for 18 micron size resolution for the assessment of the smaller part of the mandibular bone such as condylar cartilage. 9 micron resolution although possible, is extremely time consuming as well as with no further added benefit to measure the parameters. 14-micron resolution has also been reported [209]. Also by Dataviewer software, the images can be rotated into a position from where selective region of interest can be planned and later on drawn on the CTAn software. A three dimensional object can be viewed in coronal, saggital, and transaxial positions (Figures 1.4 a, 1.4 b, 1.4 c).

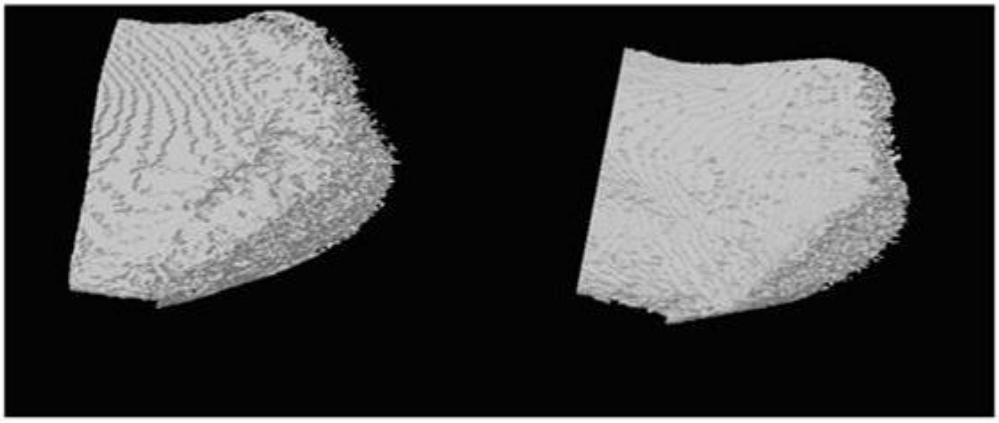

Figure 1.3 b: MicroCT Image of Left and Right Mandibular Condyle. Extracted mandibles were scanned in Skyscan 1076 MicroCT imager post-treatment using 18 μ resolution with an Aluminum filter, frame average of 3.0. After reconstruction by NRecon Software the raw 2D images were obtained. Then, by cTAN software those 2D images were processed for 3D model and cTVOL software was used to obtain the model.

Well recognized three dimensional morphometric parameters for the bone measurements are including but not limited to bone volume/tissue volume ratio

[209], trabecular thickness [209], bone surface density, trabecular plate number (Tb.N) [210] etc. Above all, bone mineral density is arguably the most popular technique probably due to the fact of the uniqueness of this tool to address the change in terms of the change in mass to the change in volume as a ratio that nullifies any ordinary or extraordinary variables associated with the others. For example, bone volume/tissue volume ratio depends on the dexterity and expertise of the extraction of the bone from the tissue surface. It may also get affected due to the aging of bone outside the fixating environment such as when being processed for sample preparation for scanning. For measuring mandibular bone mineral density we understand that assessment of the therapeutic effect on the bone mineral density of the whole hemimandible can be ambiguous as well as misleading. The most important reason being the presence of tooth. For the rat hemimandible, it is also true. Moreover, rats' incisor position is also a factor, which may impart variability to the result of the mandibular BMD. So, it is important for the mandibular measurements to exclude the dental part of the mandible from the measurement of the BMD.

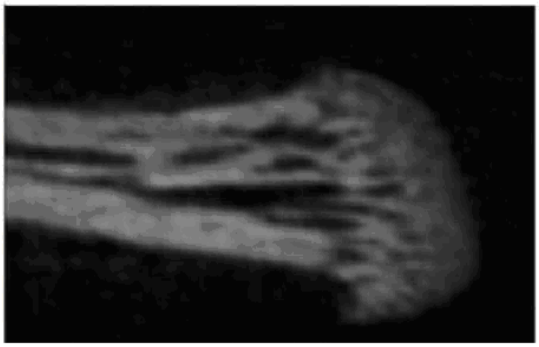

Figure 1.4 a

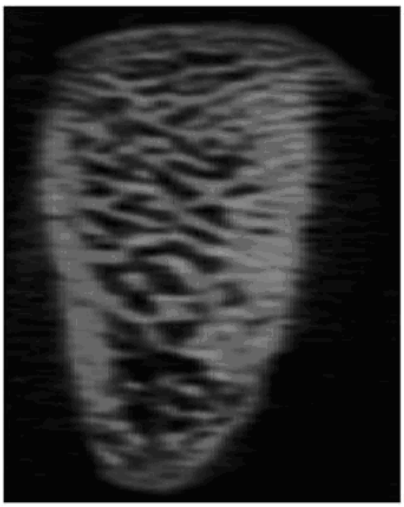

Figure 1.4 b

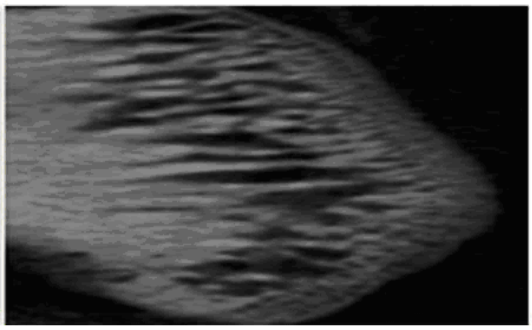

Figure 1.4 c

Figure 1.4: MicroCT Image of Left Mandibular Condyle: a. Coronal image, b. Saggital image, c. Transaxial image. Extracted mandibles were scanned in Skyscan 1076 MicroCT imager post-treatment using 18 µ resolution with an Aluminum filter, frame average of 3.0. After reconstruction by NRecon Software the raw 2D images were obtained. Then, using Dataviewer software, the 2D images are positioned in such a way so that the condylar head and neck are accessible for the region of interest selection. Thus coronal image (Figure1.4 a), saggital image (Figure 1.4 b) and transaxial image (Figure 1.4 c) positions were obtained. Transaxial images were copied later on to use them in cTAN software for region of interest determination, and subsequent measurements.

With this end in view, since we have discussed earlier that condyle is one of the major region that regulates the mandibular growth selection of condylar part carrying growth plate is important and is feasible by MicroCT. Also, higher resolution like 18 micron helps to generate 3 D images showing the trabecular bone and other structural features i.e. growth plate.

Chapter 2: Materials and Methods

2.1 Chemicals:

TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, and SYBR Green Master Mix were purchased from Applied Biosystems (Foster City, CA). Real time-PCR primers were synthesized by Integrated DNA Technologies Inc. (San Diego, CA) according to previously published sequences [211], [212], [213] and [214]. Recombinant rat growth hormone (rGH) was purchased from Prospecbio (Israel) and it was dissolved in PBS at 100 μg/ml concentration according to manufacturer's instruction. Low Intensity Pulsed Ultrasound (LIPUS) device was obtained from Smilesonica Inc. LIPUS device was validated and propagation of therapeutic ultrasound was checked every day by using ultrasonic gel. Phantoms (standard hydroxyapatite for bone mineral density calibration), and Skellites (to distinguing between left and right sides of the jaw) has been obtained from Dr. Mike Doschak's Pharmaceutical Orthopedic Research Lab.

2.2. Methods:

2.2.1 Design of Experiment:

Twenty four male Sprague Dawley rats, weighing 200 gm each, were divided into 4 groups (n=6). Group 1 was untreated control. Group 2 received rGH 5µg/day, group 3 received LIPUS for 20 minutes/day and group 4 received rGH 5µg/day with LIPUS for 20 minutes/day both into the posterior attachment of rat left Mandibular condyle. Treatments on groups 2, 3, and 4 were performed for 21 consecutive days. The right mandibular condyles did not receive any treatment and hence acted as internal control and was used for ratio measurement. The LIPUS unit has a 2.5 cm diameter transducer nominally generating a 200 milisecond duration burst of 1.5 MHz ultrasound at a pulse repetition frequency of 1 kHz delivering 30mW/cm2 temporally and spatially averaged incident intensity similar to that published in the literatures [215] and [216]. These procedures were performed under the 2.5% Isoflurane (Halocarbon, NJ, USA) inhalation anaesthesia. Animals at the designated time points (24 hours after the last treatment) were euthanized under 2.5 -3.5 % isoflurane anaesthesia. The liver and kidney tissues were excised, immediately frozen in liquid nitrogen. All of the extracted tissues were stored at −80 °C until analysis. The whole mandibles were extracted from the heads of the carcasses and muscular soft tissues were removed cautiously from the bone. Immediately after that, the extracted Mandibular bones were submerged into 10% Formalin for fixation until the completion of the other samples' extraction. Then all the mandibles were stored in −80 °C until Micro Computed Tomography (MicroCT) analysis.

2.2.2 MicroCT Measurements

2.2.2.1 MicroCT measurement of whole mandibular and condylar bone volume and bone surface area:

After the mandibles were dissected and fixed, they were imaged using a micro-CT imager (Skyscan-1076, Skyscan NV, Belgium). Briefly, the whole mandibles were taken out of the −80 °C freezer, and loaded into the imager gantry by appropriately rapping them with scotch tape to avoid movement during the scanning procedure and to avoid fuzzy appearance and poor-quality scan. Samples were at first scanned at 35μm resolution, using a tube voltage of 100kVp, a current of 100μA, and a power of 10W. The X-ray beam was hardened using a 1.0 mm Aluminum filter in order to increase beam photon energy, reduce beam hardening and edge artefacts. Three scan projections were averaged per step, through the 180° of rotation at 0.5° step increments with 1180ms exposure time. The raw image data were reconstructed using a modified Feldkamp back-projection algorithm, thresholded at an image to cross-section of 0.0–0.046 using NRecon reconstruction software (version 1.4.4). Reconstructed images were loaded to the vendor supplied histomorphometric image analysis software (CT-An, Skyscan NV, Belgium) for the whole hemimandibular bone volume, and surface area measurements. Reconstructed images were rendered into 3D representations using vendor supplied CT-Vol software. Scans with 18μm resolution were performed for analysing the left and right mandibular condylar volume, surface area, and bone mineral density with the same protocol mentioned

above. Appropriate Region of Interest (ROI) containing mandibular condylar process, Mandibular head, and Mandibular neck was selected for measuring.

2.2.2.2 Mandibular Condylar Bone Mineral Density (MCBMD) measurements

Volumetric micro-CT calibrations using phantoms of known calcium phosphate density (0.25g/cm3 & 0.75g/cm3) were performed whenever the scans were performed with different samples. The samples of a specific day were always calibrated with the phantom of that particular day only. Briefly, similar parameters for voltage, current, power, Aluminum filter thickness, frame averaging, rotation step and exposure time were used for the phantom since they were entered into the gantry along with the sample under examination. After reconstruction, the dataset were loaded into CTAn and a volume of interest (VOI) was created containing only the phantoms (Calcium hydroxyapatite) and the water gap. ROIs were selected from both the densities (0.25 and 0.75 g/cm^3) and the water gap separately and the mean total values of greyscale indices were obtained from all of them to substitute the values of the corresponding parameter of the equation. 18 micron scans were used for this procedure. Only specific measurement for the condylar region was performed.

2.2.3 Gene expression studies

2.2.3.1 RNA extraction and cDNA synthesis

Total RNA from the frozen tissues of livers and kidneys was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and the absorbance were measured at 260 nm for quantification. RNA quality was determined by measuring the 260/280 ratio. Afterwards, first-strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. In brief, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μL 10× RT buffer, 0.8 μL 25× dNTP mix (100 mM), 2.0 μL 10× RT random primers, 1.0 μL MultiScribe™ reverse transcriptase, and 4.2 μL nuclease-free water. The final reaction mix was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C.

2.2.3.2 Quantification by real time-PCR

Quantitative analysis of specific mRNA expression was performed by real time-PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). 25 μL of the reaction mix contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. The primers used in the current study were chosen from

previously published studies. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

2.2.3.3 Real time-PCR data analysis

The real time-PCR data were analyzed using the relative gene expression i.e. (ΔΔCT) method as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen [217]. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to a calibrator. The untreated control was used as the calibrator when the change of gene expression by GH and/or LIPUS treatments being studied.

2.2.4 Weight measurement

Rats were given 24 hours washout period prior to euthanization and performing subsequent extraction of the organs, measurements and analyses. Within this time they were all weighted to see whether or not the GH was being absorbed systemically resulting in increased body weight.

2.3 Statistical analysis

Data are presented as mean + standard error of the mean. Comparative, left mandibular bone volume, left mandibular bone surface area, left and right mandibular bone surface area, left mandibular condylar volume, ratio of left over right side of mandibular condylar bone volume, left mandibular condylar bone mineral density and ratio of left over right mandibular condylar bone mineral density, weight of the rats after the treatment, and gene expression across different treatment groups were analyzed using a one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc comparison. For the purpose of ratio calculation, left sides of the mandibles were divided by the right sides (untreated internal control). A result was considered statistically significant where $P < 0.05$.

Chapter 3 – Results

3.1 Effect of GH with or without LIPUS treatment on the whole left hemimandibular bone volume (MBV) (Figure 3.1)

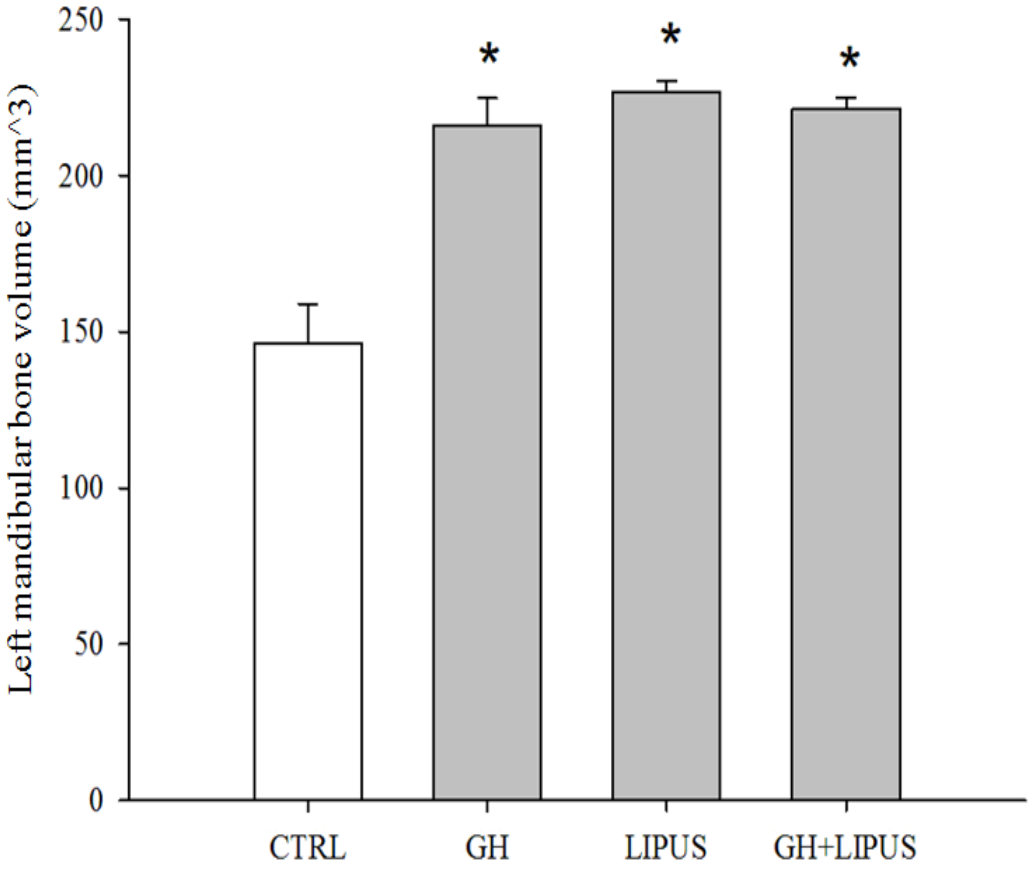

Figure 3.1: Effect of Treatments on Left Hemimandibular Bone Volume. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. 35 micron scans were performed for the whole MBV. These scanned images were then reconstructed by NRecon software using the appropriate parameters mentioned above. Regions of interest were manually selected on the right and the left sides of the whole jaws by CTAn sofware. Afterwards, CTAn software was used to perform 3D-analysis from the 2D images. Data are expressed as the Mean + SEM (n = 6). *$p<0.05$, while compared to CTRL group.

3.2 Effect of GH with or without LIPUS treatment on the left Mandibular condylar bone volume (MCBV) (Figure 3.2)

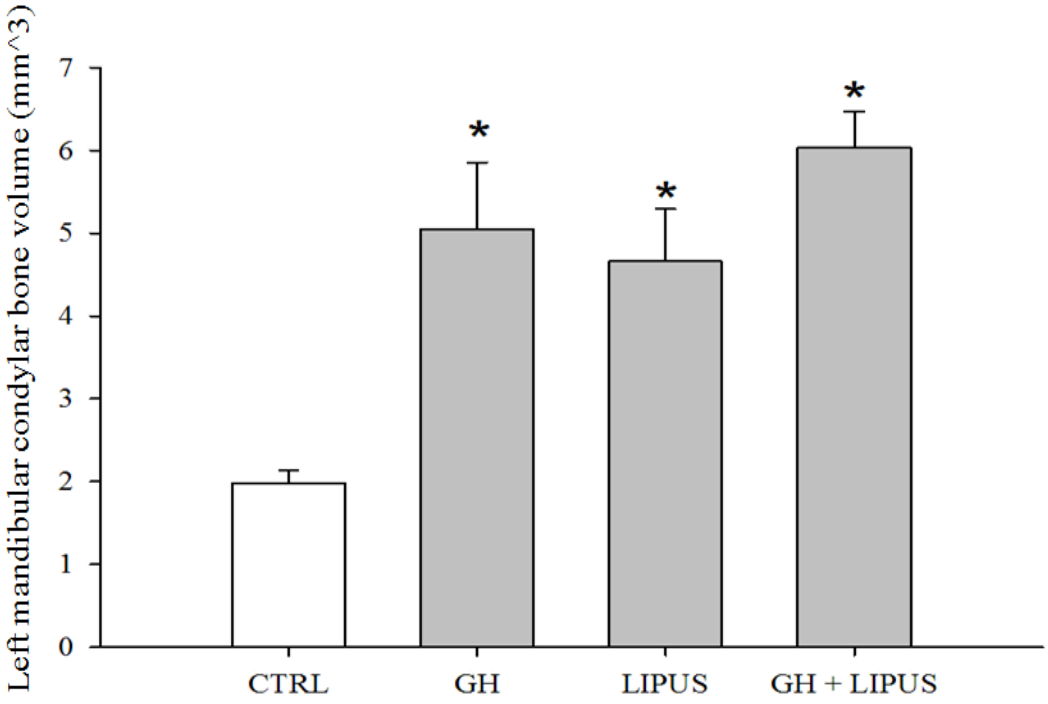

Figure 3.2 Effect of GH with or without LIPUS treatment on the left Mandibular condylar bone volume. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. 18 micron scans were performed for the MCBV. Reconstruction, model generation, and 3-D analysis were performed similarly as described in section 3.1. Data are expressed as the Mean + SEM (n = 6). *$p<0.05$, while compared to CTRL group.

3.3 Effect of GH with or without LIPUS treatment on the ratio of MBCV between left and right side (Figure 3.3)

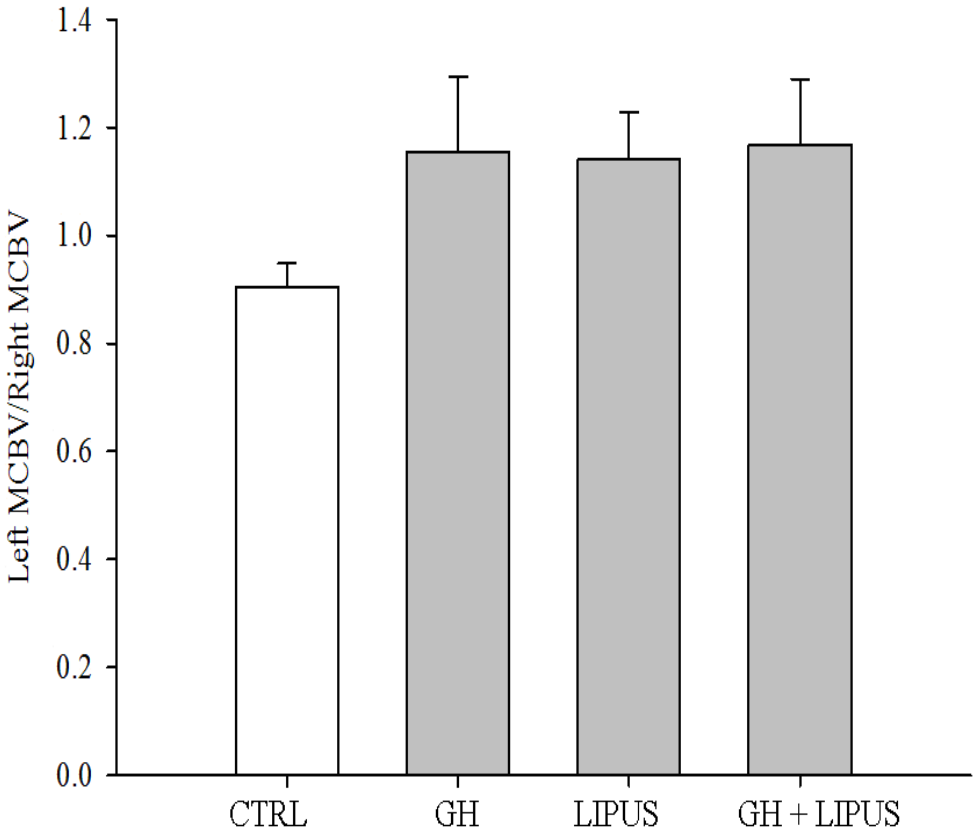

Figure 3.3 Effect of GH with or without LIPUS treatment on the ratio of MCBV between left and right side. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. 18 micron scans were performed for the MCBV, surface area and bone mineral density measurements. Reconstruction, model generation, and 3-D analysis were performed similarly as described earlier. Ratio was calculated by dividing the left side (treated side) value by the right side (untreated internal standard) counterpart of all of them. Data are expressed as the Mean + SEM (n = 6).

3.4 Effect of GH with or without LIPUS treatment on the whole hemimandibular bone surface area (MBSA) (Figure 3.4)

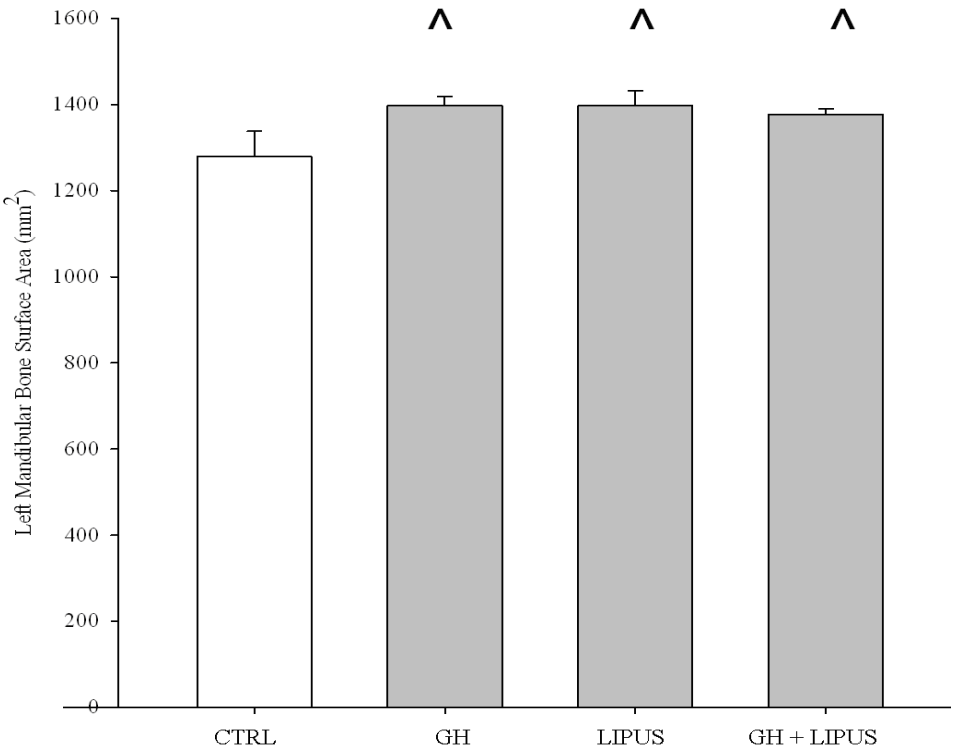

Figure 3.4: Effect of GH with or without LIPUS treatment on the whole hemimandibular bone surface area. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. Scans with 35 micron resolution were performed for the measuring whole hemimandibular Bone Surface Area (MBSA). Reconstruction, model generation, and 3-D analysis were performed similarly as described earlier. Data are expressed as the Mean + SEM (n = 6). ^$p<0.05$ vs CTRL

3.5 Effect of GH with or without LIPUS treatment on both the left and the right whole hemimandibular bone surface area (MBSA) (Figure 3.5)

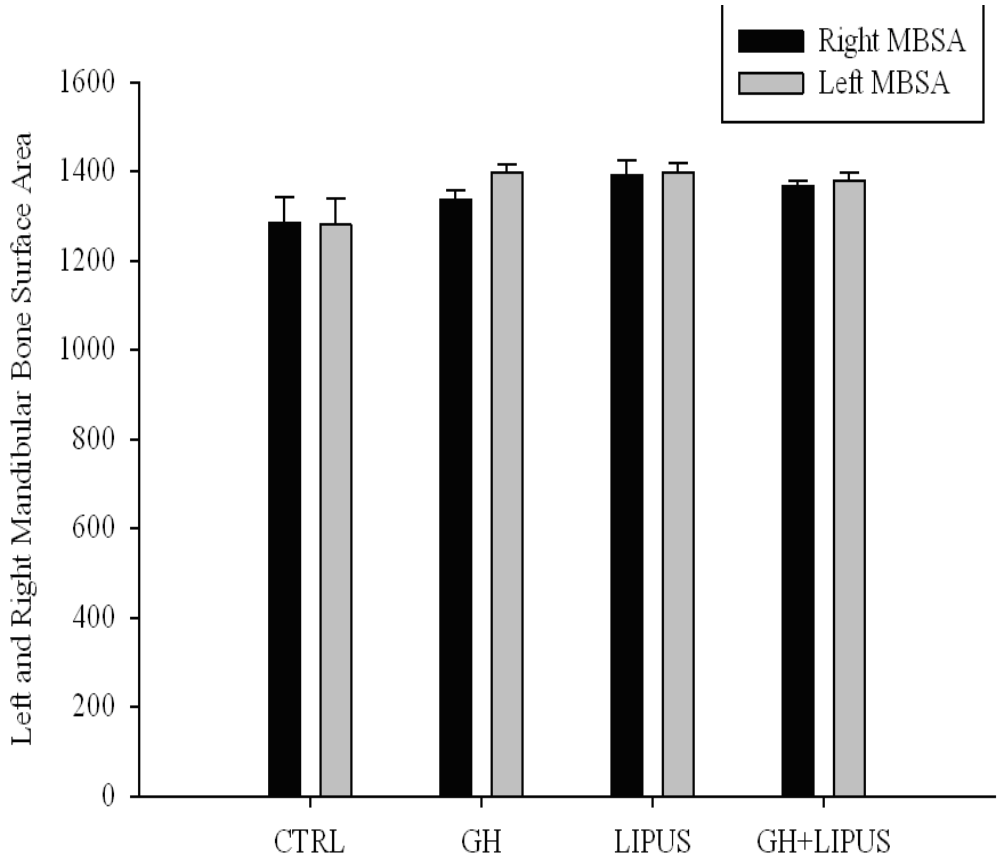

Figure 3.5: Effect of GH with or without LIPUS treatment on both the left and the right whole hemimandibular bone surface area. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. Scans with 35 μ resolution were performed for the measuring whole hemimandibular Bone Surface Area. Reconstruction, model generation, and 3-D analysis were performed similarly as described earlier. Data are expressed as the Mean + SEM (n = 6).

3.6 Effect of GH with or without LIPUS treatment on left mandibular condylar bone surface area (MCBSA) (Figure 3.6)

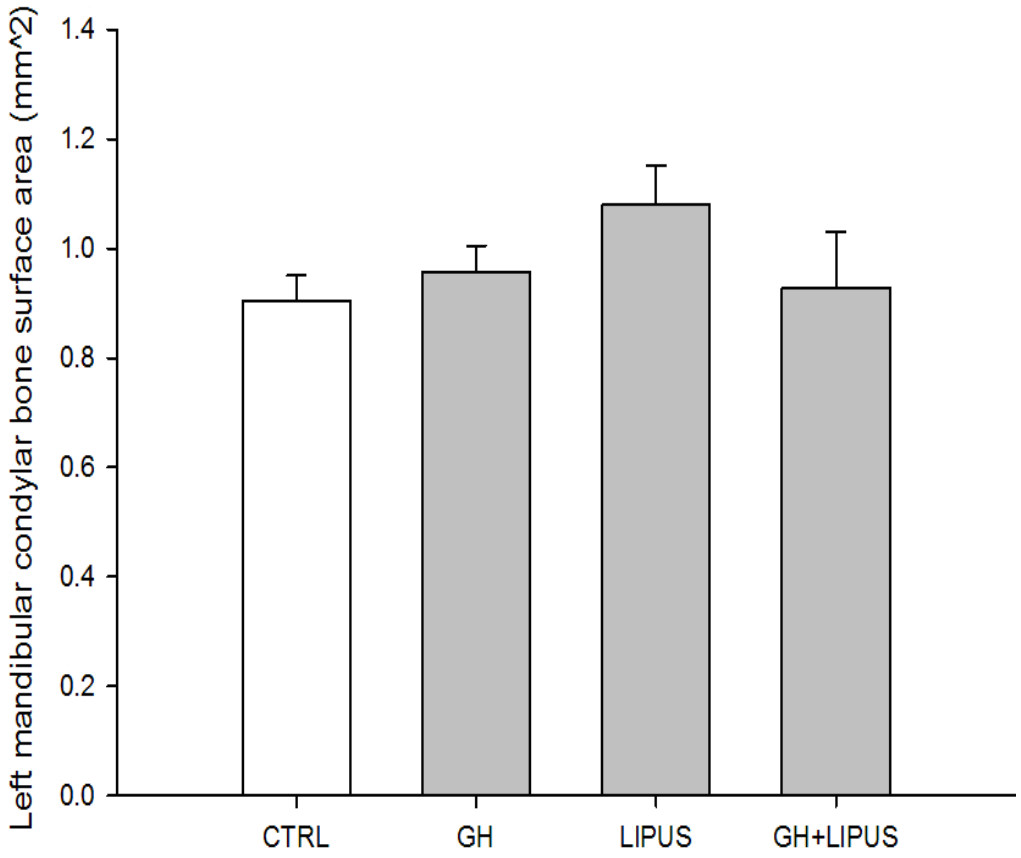

Figure 3.6: Effect of GH with or without LIPUS treatment on left mandibular condylar bone surface area. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. 18 μ scans were performed for the left MCBSA measurement. Reconstruction, model generation, and 3-D analysis were performed similarly as described earlier. Data are expressed as the Mean + SEM (n = 6).

3.7 Effect of treatment on Left mandibular condylar bone mineral density (MCBMD) (Figure 3.7)

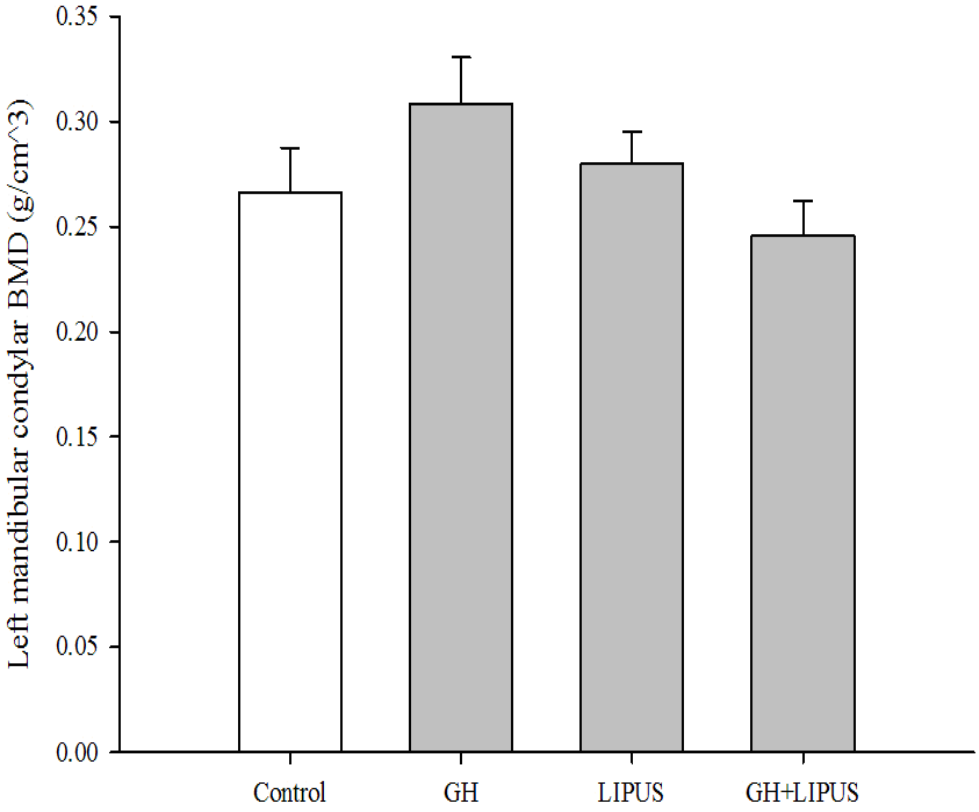

Figure 3.7: Effect of treatment on Left MCBMD. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. MCBMD was measured by MicroCT from anatomical landmark of left mandibular head and neck (with phantom Calcium Hydroxyapatite having 0.25g/cm^3 and 0.75 g/cm^3 standardized densities). Data are expressed as the Mean + SEM (n = 6).

3.8 Effect of treatment on the ratio of Left over Right MCBMD (Figure 3.8)

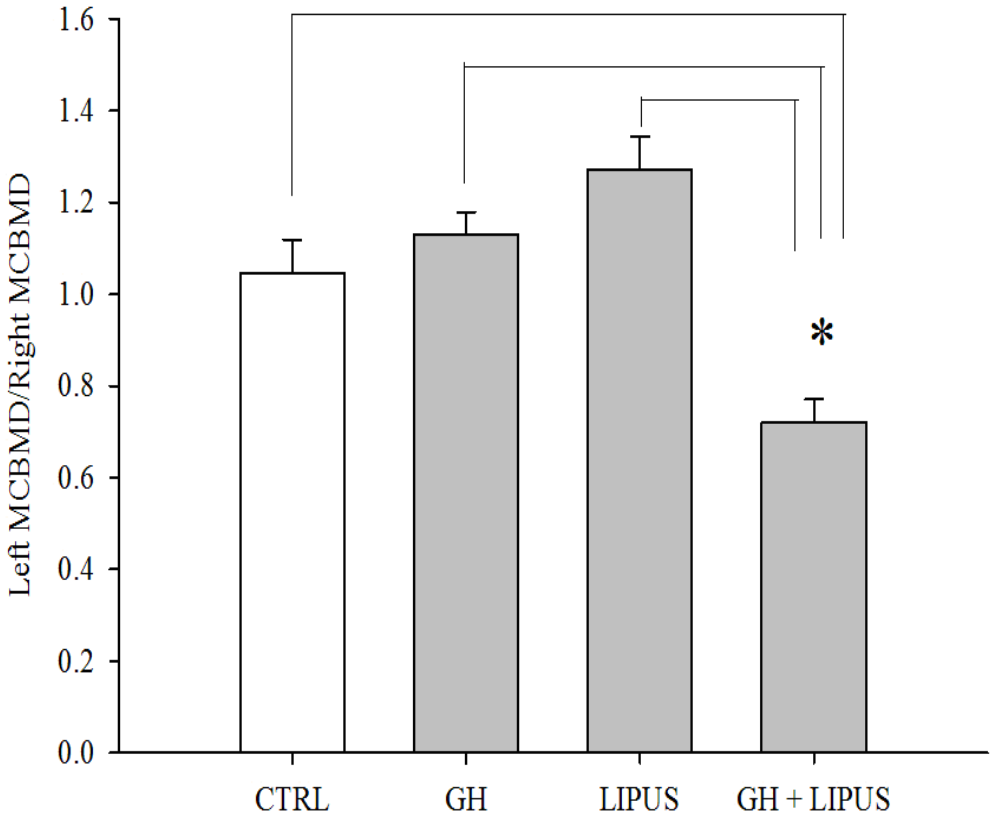

Figure 3.8: Effect of treatment on the ratio of Left over Right MCBMD. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. MCBMD was measured by MicroCT from anatomical landmark of mandibular head and neck (with phantom Calcium Hydroxyapatite having 0.25g/cm^3 and 0.75 g/cm^3 standardized densities). Ratio was measured by dividing the left by right condylar BMD. Data were expressed as the Mean + SEM (n = 6). *$p<0.05$, while compared to CTRL, GH, and LIPUS groups.

3.9 Effect of the treatments on the C-jun gene expression on the treated rats' livers (Figure 3.9).

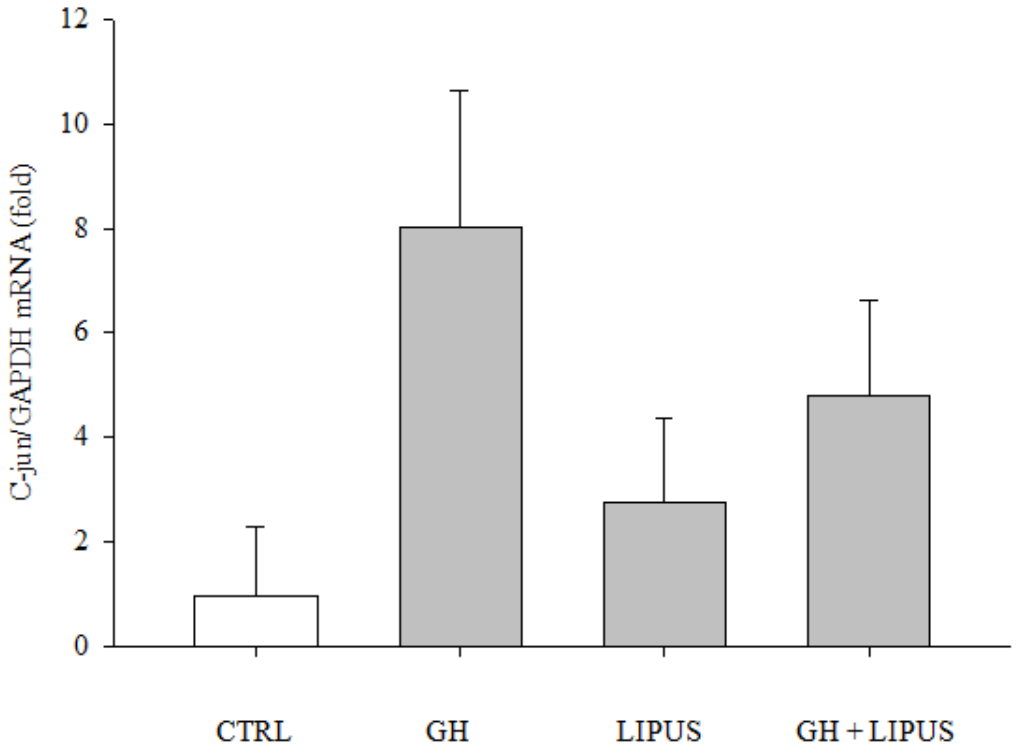

Figure 3.9: Effect of the treatments on the C-jun gene expression on the treated rats' livers. 24 male Sprague Dawley rats, with a starting weight of 200 grams were treated for 21 days of daily treatments. Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. Preparation of mRNA was performed by Trizol method and reverse transcribed. Then SYBR green was used as fluorescence detector to observe the gene expression which was measured by relative quantitative methods by using 2-^ddCT as described earlier. Results are shown as Mean + SEM (n=6)

3.10 Effect of the treatments on the weight of the rats (Figure 3.10)

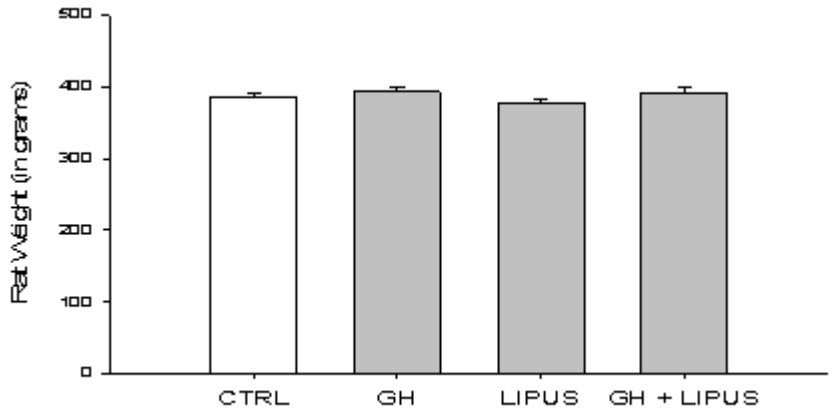

Figure 3.10: Effects of the treatments on the weights of the rats. 24 male Sprague Dawley rats, with a starting weight of 200 grams were weighed at the end of the treatment period (after 21 days of daily treatments). Rats' treatment groups are represented as CTRL = Untreated Control, GH = Group receiving 5 mcg GH daily, LIPUS = Group receiving 20 min exposure to LIPUS daily, and GH + LIPUS = Combination of GH (5 mcg) and LIPUS (20 min exposure) treatment daily. Results are shown as Mean + SEM (n=6)

Chapter 4 Discussion

4.1 General discussion

Current study investigated the individual and combined effects of GH and LIPUS on intact mandibular bone. Potent growth induction by the growth hormone may be attributed to the stimulation of mitotic activity and delaying maturation on the condylar cartilage as suggested previously [147]. GH has also been shown to be effective in the periosteam of both fractured and intact bone previously [218] in a dose below and above the dose being used in current study. Also the recommended doses of human GH to be used in Turner Syndrome and Idiopathic Short Stature are 0.05 mg/kg/day and 0.043 mg/kg/day respectively [219, 220] which is higher than the amount of dose we used for the rats in our current study. Moreover, studies performed in osteoblast-like cell lines have showed GH receptors [221] and their mitogenic response to GH [222]. Also, stimulation as well as modification of the growth pattern in the growing baboons' mandibles was showed where LIPUS was accompanied by functional appliances [190]. Calcium incorporation [223], synthesis of cell matrix proteoglycan mediated by intracellular calcium signaling [224], and inducing the efflux of potassium [150] were thought to play crucial role on the effect of LIPUS on the fractured bone.

All those results have supported the obtained results related to GH and LIPUS individual treatment in current work. To the best of our knowledge, exploring the concomitant effect of GH and LIPUS has not been performed before. Our study's effort was to minimize both the dosing and the side effects of the GH by

introducing LIPUS as a treatment adjunct. Our data shows that GH and LIPUS induced Mandibular growth complimentarily. GH and LIPUS separately increased the left MBV significantly than the untreated control (Figure 3.1). Combination therapy did not show any added effect to this growth (Figure 3.1) even though after changing the order of the introduction of GH or LIPUS application. Moreover, we found significant increase in the mandibular condylar bone volume (Figure 3.2) which is in agreement with the global measurement. Increasing bone volume is important because the mandible undergoing endochondral ossification happens to grow against the applied pressure. Similar findings obtained from the MBSA (Figure 3.4) results with no significance increase in condylar MBSA (Figure 3.6). On the other hand, there is no significant difference were observed between the left and right sides of the mandible (Figures 3.3, 3.5, 3.6). These findings dictate for the importance of having a guideline for the difference between both sides of the mandible to be expected. This would give us the ability to perform power assignment for the research and subsequent elucidation of appropriate sample size for our study.

We did not perform the bone mineral density (BMD) for the whole hemimandible since the density of the molar and incisor will affect the measurement of actual bone. Considering this, we selected the mandibular condylar head and neck as a region of interest to precisely determine the growth induced in the area adjacent to the site of GH and LIPUS application. Surprisingly enough, the BMD was found to be significantly reduced in case of combination therapy when ratio of left over right MCBMD was considered (Figure 3.8). At this standpoint we can merely

assume that there is a bone loss. Hence, this striking finding of antagonism among these two established clinical approaches gives us opportunity to think the reason of the decreased bone mass owing to concomitant endocrine and ultrasound treatment.

Although, it is evident from mice [225] and rat [226] studies that parathyroid hormone (PTH) has a potent anabolic skeletal effect by virtue of its regulation on calcium and phosphate metabolism, the combined effect of the former with LIPUS was not promising when LIPUS in conjunction to PTH was shown to decrease the total callus volumetric bone mineral density in the injured femurs of the Sprague Dawley rats after injury [226]. We did not find any study that combine such approaches on intact bone. So, participation of healing process will hinder us to even observe the similarity of our findings with the others who performed their treatment on injured bone.

Length of time of LIPUS exposure in the current study might have been shortened as 10 minutes but a study has concluded a greater response in trabecular shape and perimeter for 20 minutes instead of 10 minutes daily [227]. Nonetheless, there is no evidence or information about the effect of both the treatment durations on the BMD measurements separately or in combination. Also, GH was found to be an effective bone growth inducer in amount twice higher than the dose selected in our study [128].

Power in pharmaceutical research is importance to attain the confidence level of the obtained significant data. Using equation we can obtain the appropriate

sample size from power and vice versa. A rearranged equation has been suggested earlier for optimum sample size calculation:

$n = (\sigma_1^2 + \sigma_2^2) [\{D/(Z_\alpha + Z_\beta)\}^2]^{-1}$ where,

D = minimum difference to detect the given power

Z_α = critical value of the standard normal distribution

Z_β = power critical value of the standard normal distribution

σ_1 = standard deviation of method 1

σ_2 = standard deviation of method 2

n = number of samples to be treated by each method [228]

It is difficult to rationalize how much significant increase we might have foreseen in the rats' mandibular bone growth because we performed bone volume, bone surface area and bone mineral density. Also appropriate guideline is also absent. For our purpose, if we would have tried to achieve 1% higher mandibular growth in all those parameters we might need a higher number of samples which might render the data for mandibular or condylar bone surface area significant.

Lack of expression of C-fos and IGF-1 (data not shown), and the mode of C-jun expression in liver suggested that there is little or no systemic side effect for this local application procedure (Figure 3.9). For the C-jun gene expression, GH alone group has showed comparatively the highest expression followed by the combination of GH with LIPUS. LIPUS alone, as expected, has shown the least amount of C-jun expression (Figure 3.9). C-jun, a proto-oncogene as mentioned earlier, has been showed to express preferentially more in acute myeloid leukemia

patients than normal healthy volunteers [229]. That is why expression of this proto-oncogene is crucial in treatments associated with GH application. No site irritation or inflammation was visible throughout the treatment period. Also, total body weights of the rats at the end of the experiment were not significantly different from each other confirming the less side effects by the treatment approaches taken (Figure 3.10).

As mentioned earlier, in our RT-PCR analysis we did not find any change in the level of expression of IGF-1 in the livers of the euthanized rat. However, the serum level of IGF-1 was not performed since postnatal growth and development was shown to be more regulated by the locally produced IGF-1 instead of the circulating liver derived IGF-1 [230]. Therefore, it stays unclear whether or not IGF-1 played an important role in the regulation of Osteoprotegerin (OPG) with Receptor Activator for nuclear factor κ B Ligand (RANKL) pathway for bone resorption as suggested by a study performed on healthy and menopausal women [231]. All of those rationales suggest core understanding of the mechanism of growth induction or inhibition by these two treatment approaches which we were not interested in current study. The role of IGF1 overexpression should be tested at the in vitro level using cell culture. In this context, human chondrocyte cell lines isolated from condylar cartilage are well suited for testing the expression of several markers of growth regulation on the condylar cartilage in vitro [232]. Expression and activity of OPG/RANKL can be assessed along with the assessment of local IGF-1 production which may be helpful to limit the effort of growth induction in a quantitative manner that it induces the bone growth and

does not induce osteoclastogenesis because of excess IGF-1 production which results in subsequent OPG/RANKL expression.

We have mentioned in the relevant section for growth hormone, the dual role of the hormone has not been suggested or claimed until now. That is why, after successful finding of growth induction as well as obtaining information of specific genes expressions as mentioned above, animal study should commence. In this study, IGF-1 knocked out rats might be used to see whether the applied dose of both of the treatments induce growth in the light of bone mineral density, and other bone parameters including the gene and protein expression analysis of protooncogenes, OPG/RANKL, along with alkaline phosphatase (ALP), and Osteocalcin (OC). Reasons for using the last two biomarkers lie on the fact that ALP is one of the most common earlier bone growth markers [234] and OC is a one of the bone turnover markers [235]. The better choice of analysis would be making mRNA sample from the condylar cartilage portion [231] and measure the IGF-1 gene expression and subsequent activity measurement. Although the mRNA yield might be low, this might have given us a better idea to understand whether or not IGF-1 is playing the double role of bone formation followed by marked resorption due to concomitant combination therapy and further up-regulation of IGF-1 in mRNA and protein level. Those will help us to design appropriate experiment for the normal population. Another important suggestion can be made about the application of growth hormone which is, delivery of the hormone through radioactive labelling that can tag the condylar cartilage while applied locally in similar fashion as performed in current study.

Furthermore, possibility of interplay between different hormones in endocrine system i.e. interaction among hormones of contrasting physiological role may not be eliminated. For example, the inhibitory effect of glucocorticoids on cell proliferation, matrix production, paracrine IGF-I secretion, GH and type I IGF receptor might play a subsidiary role on the reduced bone mass however, was also a discrete topic for our study [236] because long term doses for impeding growth is required. Another important point may be attributed to the dose of GH which might be given at further below concentration so as to avoid the possible over expression of IGF-1 locally. GH might be delivered following radiolabelling since it will ensure the GH application will be directed into the condylar region without getting drained into the systemic circulation and cause untoward effects. Histomorphometric evalauation of bone loss is important to observe whether the bone is resorbed or growth is induced in the hypertrophic zone through measurements like hypertrophic cell counting, surface area determination etc.

4.2 Conclusion and future work

We found the significant increase in hemimandibular bone volume and surface area as a result of treatment with GH and LIPUS separately and in the combination of both. Combination therapy was unable to provide any further benefit than the individual treatments. On the other hand, we found a significant decrease in the mandibular condylar bone mineral density as a result of combination therapy only suggesting the combination of two growth inducers reducing the bone mass, a potential detrimentive outcome for an intact bone. Besides, the unchanged post-treatment weights for the rats' of all the treatment groups suggest the least amount of leakage of GH from the site of injection to elicit weight gain for the rats. Furthermore, insignificant and non-constitutive C-jun expression suggests fewer side effect due to local injection of GH to the posterior mandibular condylar attachment.

The results obtained from current study necessitate the following future objectives:

1. To determine the hypertrophic cell count, bone volume/tissue volume, and surface area by histomorphometric analysis to strengthen the results obtained from MicroCT
2. To study the combination of GH and LIPUS at a lower dose and or strenght that was used in the current study.
3. To study the growth induction by application of other growth factor inducers (apart from GH) along with LIPUS

4. To evaluate targeted delivery of potential growth inducers so as to direct the effect for e.g. radiolabelled tagging of the growth inducing molecule
5. To understand molecular pathways by utilizing appropriate cell cultures and by using IGF-1 knocked out animal so as to understand whether or not the IGF-1 overexpression causes bone mass reduction.

Chapter 5 References

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