INVESTIGATION OF VERTICAL MANDIBULAR DISTRACTION Oстеогенезис на мышцы жевания в модели "бокового микрофациального" дефекта

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CHAPTER 1: GENERAL INTRODUCTION

Distraction osteogenesis has been successfully used in orthopaedics for many years and more recently for the facial bones. Many studies have been conducted to explain and understand the hard tissue responses but only a few studies have looked at the mechanism of adjustment of the soft tissue in response to this technique. Soft tissue, particularly muscle, plays an important role, as it needs to accommodate and adjust well to the new position to prevent complications such as relapse. Information on short-term effects on muscles after distraction osteogenesis is available but there is very limited information on the long-term effect after distraction and after removal of the device. A comprehensive experiment to look at the mandibular ramus bones and masticatory muscles in healing and changes, for different duration on neutral fixation after distraction and after removal of the device are the next step to answer the question of relapse.

Hemifacial microsomia is a congenital deformity that has a deficiency in the amount of hard and soft tissues on one side of the face. The de novo bone traction technique known as distraction osteogenesis has become a valid surgical correction for these cases. The gradual distraction process stimulates the growth of the hard and soft tissue through the process of osteogenesis and histogenesis respectively. The deficiency of the bone and muscle tissue may adjust well during the traction process but may not be stable in the new position for the longer consolidation and remodelling period. The bone and muscle tissue that is not readily stable may cause the relapse phenomenon.
Therefore, a comprehensive animal study, which involved the vertical mandibular distraction osteogenesis was conducted to study bone and muscle tissue during the longer consolidation and remodelling periods.

Sheep were selected as the animal model based on their broader mandibular ramus and adaptability of the animals toward multiple surgical procedures. This study performed surgical correction on a surgically created defect. The intention was to create the nearest true effect of surgical correction on the affected bones and muscles. Therefore, very young lambs had a condylectomy and superficial masseter myectomy. The result was retardation of growth of the mandibular ramus height; reduced muscle bulk and a midline shift to the operated side. A ‘unilateral hemifacial microsomia like’ defect in the sheep model was successfully created and this model was then surgically corrected with a distraction osteogenesis technique. Mandibular ramus bone and masticatory muscle changes, adaptation and adjustment were studied in relation to the relapse phenomenon.

The purpose of this study was to investigate the vertical height of the mandibular ramus and masticatory muscle (masseter and medial pterygoid muscles) changes in vertical ramus distraction osteogenesis within 2, 3, and 4 months consolidation as well as 1 and 2 months after device removal.
Chapter 2 Literature Review

Distraction osteogenesis was developed in orthopaedic surgery for long bone lengthening and has been applied for the correction of craniofacial defects. The historical developments, adaptations and applications of the technique are reviewed. The distraction osteogenesis protocol and its application on the long bone and facial bone are elaborated along with the bone and muscle healing processes and the relationship to the distraction process as well as the different consolidation periods. Complications during the neutral fixation and post device removal period were revealed. The intention of the current study was to look at the relapse phenomenon during longer consolidation and remodelling periods.

Hemifacial microsomia patients benefit from distraction osteogenesis as a surgical correction technique. The aetiology, criteria and classifications of this facial defect are stated. Relapse is a complication during the long-term consolidation and remodelling periods. The bone and muscle response in distraction osteogenesis are discussed. Furthermore, there are few studies on the mandibular bone and masticatory muscle changes and their influence on relapse on longer consolidation and remodelling periods.

Chapter 3 Materials and Methods

A surgical procedure to create a unilateral hemifacial microsomia-like defect is presented in an animal model. Retardation of mandibular growth and an associated soft tissue defect on the experimental side was achieved. Distraction osteogenesis as a surgical correction to lengthen the affected mandible was demonstrated. The animals were divided into 6 groups based on the consolidation and remodelling periods of the distraction process, for example: at two, three and four months. Three of the groups, where by the device was removed and the animals had to be sacrificed, one to two months later. The evaluation of the bones was performed by radiography,
direct measurement and histomorphometry. Masticatory muscle assessments were conducted via the methods of wet weight of muscle, measurement of soft tissue landmarks, cross section and thickness using ultrasound and histopathological evaluation. The materials and methods for the bone component described in this thesis was adapted from Syed Zainal (2005).

Chapter 4 Results

The distracted bone was reported to be consolidated and stable after a 3 month or longer consolidation period. Bone histology showed that the distracted bone quality was almost the same as the original bone fragments. On the other hand, the distracted bone was noted to have a soft callus and woven bone after a 2 month consolidation period (Syed Zainal, 2005).

The masseter muscle was surgically reduced by approximately 30% after the first operation. The net change of the masseter and medial pterygoid muscles showed a tendency towards reduction in weight on the experimental side during the first months after device removal, both with 2 and 3 month consolidation periods. This was a temporary change as the weight was regained during the two months after removal with a 3 month consolidation period.

Six planes of the distracted masseter muscle showed different changes in lengths at different consolidation and remodelling periods. The anterior and posterior planes of the experimental sides showed a major reduction in length during the first month after device removal, but less change was observed on the middle and oblique planes one month after device removal with 2 and 3 months consolidation periods. The length was normalized during the second month after the removal of device within a 3 month consolidation period.
The cross section of the scan images on the proximal (origin), middle and distal (insertion) showed a reduction one month after device removal with 2 and 3 month consolidation periods. The cross section increased to the size of the control side during the second month after the removal of device with 3 months consolidation period. The thickness of the origin (proximal) of the masseter muscle showed a converse activity to the middle masseter muscles. Generally the control side of the distal masseter muscles are thicker in all groups.

Chapter 5 Discussion

The healing process of the mandibular bones and masticatory muscles (masseter and medial pterygoid) during different consolidation and remodelling periods is evaluated and compared to look at the pattern of reduction and increase in size. The reduction of the vertical height of the distracted bone was greater during the first month after removal of device with a 2 month consolidation period. The distracted bone was still at the soft callus healing stage and was unstable. The parameters for the masseter muscle were also at a stage of adjustment and not stable. On the other hand, the quality of distracted bone was the same as the original fragments for a 3 month or longer consolidation period. The masticatory muscle showed the higher percentage of changes during the first month after removal of the device with a 3 month consolidation period but normalised two months later. The muscle changes during the first month after device removal was a temporary activity as in the second month the muscle was almost back to normal.
The distraction process successfully increased the height of the vertical ramus of the mandible.
Distraction increased the anterior and posterior planes but not the middle and oblique planes.
This study found that there was no increase in the muscle mass of the masticatory muscles.
Based on the current experiment, it can be concluded that the optimum time for the device to be in place after completion of the distraction process was a 3 month or longer consolidation period. The bone and muscle healed and was stable after a 3 month consolidation period. The main body at this thesis was presented in five chapters including literature review, materials and methods, results, discussion and the conclusions.
CHAPTER 2: LITERATURE REVIEW

2.0 Distraction Osteogenesis

Distraction osteogenesis is the process of generating new bone in a space between two bone segments in reaction to the gradual traction across the bone space (Swennen et al., 2001). Uniquely, the formation of the new callus between the bone fragments continues throughout the stretching process. This technique allows the formation of a large area of bone to correct a defect in syndromic patients. It also gives an alternative to conventional orthognathic and craniofacial surgery, where there is a risk of relapse when the muscles cannot accommodate the new position. It was proposed that the slow, gradual traction during the process of distraction osteogenesis allowed concurrent stimulation of growth in the surrounding soft tissues such as blood vessels, nerves, skin, mucosa, fascia, ligaments, cartilage, periosteum and muscle (Block et al., 1993; Fisher et al., 1997; Lee et al., 1993; Makarov et al., 2001b; Schumacher et al., 1994; Shevtsov et al., 2002; Yasui et al., 1991). The response of the surrounding soft tissue to the distraction process is termed as distraction histogenesis (Cope et al., 1999). Distraction osteogenesis and histogenesis both respond in parallel to gradual traction and this factor was important in the selection of this technique.

2.1 History of Distraction Osteogenesis

This technique was first described in 1905 by Alessandro Codivilla in Bologna; It involved the elongation of the femur by fixing an external traction of 25-75kg in combination with plaster casting after an oblique osteotomy (Codivilla, 1905) (Figure 2.1). In this endeavour, he managed to elongate the femur up to 8 cm but the patient ended up with nerve problems and convulsions. Following from there, Putti (1921) (cited from Samchukov et al., 2001b) designed a unilateral external fixation device to lengthen the femur and reduce trauma from the
osteotomy by constant control of the traction process. An application of bilateral external fixation was conducted by Abbot in 1924 (cited from Samchukov *et al.*, 2001b) in the United States of America and gained acceptance among surgeons. This technique was however discredited over time because of the improper patient assessment and unsafe surgical techniques that resulted in complications such as infection, septicaemia and death (Wiedemann, 1996).

The technique was further developed by a Russian physician, Dr Gavriel A. Ilizarov in the 1950s (Ilizarov, 1988; Ilizarov, 1989). He designed a bone fixator by using a round metal frame, which was joined together by three or four threaded pins. The upper and lower bone fragments were fixed in place with two small wires in each fragment (Figure 2.2). The technique was used to lengthen the upper and lower extremities with success for many years. Ilizarov used and applied the knowledge of bone and soft tissue genesis to further supports this technique. He continued treating patients with non-union fracture until after the Second World War. His excellent work remained unknown until his results were reported in the Western scientific literature in 1979, which popularised the technique (Ilizarov *et al.*, 1979). He continued development of the technique by introducing the corticotomy technique and formulated a set of rules for performing the procedure, including a 5–7 day latency period, and a distraction rate of 1 mm per day with frequency of 4 times per day of 0.25 mm. He also suggested that just corticotomy alone to the long bones would cause less trauma to the periosteum and endosteum, and reduce complications (McCarthy *et al.*, 1992).
Figure 2.1: The combination of external frame and plaster casting for limbs lengthening, design by Codivilla.

(Adapted from Samchukov et al., 2001b).

Figure 2.2: The external ring to perform lower limbs lengthening, design by Ilizarov. One pair of crossed un-tensioned wires (A), one pair of crossed tensioned wires (B) and two pairs of crossed tensioned wires (C).

(Adapted from Samchukov et al., 2001b).
2.2 Biology of Distraction Osteogenesis

Mechanical traction between two bone ends stimulates the formation of new tissue. This is the basis for the Ilizarov effect. The effect from the tension stimulates the formation of new tissue and it also increases the blood supply. The formation of new bone or callus at the edges of two bones occurs at an early stage before the bones are distracted. As the gradual traction is applied, it causes tension on the callus and this stimulates more callus to be formed. When the expected length is obtained the traction is discontinued. The callus undergoes maturation and remodelling to become solid bone.

Basic distraction osteogenesis procedure involves 5 stages (Table 2.1):

<table>
<thead>
<tr>
<th>Stages</th>
<th>Descriptions</th>
</tr>
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<tbody>
<tr>
<td>1) Osteotomy</td>
<td>When the bone is separated surgically and the device applied</td>
</tr>
<tr>
<td>2) Latency</td>
<td>The period from the bone splitting to the onset of distraction</td>
</tr>
<tr>
<td>3) Distraction</td>
<td>The moment when the traction is applied to the callus and further development of the formed callus</td>
</tr>
<tr>
<td>4) Consolidation</td>
<td>The duration of time after the distraction has been discontinued to the time when the distractor is removed and the maturation and consolidation of the distracted callus</td>
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<tr>
<td>5) Remodelling</td>
<td>The time from first application of full functional loading to the end of bone remodelling</td>
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Table 2.1: The stages of distraction osteogenesis, osteotomy, latency, distraction, consolidation and remodelling.
(Samchukov et al., 2001a).
2.2.1 Osteotomy

Osteotomy of bone results in a loss of continuity and the mechanical integrity of the bone. This process stimulates the healing process, which triggers the grouping of osteoprogenitor cells, continues production of bone cells and creates an environment that is suitable for bone conduction. The formation of new bone starts at the fracture ends.

The normal fracture and healing process, involves six stages: impact, induction, inflammation, soft callus, hard callus and remodelling. During the impact stage, the bone experiences stress, dissipation of energy and total absorption of impact. This results in fracture. The induction stage is started soon after the impact and continues through the stage of inflammation and the duration is indefinite. This stage includes an oxygen gradient, bioelectric potentials, bone morphogenetic protein and other non-collagenous proteins for preparation of cell modulation for the healing process.

The inflammation stage occurs shortly after the impact and persists until the major pain and discomfort abates or when fibrous union develops between bone ends. The fracture results in a disruption of the blood supply, haemorrhage and formation of a fracture haematoma. In addition, oxygen and pH drop and bone necrosis and debris cause the release of lysosomal enzymes. Beside the mono- and polynuclear cell activities, there is also a rapid ingrowth of vessels and capillaries particularly periosteal vessels. The low oxygen tension and the pH stimulate formation of fibrous or cartilaginous callus and form a scaffold for cartilage and bone production and supports circulation.
The soft callus stage starts when the pain and swelling subside and the fragments bridge with fibrous and cartilaginous tissues. The vascularity and proliferation of capillaries invade the fracture callus. The osteoclasts start to remove the dead bone. The callus is electronegative relative to the surrounding bone. The oxygen saturation remains low but the pH is normalised.

The hard callus stage followed the soft callus formation through the establishment of unification of new bone. The woven bone forms when the callus changes from fibrocartilaginous tissue to fibre bone. At this stage, the pH is neutral, the callus is still electronegative, osteoclast continues to clear the dead bone and deposition of the new bone by osteoblasts is profuse. Movement or functional weight bearing further stimulates healing.

The remodelling stage is when the fibre bone slowly converts to lamellar bone and the medullary canal is then formed, the oxygen is normal and electronegativity normalises.

### 2.2.2 Latency period

Latency period is the period from osteotomy of the bone to onset of distraction. This phase involves the same stage as in early bone healing where there is disruption of blood vessels that result in a haematoma. The haematoma or blood clot covers the fracture site. The blood supply and activity of cellular proliferation increase. The blood clot is replaced by granulation tissue, which is the combination of inflammatory cells, fibroblast, collagen and capillaries. This stage (inflammation) lasts 1-3 days. A soft callus forms and lasts approximately 3 weeks. The absence of a latency period is associated with reduced callus formation and if prolonged, results in premature consolidation (McCarthy et al., 2001).
The duration of latency is controversial for facial bone distraction osteogenesis. An experimental study using 20 minipigs, demonstrated that the bone showed the same degree of stability with a 0 or 4 day latency period (Glowacki et al., 2004). Other animals studies supporting this idea, showed equal bone strength and callus formation between a latency duration of 0 and 7 days in the sheep model (Tavakoli et al., 1998). Troulis and associates stated that the same radiological density was noted in the pig model with latency periods of 0 and 4 days (Troulis et al., 2000). A shorter latency period was suggested to be sufficient for the early stage of healing process because the craniofacial bones have a rich vascular supply (Swennen et al., 2001). In a review of published studies of craniofacial distraction osteogenesis in 3278 patients, there were no difference between the application and non-application of the latency period (Mofid et al., 2001). Mandibular distraction was reported to have a latency period of 0 – 2 weeks (Aida et al., 2003). Based on the above inconsistencies the suggested optimal duration is between 5-7 days (McCarthy et al., 2001).

2.2.3 Distraction period

Following the soft callus stage, fibrocartilaginous tissue is replaced by osteoblasts to form hard callus. Cartilage starts to ossify and osteoblasts deposit new bone on the calcified cartilage matrix. For many fractures, this stage lasts 3 to 4 months. This is followed by the remodelling stage when bone fibre slowly remodels to lamellar bone and the medullary canal is reorganised.

Distraction has to occur prior to the hard callus stage. Gradual traction to the soft callus creates the tension that stimulates changes in the cellular and subcellular levels. This growth stimulating effect causes prolongation of angiogenesis with increased tissue oxygenation and
fibroblastic proliferation. During distraction, bone formation as well as spindle-shaped fibroblast-like cells occurs parallel to the direction of the vector of traction. Between days 3 to 7 post-distraction, vessel ingrowth occurs at a rate 10 times more rapidly than during normal fracture healing. Blood flow remains elevated at about 3 times that of control levels for at least 17 weeks post corticotomy (Aronson, 1991; Aronson, 1994a; Aronson, 1994b).

By the second week, osteogenesis progresses from the bone edges towards the centre of the distracted gap. By the end of the second week, mineralisation is initiated. At this stage, three distinct zones can be differentiated (Samchukov et al., 2001a) (Figure 2.3). The zones are;

1. Fibrous interzone in the middle where tensional stress is maximal. This zone consists of highly organized, longitudinally oriented, parallel bundles of collagen with spindle-shaped fibroblast-like cells and undifferentiated mesenchymal cells.

2. Mineralisation zones at the periphery (two separate zones) providing active osteogenesis throughout the distraction period. Kojimoto et al. further described the mineralisation zones as: zones of increased bone density (zone of sclerosis); and a zone of low density (zone of remodelling) (Kojimoto et al., 1988)

3. As the regeneration matures, another two distinct zones can be observed at the periphery of the mineralization zones. They are the remodelling zones.
The distraction stage involves the activation of the distractor, which applies the traction forces between the bone fragments. The traction is gradually applied to pull the fragments apart and this stimulates new bone formation between the bone fragments. The soft callus remolds into hard callus and takes approximately 3-4 months. This is followed further remoulding of hard callus with the full normal strength.
Figure 2.4: Radiograph (left) and schematic drawing (right) of a goat tibia demonstrating structure of distraction regenerate during the consolidation periods. Note two radiolucent zones of remodelling (RZ) adjacent to the residual host bone segment (RHBS) and divided by the mineralization zones (MZ).

(Adapted from Samchukov et al., 2001a).

NOTE:
This figure is included on page 16 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.5: Photomicrographs of goat tibial distraction regenerate demonstrating the three types of bone maturation during the consolidation period.

(Adapted from Samchukov et al., 2001a).
2.2.3.1 Rate and Frequency of distraction

The optimal distraction rates were between 1–2 mm per day (McCarthy et al., 2001). This had been shown to stimulate the osteoprogenitor cells and other osteoblastic activities involved in bone healing. This rate was also noted to be well tolerated and promoted the histogenesis of surrounding soft tissue (McCarthy et al., 2001).

2.2.4 Consolidation period

The consolidation stage refers to the time between the discontinuation of the traction force and when the distractor is removed. The mineralisation of hard callus occurs during this period. Assessment of the distracted bone is usually based on its radio-density. The new callus is noticeable on plain radiology after one to two months (Figure 2.4 and Figure 2.5). Another imaging technique suggested to be useful in the evaluation of new bone, during the consolidation period, is ultrasound (Juenger et al., 1999). This is a non-invasive and reliable method for evaluation of mandibular distraction (Troulis et al., 2003).

There are several assessments that can be used in deciding when to remove the device. Clinical evaluation and image assessment of distracted bone through to radiography or ultrasonography have been used to determine the time for distractor removal. The consolidation duration is arbitrarily at a minimum, twice the distraction time. Palpation of the bone and bone movement can also be undertaken during these periods (Mofid et al., 2001).

Felemovicius and co-workers conducted post-distraction bone assessment using bone scintigraphy in 20 cases, with the patient’s ages ranging from 3 months to 22 years. They
suggested a consolidation period of 10 weeks or less in children and between 10 to 14 weeks in young adults and adults (Felemovicius et al., 2000). It was reported that a 6–8 week consolidation period was used in 72% of patients involved in mandibular distraction osteogenesis for either congenital and acquired craniofacial deformities (Swennen et al., 2001).

A classification based on the radiological evaluation of regenerated bone had been proposed as a guideline for device removal (Cope & Samchukov, 2001). The classification was based on 4 different categories: length, width, density of the mineralised new bone and presence of the interzone. The interzone, which was occupied by the callus, appears more radiolucent. The absence of the interzone suggested a more mature formation of new bone within the distraction gap. The stability of the distractor fixation influenced the quality of regenerated bone. It was also shown that stable fixation resulted in complete remodelling within 10 weeks after rigid fixation (McCarthy et al., 2001). Kaban and associates conducted a correlation study of the biomechanical stiffness with radiological density and ultrasound of mandibular distraction in Yucatan minipigs (Kaban et al., 2003). They illustrated that following 24 days of neutral fixation, high clinical stability and increased bone density was evident but only showed 25.5% stiffness as compared to control.

2.2.5 Remodelling period

The remodelling stage starts at the application of full functional loading to the complete remodelling of new bone. It takes a year or more before the newly distracted bone resembles the original bone (Samchukov et al., 2001a).
2.3 Craniofacial Distraction Osteogenesis

2.3.1 Dentofacial traction

The principle of traction and expansion is not new, as dental correction by distraction was reported in 1728 by Fauchard (cited in Weinberger, 1916). A metal plate was used to anchor the teeth and caused movement of the teeth but not of the bone. Wescott introduced the use of an expander appliance in the upper jaw (Wescott, 1859). A telescopic bar and two double clasp separators were used to correct crossbites. The following year, Angell, repeated the same technique but modified it by using a threaded jackscrew connected to the premolars for palatal expansion (Angell, 1860) (Figure 2.6). The palatal expansion was further standardized by activating it twice a day for a 3 weeks, followed by a short period for new bone consolidation Goddarad (1893) (cited from Cope et al., 1999).

Figure 2.6: Angell's palatal expansion appliance on the maxillary arch.
(Adapted from Cope et al., 1999)
2.3.2 Craniofacial osteotomies

Orthognathic surgery involves an osteotomy of facial bones for correction of facial deformity. Hullihen first reported the correction of a prognathic mandible using a partial osteoplastic resection (Hullihen, 1848). Blair (1907) (cited by Cope et al., 1999) demonstrated the application of a bilateral horizontal ramus osteotomy to advance the mandible.

Mandibular corpus osteotomy has been used for the advancement of retrognathic mandibles (Converse & Shapiro, 1952). The vertical mandibular osteotomy and acute forward advancement was reported by Brown (1918) and Bruhn-Linderman (1921) (cited from Limberg, 1928; Limberg, 1925). The one step advancement process has a limitation where bone contact is poor.

Different osteotomy designs were carried out to make sure the bones were more stable by performing C-shaped and oblique L-shaped osteotomies (Limberg, 1925) and a step-like sliding osteotomy for lengthening (Limberg, 1928) or widening (Eiselberg, 1906) the body of the mandible.

2.3.3 Mandibular distraction

The first mandibular osteo-distraction was conducted by Rosenthal in 1927 with an intraoral tooth borne apparatus that was constantly activated over a 1 month period, (cited by Cope et al., 1999). Ten years later Kazanjian performed gradual incremental traction on the mandible instead of one-step expansion of bone. He performed the L-shape osteotomies in the corpus and anchored the symphysis to an “over the face” appliance (cited by Cope et al., 1999). An elastic band was used to advance the mandible. In late 1940, Crawford performed traction by
using a jackscrew to move the collapsed mandible to the correct position and maintained it in place with an occlusal splint. Distraction osteogenesis was not appreciated in the early stages due to limitations in bone handling, limited distraction devices and instability of the bone fixation (cited by Cope et al., 1999).

Mandibular distraction osteogenesis was first studied experimentally on the canine mandible (Snyder et al., 1973). A defect was created by removal of a 15 mm bone segment on one side to create a crossbite. The mandible was allowed to heal for 2.5 months. The affected mandible was then re-osteotomised and an extra-oral distractor was placed. The distractor was activated after a 7 day latency period at the rate of 1mm per day for 14 days and the occlusion was corrected. Intraoral mandibular lengthening with the same protocol was conducted in dogs, achieving a lengthening of 5 mm and 15 mm (Michieli & Miotti, 1977). They noted that the histological examination showed new callus formation and also the presence of collagenous tissues, which later matured to form lamellar bone. Mandibular distraction osteogenesis which demonstrated the different zones of callus healing was performed by Panikarovski (1982) (cited from Cope et al., 1999). The histological examination in his study showed that the central region of distracted gap was occupied by fibrous tissue, mainly collagenous fibres and capillaries oriented parallel to the distraction vector. The trabeculae were oriented longitudinally extending from the original osteotomy site towards the central zone. Kutsevliak and Sukachev further experimented with this technique on canine mandibles (Kutsevliak & Sukachev, 1984). Karp and associates continued to investigate the distraction osteogenesis on canine mandibles (Karp et al., 1992; Karp et al., 1990). They further identified four zones within the distracted gap; a central zone with fibrous tissues, a zone with new bone formation, a zone with bone remodelling and a zone with mature bone.
Although dogs have been used extensively in orthopaedic bone studies, there are problems, namely, dogs have a much greater healing capacity than humans (Martini et al., 2001). There are also ethical issues using dogs for experimental purposes in Australia.

The first reported mandibular lengthening by distraction osteogenesis in humans was performed by McCarthy on patients with craniofacial discrepancies (McCarthy et al., 1992) (Figure 2.7). Children with hypoplastic mandibles (1 with Nager's syndrome and 3 with unilateral hemifacial microsomia) were corrected by increasing the length and volume of the affected bones. The use of distraction osteogenesis has also been reported in correction of hypoplastic mandibles (Molina & Ortiz Monasterio, 1995). They reported the first bi-directional mandibular distraction (Figure 2.8) on 87 unilateral hemifacial microsomia patients and 19 bilaterally hypoplastic mandibles. They managed to correct the asymmetry of the patients’ faces. Further improvement of the technique of mandibular distraction osteogenesis was reported by McCarthy and associates, based on their 10 year experience (McCarthy et al., 1999). They reported 70 cases of distraction osteogenesis using unidirectional distractors in 33 patients and multidirectional distractors in 37 patients. Mandibular distraction osteogenesis has also been used to correct craniofacial discrepancy after injury, degenerative diseases and tumour resection.
Distraction appliances can be divided into unidirectional and bi-directional. The unidirectional extraoral device (Hoffman Mini Lengthener by Howmedica Co., Rutherford NJ) has been used to correct craniofacial deformity since 1989. Two plates with 2 pins are attached to the proximal and distal fragments and after the device is activated one vector is created (McCarthy et al., 1992).

On the other hand the bi-directional device involves 3 attachments to 3 bone fragments. Two separate corticotomies, horizontal in the ramus and vertical in the corpus, are performed via intraoral incisions. The screws are attached one on each side of the corticotomy and the central screw placed at the angle of the bone and acts as a fixed point for independent vertical and horizontal traction (Molina & Ortiz Monasterio, 1995).

Figure 2. 7: Mandibular distraction osteogenesis (unidirectional), placement of the device to hold two fragments and arrow showing the vector of distraction. (Adapted from McCarthy et al., 1992).
2.3.4 Mechanical movement in mandibular distraction

Satisfactory outcome from surgical correction of the hypoplastic mandible is associated with many factors such as three-dimensional anatomy, severity of the deformity, the positions of associated structures, muscle tension, the magnitude of movement and stability of fixation and correct distraction vector (Schendel et al., 1978; Trauner & Obwegeser, 1957; Van Sickels & Richardson, 1996). Computer tomography data and 3-dimensional (3D) CT reconstruction assists in simulation of multifocal osteotomies for correction of hemifacial microsomia patients (Kunz et al., 2003). They found these investigations useful in defining the problem and conveying the plan to the patient but multifocal 3D distraction may cause divergence from the expected outcome by interference with distraction vectors. Demann and Haug conducted a study to look at the effect of the distraction vector from the position of the device and soft tissue...
activities (Demann & Haug, 2002). They used a polyurethane skull and mandible replica to help plan the position of the distraction device parallel to body of the mandible.

Demann and Haug concluded that the position of the distractor caused minimal effect on the distraction vector but the combination of position and stimulation of soft tissue caused obvious vertical deflection (Demann & Haug, 2002). Hendrickx and co-workers investigated, by cephalometry, the movement of the proximal segment in the sagittal plane in patients treated with distraction (MD-DOS device) for mandibular lengthening. They noted that the proximal segment was anteriorly rotated, whilst the distal segment was posteriorly rotated, post distraction. They suggested the anterior rotation might be due to the realignment of the proximal segment during distraction and the masticatory muscle contraction was greater than the force from distraction. They also stated that there was anterior and inferior rotation of the distraction vector, which suggests that the masticatory muscles caused anterior deflection and posterior redirection was influenced by the distraction force (Hendrickx et al., 1999). Gonzalez and co-associates conducted a study to look at the positional changes and stability of the bone in bilateral mandibular lengthening and widening by distraction (Gonzalez et al., 2001). Their study used baboons with a distraction rate of 0.9 mm per day for 10 days and a 2 month consolidation period. They stated that during the distraction process the proximal segment moved superiorly and slightly anteriorly. During the consolidation periods the segment moved anteriorly only. The distal segment was reported to have moved anteriorly and remained stable in the anterior plane. Rotation of the proximal segment occurred at the altered angle and this was suggested to be due to muscle involvement (Gonzalez et al., 2001).
2.4 Complication Related to Distraction Osteogenesis

The long term stability of the orthognathic and craniofacial surgery is dependent on the tension created on the soft tissue (Douma et al., 1991; Ellis & Carlson, 1983; Ellis et al., 1988; Gassmann et al., 1990; Van Sickels et al., 1986). The role of muscle is the most important unsolved difficulty in lower extremity distraction osteogenesis (Paley, 1990) and problems such as relapse may be associated with lack of muscle adaptation.

2.4.1 Relapse

The relapse phenomenon in orthognathic surgery was defined as the tendency of bone and surrounding soft tissue to return to the pre-surgical condition and is a frequent but unpredictable complication (Van Sickels et al., 1988). This phenomenon leads to problems in dental occlusion, extended duration of treatment and affects aesthetics. Distraction osteogenesis, concurrent with soft tissue manipulation, was suggested to allow bone to regenerate with an even consistency and to prevent relapse (Schendel & Epker, 1980).

Review of the literature on craniofacial distraction illustrated a lack of long term data on skeletal relapse (Swennen et al., 2001). However, their review found that 30% of cases showed skeletal relapse. Another assessment was conducted by questionnaire involving 3,278 craniofacial distraction cases, results obtained showed that 64.8% of respondents experienced relapse (Mofid et al., 2001). They also stated that relapse occurred at less than 6 months post-distraction. The surgeons in this study suggested that relapse was not due to the consolidation period or distraction process but more a factor of growth. Cho and associates also reported the occurrence of relapse 6 months after bimaxillary distraction in two out of nine patients (Cho et al., 2001).
Evaluation of mandibular distraction with 3-D CT imaging, showed signs of relapse in 50% of cases after 1 year (Huisinga-Fischer et al., 2003). They also stated that relapse seemed to have a progressive character after 3 years post distraction as compared to 15 weeks post distraction and they suggested that this was due to the remodelling activity of new bone. This has been supported in a study by Ko and associates, who found that relapse occurred in 30% of cases at one year after mandibular distraction with a multidirectional device (Ko et al., 2004). Where as, the angle of the mandible influenced the occurrence of relapse in a study by Van Strijen and associates. In this study 57% of cases with a high mandibular angle had a higher chance of relapse when compared to lower angle cases (van Strijen et al., 2004). However, follow up in 106 patients after 3 months to three and half years was reported as not showing signs of relapse (Molina & Ortiz Monasterio, 1995). The long-term study by Del Santo also stated that there was no relapse after about 1 year post distraction (Del Santo et al., 2000).

(McTavish et al., 2000) conducted a study to investigate relapse in the sheep mandible with distraction osteogenesis with remodelling periods of 3, 6, 9, and 12 months. They stated that there were no relapses at the 12 month remodelling period. In this study the osteotomy site was at the body of the mandible (diastema area), which is not surrounded by many muscles and it was believed that there was not much muscular pressure to induce relapse. Rachmiel and associates conducted a study on the distraction of the mid face of sheep and they noted that relapse occurred in the first 3 months post consolidation period (Rachmiel et al., 1995). Conversely, this study has showed relapse but still there is little muscle attachment to the mid face. Vertical mandibular distraction osteogenesis has a high chance of relapse. The superficial, middle and deep masseter and the medial pterygoid muscles surround the mandibular ramus and the lines of action are at right angles to the line of distraction.
2.4.2 Muscle Responses

Distraction osteogenesis involves 2 major physical manipulations; detachment and elongation of the masticatory muscle (Liu et al., 2003). The muscles were known to adapt well, but the rate of the distraction that is suitable for hard tissues may not be suitable for muscle adaptation (Lindsey et al., 2002). The adaptation and proliferation of muscle was demonstrated to be influenced by mechanical variables such as the different rates of distraction and the length of the distracted gap (Castano et al., 2001; Simpson et al., 1995). In addition it was also reported to depend on the age and maturity of the animal (Hayatsu & De Deyne, 2001). The gradual distraction process causes a series of changes in the muscle as they adjust to the new position.

There are several parameters and methods that have been used to study the adaptation and changes in muscle related to the distraction process of limbs (Day et al., 1997; De Deyne, 2002; Fink et al., 2000; Lee et al., 1993; Lindsey et al., 2002; Makarov et al., 2001a; Makarov et al., 2001b; Schumacher et al., 1994; Williams et al., 2001) and masticatory muscles (Castano et al., 2001; Fisher et al., 1997; Tuz et al., 2003; Xiao et al., 2002) (Figure 2.9 and Figure 2.10). Gross assessments such as weight, length, cross section and thickness of muscle have been used as a basis of investigation. Histology, histochemical, immunohistochemistry, clinicopathology and molecular studies have also been used to investigate the muscle adaptation during distraction and post distraction periods.
Investigation of Vertical Mandibular Distraction Osteogenesis on Masticatory Muscles in ‘Unilateral Hemifacial Microsomia Like’ Defect in the Sheep Model

Figure 2. 9: Mandibular distraction osteogenesis on canine mandible, (an oblique osteotomy was performed). The masseter muscle illustrated in this figure was smaller than normal.

(Adapted from Fisher et al., 1997).

Figure 2. 10: Mandibular distraction osteogenesis on porcine mandible. An angle osteotomy on mandible. The muscles were perpendicular to the distraction vector. The movement of the bone fragments (arrows).

(Adapted from Castano et al., 2001).

NOTE:
This figure is included on page 29 of the print copy of the thesis held in the University of Adelaide Library.
2.4.2.1 Level of Changes in the distracted muscle

The sites of muscle changes are related to the orientation of the distraction device and the anatomical site of muscles involved. The changes are also reported to be more at the musculotendinous junction in the lower limb (tibial) muscles (Sun et al., 1996; Swennen et al., 2001). Other studies on lower limb (tibial) muscles showed that changes occurred throughout the muscle fibres (Schumacher et al., 1994; Yasui et al., 1991). Muscle changes also depend on the site of the osteotomy; for example in long bones, osteotomy at the diaphysis level resulted in distinctive muscle sclerosis and increased fibroblast proliferation where as metaphyseal osteotomy showed more of regeneration (Makarov et al., 2001a). They also noted that the bi-focal lengthening maintained the architecture of muscle fibres (Makarov et al., 2001a).

The effects of distraction forces on the masseter muscles are different dependent on where the device was fixed in relation to the muscles position (Castano et al., 2001). The masticatory muscles in dogs which were parallel to the distraction vector were reported to show transient atrophy, regeneration and hypertrophy when compared to the muscles which were oriented perpendicular to the vector (Fisher et al., 1997). Xiao and co-workers conducted the distraction experiment on the dog's mandible and their results supported this finding (Xiao et al., 2002).

2.4.2.2 Weight of the distracted muscle

The distracted limb muscle has been noted to increase in weight during the distraction process but return to normal when the distraction ends (Schumacher et al., 1994). They also suggested that the early increase in weight might be due to muscle oedema and increased proliferation of new muscle cells. Increase in the tissue volume and weight were also suggested to be due to
an increase in endomysial and perimysial fibrosis (Simpson et al., 1995). In contrast, there is no difference in net weight of the lower limb muscles between experimental and control sides (Sun et al., 1994).

The normal wet weight of masticatory muscles in sheep has been studied by De Jongh and co-workers (De Jongh et al., 1989). They conducted this study on a local breed (Tessellar), female and approximately 1½ years old. They reported that the wet weight of superficial masseter was 34.2 grams, the deep masseter was 77.5 grams and the medial pterygoid was 30.5 grams. In contrast, mandibular distraction osteogenesis in rats showed that the weight of masseter muscle was smaller on the operated side compared to the control side (Liu et al., 2003). This study also stated that the distracted masseter muscle gained less weight than the control sides.

The above studies were conducted in normal and non-growing animals. The reduction in muscle mass might be due to disuse atrophy. The perpendicular vector orientation of distraction in relation to masseter muscles may cause reduction in its bulk. Distraction osteogenesis of the human mandible has been reported to induce soft tissue lengthening and increase in its volume (McCarthy et al., 2001; Polley et al., 1997). On the other hand the soft tissue was reported to reduce in volume in relation to the distraction process (Marquez et al., 2000). This phenomenon was demonstrated in conventional surgical correction, as the movement is toward the normal side, soft tissue contour increased and the hypoplastic soft tissue on the affected side is further stretched and attenuated (Kaban et al., 1998). This was solely an observation and not an objective measurement.

### 2.4.2.3 Length of distracted muscle

Lengthening of the limb by distraction osteogenesis demonstrated an increase in muscle length (Hayatsu & De Deyne, 2001). Muscles were shown to increase in length at the muscular part...
and not at the tendinous part (Sun et al., 1994). In contrast De Deyne et al., conducted an investigation to look at the effects of different distraction rates on anatomical site of muscle in lengthening (De Deyne et al., 2000). They noted that slow distraction (0.7mm per day) induced an elongation both at the tendinous part and muscle at rest and the faster rate of 1.4 mm per day only resulted in elongation of muscle component and not the tendon. The muscle portion, with the contractile component, is adjusted by adding or reducing the number of sarcomeres. Studies on the limbs of the dogs, showed that by increasing the number of new sarcomeres resulted in an adjustment to a new length (Makarov et al., 2001a). Sarcomeres showed an increase in number (Simpson et al., 1995) but not in length (Williams et al., 1998) (Figure 2.15).

2.4.2.4 Cross section and thickness of distracted muscle

Imaging techniques have become an important tool in neuromuscular evaluation. Ultrasound imaging uses an echo, which is sent and then received to create a sound wave that forms an image. The transducer is made from material which can release and receive the signal from the activity of the sound wave. The water levels in different types of tissues allows for different penetration and reflection of energy. These differences create the edges of muscle and other surrounding structures. Ultrasonography is a non-invasive, easy and economical technique for continuous assessment of the muscles of the head and neck (Ariji et al., 1994). Ultrasonography is used to detect swelling in the head and neck region (Siegert, 1987) and to evaluate swellings and pain (Bakke et al., 1996). The hyperechoic bands and hypoechoic areas will give an indication of local tissue activity (Ariji et al., 1994). The measurement of masseter muscle thickness (Raadsheer et al., 1996) and volume (Benington et al., 1999) has been reported using an ultrasonography method. The thickness of masticatory muscles was noted to be related to the morphometry of the face and other functional factor such as bite force.
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(Literature Review) (Raadsheer et al., 1999). Ultrasound has been shown to be a consistent diagnostic method for the assessment of cross-sectional proportions and areas of muscles of the head and neck (Emshoff et al., 1999).

Furthermore, this technique was stated to be a reproducible and reliable procedure in measuring the area of the middle and lower masseter (Bertram et al., 2003b; Emshoff et al., 2002; Prabhu & Munshi, 1994) and also the anterior masseter (Bertram et al., 2003a). Emshoff and associates, stated that standardization of the positions of transducer, position of the scan area, biting position and resting are important elements for a better diagnostic procedure (Emshoff et al., 2003). Emshoff and Bertrams also used ultrasound scanning to evaluate the effects of implants (such as splint stabilisers) on masticatory muscles in the correction of craniofacial syndromic patients (Emshoff & Bertram, 1995; Emshoff & Bertram, 1998). The normal left or right masticatory muscles has minimal bilateral differences and in hemifacial microsomia patients, the normal side does not demonstrate a compensatory mechanism (Huisinga-Fischer et al., 2001). Huisinga-Fischer and associates studied the long-term results of mandibular distraction in hemifacial microsomia patients using three-dimensional (3-D) C.T.

The volume of the affected side was noted to have increased 3 years after the lengthening process, compared to the contra-lateral side (Huisinga-Fischer et al., 2003). They also stated that relapse occurred 1 year after distraction. In addition, the normal growth of the unaffected side and affected side occurred simultaneously but resulted in an increase in asymmetry 15 weeks post-distraction. The symmetry was not corrected at this stage because the affected side was retarded in growth to start with. Therefore the distraction osteogenesis and continuing growth were not able to catch up with the normal side.
Computerised tomographic scans of the mandibular distraction osteogenesis of humans showed an increase in medial pterygoid muscle volume and the bilateral distraction of mandible showed almost twice the volume of this muscle if compared to the pre-distraction image (Mackool et al., 2003).

2.5 Cellular Changes of Skeletal Muscle.

2.5.1 Normal histology of muscle

Skeletal muscles are composed of muscle fibres, known as muscle cells or myofibres. The muscle fibres are elongated with multinucleated syncytia with a length of up to 5 cm long. Muscle fibres appear in cross section as having hexagonal shape (irregular polygons) with slightly rounded edges. The fibre size is usually shown as the lesser transverse diameter (Brooke & Kaiser, 1970) and has a diameter in the range of 10-80 μm (Adams et al., 1962). Muscle fibres vary in size depending on age, exercise, nutritional status, location and function of muscle and also species of animals (Jubb et al., 1991).

Muscle fibres attached together in a bundle are termed fasciculi and are arranged parallel to each other (Figure 2.11 and Figure 2.12). They are enveloped both individually and in groups by connective tissue sheaths. The engagement of several hundred muscle cells will form the primary, secondary and tertiary bundles. The connective tissues, which cover the outer most muscles, are known as epimysium. Its projection into the primary, secondary and tertiary bundles, are termed the perimysium. The extension of the perimysium between the muscle cells is called endomysium. These connective tissues support the existence of other structures such as nerves, fibroblasts, capillaries and other mononuclear cells (Adams et al., 1962). Myofibrils are built up by a series of filaments, which have two different sizes. The thin filament
or actin (60 Å wide) is formed by tropomyosin and tropinin and the thick filament of myosin (160 Å wide) (Figure 2.13). The actin and myosin are the contractile component. The contractile unit or sarcomere is about 20μm long.

2.5.2 Muscle injury and healing

The healing of muscle injury has been documented by Jarvinen and co-workers (Jarvinen et al., 2000). They stated that the pathobiology of the injured muscle involves three phases. The first phase (Destruction phase), is characterized by hematoma, myofibres necrosis, and infiltration of inflammatory cells. Second phase (Repair phase), consisting of phagocytosis of the necrotized tissues, regeneration of the myofibres, production of connective tissues scar and growth of capillaries. The third phase (Remodelling), involves maturation of regenerated myofibres, contraction and reorganization of the scar tissue, and restoration of the functional capacity of the repaired muscle (Figure 2.14). The extent of regeneration of injured muscles varies, but in most situations the process involves necrosis of mature myofibres, infiltration of inflammatory cells and phagocytosis of damaged muscle, revascularization, proliferation of muscle, differentiation and fusion and reinnervation (Grounds, 1991).
Figure 2.11: A drawn illustration of the small fascicle of the myofibre and structures. Perimysium (1), endomysium (2), individual myofibre (3), elongated nuclei (4), contractive myofibres (5), small myosatellite cells (6) and capillaries (7).

(Adapted from Mastaglia & Walton, 1982).
Figure 2. 12: Normal muscle fibres (M) and muscle spindle (S), presence of several muscle fascicles and perimysium (P) x 350; H&E.
(Adapted from Swash & Schwartz, 1991).

Figure 2. 13: The contractile component of the skeletal muscle (sarcomere). The micro filament known as actine (A) and myosin (M).
(Adapted from Makarov et al., 2001b).
2.6 Muscle healing in distraction osteogenesis

2.6.1 Destruction phase

The pressure increases from the traction process, resulting in stretching of the muscle fibres and if the tolerance limit is exceeded, muscle fibres rupture (Calandriello, 1975). It also been documented that constant pressure and stretching of lower limb muscles with a tissue expander will cause thinning of muscle fibres in rats (Kim et al., 1993). The ruptured muscle fibres resulted in distortion of the contractile components (Simpson et al., 1995). The muscle contraction creates gaps between the rupture sites, the gap fill with the blood from the torn vessels and a haematoma develops.

2.6.2 Necrosis of myofibres

Muscle fibre damage involves the breakdown of the myofibre plasma membrane and exposure of the sarcoplasm. Necrosis starts at the rupture site and extends the whole length (Jarvinen et al., 2000) but condensation of cytoskeletal material at a contraction band limits the process. Review of muscle degeneration and regeneration following trauma showed that the damaged muscle will undergo hyaline degeneration and this results in loss of the striations (Allbrook et al., 1966). On the contrary, Kim and co-workers in their experiment on expansion of limb muscle with a muscle expander noticed that the muscle’s striation persisted (Kim et al., 1993). Their study did not involve sufficient expansion force that would have lead to muscle degeneration. Their study also only involved traction of soft tissues without osteotomy and elongation of bone.
2.6.3 Inflammation

The ruptured blood vessels allow blood-borne inflammatory cells to infiltrate the damage site. The chemoattractants released from the necrotic muscle fibres further attract inflammatory cells. The inflammatory cells, such as macrophages and mononuclear cells then act as chemotactic signals to attract more inflammatory cells from blood circulation to the injured sites (Tidball, 1995; Tidball, 2005). Mononuclear cells infiltrate the broken site 12 hours after injury. On day four after muscle injury, there was a reduction in the number of mononuclear cells. The polymorphonuclear cells especially leucocytes predominate in the immediate stage but were replaced by monocytes. These cells soon changed to macrophages, which are involved with proteolysis and phagocytosis of the necrotic tissues. In phagocytosis the basal lamina of the muscles were not attacked by macrophages.

2.6.4 Repair and Remodelling Phase

The basal lamina forms the scaffold for new muscle formation. The new myoblasts (basophilic cytoplasm) were arranged and started to form a new muscle at days 4 to 6 post injury. The myotubes occupied by myofibrils with central nuclei and cytoplasm was noted around 2 weeks post injury, indicating that healing was taking place. The myoblasts were divided by mitotic division and formed sarcoblasts that were responsible for development of new muscle fibres. The muscle healing process takes about 3 weeks to complete (Allbrook et al., 1966).
Figure 2. 14: Mechanism of skeletal muscle adapted in distraction process. Myofibril proliferation and regeneration to bridge the damage muscle. (Adapted from Makarov et al., 2001b).

Figure 2. 15: The traction process caused the contractile unit to increase in number as adaptation to the new length. Circle shows the new sarcomere. (Adapted from Makarov et al., 2001b).
Figure 2. 16: Mechanism of the adaptation of skeletal muscle, which involved the connective tissues. Healing by fibrous tissues formation (sclerosis).
(Adapted from Makarov et al., 2001b).

2.7 Histopathology of distracted muscle

Histopathological studies of muscle involved in callus distraction have been conducted in the lower limb and craniofacial area during the distraction, consolidation and remodelling periods. The studies generally looked at the degeneration and regeneration activity of muscle structures in various distraction protocols. The muscle fibre changes were shown to undergo dystrophy (Makarov et al., 2001a), atrophy (Fink et al., 2001; Lee et al., 1993), degeneration (Fink et al., 2001), necrosis (Lee et al., 1993; Simpson et al., 1995), fibroblast proliferation, sclerosis (increased connective tissue at the perimysium and endomysium levels) (Figure 2.16) (Makarov et al., 2001b) (Figure 2.17 and 2.18) and regeneration (Castano et al., 2001; Makarov et al., 2001a).
The lower limb muscles and the face muscles responded differently as the anatomy and the muscle forces act differently (Castano et al., 2001). Contracture was reported to occur during tibial distraction and was related to muscles that involved 2 joints (Paley, 1990).

2.7.1 Histopathology

The traction force caused the rupture of some muscle fibres when it exceeded their tolerance. Some of the damaged fibres degenerated and can be described as pale–staining liquefied or hyalinised acidophilic cytoplasm and showed loss of striation with H and E stain (Lee et al., 1993). Lee and co-workers conducted a study of the lower limbs of rabbits to look at the muscle changes, at different percentages of the total increment, during the distraction period. Their study also demonstrated that with 20% or more lengthening, there was internalisation of nuclei and endomysial fibrosis, which is indicative of irreversible damage of muscles. Other studies on the lower limb distraction in other species of animals; rabbit models (Lee et al., 1993; Simpson et al., 1995; Sun et al., 1994; Williams et al., 1999) and goat models (Makarov et al., 2001a) showed the same finding. Van der Meulen and co-workers conducted an experiment to look at the effect of distraction rates on the digastric muscles in mandibular distraction osteogenesis on sheep models (van der Meulen et al., 2005). They experimented the distraction rate at 1mm and 3 mm per day and achieved 21 mm distraction gaps. Their study noted that the distraction process only distracted muscles half of the length of the distracted gap. They concluded that the distraction rate of 3mm per day resulted in maladaptation when compared to the rate of 1mm per day. In contrast, the distraction osteogenesis in porcine mandibles suggested that the faster rate of 2–4 mm per day of distraction induced more myocyte proliferation (Castano et al., 2001). In addition, Lindsey and co-workers conducted a
study to look at the effect of limb lengthening at 30% and found that the muscle adapted well by increasing fibre length and producing additional sarcomeres (Lindsey et al., 2002). Muscles were found to adapt well at high distraction frequency (Shilt et al., 2000). They conducted distraction on the tibia of rabbits at a rate of 1.05 mm/day and compared the automated high frequency distraction process using 1,440 increments per day to 3 increments per day, performed manually. However another study showed no sign of muscle inflammation or necrosis (Kim et al., 1993). This study was conducted using a muscle expander to elongate the femoral muscles in rats.

Fink and associates conducted a study to investigate the muscle changes after immediate post distraction and with a consolidation period of 25 days. The mixture of pathological activity, such as tissue degeneration, cytoarchitectural changes related to myogenic damage (target fibres, central cores, minicores), endomysial and perimysial fibrosis and regeneration was noted in both periods but was predominantly evident during the immediate post-distraction period. The necrotic muscle was shown to become shorter, surrounded by inflammatory cells and some underwent phagocytosis. They stated that the degenerative process occurred when the muscle underwent ischemia, which was followed by necrosis and sclerosis. After a 25 day consolidation period, satellite cells and myoblast myogenesis were more prominent (Fink et al., 2001). They also reported that both type I and II muscle fibres atrophied, as demonstrated by ATPase staining of distracted muscles. This meant that there is no predisposition to atrophy with the two types of muscles during distraction. The transformation of muscle fibres was reported in slow electrical stimulation and increase of the load or pressure on the muscle (De Deyne et al., 1999). They also stated that increased loading on muscles would cause fast twitch fibres to take on the properties of slow twitch fibres.
Histomorphometric evaluation of masseter muscle in distraction of rabbit mandibles has been studied after a one month consolidation period (Tuz *et al.*, 2003). They noted that there were areas of interstitial oedema, inflammatory activity with focal and mild lymphocyte and macrophage activity. They also observed there was evidence of atrophy and hypertrophy of muscle fibres. It is known that reduction in nerve function will lead to some degree of muscle atrophy. Fisher *et al.*, (1997) also reported that atrophy and/or hypertrophy occurred in the masticatory muscles of dogs during the distraction process and up to 48 days consolidation period. Hypertrophy is believed to give long-term stability for patients.

Regeneration has been stated to occur by proliferation of muscle cells (Calandriello, 1975; Castano *et al.*, 2001; Makarov *et al.*, 2001a) (Figure 2.19). Castano *et al.*, 2001 studied the proliferation of myocytes by using immunohistochemistry to localize proliferating cell nuclear antigen (PCNA) with antibodies against it. Myoblasts proliferate to produce more sarcoblasts in the development of new sarcomeres and it has been characterised by using bromodeoxyuridine, a thymidine analogue that is integrated for the duration of cell division, and desmin as a specific marker (Day *et al.*, 1997). Muscle cell proliferation was found to have increased during the distraction period (Fink *et al.*, 2001). The lengthening of rabbit tibiae was stated to result in myocyte hyperplasia (Day *et al.*, 1997).

The distraction process was shown to produce a dramatic up-regulation of a gene, GADD45, causing growth arrest and DNA destruction (Caiozzo *et al.*, 2002). The RNA and DNA ratio was decreased in the muscles perpendicular to the vector but not affecting the parallel muscles (Fisher *et al.*, 1997). The DNA synthesis in the distracted muscle increased at a higher distraction rhythm (Mizumoto *et al.*, 1996). DNA content is an indication of nuclear mass and is
not a good indicator of muscle proliferation as other inflammatory cell infiltration also contributes to the elevation of DNA in muscles. Muscle adaptation is also influenced by the insulin-like growth factor-1 which is released during muscle stretching (De Deyne, 2002).

NOTE:
This figure is included on page 45 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.17: Degeneration of the muscle fibres and sclerosis, which appeared as light pink within muscle fibres (arrows).

(Adapted from Makarov et al., 2001b).
Figure 2. 18: Increased fibrous tissues between the perimysium (arrows) and endomysium (yellow arrow heads). (H&E; X80).
(Adapted from Makarov et al., 2001b).

Figure 2. 19: Regeneration occurred at the musculotendinous junction (arrows), tendon (T) and Muscle (M).
(Adapted from Makarov et al., 2001b).
2.7.2 Histomorphological evaluation

Semi-quantitative analysis of histopathological changes on distracted muscles of lower extremities has been conducted using a scoring method (Lee et al., 1993). They identified muscle fibre sizes, internalisation of nuclei, degeneration, regeneration and endomysial fibrosis as parameters and scored them 0 as normal, 1 as mild, 2 as moderate and 3 as severe changes. More characteristics of muscle changes were identified and levels of scoring were added in the study of frequency and percentage of lengthening in the lower limbs of goat (Makarov et al., 2001a). They studied the effects by assessing the focal dystrophy, necrosis, atrophy, necrosis, fibroblast proliferation, sclerosis and regeneration. They also scored one plus, up to five plus for dystrophy, necrosis, fibroblast proliferation and sclerosis; scoring one plus to four plus for muscle atrophy; and zero plus to two plus for muscle fibre regeneration.

2.8 Lack of Distracted Muscle Adaptation

The strengths and weaknesses of the muscle are related to the ability of the muscle fibres to add the contractile component in adjustment to the new length. The appropriate rate will minimise muscle damage and allow sufficient adjustment of sarcomeres to new lengths (Williams et al., 1994). Studies of lower limbs has shown that the weakness of muscle was due to the failure of the muscle contractile component to proliferate at higher rates of distraction and this will lead to over stretching of the present contractile component (Williams et al., 2001).

In contrast, mandibular distraction of the pig mandible showed that a higher distraction rate (2-4mm) induced more proliferation of muscle cells (Castano et al., 2001). These conditions also resulted in increased collagen type III in the perimysial area (Williams et al., 1998). Increased
deposition of the collagen perimysially will cause over stretching of the muscle leading to contracture and a reduced range of joint movements (Williams et al., 1999). The reduced range of motion may be in part the role of perimysium adjustment to limb lengthening. It was also noted that connective tissues have different adaptation capacities as compared to the contractile component in the distraction protocol of lower limbs (Williams et al., 1998). Over flexion and extension of a joint will result in reduction of sarcomeres and mobilisation will allow a muscle to return to normal status within 4 weeks (Williams & Goldspink, 1971).

Muscle weakness has been shown to start after surgery, during the early stages of distraction but consistently regain strength as the distraction of bone progresses (Oey et al., 1999). Muscle weaknesses are also related to nerve conduction capacity, as it has been shown that the post-lengthening weakness resulted from neuropathy (Polo et al., 1997). It was noted that a denervation process occurs during the distraction process and reinnervation will take place throughout the consolidation period (Fink et al., 2001). The stretched muscle shows a lesser degree of contraction when weak but the contraction becomes stronger, as a result of collateral or axonal enervation rather than the number of functional axons (Polo et al., 1997). Nerve supply to muscles not only influences the contraction activities but also the muscle size.

There are still few studies to investigate the long term effects on both surrounding hard and soft tissues, throughout the consolidation and remodelling period, in hypoplastic mandibular distraction osteogenesis in unilateral hemifacial microsomia (Figure 2.20).
Figure 2. 20: Unilateral hemifacial microsomia patient (above) and the same patient with the device in place on the right mandible that was treated with distraction osteogenesis.

(Adapted from McCarthy et al., 1992).

2.9 HEMIFACIAL MICROsomia

Hemifacial microsomia (HFM) is a condition which involves deformity of the lower one half of the face and has a prevalence of 1 in every 56000 live births (Walker et al., 2004). This second most common condition after cleft lip and palate is a congenital syndrome, which presents with hypoplasia of the craniofacial skeletal and soft tissues. The key features are small jaw, some degree of loss of jaw joint, under development of ear, reduced muscle mass and facial clefts (Cousley & Calvert, 1997). The deformity has been called various terms. The term “Dysostosis
Otomandibulars” has been used in continental Europe, first and second branchial arch syndrome (Grabb, 1965), oculoauricularvertebral dysplasia (Gorlin et al., 1963), oculoauriculo-vertebral spectrum (Cohen et al., 1989), lateral facial dysplasia (Ross, 1975) and McCarthy uses the term unilateral craniofacial microsomia. The term hemifacial microsomia was coined by Gorlin and Pindborg (Gorlin et al., 1964) and was also recommended by Mulliken (Mulliken & Kaban, 1987).

2.9.1 Aetiology of Hemifacial Microsomia

There are several theories of development of this syndrome. This syndrome is a manifestation of a defect of the first and second branchial arches (McKenzie & Craig, 1995). There are two pathological theories to explain the mechanism of development of hemifacial microsomia, a defect of the vascular system (Poswillo, 1973) or of neuroectodermal cell migration (Johnston & Bronsky, 1991). The facial structures that derive from the first branchial arches are described below.

2.9.1.1 Branchial arches and facial development

Early development of oral cavity occurs at the late third prenatal week as an excavation or invagination of the tissues underlying the forebrain (Figure 2.21). This excavation will differentiate to form the oral cavity and the surrounding tissue will grow into facial structures. The protruded tissues horizontally surrounding the oral opening are the branchial arches. These arches form the external facial structure and internal oropharyngeal structure. The facial tissues derived from the first branchial arch are lower jaw, cheeks and overlying tissues of forebrain and forehead.
The branchial arches develop in pairs, which consist of epithelial enveloped mesoderm. The external surface is covered by ectoderm and the internal layer covered by endoderm. Each branchial arch is supplied by neural crest cells under the epithelium that envelop a core of mesodermal cells. The paired mandibular prominences develop into the lower jaw. These buds continue to grow to form the components of the facial structure such as facial bones, cartilage, nerve, vessels and muscles. The muscles further develop and grow in stages. The myotomes are formed within the mandibular arch and penetrate the second visceral arches (Noden & de Lahunta, 1985).

In the first stage, myoblast proliferation begins in 5th and 6th week of embryological development. They appear as spindle shaped cells and continue to develop into myofibres and satellite cells. The myogenic cells are arranged at the site of origin and insertion (Figure 2.22). The second stage involves the formation of a sarcoplasmic membrane that will group the multinucleated myofibril into bundles. The third stage is development of contractile filaments (actin and myosin) and ends with the formation of the T-tubular system. The fourth stage involves further envelopment of the myofilament and increase in the number of myofibrils and nuclei. The final stage is the growth of a basal lamina and an additional sheath of collagen, fibroblasts and capillaries investing each developing muscle. Development continues to grow rostrally and dorsally during 10 weeks of embryological development (Figure 2.23). Migration happens under the surface ectoderm and differentiates into the 4 masticatory muscles and is innervated by the motor branches of 5th cranial nerve (Lewis et al., 2001). The grooves from the first branchial arches are inverted to create external auditory meatus or ear canal.
Figure 2.21: Sagittal view of the brachial region at 4 weeks. Observe the blood vessels that arise from the heart below and passes through each branchial arch.

(Adapted from Avery, 1994).

Figure 2.22: Development of masticatory muscles. Establishment of masseter, temporalis and medial and lateral pterygoid muscles at 10 weeks.

(Adapted from Avery, 1994).
Figure 2. 23: Facial muscles overlying skull and positioned almost at final location at 10 weeks
(Adapted from Avery, 1994).

NOTE:
This figure is included on page 53 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2. 24: Facial blood supply of internal carotid artery by the stapedial artery, which is related to the common and external carotid arteries at 7 weeks.
(Adapted from Avery, 1994).

NOTE:
This figure is included on page 53 of the print copy of the thesis held in the University of Adelaide Library.
Each branchial arch is supplied by blood vessels dorsally from the inferiorly located heart. The growth of the blood vessels happens at different times and some vessels such as the fifth arch disappear but the third, fourth and sixth arch vessels continue to develop. The first and second vessels disappear at the fifth week and the third branchial arch arteries continue to supply the blood to the face. These blood vessels form the common carotid artery. The common carotid artery divides into the internal carotid, which supplies blood to the brain and external carotid, which supplies blood to the ventral parts of the face. The internal carotid at the ear level, branches to form the stapedial artery, which supplies blood to the upper face and palate between 6 – 7 weeks prenatal age (Figure 2.24). The stapedial artery separates from the internal carotid artery at seven weeks and discontinues its blood supply of face and palatal tissues. The external carotid will take over supply of blood to the face and palatal region. This change is very critical because a loss of blood supply will result in poor oxygen and nutrition to the facial area. This may end up with underdevelopment and retardation of growth of certain facial components. The development of hemifacial microsomia syndrome is also stated to be caused by the haemorrhage and haematoma of the stapedial artery. The development of this phenomenon may happen in 6–7 weeks of prenatal age. The seventh week is critical because that is the maximal growth for the lip and palate (Avery, 1994). This abnormality of the vasculature, that results in haemorrhage and hematoma in the stapedial artery area may interfere with the normal development of the facial structures (Poswillo, 1968). Animal experimentation indicates that the pattern of defects caused by haemorrhaging of the stapedial artery results in facial underdevelopment and deformity (Poswillo, 1973).
Chemicals such as thalidomide and retinoic acids were known to induce and cause the facial defects which are characteristic of hemifacial microsomia (Johnston & Bronsky, 1991). This acid is known to destroy neural crest cells and interrupt cell migration. Neural crest cells within the mesenchyme are the major component of the branchial arches. It has been speculated that the retinoic acid changes the arrangement and migration of neural crest cell and results in the abnormalities. If 10 day old pregnant mice are injected with triazine this results in bilateral micrognathia, a low-set ear pinna and abnormal middle and inner ear, facial nerve, nervous system and stapedial artery. Furthermore, that study also showed that 30% of unilateral haematomas were present in their study (Louryan et al., 1995). There is some disagreement on this concept of haematomas as the effect might be acting directly on the branchial arch mesenchyme and the stapedial artery may not involved (Cousley & Calvert, 1997).

Genetic transmission is not involved in hemifacial microsomia and most cases are sporadic and isolated (Kearns et al., 1999). For example, it has been shown that out of 102 affected patients only 4% had a parent or sibling with signs of hemifacial microsomia (Marsh et al., 1986). A study on 82 hemifacial microsomia patients and parents showed that 44% had a positive family history of facial malformation and 8% of siblings were affected (Rollnick & Kaye, 1983). An autosomal dominant gene has been suggested although the occurrence on a first degree relative was only 2-3% (Kaye et al., 1992). The autosomal dominant is a gene on one of the non-sex chromosomes that is always expressed, even if only one copy is present. The chance of passing the gene to offspring is 50% for each pregnancy. Recently, the human chromosome 14 (q32) was reported in a family with this defect (Kelberman et al., 2001).
The hemifacial microsomia strain mouse (Hfm) show the defect as a small ear or an asymmetric jaw. It has been noted that a chromosome 10 is involved and 25% of the progeny are heterozygous for the transgene (Naora et al., 1994) (Figure 2.25). Cousley and associates conducted a study to look at the validity of the Hfm transgenic mouse as a model for hemifacial microsomia (Cousley et al., 2002). The Hfm model showed evidence of low penetrance and recurrence levels and only Hfm heterozygotes were viable and express the HFM phenotype. Furthermore, this evaluation suggested that the HFM anomalies might have a genetic influence which caused mesenchymal disruptions and possibly haemorrhages of stapedial artery. Identification of specific gene directly linked to hemifacial microsomia will certainly provide answers as to the contribution of genetic factor to this abnormality.

Figure 2.25: Hfm transgenic mouse. Small ear pinna (arrow) (A), Embryo with smaller pinna (arrow) (B), Midline shift to the right side (C), and underdevelopment of mandibular arch (D).

(Adapted from Naora et al., 1994).
2.10 Structural Deficits in Hemifacial Microsomia

2.10.1 Hard Tissues

Hypoplastic mandibles resulting from asymmetric growth and development of the mandible are characteristic of this syndrome (Kearns et al., 1999). The affected mandibles were much shorter, narrower and extrusive at an early stage and became progressively more asymmetrical as the normal side continues to grow. The hypoplastic mandible can be small with a normal shape of the ramus and temporomandibular joint, to total absence of both of these structures. The nasal, maxilla, zygomas and orbits usually overdevelop inferiorly and rostrally from the cranial base. If there is no surgical correction of this condition the end growth stages are variable. It generally results in a short mandible, displaced toward the medial side or totally without a ramus. The ramus was usually present with the flat shape of the body and the chin positioned towards the abnormal side.

2.10.2 Soft tissues

The soft tissue influences the growth of the skeleton. According to Moss and Salentijn, the functional matrix for the growth of the craniofacial skeleton is influenced by the functions of the attached neuromuscular tissue and the associated spaces (Moss & Salentijn, 1969). Mandibular growth is dependent on the development of the muscles of mastication and the eruption of teeth (Enlow, 1982). In hemifacial microsomia, the extent of hypoplasia of specific muscles of mastication predicts the extent of movement in the facial bone origin and insertion (Kane et al., 1997). Besides underdevelopment of facial muscle, other structures involved were the ear, facial nerve, subcutaneous tissues and other structures of the face. Patients may present with a reduced volume of subcutaneous tissues and masticatory muscles. Macrostomia
and skin tags also may present along a line from tragus of the ear to the commissure of the mouth (Kearns et al., 1999). There may be interference with the function of the trigeminal nerve (Kaban et al., 1998) and in more than 25% of hemifacial microsomia patients (Luce et al., 1977). External ear deformities has been graded by using the system described by Meurman, and modified by Marx (cited from Kearns et al., 1999).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mild hypoplasia, obvious malformation but with all the structures present</td>
</tr>
<tr>
<td>II</td>
<td>Atresia of the external auditory canal and cartilaginous remnant vertically orientated</td>
</tr>
<tr>
<td>III</td>
<td>Absent auricle, the lobular remnant displace anteriorly and inferiorly</td>
</tr>
</tbody>
</table>

Table 2.2: External ear classification system by Meurman and Marx. 
(cited from Kearns et al., 1999).

The temporalis and pterygomasseteric muscle group may be underdeveloped in hemifacial microsomia syndrome. There are correlations between the severity of the facial bone abnormality and of muscles abnormality. The development of associated muscle influences the growth of the bone, for example the temporalis muscle and coronoid process and pterygomasseteric muscles and the mandibular ramus.
The classification of hemifacial microsomia, was first described by Pruzansky based on the degree of mandibular and temporomandibular joint discrepancy (Pruzansky, 1969). He classified three types of hemifacial microsomia. Type II was subclassed into A and B (Kaban et al., 1988).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Miniature mandible and TMJ, all structures present but hypoplastic</td>
</tr>
<tr>
<td>Type II</td>
<td>Characterized by a small abnormally shaped ramus and an underdeveloped displaced TMJ</td>
</tr>
</tbody>
</table>
| Type IIA       | 1) The glenoid fossa is in an acceptable functional position in reference to the opposite TMJ.  
2) The joint is adequately positioned for symmetrical opening of the mandible and, therefore, does not require TMJ reconstruction. |
| Type IIB       | 1) The TMJ is abnormal in form and location, being medial and anterior.  
2) TMJ and ramus reconstruction are necessary |
| Type III       | Absence of the mandibular ramus and glenoid fossa |

Table 2.3: Classification of hemifacial microsomia Pruzansky for Type I, II and III Kaban for Type II A and B.  
(Adapted from Kearns *et al.*, 1999).
The OMENS anatomical classification was used in early 1990. This classification included more skeletal and soft tissue discrepancies in hemifacial microsomia cases. OMENS signifies Orbital dystopia, Mandibular hypoplasia, Ear (external), Nerve (cranial) and Soft tissue discrepancy. The orbit is graded based on size and position, the ear is categorised according to Meurmann (Meurmann, 1957) and the facial nerve based on the affected nerve branches.

Vento, LaBrie and Mulliken classified the components of the OMENS as

Mandible (M)

M0: Normal mandible

M1: The mandible and glenoid fossa are small with a short ramus

M2: The mandibular ramus is short and abnormally shape.
   Subdivisions A and B are based on the relative position of the condyle and temporomandibular joint (TMJ).

M2A: Glenoid fossa is in an anatomically acceptable position with reference to opposite TMJ

M2B: TMJ is inferiorly, medially and anteriorly displaced, with severely hypoplastic condyle.

M3: Complete absence of ramus, glenoid fossa, and TMJ.

Submental vertex views were used to determine mandibular Type 2A from Type 2B (Kaban et al., 1988; Meurmann, 1957; Mulliken & Kaban, 1987). The early classification system did not include the normal mandible for example as Type 0.
Classification was based on deficiency of subcutaneous and muscle masses. The other tissues such as the cranial nerve and ear defect were not included.

S0: No obvious soft tissue or muscle deficiency
S1: Minimal subcutaneous or muscle deficiency
S2: Moderate subcutaneous or muscle deficiency
S3: Severe subcutaneous or muscle deficiency

The OMENS classified each category on the grading to reflect the severity and the total of the score can be calculated to look at the severity of the patient’s defect. Comparison of the OMENS and SAT classification system showed that the OMENS system gives more detail and better definition by including the non-craniofacial abnormality (Cousley, 1993). This classification system was further supported by Horgan and associates (Horgan et al., 1995). They stated that 55% of hemifacial microsomia patients had abnormalities of other systems such as the central nervous system, cardiac and bone defects.

The distracted mandible of unilateral microsomia continues to grow at a slower rate than that on the contralateral side (Hollier et al., 1999). On the other hand, the distracted ramus height of unilateral microsomia was noted to be stable and its growth parallel the contralateral side (Polley et al., 1997). Grayson and co-workers stated that “the growth pattern is variable and modulated by both the original genetic predisposition of the native bone as well as the accompanying soft tissues and functional matrix” (Grayson et al., 1997).
2.11 Development of the animal model for this study

A review of craniofacial distraction osteogenesis stated that 70% involved mandibular distraction (Swennen et al., 2001). Hemifacial microsomia patients contributed 34% of the cases and others included syndromic conditions, dentofacial deformities and sleep apnoea problems. This technique has been used primarily in the surgical correction of the hypoplastic mandible in hemifacial microsomia. Although the mandibular distraction has become one of the choices in the surgical correction of hemifacial microsomia there is very limited study on the long-term stability of mandibular distraction osteogenesis. Hence the development of a study of mandibular distraction osteogenesis in a sheep model, in which a surgically created defect similar to unilateral hemifacial microsomia was first created.

The ‘hemifacial microsomia like’ defect was a retardation of growth on part of mandible by a condylectomy and reduction of the muscle surgically. The unilateral condylectomy and superficial myectomy of the masseter muscle from the same side was performed on ten weeks old lambs resulting in varying degrees of a hemifacial microsomia like defect (Miyamoto et al., 2001b; Miyamoto et al., 2002; Miyamoto et al., 1999). They observed that at three months post surgery, the lamb’s mandible showed a midline shift (3.5mm) toward the operated side, a shorter mandibular ramus (vertically short) and was laterally wider. The surgically created defect without causing the severe functional abnormality to the animal will allow this to be used in surgical research. The current investigation followed the same surgical protocol to create the ‘unilateral hemifacial microsomia like’ defect and the hypoplastic mandible is corrected by vertical mandibular ramus distraction osteogenesis to investigate the bony and related soft tissue changes.
2.11.1 Sheep as Animal Model

The Sheep (Australian Merino breed) was selected for this experiment. It belongs to Phylum Chordata, Subphylum Vetebrata, Class Mammalian, Sub-class Theria, Order Artiodactyla, Sub-class Ruminata. Infraorder Pecora, Family Bovidae, Genus Ovis (Bosanquet, 1988). They have a relatively similar temporomandibular joint to humans and sheep give opportunities to compare treatment intervention to the masticatory system (Bosanquet & Goss, 1987). Comprehensive examination of masticatory apparatus of the sheep showed that the mandibular condyles are situated above the tooth row, which is relatively similar to human where the temporomandibular joint is much higher than the occlusal plane (Finn, 1994). These positions provide better protrusion of the lower jaw over the upper jaw (Dyce et al., 1987). These give an indication for better functional movement and constant grinding of the sheep as herbivore. Sheep grind food by lateral movements of the jaw. This is due to the presence of a posterior inclination of the condyle head that allows lateral movement (Finn, 1994). A similar situation exists in human (Iyde et al., 1991). In carnivores the movement of jaws is more of a hinge movement. Lateral grinding is stopped by interfering carnassial blades (Scapino, 1965). Omnivores have an articulation that allows both lateral and vertical movement of jaw (Dovitch & Herzberg, 1968).

Australian Merino sheep anatomy is well described in a text book on sheep (May, 1970). The anatomy of the masticatory muscles of different species of animals has been described in several studies (Dyce et al., 1987; Noble, 1973; Schumacher, 1971). The masticatory muscles are a component that originates from the first branchial arch and are innervated by the motor branch of fifth nerve. These muscles are the masseter, temporalis, medial and lateral pterygoids. A temporo-masseteric superficial plane and a pterygoid deep plane are present in man. This is also present in domestic sheep (May, 1970).
The masseter muscle is the major masticatory muscle. It forms 3 layers; superficial, middle and deep. The superficial masseter has a prominent “tear drop shape.” It originates from the facial tuber and extends posteriorly into a broad insertion on the lateral mandibular ramus; caudally, ventral and at the angle of the mandible. The middle masseter originates from the facial tuber and extends along the crest of maxillary and malar bones caudal to the zygomatic arch (Figure 2.26). The insertion of the middle masseter is along the caudal, ventral and angle of mandibular ramus. The deep masseter originates from the infra orbital rim and the zygomatic arch. Its fibres extend ventrally and make an insertion at the mandibular body anteriorly and obliquely up to the middle of the mandibular ramus.
The medial pterygoid was located on the inner side of the mandibular ramus. It is divided into superficial and deep layers. The deep layer originated from the medial lamina of the pterygoid process of the sphenoid bone caudal to the hard palate and reaches the pterygoid hamulus. The muscle obliquely extends ventrally before it inserts into the ventral edges of mandibular ramus, body and angle. The superficial medial pterygoid is attached from the fossa between the lateral and medial laminae of the pterygoid process. The insertion of this muscle is into the deep layer of medial surface at the vertical and angle of the mandibular ramus (Figure 2.27).

NOTE:
This figure is included on page 65 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.26: Right middle masseter muscle (dot arrow), which is almost parallel to the vertical outline of the mandibular ramus (solid arrow).
(Adapted from Finn, 1994).
2.11.2 Biomechanics of the sheep masticatory system

The biomechanics of the sheep mandible have been studied to look at the mechanical loading which helps to explain the mandibular shape and to understand the tolerance of jaw shape to the stresses during grinding foods (De Jongh et al., 1989). They showed that both ipsilateral and contralateral sides are functional during grinding in mastication and rumination.

The motions involved in chewing are complex. The sheep moves the symphysis in a triangular path in every cycle (De Jongh et al., 1989). There were 3 phases to complete the cycle; (1) First (closing) phase: symphysis passes far laterally toward the side where food was being chewed (ipsilateral side), (2) Second phase: Long and almost horizontal power stroke direct to the opposite side (contralateral side) and (3) Third phase: the mouth opens again in which the gap does not surpass 15°. The sheep were chewing on both sides of the jaws randomly and with no preferential side. Several hundred cycles (approximately 590 cycles for mastication and
654 cycles for rumination) are involved in the chewing pattern and changing side every 20 cycles (De Jongh et al., 1989).

De Jongh et al also noticed that the muscle activity was activated at random without fixed stages. The masseter and pterygoid muscles showed higher activity during the grinding process. They also stated the muscle activity was more regular during rumination than in mastication but higher firing amplitudes are recorded during rumination than in mastication.

De Jongh and associates (De Jongh et al., 1989) also demonstrated the maximum stress distribution on the sheep mandible (Figure: 2.28). Finn showed that the maximum mechanical force is generated at the molar base (Finn, 1994). He also stated that the estimated condylar reaction force is between 200-900 N depending on the position of the bite point. In humans it has been estimated the temporomandibular force is 513 N (Koolstra et al., 1988).

2.11.3 Current research on mandibular distraction osteogenesis in sheep

There are a total of 85 mandibular distraction osteogenesis experiments published to date which involved six species of animals; 41 in dogs (48.2%), 13 in sheep (15.3%), 11 in rabbits (12.9%), 11 in pigs 12.9%), 5 in monkeys (5.9%) and 4 in rats (4.7%) (Swennen et al., 2002). Sheep were the second most common species of animal that has been used for mandibular distraction experiments. All together, there were a total of 13 sheep experiments, which have been conducted on 213 sheep. Twelve experiments were performed to study mandibular lengthening and one on vertical distraction. Sheep were used for experimenting with various research components including testing new devices, new applications, histology, molecular
About half of the total of sheep used and more than half of the research was conducted on growing sheep. Growing animals were distracted at the rate of 0.5-1 mm per day and non-growing were distracted at an average 1 mm per day (range 0.5 – 4mm per day). The latency period was range from 0 day, 4 days and 5-7 days, where most of the studies are using the latter range. The consolidation period ranged from 1 – 6 months. The review recorded only two animal deaths in 13 sheep experiments (involving a total of 213 sheep); these were due to anaesthetic complications (Swennen et al., 2002).

There are experimental limitations when relying on animal models to mimic of what is occurring in humans. It is important for proper observations to be made. Small animals such as rabbits and rats are not suitable for surgical experimentation because of their small size and marked anatomic differences with respect to humans. Merino sheep has been suggested to have more similarity in size and surgical anatomy to humans (Bosanquet & Goss, 1987).

The ‘unilateral hemifacial microsomia like’ defect, surgically created in sheep was used as an animal model to study the long term effects in vertical mandibular distraction osteogenesis on masticatory muscles. In this current study the focus was more toward the masseter and medial pterygoid muscles. These two muscles envelope the lateral and medial side of the mandibular ramus and receive more effect from the distraction process (Figure 2.29). Therefore, it would be interesting to invistigate the adaptation of the masticatory muscles during and following the vertical mandibular ramus distraction osteogenesis.
Figure 2.28: Graphic presentation of the stress distribution on sheep mandible when the masticatory muscle is contracted at the maximum level, focus mainly at the insertion of muscles.

(Adapted from De Jongh et al., 1989).

Figure 2.29: The anticipated movement (arrow) of the proximal and distal fragments when the device is activated. The masseter muscle is expected to experience a direct effect from the distraction process as the outline is parallel to the distraction vector.

(Adapted from Finn, 1994).
2.12 OBJECTIVES AND HYPOTHESIS

General and specific objectives of this study were:

General Objectives

1) To assess the development of the surgically created ‘hemifacial microsomia like’ defect as a model for vertical mandibular distraction osteogenesis in sheep.

2) The effect of distraction of the bony vertical ramus of the mandible in this experiment has been previously reported (Syed Zainal, 2005).

3) To study the adaptation of the masticatory muscles during vertical mandibular ramus distraction osteogenesis

4) To evaluate the adaptation of the masticatory muscles after distraction in different consolidation periods when neutral fixation was applied.

5) To evaluate masticatory muscle adaptation after device removal during the remodelling period.

6) To investigate the optimum time for both the neutral fixation and removal, as part of recommendations for the vertical mandibular distraction osteogenesis protocol.

Specific Objectives

1) Does increase of the vertical mandibular height by distraction osteogenesis increase the mass of the masseter and medial pterygoid muscles?

2) Does increase of the vertical mandibular height by distraction osteogenesis alter the length of the anterior, middle, posterior and oblique plane of the masseter muscles?
3) Does increase of the vertical mandibular height by distraction osteogenesis alter the cross sectional area and thickness of the proximal, middle and distal of the masseter muscle?

4) Does increase of the vertical mandibular height by distraction osteogenesis induce histopathological changes in the l of the masseter and medial pterygoid muscles?

5) Does the process of detachment of the muscle during the surgical placement result in the repositioning of the masseter muscle?

6) Does neutral fixation following distraction with different consolidation periods (2 months, 3 months and 4 months) give greater stability to the masseter and medial pterygoid muscles?

7) Does removal of the device after distraction and neutral fixation result in changes to the masseter and medial pterygoid muscles?

8) Is ultrasound a clinically effective assessment tool to evaluate the condition of the distracted muscles?

Null Hypothesis

There are no gross and histological changes in the masseter and medial pterygoid muscles during distraction, neutral fixation and after removal of device between experimental and control and between groups.
CHAPTER 3: MATERIALS AND METHODS

3.0 Animal Model for “Hemifacial Microsomia Like Defect”
This experiment was conducted after receiving an ethical clearance from the Animal Ethics Committee of the Institute of Medical and Veterinary Science (IMVS) (approval number: 12/02 and 31/03) and the University of Adelaide (approval number: S-02-2002). This experiment follows the guidelines of the Australian Code of Practice for the care and use of animals for scientific purposes published by National Health and Medical Research Council (NHMRC).

3.1 Animal
Thirty just-weaned and castrated male lambs (10 weeks old) were purchased from the Veterinary Division of IMVS animal experiment resources at Gilles Plains, South Australia. These lambs are available in the late autumn and spring seasons. The lambs were brought to the Gilles Plains Farm and kept there for at least one week for adaptation and observation before being subjected to this study. The lambs were then brought to the animal research theatre of IMVS, Frome Road, several days before surgery.

3.2 Surgical Protocol
The lambs were fasted 24 hours before anaesthesia and surgery. All lambs were weighed pre-surgery and given penicillin 20 mg/kg body weight and an analgesic (Flunixil® (Flunixin meglumine)) 1.1 mg/kg body weight intramuscularly. Inhalation induction of 3 - 5% halothane via a nasal mask or thiopentone sodium at a dosage of 12.5 mg/kg body weight was given intra jugularly for induction and followed by endotracheal intubation with a tube size 6.5. The endotracheal tube was secured snugly to the mandible to avoid tube dislodgment. Anaesthesia was maintained with 2% halothane throughout surgery. The head of the sheep was shaved on the right side at the preauricular site and lower border of the mandibular body. Betadine® scrub
was used to scrub the surgical site and it was then dried with a paper towel. Betadine solution was used to spray the surgical site. The surgical site was draped. The preauricular and submandibular area was finally injected with local anaesthetic (2.2 ml, 2% lignocaine with 1:80,000 adrenaline).

A five-centimetre preauricular skin incision was made (Figure 3.1). The subcutaneous tissue and muscles were bluntly dissected to expose the capsule of the temporomandibular joint (Figure 3.2A), sigmoid notch, and anterior-lateral surface of mandibular ramus. The periosteal layer of the neck of the condyle was incised and elevated with a periosteal elevator, to preserve it. The periosteal elevator was used to detach the intra-articular disc at the lateral pole. Then a fissure burr was used to make several holes on the neck of the condyle about 1 cm from the sigmoid notch. These holes were joined together to make a straight horizontal cut. A chisel and mallet was used to complete the condylectomy. The condyle was then removed (Figure 3.2B) and any sharp edges were smoothed. The muscle, subcutaneous and skin were closed in layers.
Figure 3.1: Preauricular skin incision to approach to the right condyle (arrow head) and submandibular incision to expose the superficial masseter muscle (small arrow) in the first surgery.

Figure 3.2: Exposure of the right condyle (arrow) (A) and the right condyle after removal (B).
The second procedure performed at this first operation was superficial masseter myectomy to reduce the muscle bulk, to stimulate the reduced muscle mass in hemifacial microsomia. A 7 cm skin incision, was made about one third of the way along the lower posterior border of mandibular ramus, extended to the posterior ramus. Subcutaneous tissue was cut and bluntly dissected. Care was taken not to damage the parotid duct. The parotid duct can be distinguished by its clear tubular structure, with fine capillaries on the outer surface of the tube (Figure 3.3). Subcutaneous tissues holding the parotid duct, facial arteries and veins and facial nerve were carefully retracted anteriorly and superiorly. The whole superficial masseter belly at its insertion and tendon of origin was exposed (Figure 3.4 A). Incision of the posterior border of the superficial masseter was made and the muscle was elevated and detached from the attachment at the mandibular bone posteriorly and the middle masseter by using a periosteal elevator and blunt dissection with dry gauze (Figure 3.4B). The superficial masseter myectomy was completed by cutting the tendon, anteriorly at its origin. The superficial masseter was removed (Figure 3.4C). The incision was closed in layers. Vicryl® size 3.0 was used to suture the muscle and the subcutaneous layers with a simple continuous suture pattern. Silk size 2.0 was used to suture the skin with an interlocking suture pattern.

These lambs were allowed to recover from anaesthesia and surgery in the recovery pen. Penicillin was given at 20mg/kg body weight intramuscularly once a day, for 7 days and analgesic (Flunixil® (Flunixin meglumine)) was given 1.1 mg/kg body weight intramuscularly once daily for the first 3 days.
Examination and monitoring of the animal’s eating behaviour and the surgical site was done daily until the animal was transferred back to the farm at Gilles Plains, usually after one week. The lambs were fed with lucerne, hay and sheep pellets. Based on Animal Ethics regulations any sheep which loses more than 15% of its body weight should be killed.

For the next three months after the first operation the lambs were kept at the farm. They were observed to develop normally except for a short right mandibular ramus and deficient muscle tissue as a direct effect of the first operation (Figure 3.5). Thus the second operation was mandibular distraction osteogenesis of the right mandible to restore it to its previous length as described in the next section.
Figure 3.3: The right parotid duct (arrow), which has been identified before completion of the right superficial masseter myectomy.
Figure 3. 4: The right superficial masseter muscle (arrow) (A), the blunt dissection process to separate the superficial layer from the middle masseter (B), the right superficial masseter (arrow) after removal and exposed fascia of the middle masseter muscle.
Figure 3.5: The right midline shift resulted from condylectomy and superficial masseter myectomy.

Figure 3.6: The mandibular osteotomy and the placement of the bone-born Mathys mandibular distractor (Mathys Australia Pty. Ltd, AUS- Kensington, N.S.W).
3.3 Mandibular Distraction Osteogenesis Protocol

3.3.1 Age of Animal

The lambs were subjected to second surgery 3 months after the first operation, at approximately six months of age. At this stage the ‘unilateral hemifacial microsomia like’ defect of the mandible was apparent (Figure 3.5). The mandibular ramus was short and resulted in the right sided midline shift. The right side masseter was deficient. The mandibular and soft tissue components of unilateral hemifacial microsomia was shown at this stage and it can be classified as a Type I Kaban and a type 00 M1 E0 N0 S1 O.M.E.N.S.

3.3.2 Surgical Placement of Distractor

The lamb was fasted for 24 hours and anaesthesia induced with thiopenthione sodium at a dosage of 12.5 mg/kg body weight. The lambs were intubated and anaesthesia was maintained by 2% halothane throughout the operation. The head of the sheep was shaved on both the right and left sides. The surgical area was scrubbed with Betadine® scrub, dried with a paper towel and sprayed with Betadine solution. The surgical area was then draped. A seven-centimetre preauricular skin incision was made and the subcutaneous tissue was cut. Fibrous tissues were noted in the area of previous surgery and it was quite firm and highly vascularised with multiple capillary vessels. Blunt dissection was used to clear the surgical area. The muscle outline was identified, dissected and retracted to expose the temporomandibular joint, mandibular ramus, sigmoid notch and coronoid process. The periosteum was incised at the superior ramus and elevated with a periosteal elevator, to preserve it. The muscle attachment was detached from the mandibular ramus and extended until the anterior border of the mandible edge was reached. The base of the coronoid process was identified and coronoidotomy was performed using the fissure burr. The coronoid process was not removed.
The coronoidotomy was performed to reduce the masticatory forces. The condyle was examined and if it had regenerated, a second condylectomy was performed. Two marker screws were placed where the proximal screw was placed 1 cm from the sigmoid notch and 1 cm from posterior edge of mandibular ramus. The distal marker screw was placed 1.5 cm from the proximal marker screw.

A bone-born Mathys Mandible Distractor (Mathys Australia Pty. Ltd., Kensington, N.S.W.) was positioned and the skin incision at the ventral border of mandible was placed to allow the activator of the distractor to protrude. The proximal and distal plate of the distractor was pre-placed and the holes for the screws were drilled. The distractor was screwed to the mandibular ramus. A series of holes to outline the osteotomy were made on the horizontal mandibular ramus. The distractor was unscrewed and chisels and a mallet were used to complete the osteotomy cut. Both distractor plates were screwed back using the original holes (Figure 3.6). The distractor was checked by winding the activator and making sure the two plates were close together before the incision was closed. The surgical area was irrigated with normal saline and Betadine solution. The subcutaneous and muscle layers were sutured using 3.0 Vicryl®, with a simple continuous suture pattern. The skin was closed with 2.0 silk, with an interlocking suture pattern. The oral cavity was flushed with normal saline to clear away regurgitated rumen contents. Betadine solution was applied on the gingivae of the oral cavity. A 1.5 cm gingival incision was performed, 0.5 cm anterior to the first pre molar. The gum was elevated by using the periosteal elevator. A hole was drilled and the third marker screw was then placed.
The lamb was turned on the left side and the same procedure on the temporomandibular joint was performed. Subcutaneous tissue and muscle were bluntly dissected and retracted to expose the left mandibular ramus. Marker screws were placed 1 cm from the sigmoid notch and 1.5 cm from the posterior edge of mandibular ramus. A distal marker screw was placed 1.5 cm from the proximal screw. The surgical incision was closed, the same as on the right side. The oral cavity was irrigated to clear away regurgitated rumen contents by flushing it with normal saline. Betadine solution was applied on the gingival of oral cavity. The animal was given Penicillin 1 ml (20 mg/kg body weight) once a day for 7 days and analgesia, Flunixil® (Flunixin meglumine) a non steroidal anti inflammatory 1.1 mg/kg body weight daily for 3 days. Examination and monitoring was done on a daily basis.

3.3.3 Latency Period

The latency period was seven days for this experiment. It was started on the day one post operation. Throughout this period the activator of the distraction device was not yet activated.

3.3.4 Distraction Period

At eight days post operatively, the activator was activated using a screwdriver. One complete turn will give a 0.5 mm gap. The chosen rate was 1mm daily and the frequency was once daily. The activator was turned twice (counter clockwise), once a day for ten days. A lateral jaw radiograph was taken to measure the distance between both right marker screws at day 18 post operatively after 10 days of activation. In some animals, the gap was not 10 mm, so the distractor was activated again daily until a 10 mm distraction gap was achieved (Figure 3.7). The distraction process also corrected the midline (Figure 3.8)
Figure 3. 7: The lateral cephalogram before (A) and after distraction (B). The right midline shift was corrected when 10 mm gap showed on radiograph.
Figure 3.8: The midline shifting toward the right side resulted from condylectomy and superficial myectomy (A), the distraction process corrected the midline (B).
3.3.5 Consolidation and Remodelling Periods

Initially, a pilot study was conducted to look at the pattern of masticatory muscle changes in different consolidation and remodelling periods. Eight animals were selected in eight identified groups (Table 3.1) (Figure 3.9).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number (n)</th>
<th>Consolidation period and Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1a</td>
<td>1</td>
<td>Sacrificed post-osteotomy and pre-distraction</td>
</tr>
<tr>
<td>Group 1</td>
<td>1</td>
<td>Sacrificed immediately post-distraction</td>
</tr>
<tr>
<td>Group 2a</td>
<td>1</td>
<td>Consolidation with device 2 months after distraction completed</td>
</tr>
<tr>
<td>Group 2</td>
<td>1</td>
<td>Consolidation with device 2 months, device removed then sheep sacrificed after 1 month. Total 3 months post-distraction</td>
</tr>
<tr>
<td>Group 3</td>
<td>1</td>
<td>Consolidation with device 3 months</td>
</tr>
<tr>
<td>Group 4</td>
<td>1</td>
<td>Consolidation with device 3 months, device removed then sheep sacrificed after 1 month. Total 4 months post-distraction</td>
</tr>
<tr>
<td>Group 5</td>
<td>1</td>
<td>Consolidation with device 3 months, device removed then sheep sacrificed after 2 months. Total 5 months post-distraction</td>
</tr>
<tr>
<td>Group 6</td>
<td>1</td>
<td>Consolidation with device 4 months.</td>
</tr>
</tbody>
</table>

Group 1a and 2a was not included in the analysis for soft tissues data.

Table 3.1: Animal for pilot study, number of sheep per group (n) and the description of the groups.
Figure 3.9: The experimental design for the pilot study, which involved one animal each for eight groups.
The experimental design was refined after review of the outcome of the pilot study. Results of this pilot study showed there were no gross and histopathological changes of the soft tissues in pre-distracted animals (results not shown). Based on the above results, only 6 groups (excluding 1a and 2a) were further investigated. The refined experimental protocol was to focus on these groups where 4 animals were randomly assigned in each group (except for group 1 where only 3 animals were involved). The groups are as stated below;

Group 1: Immediately post-distraction (n=3).

Group 2: Consolidation period for 2 months with remodelling period of 1 month (n=4).

Group 3: Consolidation period for 3 months (n=4).

Group 4: Consolidation period for 3 months with remodelling period of 1 month (n=4).

Group 5: Consolidation period for 3 months with remodelling period of 2 months (n=4).

Group 6: Consolidation period for 4 months (n=4)

The animals that were involved in a remodelling period were subjected to distractor removal after the identified consolidation periods. During the experimental period, animals were kept in an individual pen for about one week after first surgery and about 20 days after the second operation. Later, the lambs were kept in a group pen. All lambs were fed lucerne chaff, hay and sheep pellets throughout the experimental period.

3.3.6 Removal of the distractor

Similar surgical protocol for insertion was followed for the removal of the distractor. The sheep was intubated and shaved before the ultrasound was performed. The distractor was then surgically removed. Some screws were well osseointegrated requiring bone removal. The
distance of the distracted gap on the distractor was confirmed by direct measurement. The incision was then closed in layers. Lateral cephalometric X-rays were obtained.

3.4 Gross Assessment of Soft and Hard Tissues Pre and Post Mortem.

Gross assessment of the hard and soft tissues was conducted by 2 methods. The first method was to scan the masseter using an ultrasonography technique. The second method was to weigh both the experimental and control sides, masseter and medial pterygoid muscles at sacrifice. Measurement was done in situ for 6 identified points on the masseter muscles. The mandibular bone was dissected, measured and bone histological study was conducted by co-researcher, Dr. Syed Zainal. The method and results of Dr. Syed Zainal’s study are presented in this thesis because it is useful to compare studies on both hard and soft tissues changes in the same animal models.

3.4.1 Ultrasonography (scanning) of masseter muscles

Ultrasound examination included bilateral scanning of the masseter muscle at three levels. The areas of scanning were identified by dividing the distance between the zygomatic arch and the mandibular angle into three equal parts. The masseter muscle was scanned at 3 levels; proximal, middle and distal (Figure 3.11). The scanned masseter was traced to attain the cross sectional area (mm²). The greatest depth was measured to obtain the thickness (mm) of the masseter muscle. The measurement was done twice so that the reliability and error could be calculated. The transverse ultrasonographic images were obtained using the linear scanner (B-scan) with 7.5 MHz transducer. The transducer was attached to an echocamera (Aloka, model SSD 650 cl) (Figure 3.12). The sonogram was conducted by a Registrar in the Oral and Maxillofacial Surgery Unit. The animal was anaesthetised before performing the scanning. The
lamb was positioned on the left or right lateral side. The wool on the left and right lateral face was shaved using the calliper blade size 40. The scanning area was marked and a water-based gel was used to obtain maximal contact. The transducer was placed on the masseter with light pressure and was positioned perpendicular to the cortex of ramus bone. The transducer was rotated until the image of ramus appeared as a sharp white line. The scanning was done on the relaxed masseter muscle.

3.4.2 Euthanasia procedure

Prior to sacrifice, the sheep were anaesthetised and subjected to ultrasound and lateral cephalometry X-rays. Animals were killed by intra-venous injection of the barbiturate (Lethabarb® at 0.5 ml/kg body weight).

3.4.3 Anatomical Descriptions of Masseter and Pterygoid Muscles

The cheek skin and the facial tissue were dissected to expose the masseter muscles. The masseter muscles were examined and the length of identified points were measured (3.4.6) and the dissected masseter and pterygoid muscles were weighed (3.4.7). The masticatory muscles, namely the masseter and medial pterygoid, were harvested for further investigation to look at the effect on soft tissue.

The right and left mandibular ramus were dissected. The distractor was removed, if still present but sacrifice may or may not be concurrent with distractor removal. The distance between the screws (AB, BC and AC) were measured directly using callipers (Figure 3.10). The mandibular ramus was then sectioned using a bench saw, to include the entire distracted segment, which included 1 cm above and below.
Figure 3.10: Placement of marker screws A, B and C on the mandible.

3.4.5 Post mortem description

Gross pathological appearance of the masseter muscle was noted at post mortem. The pathological changes at the site of surgery were described. The post mortem examination was performed and recorded by examination of the masseter muscle and the site where the distractor was placed or removed.

3.4.6 Length of Masseter Muscles

The left and right masseter was exposed and the direct measurement was conducted based on 4 planes (Figure 3.13). The AB was the anterior plane of masseter muscle, CD was the middle plane of masseter muscle, EF was the posterior plane of masseter muscle and AD was the oblique plane of masseter muscle. The measurement was done twice using callipers (Leibinger, 35-01600; Japan) and the interval between the two readings was 5 minutes. The analysis was conducted on the first measurement, the second measurement was then used to test for error of the method.
3.4.7 Weight of Masseter and Medial Pterygoid Muscles

The masseter and medial pterygoid muscles on the left and right side were completely dissected out. The origin of the masseter muscle was dissected first, followed by the anterior attachment before the rest of the masseter was detached from the mandibular ramus. For the medial pterygoid muscle, the insertion was dissected first and followed by the rest of the muscle. The muscle was weighed twicely with digital scales.
A : The proximal level

B : The middle level

C : The distal level

Figure 3. 11: The placement of transducer to scan 3 levels of the masseter muscle; proximal, middle and distal.
Figure 3. 12: The ultrasound machine, Aloka (Echo camera), model SSD 650 cl. with transducer 7.5 MHz.
The attachment of muscle between maxillary process of zygomatic arch

The attachment of masseter middle masseter muscle almost at middle mandibular ramus

The attachment of muscle at the ventral posterior (angle of mandibular ramus)

Superior point of maxillary crest between the facial tuber and infraorbital rim

Facial tuber of the maxilla

The most ventral anterior of muscle attachment

Figure 3. 13: The six anatomical points to measure four landmarks on the masseter muscle.
3.5 Histological Examination

3.5.1 Muscle tissue sampling

The experimental (right) and control (left) sides were dissected from the bone. The masseter and medial pterygoid muscles were put in a 10% formalin solution and transferred to the Oral Pathology Laboratory at the Dental School for further processing. The experimental and control sides of the masseter and medial pterygoid muscles were further divided into 3 levels; proximal, middle and distal (Figure 3.14). The longitudinal and transverse orientations of approximately 5 mm in thickness were obtained from each level of muscle and kept in a different labelled cassette. All the muscle tissues were further fixed in a 10% formalin solution for 5 days.

Samples of masseter muscles were taken at the centre and about 1.5 cm from the posterior mandibular ramus and anterior border of the masseter. This was to avoid damaged muscle tissue, which might have undergone changes from the first, second, and third surgical procedures rather than the distraction process. The tissues from the masseter and medial pterygoid muscles were further processed using a Shandon Citadel 2000 automatic tissue processor (Shandon Industries, Pittsburgh, Pennsylvania). The muscle tissues were then dehydrated in graded alcohols (70%, 80%, 90%, 3 series of 100%) and 3 series of Histolene (Appendix 2). Muscle specimens were embedded in paraffin wax using a Reichert-Jung Histostat with care to make sure the longitudinal and cross section samples of muscle were correctly oriented. Paraffin blocks were attached to a Leitz 1512 Microtome and a series of 5 μm ribbons were cut.
The sections were then put on the pre-heated glass slide with water on the hot plate set at 50°C. The section was oriented and the water was removed. The glass slide with the section was arranged vertically in the rack before drying in the 30°C incubator. Three sections were obtained from the proximal, middle and distal levels of the masseter and medial pterygoid muscles for both experimental and control sides. Each slide was stained with Mayer’s haematoxylin and eosin (Appendix 2) for histomorphometric study as described below.

3.5.2 Histomorphometry evaluation

A histomorphometric study was carried out to obtain semi quantitative values at 100x magnification. Traditional morphometry using a grid method (1μm²) was performed to assess the muscle structure changes and adaptation in relation to distraction osteogenesis during the period of consolidation and remodelling. An ordinal scoring method was used to assess the histopathological changes; dystrophy (Figure: 3.16), atrophy (Figure: 3.17), necrosis (Figure: 3.18), proliferation of fibroblasts (Figure: 3.19), sclerosis (Figure: 3.20) and regeneration (Figure: 3.21). The histology of the normal (control) muscle (Figure 3.15) was also examined. Each section was read twice.
The score protocol is shown in Table 3.2.

<table>
<thead>
<tr>
<th>SCORE CHANGE</th>
<th>NORMAL</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dystrophy</td>
<td>None</td>
<td>Up to 5 foci/grid area</td>
<td>Up to 10 foci/grid area</td>
<td>&gt; 10 foci/grid area</td>
</tr>
<tr>
<td>Atrophy</td>
<td>None</td>
<td>Up to 10 fibres/grid area</td>
<td>Up to 15 fibres/grid area</td>
<td>&gt; 15 fibres per grid area</td>
</tr>
<tr>
<td>Necrosis</td>
<td>None</td>
<td>Up to 5 fibres/grid area</td>
<td>Up to 10 fibres/grid area</td>
<td>&gt; 10 fibres/grid area</td>
</tr>
<tr>
<td>Proliferation of fibroblast</td>
<td>None</td>
<td>Up to 5 foci/grid area</td>
<td>Up to 10 foci/grid area</td>
<td>&gt; 10 foci/grid area</td>
</tr>
<tr>
<td>Sclerosis</td>
<td>None</td>
<td>Mild focal</td>
<td>Intermediate</td>
<td>Multifocal</td>
</tr>
<tr>
<td>Regeneration</td>
<td>None</td>
<td>Up to 5 fibres/grid area</td>
<td>6 – 10 fibres/grid area</td>
<td>&gt; 10 fibres/grid area</td>
</tr>
</tbody>
</table>

Table 3.2: Definition of the score for histopathological changes in masseter and medial pterygoid muscles.

Figure 3.14: The three levels of sampling at proximal, middle and distal levels of the masseter muscle.
Investigation of Vertical Mandibular Distraction Osteogenesis on Masticatory Muscles in ‘Unilateral Hemifacial Microsomia Like’ Defect in the Sheep Model

Figure 3. 15: Normal architecture of masseter muscle. Normal muscle fibres (M), perimysium (P), collagen deposition (C), artery (A) and vein (V). H&E (100x).

Figure 3. 16: Muscle dystrophy—presence of various sizes of muscle fibres within one fascicle. Normal muscle fibres (M), atrophic muscle (m), perimysium (P) and fat tissue (F). H&E (100x)
Figure 3. 17: Muscle atrophy (a), generalize uniform reduction in fibre size with increased endomysium (E), perimysium (P) spaces and collagen deposition (C). H&E (100x)

Figure 3. 18: Muscle necrosis, presence of inflammatory cells and phagocytic process. Normal muscle fibres (M), necrotic fibres (N), fat tissue (F) and regeneration (R). H&E (100x).
Figure 3. 19: Proliferation of fibroblasts (arrow) increase in number of fibroblast nuclei (fb) between connective tissues at perimysium (P) and endomysium (E). Normal muscle fibres (M). H&E (400x).

Figure 3. 20: Sclerotic muscle, increased inter fascicle space and muscle fibre with connective tissues (collagen). Normal muscle fibres (M) and perimysium (P). H&E (100x)
Figure 3. 21: Regeneration of muscle (R), presence of new muscle cells (arrow) within damaged muscles. Normal muscle fibres (M). H&E (400x)

3.6 Statistical analysis

This study involves metric and non-parametric data. The metric data were in the measurements of the weight of masseter and medial pterygoid muscles, length of the landmarks of masseter muscles and the ultrasonographic crosssectional as well as thickness of the masseter muscles. Meanwhile, non-parametric data was for the ordinal scoring of histopathological parameters. For the consolidation analysis of these data, a biostatistician from the Department of Biostatistic, University of Adelaide and a senior biostatistician who involved in many biological studies during his tenure with the University were consulted. A wide range of statistical analyses including paired t-test as well as uni and multivariate analyses within and across all groups were taken into consideration. Based on this discussion, independent t-test was suggested to consolidate the matric data due to small sample size and the data were also compared on both net differences and the basis of relative as a percentage basis relative to the
control side. Catagorical non-parametric data was analysed using a Wilcoxon test and Kruskal-Wallis test as further elaborated in the following sections.

### 3.6.1 Metric Data

A paired t-test was carried out to test the difference between the control (left) and experimental (right) sides within the groups. An analysis of variance (ANOVA) and the appropriate post hoc test (Tukey’s HSD) were conducted to analyse the percentage differences between the control and experimental sides between the 6 groups. Data that were significantly different had a probability value lower than 0.05.

### 3.6.2 Non-parametric Data

A Wilcoxon test was conducted to investigate the differences in histology scoring between the control and experimental sides at 3 levels; proximal, middle and distal levels of the masseter and medial pterygoid muscles at a 90% significance level ($p \leq 0.1$). A Kruskal-Wallis test was also performed to compare each experimental side variable between 6 groups. The significant difference was at $p \leq 0.1$. The p value was choosen in order to establish some association and relation in the histopathological changes at different level and time interval.

### 3.7 Mandibular Ramus (Hard tissues components)

The following sections (section 3.7.1- 3.7.5) were adapted and modified from Syed Zainal (2005).
3.7.1 Radiographic procedure

Right and left lateral cephalometry was performed bilaterally using a custom designed head frame (Figure 3.22 and Figure 3.23) to standardise the radiographs. All cephalograms were taken using the protocol developed by Ma (Ma, 2002). A specially designed cephalostat was used, incorporating a head frame and holder to stabilize the sheep head. The focal point to film distance was 150cm and the object (mid-sagittal plane) film distance was 10.5 cm. The voltage used for the lateral cephalograms was 60kV (15 mA).

X-ray measurement of distances AB, BC and AC (Figure 3.10) of the dry skull were first taken and compared with direct skull measurements to identify the magnification effect. X-ray measurement of distances AB, BC and AC both on the right and left mandible were measured using the same metal ruler (Penguin Brand, China) and repeated twice for the same distance before the mean value was undertaken. Measurements of the control side were obtained to determine the normal growth rate of the mandible. The mean X-ray measurements of the left and right sides were compared at pre-distraction, post-distraction and at sacrifice.

Direct measurement of AB distances of both left and right was recorded during surgery at the pre-distraction stage. These were compared with the distance obtained at sacrifice using a hand-held vernier calliper (Leibinger) to the nearest 0.5 mm. The same post-operative protocol for the first surgical procedure was applied.
Figure 3. 22: Lateral cephalometry using cephalostat head frame.
Figure 3.23: The sheep positioned in cephalostat head frame (A) and the result of lateral cephalometry X-ray (B). The white tape in (A) is holding the ruler in place. The marker screws, distance and the distracted gap can be visualized on radiograph.
The right and left mandibular ramus were dissected. The distractor was removed if still present. Sacrifice may or may not be concurrent with distractor removal. The distances between the screws (namely AB, BC and AC) were measured directly using callipers (Figure 3.24). The mandibular ramus was then sectioned using the bench saw, to include the entire distracted segment, which included 1 cm above and below the osteotomy site.

3.7.2: Tissue processing

The bone blocks were sectioned coronally into three equal sections (Figure 3.25). All tissue blocks were placed in buffered formalin solution for 24-48 hours. This was then followed by immersion in 70% alcohol for 48 hours. Finally the blocks were placed in 10% ethylene diamine tetra-acetic acid for decalcification (pH 6.5-7.5). The specimens were radiographed pre-decalcification and then periodically to ensure full decalcification (52kV, 8 mAS).

After about 4 months decalcification was completed, the blocks were then transferred back into a 70% alcohol solution. The middle and posterior blocks were subjected to a dehydration process by passing them through a graded series of incremental alcohol solutions (80%, 90% and 100%) over a few days as per the IMVS protocol. The anterior blocks were stored in 70% alcohol for future study. The alcohol was then removed via the clearing process by immersion in chloroform for an hour and then via a histoclear infusion. Infiltration with paraffin wax (Paraplast) was then performed under vacuum (25 inches of mercury) at 60°C.
All tissue specimens were mounted on wooden blocks. Sectioning was performed coronally from the anterior aspect for the middle segment. The posterior segment was sectioned sagittally from the lateral aspect. Sections were 5 μm in thickness using a sledge microtome (Leica, Germany).

Figure 3.24: The marker screws A, B and C in circles. Distances between screws AB, BC and AC were measured directly on the specimen.

Figure 3.25: Bone blocks sectioned coronally, most anterior on the right.
3.7.3 Histological analysis

The protocol set by was adapted for staining of specimens with haematoxylin and eosin (Lillie, 1965) (see Appendix 3). The cellular changes were observed using a light microscope. Soft and hard tissue changes, particularly bony maturation, were analysed and related to the consolidation period.

3.7.4 Histomorphometry

Using the same methods applied in the pilot study by J. Varughese (Varughese, 2002), histomorphometric analysis was applied. The software package Quantimet (Leica, Cambridge) measures the contrast differences between soft and hard tissues. Van Gieson’s staining was used to enhance contrast between the tissues for Quantimet analysis. Van Gieson basically identifies hard tissue with dark red/orange staining while soft tissues are stained yellow (Figure 3.24).

The sampling routine involved selecting one section after every twenty consecutive sections for histomorphometric analysis. This protocol was used for all tissue blocks of the middle and posterior-ramus segments. The specimens were coded (for example, Group 3) and therefore no information about the treatment group was known during the analysis.

3.7.5 Quantimet analysis

Quantimet analysis was used to perform bone histomorphometry. The Quantimet analysis Q500MC program is a software program utilizing the CCD camera, which is set at a fixed magnification (10 times) and distance from the analysed specimen. This calibration value was kept constant at 25 μm for all histoquantification measurements. Slides were placed on an X-ray viewer as a light source and the camera recorded the image with a zoom lens attached.
Materials and Methods

The image was first scanned as a black and white image. The region of interest was then marked out (Figure 3.26). This area was then scanned again and the contrast levels manipulated to obtain a reasonable reflection of the trabecular bone. Any editing of unwanted areas was easily performed at this stage.

The Quantimet Q500MC measures certain features by creating a binary representation of the original image on an X and Y pixel array (Leica Q500MC User Manual). By setting the binary threshold to a grey level, it detects the entire bone matrix but none of the background. The total pixel count of the binary image and the perimeter pixel count of the binary image are then calculated. These two values and the total measuring frame (X and Y) can be applied to formulae based on trabecular bone being composed of vertical plates with interconnecting rods.

The following parameters for bone structure were then obtained from the raw pixel data (Varughese, 2002):

1. Bone volume/ tissue volume
2. Bone surface/ tissue volume
3. Bone surface/ bone volume
4. Trabecular thickness
5. Trabecular separation
6. Trabecular number
Figure 3.26: Bone specimen from an experimental side to demonstrate the distracted gap, which is the zone of interest for the histomorphometric study. Van Gieson actual size.