THE UNIVERSITY OF MANITOBA

ATTEMPTED TRAUMATIC INDUCTION OF ECTOPIC BONE IN RATS

by

Mark R. Reisdorf

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

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Master of Science

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ABSTRACT

Ectopic bone (EB) development is a common result of severe contusions, supracondylar fractures, hip arthroplasties and head and spinal cord injuries and often impairs function of the proximate musclulature. Conventional management has limited effectiveness in the reduction of EB and experimental management research has been in part limited by the fact that it is very costly and labour intensive to induce ectopic bone in laboratory animals. A protocol has been reported in which EB was induced in the thighs of rabbits via systematic passive mobilization (PM) of an immobilized knee (Michelsson, Granroth & Andersson, 1980).

The purpose of this study was to determine whether EB could be consistently induced traumatically in rats with a protocol that was less labour intensive than that used with rabbits. This study consisted of 3 experiments. The purpose of the first 2 experiments was to devise a technique to immobilize the knee joints of rats with externally applied splints. Experiment 1 attempted to unilaterally immobilize the right knee of 2 rats with a plaster cast and with a malleable metal splint both of which the rat was easily able to shed by using the unrestricted hind limb to pull the splints over the foot. Attempts to prevent this problem involved the use of a collar as well as a copper tube with a 90 degree bend, both placed over the ankle to prevent the metal splint from being pulled past the foot of the rats. Both of these systems kept the splints on the thigh, however both were also pulled against the foot so tightly that circulation was cut off distally. In Experiment 2, the knees of 3 rats were bilaterally immobilized by placing them in denim harnesses and suspending their hind limbs with their knees splinted in extension. This procedure was observed to effectively immobilize their knees for as much as 16 consecutive days. Experiment 3 attempted to utilize this immobilization system with smaller rats for the potential implementation of passive mobilization (PM) of the knees to induce EB in the rats thighs. This resulted in 4 of the first 5 immobilized rats dying within 7 days at which time Experiment 3 was halted. It was concluded that these rats most likely died from renal failure due to excessive pressure from the harness and that the smaller rats (200 g) used in Experiment 3 were less able to cope with stress than those of Experiment 2. Pressure on the kidneys could be avoided by using

tail suspension and a non weight bearing harness to anchor the splints. Larger rats (+400 g) may still provide a good model for this method of EB induction. Induction of EB via PM is laborious; however, it is presently the best induction system to study the process of EB development.

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LIST OF ABBREVIATIONS

BMP	BONE MORPHOGENETIC PROTEIN
DBM	DEMINERALIZED BONE MATRIX
EB	ECTOPIC BONE
EO	ECTOPIC OSSIFICATION
PM	PASSIVE MOBILIZATION
MOT	MYOSITIS OSSIFICANS TRAUMATICA

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Chapter 1
INTRODUCTION

Normal creation of new bone occurs during fetal osteogenesis, post fetal adolescent bone growth, as a reaction to loading and in the healing of fractures. This bone formation occurs within the skeletal system and functions to maintain the integrity of the skeleton. A second, pathologic type of osteogenesis is ectopic (or heterotopic) bone (EB) formation, which is development of bone outside of the skeletal system (Connor, 1983). The development of ectopic bone typically occurs within muscles, fascia and tendons (Anderson, 1976). It has also been found in tonsils, lungs, ovaries, kidneys, arterial walls, meninges of the brain and severely damaged eyes (Hartley & Tanz, 1951; Urist & McLean, 1952).

Ectopic bone formation is related to several specific conditions which result from a variety of causes. The nomenclature of these conditions are frequently misnomers based on outdated theories about their etiology. An example of this is that of myositis ossificans.

The term "myositis ossificans" strictly interpreted is ossification of inflamed muscle. This term can be misleading because muscles may ossify with or without inflammation during myositis ossificans (Ackerman, 1958). There are two major categories of this disease, myositis ossificans progressiva (MOP) and myositis ossificans circumscripta (MOC). The progressive form of myositis ossificans (also known as fibrodysplasia ossificans progressiva) is a rare inherited disease which involves gradual ossification of muscles and fascia as the afflicted person ages (Connor, 1983).

Myositis ossificans circumscripta (MOC) is ectopic bone which is limited to a specific region. Lewis (1923) categorized MOC into 3 major forms. First, there is the traumatic form known as myositis ossificans traumatica (MOT). This condition can be the result of a single trauma or repeated trauma. Second, is the non-traumatic form where no history of trauma can be traced. This may be simply referred to as non-traumatic myositis ossificans. Finally there is the neurogenic or idiopathic form which results from head or spinal cord injuries. This condition is frequently termed para-osteoarthropathy (POA). Each form has several less commonly used names.

Diagnostic techniques of this condition have included radiography, bone scanning, computed tomography and ultrasound (Booth & Westers, 1989). A proper diagnosis of EB is essential for proper management (Ackerman 1958).

Conventional management involves cold, elevation, rest and possibly short-term immobilization (Jackson & Feagin, 1973; Carlson & Klassen, 1984). Clinical experience has shown that during the acute stage, common therapeutic treatments such as exercise, heat, massage and ultrasound will increase the likelihood and severity of EB development (Ellis & Frank; Varma, 1967; Slover, 1988). Another commonly employed (yet not recommended) form of therapeutic management is aggressive attempts to mobilize an immobilized joint (Ivey, 1985). This is the basis of an effective technique for experimental induction of EB (Michelsson, Granroth & Anderson, 1980).

Experimental management techniques have involved administration of radiation, diphosphonates and nonsteroidal anti-inflammatory drugs (Ahrengart, 1988). These techniques have had varying degrees of success and all may have serious deleterious effects (Neuhauser, Wittenborg & Cohen, 1952; Plasmans, Kuypers & Sloof, 1978; Canadian Pharmaceutical Association, 1989).

Methods of ectopic bone induction in laboratory animals have included injection of chemical or cellular substances into a muscle or application of trauma (Neuhof, 1917; Levander, 1938; Michelsson et al, 1980). One traumatic induction method which has proven very successful is intermittent passive mobilization of an immobilized joint (Michelsson et al, 1980). Thus far, this technique has been successfully applied to rabbits (Michelsson & Rauschning, 1983, Ahrengart, Lindgren & Reinholdt, 1988). Despite the success of this technique it is very labour intensive for the experimenter. A variation of this technique by Ahrengart et al, (1988) has reduced the amount of labour required to traumatically induce ectopic bone and it is plausible that additional variations may further reduce this labour requirement.

No attempts to apply the protocol of Ahrengart et al. (1988) to rats have been reported in the literature, despite their demonstrated ability to develop ectopic bone experimentally (Selye, Mahajan & Mahajan, 1967, Salah & Pritchard, 1969). The fact that rat experimentation is far less expensive than that of rabbits warrants an attempt to apply the induction technique devised by Michelsson et al. (1980) and as revised by Ahrengart et al, (1988) to rats.

STATEMENT OF THE PROBLEM

The purpose of this study was to determine whether EB could be consistently induced traumatically in rats with a protocol that was less labour intensive than protocols previously used with rabbits.

HYPOTHESIS

The protocol of EB induction via systematic passive mobilization of an immobilized joint, which has been successfully used in rabbits (Michelsson et al, 1980; Michelsson & Rauschning, 1983; Ahrengart et al, 1988), can be adapted for use with rats.

RATIONALE

A procedure for the experimental induction of EB in laboratory animals which minimizes laboratory expenses and labour requirements of the experimenter is useful as it will make further study of treatment for ectopic ossification more feasible. Michelsson, Granroth and Andersson (1980) devised a simple reproducible method to induce EB in rabbits via passive mobilization of an immobilized joint. Furthermore it is well established that joint immobilization results in a gradual increase in the stiffness about a joint (Goldspink, 1977, Williams & Goldspink, 1984, Williams, Catanese, Lucey & Goldspink, 1988).

Allowance of time for an increase in tissue stiffness from immoblilization to develop prior to the commencement of manipulation sessions would likely increase the degree of trauma induced by each manipulation session. As a result, fewer manipulation sessions may be needed to produce traumatically induced ectopic bone in these animals. The fact that rats can develop ectopic bone due to other causes (Selye, Mahajan & Mahajan, 1967; Salah & Pritchard, 1969) and that they are less expensive to purchase and maintain than rabbits suggest that rats are a logical experimental alternative to rabbits.

DELIMITATIONS

Delimitation 1.

Several methods have been used to induce ectopic bone. However, this study was based upon the method of Michelsson et al. (1980) and revised by Ahrengart, Lindgren and Reinholt (1988).

Delimitation 2.

This study limited itself to 3 main progressive experiments directed toward the development of an efficient protocol for the experimental induction of ectopic bone.

LIMITATIONS

Limitation 1.

The conclusions derived from this study apply to rats, and the validity of relating these findings to humans or other animals is unknown.

Limitation 2.

The difficulty of arranging x-rays of live animals prevented pretest measurements of the area of soft tissue calcification and it was presumed that soft tissue calcification was not present in the thighs of the rats prior to the experiment. This presumption was supported by health profile test results of prominent laboratory animal supply companies which suggested that gross lesions were highly unlikely in these animals.

DEFINITION OF TERMS

Bone

As used in this study, bone refers to specialized connective tissue which contains osteocytes embedded in a calcified intercellular matrix.

Ectopic Bone (EB)

As used in this study, ectopic bone refers to bone in an abnormal location, that is, bone not within the skeletal system. This term may be used synonymously with heterotopic bone.

Ectopic Ossification (EO)

As used in this paper, ectopic ossification refers to the process of ectopic bone formation. This term may be used synonymously with heterotopic ossification.

Heterotopic Bone (HB)

See ectopic bone.

Heterotopic Ossification (HO)

See ectopic ossification.

Immobilization Period

As used in this paper, the immobilization period is the time period from the initial application of knee splints to the final removal of the splints. It is during this period that passive mobilization will occur.

Manipulation Session

As used in this paper, a manipulation session is the intermittent passive mobilization (PM) of an otherwise immobilized joint through a full range of motion.

Myositis Ossificans

As used in this paper, myositis ossificans refers to ectopic bone within muscle tissue.

Myositis Ossificans Circumscripta (MOC)

As used in this study, myositis ossificans circumscripta is a localized tumour of ectopic bone within muscle tissue. This type of myositis ossificans is often, but not always, the result of trauma.

Myositis Ossificans Traumatica (MOT)

As used in this paper, myositis ossificans traumatica is a localized tumour of ectopic bone within muscle tissue which is the result of a known trauma at the site of the tumour.

Neurogenic Ectopic Bone (NEB)

As used in this paper, neurogenic ectopic bone is ectopic bone which is caused by trauma to the central nervous system. This condition is also known as para-osteoarthropathy, neurogenic ossifying fibromyopathy, osteosis neurotica (para-articularis) and myositis ossificans circumscripta neurotica (Couvee, 1971).

Passive Mobilization (PM)

As used in this paper, passive mobilization refers to mobilization of a joint by an externally applied force with no conscious active participation on the part of the subject receiving the mobilization. When applied to an animal, this procedure is performed when it is under general anaesthesia.

Radiographic Maturity

As used in this paper, radiographic maturity is the stabilization of the size of ectopic bone as evidenced by radiography.

Remobilization Period

As used in this paper, the remobilization period refers to the time period from the final removal of the splints to the killing of the animals.

Chapter 2
REVIEW OF THE LITERATURE

INTRODUCTION

The localized formation of bone in an abnormal region (ectopic bone) is a common result of traumatic incidents such as contusions, humeral fractures, arthroplastic surgery and head and neck injuries (Connor, 1983). Ectopic bone (EB) may also develop with no history of trauma or in a non-localized manner as a result of a rare genetic condition known as myositis ossificans progressiva (MOP).

Incidents of EB have been reported in all regions of the body in people of all ages. Cases of infants with EB have been reported, however they are typically due to intravenous administration of calcium salts or to fat necrosis (Connor, 1983). The youngest known cases of EB are those of a 2-year 11-month old boy with an ossified deltoid (Wilkes, 1976) and a 53 day old girl with an ossified tibialis anterior (Pazzaglia, Beluffi, Colombo, Marchi, Coci & Ceciliani, 1986). Both of these reports stated that the condition was MOT, however neither provided an account of a traumatic incident which may have preceded the lesion.

EB tumours may be very debilitating and difficult to manage effectively with currently used treatment methods. Research of new management techniques is necessary if more productive treatment methods are to be employed. For such research to be effective, certain factors related to EB must be addressed. These factors include etiology, pathogenesis, diagnostic techniques, conventional management, experimental management and experimental induction techniques.

ETIOLOGY OF ECTOPIC OSSIFICATION

Introduction

Ossification of soft tissue is a phenomenon which may arise from a variety of causes. Two major factors related to the presence of this condition include direct trauma to the eventual site of ossification and neurological trauma. The most common sites of EB following direct trauma include the quadriceps muscle following a severe contusion (Jackson & Feagin, 1973),

the brachialis muscle following an elbow dislocation or supracondylar humeral fracture (Thompson & Garcia, 1967) and in the muscles about the hip following hip replacement surgery (Elmstedt, Lindholm, Nilsson & Tornkvist, 1985). Ectopic bone is common to people who are comatose, paraplegic and tetraplegic (Hernandez, Fourner, De La Fuente, Gonzalez & Miro, 1978; Mital, Garber & Stinson, 1987). Ectopic bone may also develop as a result of tetanus (Jajic & Rulnjevic, 1979) or with no known history of trauma (Ogilvie-Harris & Fournaiser, 1980).

Blunt Traumatic Causes

Ossification due to trauma was first described by Otto in 1816, and by 1914 more than 500 cases of myositis ossificans traumatica (MOT) had been reported (Oliver, 1914). The number of cases currently reported is unknown. Patients with MOT are predominantly adolescent or young adult males (Hughston, Whatley & Stone, 1961). A report by Ellis and Frank (1966) stated that 33 of 37 patients with MOT about the quadriceps were less than 30 years of age, although the age range was 13 - 64.

Certain regions of the body are much more susceptible to MOT than others. Although bone formation may occur in virtually any skeletal muscle, the most common sites are the brachialis and quadriceps femoris muscles (Sumiyoshi, Tsuneyoshi & Enjoji, 1985). This is demonstrated by the findings of Schultz (1910) who collected records of the German army over a 10 year period and found that of 233 MOT cases, only 3 did not occur in the brachialis or quadriceps.

The brachialis is a frequent site of MOT following elbow dislocation (Hait, Boswick & Stone, 1970; Mohan, 1972). Thompson and Garcia (1967) estimated that 3% of all elbow dislocations result in MOT. These authors also stated that an elbow dislocation with fracture was 5 times more likely to develop MOT as without fracture, thus relating severity of injury to likelihood of ectopic ossification (EO).

Ossification within the quadriceps muscles is typically the result of trauma incurred during sports activities (Tredget, Godberson & Bose, 1977; Antao, 1988). Ellis and Frank (1966) reported that 33 of 37 patients with MOT in the quadriceps had been injured while playing soccer. Jackson and Feagin (1973) also reported that soccer players and American football players frequently received severe quadriceps contusions. Thorndike (1940) reported

21 of 25 cases of MOT which were caused by football injuries, with most occurring in the quadriceps. Jackson and Feagin (1973) related the likelihood of developing MOT to the severity of the contusion. It was noted that moderate to severe quadriceps contusions had disability periods ranging from 33 to 180 days, yet no correlation between the onset of MOT and the length of the disability period was found.

According to Ellis and Frank (1966) a patient who presents with a tender thigh with less than 90 degrees of flexion within 24 hours post trauma will usually develop ectopic bone. This observation was not supported by Rothwell (1982) who noted that among 28 patients who met the above criteria, only 7 (25%) developed MOT.

MOT is also known to occur following repeated trauma. In these cases the site of ossification varies considerably. These injuries are typically vocationally related and include the thigh adductors in horse riders, pectoral and deltoid muscles in infantrymen and the thighs of cobblers (Fout & McLeod, 1977, Connor, 1983).

Surgically Related Causes

Ectopic bone formation is a common result of hip surgery, particularly with total hip replacement (THR) and is typically referred to in surgical literature as heterotopic ossification (HO) (Bijovet, Nollen, Sloof & Feith, 1974; Coventry & Scanlon, 1981; Ritter & Gioe, 1982; Ayers, Evarts & Parkinson, 1986; Kjaersgaard-Anderson & Schmidt, 1986). The reported frequencies of this condition have ranged from 10% to 90% (Elmstedt, Lindholm, Nilsson & Tornkvist, 1985). Kjaersgaard-Andersen, Sletgaard, Gjerloff and Lund (1990) have suggested that the use of anti-inflammatory drugs may account for the wide range of reported incidences. The etiology of this disorder is not known, however Ahrengart and Lindgren (1984) suggested that it is related to trauma from the surgical retraction of muscles which surround the hip.

More recently, it has been shown that factors additional to soft tissue trauma may have a role in the development of HO. Maloney, Krushnell, Jasty and Harris (1991) reported a significantly lower incidence of post-THR HO when the femoral prosthesis was cemented to bone than when uncemented. The authors suggested that the cement may provide a seal around the femoral neck to prevent particulate bone debris and bone marrow from being released into

the surrounding soft tissue which might otherwise help stimulate HO. A satisfactory treatment for this condition has yet to be documented (Kjaersgaard-Anderson & Ritter, 1991).

Post surgical development of EB may occur within abdominal muscles cut by a scalpel. Classen, Wiederanders and Herrington (1960) stated that this can generate considerable discomfort to the patient.

Cases of EB as a result of tetanus have been reported (Gunn & Young, 1959; Pitts, 1964; Thorseth, 1968; Femi-Pearse & Olowu, 1971; Odiase, 1975; Mitra, Sen & Deb, 1976; Jajic & Rulnjevic, 1979; Asa, Bertorini & Pinals, 1986). The pathogenesis of this condition is not well described and is presumed to be the result of muscle damage caused by an extended period of contracture (Jajic & Rulnjevic, 1979). This condition is considered to be extremely rare even in countries where tetanus is endemic (Mitra et al, 1976).

Neurogenic Causes

Ectopic bone formation due to head injuries, particularly with comatose patients was first described by Roberts (1968) and has been further discussed by others (Garland, Blum, Waters & Downey, 1980; Sazbon, Najenson, Tartakovsky, Becker & Grosswasser, 1981; Garland, Razza & Waters, 1982; Mital, Garber & Stinson, 1987). The reported incidences of this condition have ranged from 11% (Garland et al, 1980) to 77% in long term (1 - 59 months) coma patients (Sazbon et al, 1981). Although muscle spasm is common with comatose patients, Mital et al, (1987) speculated that an unknown neurogenic factor due to coma, not muscle spasticity was the cause of the EB formation. The EB produced from this neurogenic factor has been referred to as idiopathic ectopic bone (IEB) by Garland et al, (1982).

Garland, et al, (1982) stated that IEB about a joint develops in a single plane in relation to the joint, whereas EB induced via blunt trauma or surgery tends to be periarticular. Furthermore, the authors suggested that passive mobilization of the affected joint is not contraindicated with IEB whereas it is with MOT.

IEB is also commonly associated with paraplegia and tetraplegia and is typically referred to as para-osteoarthropathy (POA) (Couvee, 1971; Rossier, Bussat, Infante, Zender, Courvoisier, Muheim, Donath, Vasey, Taillard, Lagier, Gabbiani, Baud, Povezat, Very & Hachen, 1973). Hernandez, Fourner, De La Fuente, Gonzalez and Miro (1978) reported a POA

incidence of 20% based upon the evaluation of 704 paraplegic and tetraplegic patients. Lal, Hamilton, Heinemann and Betts (1989) have identified older age and complete spinal lesions as clear risk factors in the development of this condition. The etiology of this condition is not understood and generally considered idiopathic, however Silver (1969) suggested that trauma due to pressure sores may have a role in its development.

Unknown Causes

Cases of MOC have been known to occur with no history of trauma (Paterson, 1970; Samuelson & Coleman, 1976, Jones & Ward, 1980). Geschicter and Maseritz (1938) reported a series of 25 cases of localized EB in which only 15 (60%) of the patients had a definite history of trauma. The term pseudomalignant myositis ossificans is often used in these cases because they present similar clinical signs to those of an osteosarcoma (Ogilvie-Harris & Fournaiser, 1980). Paterson (1970) discussed 4 cases of MOC without prior trauma in which osteosarcomas were initially suspected. Biopsies proved the tumours to be benign and complete recovery occurred in all cases. Little is known about the pathogenesis of this condition (Shea, 1967).

Summary

EB induced via blunt trauma (MOT) is most likely to occur in adolescent or young adult males (Hughston et al, 1961). The most common sites of MOT include the brachialis muscle following elbow dislocation or humeral supracondylar fracture (Thompson & Garcia, 1967) and in the quadriceps following contusion (Antao, 1988). EB is common to total hip replacement patients, possibly the consequence of surgical retraction of the musculature surrounding the hip (Ahrengart & Lindgren, 1984). EB is also common as a result of head and spinal cord injury (Garland et al, 1982; Lal et al, 1989). It is thought that some type of neurogenic factor is the cause of this condition (Mital et al, 1987). Benign EB tumours have resulted from tetanus (Asas et al, 1986) and also with no history of trauma (Jones & Ward, 1980). Very little information has been provided about the pathogenesis of either of these conditions.

PATHOGENESIS OF ECTOPIC OSSIFICATION

Introduction

Traumatically induced EB is typically preceded by inflammation and the development of a hard palpable mass in the muscle within a few days following the initial trauma. The overlying skin is usually reddish-blue or blue. It is very common for MOT patients to have received a second blow to the injured area a few days after the initial blow thus prompting them to seek aid (Hughston et al, 1961).

Molloy and McGuirk (1976) stated that some patients with minimal signs and symptoms following the initial trauma may still develop MOT during the next weeks or even months. Furthermore, the bony mass is easily irritated until it is mature and attempts at excision prior to this time may cause further ossification.

Pathogenesis

Pathogenic theories include calcification and ossification of a haematoma (Geschickter & Maseritz, 1938; Gilmer & Anderson, 1959) and periosteal tearing or rupture with a resultant release of osteoblasts into the overlying muscle (Zaccalini & Urist, 1960). Ossification of a haematoma is no longer an accepted theory, as haematomas rarely ossify (Connor, 1983). Periosteal damage is not required to cause EB formation as post surgery patients developed EB after incision of their abdominal muscles (Classen, 1960). Animal experiments have also demonstrated that periosteal damage is not required to induce EB (Selye, Mahajan & Mahajan, 1967; Michelsson, Granroth, & Andersson, 1980). Despite the fact that periosteal damage is not essential, EB is more likely to develop when there is disruption of bone, periosteum and muscle together (Zacalini & Urist, 1960; Hirano & Urist, 1991).

Current theories of MOT pathogenesis involve the concept of differentiation of certain mesenchymal cells, found within skeletal muscle, into cartilage and bone. This theory was first postulated by Virchow as early as 1884, though it received little support until it was promoted by Mallory (1933). Recent animal experiments have demonstrated the existence of mesenchymal stem cells within connective tissues which may differentiate into osteoblasts. These cells,

described as inducible osteoprogenitor cells (IOPC), are abundant in a variety of connective tissues (Urist, Nagakawa, Nakata & Nogami, 1978).

Another type of osteogenic precursor cell, described as determined osteoprogenitor cells (DOPC), are capable of self replication, will produce bone spontaneously, and can develop differentiated cells (Friedenstein, 1973, 1976). Determined osteogenic precursor cells are found only in bone marrow and cannot migrate in blood whereas IOPCs, which are also produced in bone marrow, are capable of migrating in the bloodstream and are therefore prevalent in a variety of tissues (Anderson, 1976). The relative importance of local IOPCs compared to blood borne IOPCs in EB formation is not yet known.

The IOPCs do not produce bone spontaneously but require stimuli (Friedenstein, 1968; Owen, 1970; Chalmers, Gray & Rush, 1975). It has been hypothesized that stimuli required to activate IOPCs include the inflammatory process which results from trauma and a bone inducing factor known as bone morphogenetic protein (BMP) (Urist, 1969, Harada, Oida & Sasaki, 1988). Localized trauma causes a proliferation and migration of IOPCs into the injured region as part of the inflammatory process (Nilsson, Bauer, Brosjo & Tornkvist, 1986; 1987). Urist (1969) stated that skeletal muscle is the most likely tissue to produce EB in response to the presence of BMP. Bone morphogenetic protein is thought to be responsible for the differentiation of IOPC into osteoblasts, however the mechanism of this process is unknown (Kubler & Urist, 1990).

The amino acid sequence of BMP has been determined and BMP has been cloned through recombinant DNA technology (Reddi & Cunningham, 1991). BMP holds tremendous promise for future application in the engineering and fabrication of prosthetic devices based upon architectural design principles (Reddi & Cunningham, 1991).

BMP is currently being tested with bioceramic materials such as hydroxyapetite blocks. This combination has been used to successfully fuse lumbar bodies in rabbits (Ragni & Lindholm, 1991). This approach may provide a desirable alternative to traditional grafts because sufficient quantities can be obtained and only one operation would be necessary. Implantation of BMP has also been shown to be effective in the repair of non-union fractures in dogs (Tiedeman, Connelly, Strates & Lippiello, 1991).

Several factors related to BMP have not yet been satisfactorily explained or even discussed in the literature. These issues include how and where BMP is synthesized in the body, if it is stored in an active form and in which tissues it is stored. Another problem is how BMP becomes present at a local site of injury (particulary in soft tissue), whether it was previously present at that site or whether it migrated to that site. Little information is available about how endocrinological factors influence BMP. Finally, the parameters of BMP within the various tissues of people with myositis ossificans progessiva, and if they produce BMP spontaneously within their soft tissue has not been examined. It is possible that these people may provide valuable information about BMP.

Preliminary evidence from recent studies indicated that mature embedded fibroblasts which surround muscle fibers may actually differentiate into chondroblasts (Yabu, Takaoka, Hashimoto & Fujita, 1991) or osteoblasts (Okamato, Horisaka, Matsumoto, Yoshimura, Kawada, Yamashita & Takagi, 1991) when exposed to certain BMP subcomponents. MOT typically progresses along muscle sheaths and within the collagenous tissue of a muscle bundle (Hardy & Dickson, 1963). As the lesion increases in size muscle fibers are compressed and destroyed but not invaded (Paterson, 1970; Kewalramani & Orth, 1977).

Morphology

The histological appearance of MOT is identical to that of IEB (Kewalramani & Orth, 1977). Ackerman (1958) described 3 histological zones within a benign EB tumour as seen with a haematoxylin and eosin stain (Figure 2.1). The first is a central region containing many mitotic undifferentiated cells and appears identical to a sarcoma (Figure 2.2). This zone is surrounded by a second layer of oriented osteoid with osteoblasts (Figure 2.3). The peripheral zone, which has the greatest mineral content, consists of lamellar trabecular and often compact bone, and is encapsulated by fibrous connective tissue (Figure 2.4) (Ackerman, 1958, Lieberman, Barzel, De Vries & Ellis, 1967). The 2 outer layers contain osteoblasts, osteocytes and osteoclasts which are morphologically and functionally similar to those found in skeletal bone (Bagi & Miller, 1991). Many authors have referred to the importance of these zones for diagnosing a biopsy, however the development of these zones is poorly understood (Shea, 1967, Paterson, 1970, Sumiyoshi, Tsuneyoshi & Enjoji, 1988).

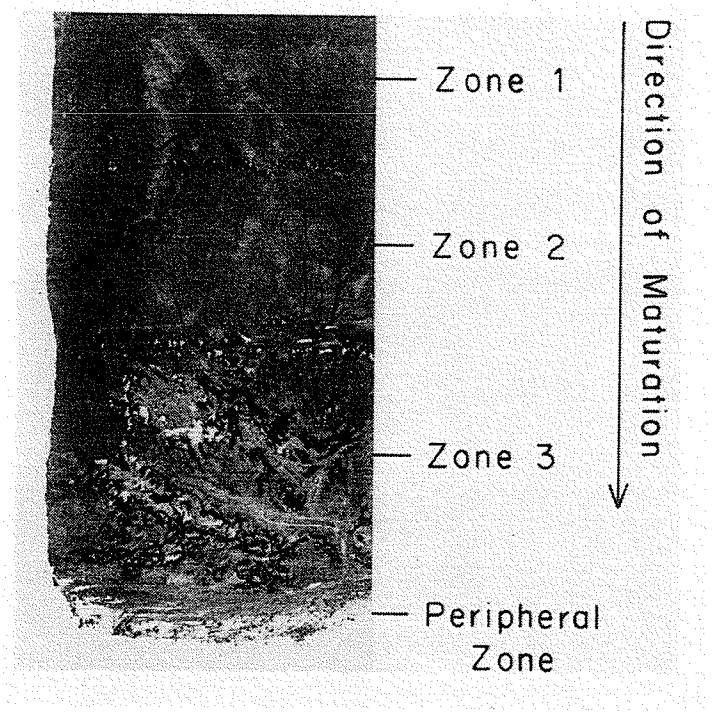


Figure 2.1 Zones of an MOT tumour (from Ackerman, 1958).

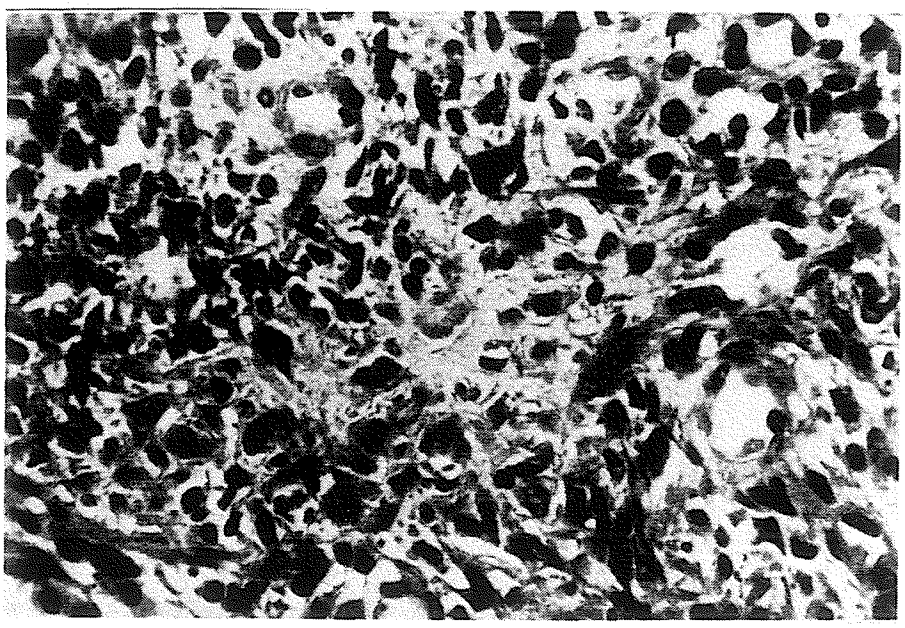


Figure 2.2 Highly cellular innermost zone of an MOT tumour (from Ackerman, 1958).

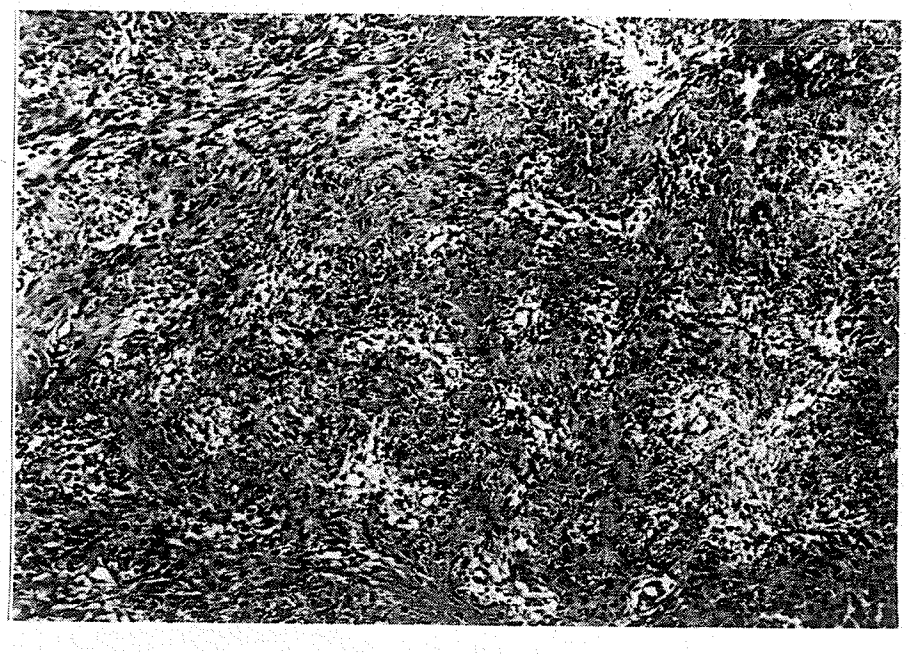


Figure 2.3 Middle zone of an MOT tumour with osteoid (from Ackerman, 1958).

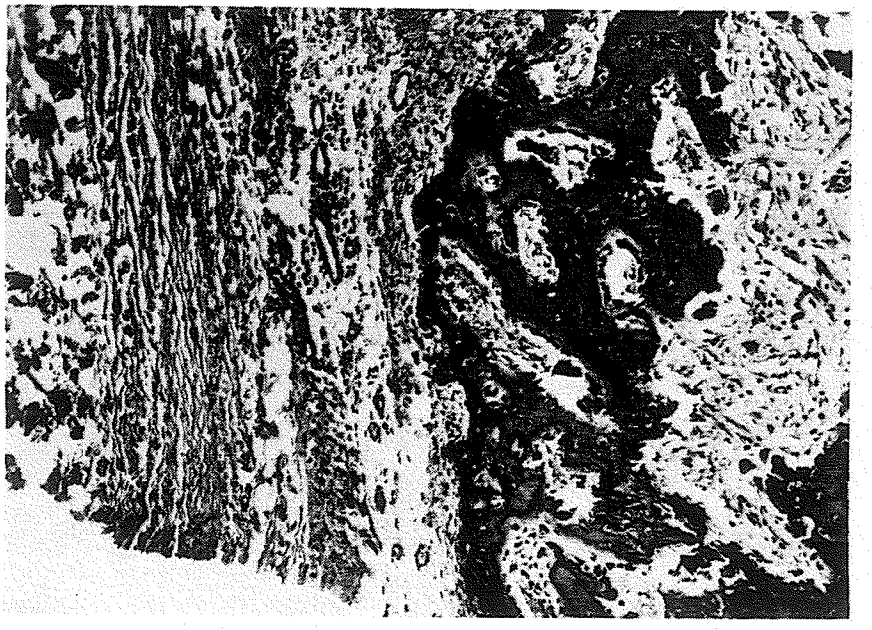


Figure 2.4 Outer zone of an MOT tumour with well developed bone (from Ackerman, 1958).

Summary

The pathogenesis of EB is poorly understood and many explanatory theories have been proposed. Theories less well accepted include haematoma ossification (Gilmer & Anderson, 1938) as well as the release of osteoblasts following periosteal disruption (Zaccalini & Urist, 1960). Predominant theories propose that undifferentiated mesenchymal cells migrate through blood vessels to the site of injury (Nilsson et al, 1986; 1987). Once at this location, the unique local tissue environment causes these cells to differentiate into osteoblasts (Harada et al, 1988). Recent evidence has indicated that mature fibroblasts located within the local tissue may differentiate into osteoblasts (Okamato, et al, 1991). Benign ectopic bone tumours have a distinctive zonular arrangement containing 3 different cellular regions (Ackerman, 1958).

DIAGNOSIS OF ECTOPIC BONE

Introduction

Connor (1983) has referred to 3 factors which should suggest the presence of MOT as opposed to an ostoeosarcoma. First, MOT requires a history of significant local injury. Second, clinical and radiological evidence of ossification must be present within two months post trauma but not progressing after one year. Finally, MOT usually occurs in the muscles of proximal segments of limbs, in particular the brachialis and quadriceps femoris. If these criteria are not met the diagnosis of MOT is less certain. EB can be detected with a variety of imaging techniques and tissue biopsy will confirm benignity.

Clinical Examination

Clinical examination of EB lesions frequently arouses suspicion of a malignant tumour (Pack & Braund, 1942; Fine & Stout, 1956; Ackerman, 1958; Jeffreys & Stiles, 1966, Rooser, Herlin, Rydholm & Akerman, 1989). Osteosarcomas, however, have an intimate connection between the lesion and the adjacent skeletal bone which is not the case with benign EB (Norman & Dorfman, 1970). Fine and Stout (1956) stated that osteosarcomas completely separated from skeletal bone are extremely rare. Furthermore an osteosarcoma contains its most mature, dense

bone in its center and has a fibrotic cortex, which is the reverse of a benign EB tumour (Mirra, 1990). Ackerman (1958) stated that if histological analysis of a biopsy sample clearly shows the three zones of cell differentiation previously described, the EB is classified as benign. Radical surgery, including amputations and high dosage radiation therapy, have been performed on people with benign EB tumours that were improperly diagnosed as osteosarcomas (Fine & Stout, 1956; Ackerman, 1958; Shea, 1967).

Soft tissue calcification with a history of recent trauma should indicate that a biopsy is unnecessary (Connor, 1983). A diagnostic biopsy is likely to irritate immature ectopic bone, stimulate further ossification and delay recovery (Tredget et al, 1977). Tissue biopsy is only considered to be justified when a large tumour-like mass is located in a region uncommon for MOT and not fulfilling the criteria of a typical MOT tumour (Connor, 1983).

Imaging Techniques

EB is normally confirmed radiographically. Radiographs usually show ectopic calcification from 2-4 weeks after the trauma. Radiographic maturity (stabilization of EB development as evidenced by radiography) usually occurs by 4-5 months following the initial trauma (Molloy & McGuirk, 1976; Deluca, 1985).

The inability of radiographs to detect EB in its early stages has prompted experimentation with other imaging techniques. Bone scanning with 99 m technetium has shown some potential in early detection of EB (Suzuki, Hisada & Takeda, 1974; Freed, Hahn, Menter & Dillon, 1982; Drane, 1984; Tyler, Derbekyan & Lisbona, 1984). The validity of this technique has been questioned because of its inability to differentiate between soft tissue calcification and unusually high soft tissue activity (Zeanah & Hudson, 1982; Booth & Westers, 1989).

Computerized tomography (CT) to reveal EO was originally advocated by Zeanah and Hudson (1982). This relatively new technique has demonstrated a greater ability to detect ectopic calcification than conventional radiography (Amendola, Glazer, Agha, Francis, Weatherbee & Martel, 1983, Heiken, Lee, Smathers, Totty & Murphy, 1984, Bressler, Marn, Gore & Hendrix, 1987). Ultrasound as a diagnostic tool for EB has been promoted by Kramer, Kurtz, Rubin and Goldberg (1979), Kirkpatrick, Roman and Rovere (1987) and Fornage and Eftekhari (1989). Kirkpatrick et al. (1987) stated that they were able to detect MOT with

ultrasound one week after trauma, whereas radiographs taken at the same time were negative. Thomas, Cassar-Pulicino and McCall (1991) have stated that both CT and serial ultrasonography (3.5 MHz or 7.5 MHz) can clearly define the 3 zones of cell differentiation described by Ackerman (1958). The role of magnetic resonance imaging for the diagnosis of benign EB tumours has not been established in the current literature.

Summary

Indicators of whether an EB tumour is benign include history, location and rate of progression of the tumour. If these factors indicate benignity, a tissue biopsy is generally not recommended (Connor, 1983). Radiography is the most commonly used technique to detect the presence of EB (Deluca, 1985). Other imaging methods that have been used very effectively for this purpose are computerized tomography and ultrasonography (Thomas et al, 1991).

CONVENTIONAL MANAGEMENT OF ECTOPIC BONE

Introduction

Early conservative treatment of cold, compression, elevation and rest is recommended when haematoma formation is suspected after trauma (Lipscombe, Thompson & Johnston, 1976; Gray, 1977; Carlson & Klassen, 1984, O'Donoghue, 1984). If possible, the traumatized limb should be immobilized for up to 48 hours, depending upon the severity of the contusion (Jackson & Feagin, 1973). Once an initial trauma has occurred it is essential to protect the injured part, as it is much more susceptible to further injury. Hughston et al, (1961) stated that, typically, athletes do not seek medical attention until they have received a second blow a few days after the initial trauma. Compression as a means to minimize the inflammatory process following trauma has been widely accepted, however it has not been discussed in the EB related literature as a method to prevent EB formation.

Contraindications

During EB management, it is critical to minimize activity, local heat massage and therapeutic ultrasound, as they may promote ossification (Ellis & Frank, 1966; Hait et al, 1970;

Slover, 1988). The danger of massaging a haematoma, especially in the acute stage, has been discussed by several authors (Ellis & Frank, 1966; Varma, 1967; Mohan, 1972). Both Varma (1967) and Mohan (1972) lamented the fact that in India, the people trusted local bone setters and masseurs who massaged their elbows after suffering trauma to the region. Mohan analyzed 200 cases of MOT at the elbow, 90 of which required surgery, and reported that almost all had a history of massage.

Another consideration is that therapeutic mobilization of a joint which has been immobilized may induce MOT (Ivey, 1985). This is especially apparent during passive mobilizations which traumatize both muscle and connective tissue about the joint. This trauma is a sufficient stimulus for inducible osteogenic precursor cells to react and form EB (Ivey, 1985).

Surgery

Certain circumstances may warrant surgical excision of the lesion. According to Lipscombe et al. (1976) excision should be performed when a large mass of EB is painful, causes muscular weakness or limits joint motion. Hait et al. (1970) stated that surgical excision should take place if the ossified mass is predisposed to further injury or has breakable protrusions. However, it is essential that the entire ectopic bone mass is mature before surgery is performed, as early surgery may cause further ossification (Venables, 1967). Maturation may take 12 months or more to occur.

Summary

Conventional management of EB includes conservative procedures such as cold, elevation, rest and protection from further trauma (Carlson & Klassen, 1984). Massage, heat, therapeutic ultrasound and forcible stretching of the muscle are contraindicated (Ellis & Frank, 1966; Mohan, 1972; Slover, 1988). Surgical excision of the tumour prior to maturity may simply result in its recurrence (Venables, 1967).

EXPERIMENTAL MANAGEMENT OF ECTOPIC BONE

Introduction

Recently, a variety of experimental treatment methods have been tested. These experimental treatments have included the administration of radiation therapy, diphosphonates, nonsteroidal anti-inflammatory drugs (NSAIDs) and other substances.

Radiation

Low-dose radiation therapy for the reduction of EB has been evaluated by Coventry and Scanlon (1981), Ayers et al, (1986) and Ahrengart et al, (1988) who reported that radiation exposure of 2000 rads or less was effective in the prevention of EB formation following total hip replacement (THR). Coventry and Scanlon (1981) stated that radiation therapy is a reasonable risk for elderly THR patients with a relatively short life expectancy. Radiation-induced osteosarcoma latency periods average 11 to 12 years (Kim, Chu, Woodward, Melamed, Huvos & Cantin 1978).

Neuhauser, Wittenborg, Berman and Cohen (1952) have demonstrated that radiation dosages greater than 2000 rads cause severe bone contour changes in children. The two considerations of latent osteosarcoma and bone deformation make radiation therapy inappropriate for young people (Coventry & Scanlon, 1981), who account for most cases of MOT (Hughston et al, 1961).

Diphosphonates

The ability of the diphosphonate ethane-1-hydroxy-1, 1-diphosphonate (EHDP), to inhibit mineralization of an osteoid matrix (Francis, Russell & Fleisch, 1969; King, Francis & Michael, 1971; Russell & Smith, 1972; Plasmans, Kuypers & Sloof, 1978) generated interest in the potential of EHDP to prevent and treat myositis ossificans progressiva (Basset, Donath, Macagno, Preisig, Fleisch & Francis, 1969; Russell, Smith, Bishop & Price, 1972; Freed, Hahn, Menter & Dillon, 1982; Thomas & Asmutz, 1985). Although EHDP inhibits osteoid mineralization it does not affect osteoid production. Consequently, the increased unmineralized

osteoid may produce an osteomalacia-like condition in skeletal bones (King, Francis & Michael, 1971; Plasmans, Kuypers & Sloof 1978).

Bijvoet, Nollen, Sloof and Feith (1974) and Thomas and Asmutz (1985) reported that EHDP delayed but did not prevent mineralization of ectopic osteoid following total hip replacement. Ahrengart, Lindgren and Reinholdt (1988) reported that EHDP had no effect on traumatically induced EB in rabbits. Adverse effects of diphosphonates include bone weakness and diarrhea and their effectiveness in treating MOT has not been established (Booth & Westers, 1989; Canadian Pharmaceutical Association, 1989).

Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, diclofenac and ibuprofen have received consideration as substances for the reduction of ectopic bone. The discovery that indomethacin impaired Haversian remodelling (Sudmann, 1975, Ro, Sudmann & Martin, 1976; Sudmann & Bang, 1979; Allen, Wase & Bear, 1980) stimulated interest in the ability of indomethacin to prevent EB formation (Ritter & Sieber, 1977). Studies with animals (Nilsson, Bauer, Brosjo & Tornkvist, 1986; 1987 ; Ahrengart et al, 1988; DiCesare, Nimni, Peng, Yazdi & Cheung, 1991) and humans (Almsbakk & Roysland, 1977; Ritter & Sieber, 1977; Ritter & Gioe, 1982; McLaren, 1990) have shown that indomethacin does inhibit EB formation when administered in sufficient doses.

Indomethacin administration may produce a number of adverse effects which include the induction and increased severity of osteoarthritis and reduced torsional strength of bones (Sudmann, 1975; Ronnigen & Langland, 1977; Tornkvist, Lindholm, Netz, Stromberg & Lindhom, 1984). Other deleterious reactions to indomethacin frequently include severe headaches and bouts of depression, blurred vision, dizziness, lightheadedness, gastrointestinal pain, nausea and diarrhea (Goodwin & Gilman, 1975; Modell, Schild & Wilson, 1976; Meyers, Jawetz & Goldsfein, 1980; Canadian Pharmaceutical Assosciation, 1992, Speight, 1989). The adverse effects of indomethacin prompted Sudmann (1975, pp. 91) to state "...the indications for using this powerful anti-inflammatory may have to be reassessed.".

According to the Canadian Pharmaceutical Association (1992) the NSAID diclofenac has the same efficacy as an equal dose of indomethacin but causes less severe central nervous system

side effects. This is supported by Nilsson et al, (1987) who found that both diclofenac and indomethacin, when administered in doses of 3 mg/kg/day, decreased EB formation in rats by 15%. Indomethacin doses above this level were toxic to rats (Tornkvist et al, 1984). Diclofenac doses of 6 mg/kg/day decreased EB formation by 30% with no toxic effects although toxic effects were produced at a dose of 12 mg/kg/day (Nilsson et al, 1989).

Interest in the NSAID ibuprofen as an EB inhibitor was generated by Tornkvist, Nilsson Bauer and Lindholm (1983). The authors showed that ibuprofen significantly reduced the content of bone calcium and phosphate. Elmstedt et al, (1985) determined that administration of ibuprofen decreased the incidence of EB formation in post THR patients. As with indomethacin, ibuprofen reduces torsional strength of skeletal bone (Tornkvist et al, 1984). Adverse effects of ibuprofen include nausea, heartburn, epigastric pain, dizziness, headaches, skin rash, reduced visual acuity and visual field defects (Goodwin & Gilman, 1975) The incidence of these effects is lower with ibuprofen than with indomethacin or diclofenac (Canadian Pharmaceutical Association, 1992).

Recent studies have indicated that aspirin may be an effective inhibitor of EB for THR patients (Freiberg, Cantor & Freiberg, 1991; Pagnani, Pellici & Salvati, 1991). Pagnani et al, (1991) reported a 3% incidence of severe EB in THR patients who were administered aspirin compared to a 48% incidence in THR patients with no medication.

Other Experimental Drugs

Other substances which have been used to inhibit EB formation include prednisolone (Ahrengart et al, 1988), prednisone, edetic acid (Liberman, Barzel, De Vries & Ellis, 1967), and calcitonin (Bethel & Doran, 1979). Ahrengart et al, (1988) determined that rabbits treated with prednisolone developed less traumatically induced EB than rabbits treated with EHDP, radiation or indomethacin. Prednisolone is a hydrated derivative of the powerful glucocorticoid prednisone, which may cause muscle weakness and atrophy, osteoporosis, fractures and psychic changes such as mood swings and depression (Modell, Schild & Wilson, 1976; Canadian Pharmaceutical Association, 1992). Both calcitonin and especially edetic acid are extremely dangerous drugs which cause a rapid decrease of serum calcium levels (Goodman & Gilman, 1975; Modell et al, 1976; Meyers et al, 1980).

Summary

Several approaches to experimental management of EB have been examined. Low dose radiation therapy has been used with some success in elderly THR patients with a short life expectancy, but is considered inappropriate for young people (Coventry & Scanlon, 1981). Diphosphonate therapy has been shown to have little effect on the process of EB formation (Booth & Westers, 1989). Usage of NSAIDs such as indomethacin, diclofenac and ibuprofen have shown some inhibitory effect on EB, however many adverse effects are associated with these drugs (Tornkvist et al, 1983; Tornkvist et al, 1984; Nilsson et al, 1987). Recent research has indicated that aspirin may have potential for the prevention of EB (Pagnani et al, 1991).

EXPERIMENTAL INDUCTION OF ECTOPIC BONE

Introduction

Effective research of treatment methods for MOT requires that effective and feasible methods are available to artificially induce EB in animals. Animal models have been the source of virtually all of the information obtained about the pathogenesis of EB. Rabbits have been the most popular of animals for these studies as they are considered to be especially prone to ectopic ossification (EO) (Zaccalini & Urist, 1960). Rats have also displayed a tendency to develop EO (Selye et al, 1967; Salah & Pritchard, 1969) whereas mice have exhibited resistance to EO (Wlodarski, 1971; Anderson, 1976). Four approaches to experimental induction of EO which have been utilized include; implantation of non-osseous substances, implantation of DBM or BMP, blunt trauma and passive mobilization of an immobilized joint.

Induction via Implantation of Non-Osseous Substances

Experimental induction of EB was initiated in 1917 when Neuhof discovered that fascial transplants to the urinary bladders of dogs consistently resulted in bone formation within the transplant (Neuhof, 1917). This observation was also made by Phemister (1923), which in turn prompted experiments by Huggins (1930, 1931). In the studies of Huggins (1930, 1931), grafts of growing bladder epithelium were transplanted into the rectus femoris of dogs resulting in EB. Similar results with guinea pigs have been obtained by Constance (1954). Anderson, Merker

and Fogh (1964) discovered that implantation of human amniotic cells in the muscles of mice resulted in EO. This has been repeated in subsequent experiments (Anderson, 1967; Anderson & Coulter, 1967).

Levander (1938) induced EB by injecting an alcoholic extract of bone into rabbit muscles. The significance of the use of bone tissue as an implant material was later disputed based on the results of studies by Heinen, Dabbs and Mason (1949); Hartley and Tanz (1951) and Bridges and Pritchard (1958). These studies showed that EB could be developed by injecting substances such as alcohol, calcium chloride or quinine, into muscles of rabbits. The EB induced from these procedures was thought to be the result of a traumatic reaction to the presence of irritating chemicals.

Induction via DBM or BMP Implantation

The recent discovery of BMP and inducible osteoprogenitor cells have renewed interest in tissue implantation to induce EB. This approach was made popular by the work of Urist, Hay, Dubuc and Buring (1969), Chalmers et al. (1975) and Urist et al. (1978) who showed that intramuscularly implanted demineralized bone matrix (which contained BMP) would induce woven, trabecular EB in a variety of animals. It has been shown that EB produced from an implantation technique is initially woven and becomes lamellar (Okamato et al, 1991). DBM removed from one species of animal could be implanted into another species and still produce ectopic ossification (Anderson, 1976). Kawai and Urist (1988) further demonstrated that the amount of EB formed was directly proportional to the amount of BMP implanted.

Induction via Blunt Trauma

An approach to EB induction that has received limited attention is induction by blunt trauma. This approach provides an opportunity to simulate the injury mechanism that would be encountered in a real life setting. Experiments involving violent blunt trauma have attempted to replicate the mechanical forces that typically cause MOT in humans. One such study was by Zaccalini and Urist (1960) in which the thighs of rabbits were placed in a percussion chamber and struck by a hammer. The experimenters tried a variety of protocols (somewhat haphazardly) to induce the trauma. The protocol which provided the highest rate of success (50%) for

inducing EB involved striking the anterior thigh 10 times. Several of the rabbits died due to crush syndrome or fat embolism. This study did not provide a clear description of the joint positions of the animals while in the percussion chamber.

The induction of MOT in dogs via blunt trauma was reported by Collins, Stone, Harkness and Bliven (1965). This brief account merely stated that the thighs of anaesthetized dogs were placed under a percussion chamber, blows were struck and that heterotopic ossification occurred. The authors did not specify their methodology or results. Walton and Rothwell (1983) applied blunt trauma to the lateral aspect of the thighs of sheep. The authors explained that sheep thighs were selected because they were morphologically similar to human thighs. The sheep were killed at intervals ranging from 3 weeks to 3 months post trauma. The results showed that although all of the traumatized thighs developed periosteal bone, only 16.5% developed heterotopic ossification.

Induction via Passive Mobilization

Michelsson, Granroth and Andersson (1980) devised a model to induce EB in the hind limbs of rabbits. One knee of each rabbit was immobilized with a plastic splint for 5 weeks. During this period the splint was removed daily for a 5 minute session of passive mobilization (PM) of the knee. Within 5 weeks all of the rabbits had developed EB with the 3 distinct histological zones as described by Ackerman (1958). Michelsson et al. (1980) noted that the EB continued to develop after the manipulation sessions were discontinued.

This more humane and successful method prompted other studies to attempt variations of this protocol. Michelsson and Rauschning (1983) used a similar protocol with the exception that several groups of rabbits were used and the frequency of manipulation sessions varied from twice daily to once weekly. The length of treatment ranged from 1 - 5 weeks. Some of the rabbits were given a muscle relaxant (diazepam) during the immobilization period, and another group had their femoral nerves resected. It was generally found that both the frequency of mobilization and the length of the treatment period were positively correlated to the degree of ossification. It was noted that all of the EB specimens were located in the vastus intermedius. Michelsson (1992) has suggested that a periosteal reaction plays a large role in the pathogenesis of EB following PM.

The above study also indicated that manipulation in conjunction with either a muscle relaxant or femoral nerve resection both failed to reduce the degree of ossification. This finding relates to the work of Lakie, Tsementzis, Walsh and Wright (1979) who reported that in humans, neither muscle relaxants or general anaesthesia decreased muscle tone below the level of when the subject was conscious and relaxed. The fact that femoral nerve resection had no effect with this induction method reflects observations of Goldspink, Tabary, Tabary, Tardieu and Tardieu (1974). These authors reported that the soleus muscles of cats reacted to immobilization in a similar manner whether they are innervated or denervated.

Ahrengart, Lindgren and Reinholdt (1988) used a variation of this basic induction method. In this experiment, the right hind limbs of rabbits were immobilized with splints and manipulation sessions occurred every second day while the animals were under anaesthesia. The manipulation sessions consisted of repeated flexion and extension of the knee until a maximum range of motion was obtained. The splints were removed after two weeks at which time the animals received 8 manipulation sessions. The rabbits were killed 30 days after the initial application of the splints. Additionally, the rabbits in the experimental groups were treated with one of the following: radiation, indomethacin, prednisolone or EHDP. This protocol resulted in EO in all of the animals and furthermore, indomethacin, radiation and prednisolone all reduced both EO and joint stiffness.

The authors stated that most of the EB was formed in the vastus intermedius, possibly due to the fact that because this muscle is the shortest quadricep muscle, it may sustain the greatest strain for a given joint movement. It was also noted that without PM, an immobilized limb will recover completely and have no EO. This is the only study that has applied this methodology of EB induction to evaluate treatments for EO, yet it indicates a tremendous potential for further research of other treatment methods.

Michelsson et al. (1980) was the first to systematically investigate the effect of intermittent passive mobilization of a shortened immobilized muscle. None of the authors who have employed this EB induction technique discussed the mechanism of trauma to the tissues of a musculoskeletal unit from PM and how the different subcomponents of the unit are affected. Factors which warrant consideration include; the process of muscle unit shortening, muscle unit

extensibility (before and after immobilization), how these subcomponents are traumatized and their relative role in the subsequent inflammatory process.

Increased resistance to passive stretch following muscle immobilization in a shortened position has been documented (Goldspink, 1977; Williams & Goldspink, 1984; Williams, Catanese, Lucey & Goldspink, 1988). Muscles that are immobilized in a shortened position degenerate to a much greater degree than when immobilized in a lengthened position. When a muscle is immobilized in a shortened position, there is a reduction of the cross sectional area of the whole muscle (Goldspink 1977), the cross sectional area and length of each muscle fiber (Spector, Simard, Fournier, Sternlicht & Edgerton, 1982) and the number of sarcomeres in each fiber (Williams, Catanese, Lucey & Goldspink, 1988). Reduced muscle fiber length also occurs when a muscle contracts within a short range of motion (Williams, et.al, 1988).

Immobilization of muscle in a shortened position also produces a relative increase in the amount of connective tissue in the endomysium and the perimysium which causes an increase in the tensile stiffness of the muscle unit (Williams & Goldspink, 1984). According to Tsuchiya (1988), passive stiffness of a muscle is directly dependant upon sarcomere length. All of these effects are minimized during immobilization if the muscle is placed in an elongated position (Spector et al, 1982). Furthermore muscle protein content may actually increase under these circumstances (Goldspink, 1977; Booth, 1978), even when the muscle is denervated (Goldspink, 1978).

These factors warrant consideration during clinical application of casts, particularly to the knee and elbow joints, and especially if the possibility of EB development is a concern. Fibers of the vastus intermedius and the brachialis are very short relative to those of other muscles surrounding them (Ahrengart et al, 1988). This may be why these muscles are more prone to develop EB than other muscles upon remobilization of the joint following removal of the casts.

The experimental schedule of 3 sessions of PM per week for 2 weeks used by Ahrengart et al, (1988) considerably reduced the labour time of the experimenter when compared to that of Michelsson et al, (1980) who used a schedule of 5 manipulation sessions per week for 5 weeks. None of the 3 authors who used this EB induction model allowed a substantial immobilization period to occur prior to initiating the manipulation sessions. It is plausible that

by allowing some time for immobilization changes to occur before initiating manipulation sessions, each manipulation session will be more traumatic and therefore fewer manipulation sessions will be required to induce EB. If this is the case, then the labour time of the experimenter can be further reduced when employing this EB induction technique.

Rats may be an economical alternative to rabbits because they are less expensive to purchase and maintain and use less laboratory space. Current animal housing fees for an individual rat are approximately half of those for an individual rabbit. Rats have also demonstrated the ability to develop EB (Selye et al, 1967; Salah & Pritchard, 1969). No studies have been reported in the literature which have applied the EB induction technique of Michelsson et al. (1980) to rats. This would require a method to immobilize the knees of rats with an externally applied device, which has not been reported in the literature.

Summary

Further research of the pathogenesis and management of EB requires that EB can be effectively induced in animals. EB has been induced in animals through the implantation of chemical irritants (Bridges & Pritchard, 1958), non-osseous tissue components (Anderson, 1967) and osseous tissue components such as DBM and BMP (Okamato et al, 1991). Attempts to induce EB via blunt trauma have produced a low incidence of EB and a high mortality rate in the animals (Zaccalini & Urist, 1960). EB has also been induced in the vastus intermedius muscles of rabbits via passive mobilization of the knee joint (Michelsson et al, 1980). The mechanisms by which this occurred were not examined. This procedure has been highly successful, but it is very labour intensive and expensive. It is possible that the labour required to implement this basic technique could be reduced. In addition, there may be advantages to the use of rats instead of rabbits for the employment of this technique.

SUMMARY OF LITERATURE REVIEW

Ectopic (or heterotopic) bone (EB) is bone which is located outside of the skeletal system (Connor, 1983). EB development is a common result of severe contusions, supracondylar fractures, hip arthroplasties and head and spinal cord injuries. EB is most commonly located

in muscle, however it has been reported to occur in many different types of soft tissue (Hartley & Tanz, 1951).

The pathogenesis of EB is associated with inflammation wherein undifferentiated mesenchymal cells migrate to the site of injury and differentiate into osteoblasts which initiate bone formation (Nilsson et al, 1986; 1987). There is recent evidence that mature fibroblasts already embedded in the traumatized tissue differentiate into osteoblasts (Okamato et al, 1991). A benign EB tumour is composed of lamellar trabecular bone with 3 distinct histological layers from the center to the periphery (Ackerman, 1958). Progression of the tumour occurs along the internal surface of epimysium with subsequent evagination and destruction of the adjacent muscle fibers resulting in functional impairment of the affected muscle (Hardy & Dickson, 1963).

The presence of EB is most frequently determined by radiography (Deluca, 1985). Other imaging techniques which have been effective for this purpose include computed tomography and ultrasonography (Thomas et al, 1991). A proper diagnosis of this condition is essential for proper management (Ackerman 1958).

Conventional management involves conservative attempts to minimize the inflammatory process. This may include the application of cold, elevation, rest and possibly short term immobilization, all of which have very limited effectiveness (Jackson & Feagin, 1973; Carlson & Klassen, 1984). Clinical evidence has shown that during the acute stage, therapeutic treatments such as exercise, heat, massage and ultrasound will increase the likelihood and severity of MOT development (Ellis & Frank; Varma, 1967; Slover, 1988).

Experimental management techniques such as administration of nonsteroidal anti-inflammatory drugs and other substances have had varied success in the inhibition of EO with the potential for the development of adverse effects (Booth & Westers, 1991). Recent studies have indicated that aspirin may be an effective inhibitor of EB (Freiberg et al, 1991; Pagnani et al, 1991). Experimental management research has been limited by the fact that it is very costly and labour intensive to induce ectopic bone in laboratory animals.

Several methods of experimental induction of EB have been attempted, usually in either rabbits or rats. A protocol has been developed to consistently induce EB in the thighs of rabbits via systematic passive mobilization of an immobilized knee (Michelsson et al, 1980). Although this protocol is quite labour intensive, this may be reduced with certain modifications. With

these modifications, this technique may also work with rats, which are less expensive than rabbits.

Chapter 3
METHODS AND PROCEDURES

INTRODUCTION

The purpose of this study was to determine whether ectopic bone could be induced in rats by applying passive mobilization of an otherwise immobilized joint. The initially proposed protocol that was submitted with the animal care utilization application (Appendix A) was as follows:

The first part of this study would use 4 rats to monitor and refine the knee immobilization technique which was to be used in the main study. This would occur over an 8 day period after which the animals would be used as part of the main study. During the 8-day pre-study phase the effectiveness of the splints would be closely monitored and any problems addressed. After the initial 8 days the rats would continue to be splinted as part of the main study.

PROPOSAL FOR THE MAIN STUDY

Rats

Twenty-two male Sprague-Dawley rats (including the 4 from the pre-study) with an initial body mass of approximately 350 grams will be used. The rats will be group housed in wire cages with indirect bedding and fed stock rodent chow and water ad libitum throughout the experiment.

Immobilization

At the commencement of the study all of the rats will be anaesthetized with methoxyflourine and an external splint will be installed over their right knees. These splints will immobilize the knees in a fully extended position, but will not restrict hip or ankle motion. The splints will be made of a malleable aluminum exterior with a thin dense foam interior and will be secured with metallized automobile body tape. These splints will be easily adjustable, removable and replaceable and will be resistant to the gnawing of the rats.

Manipulation Sessions

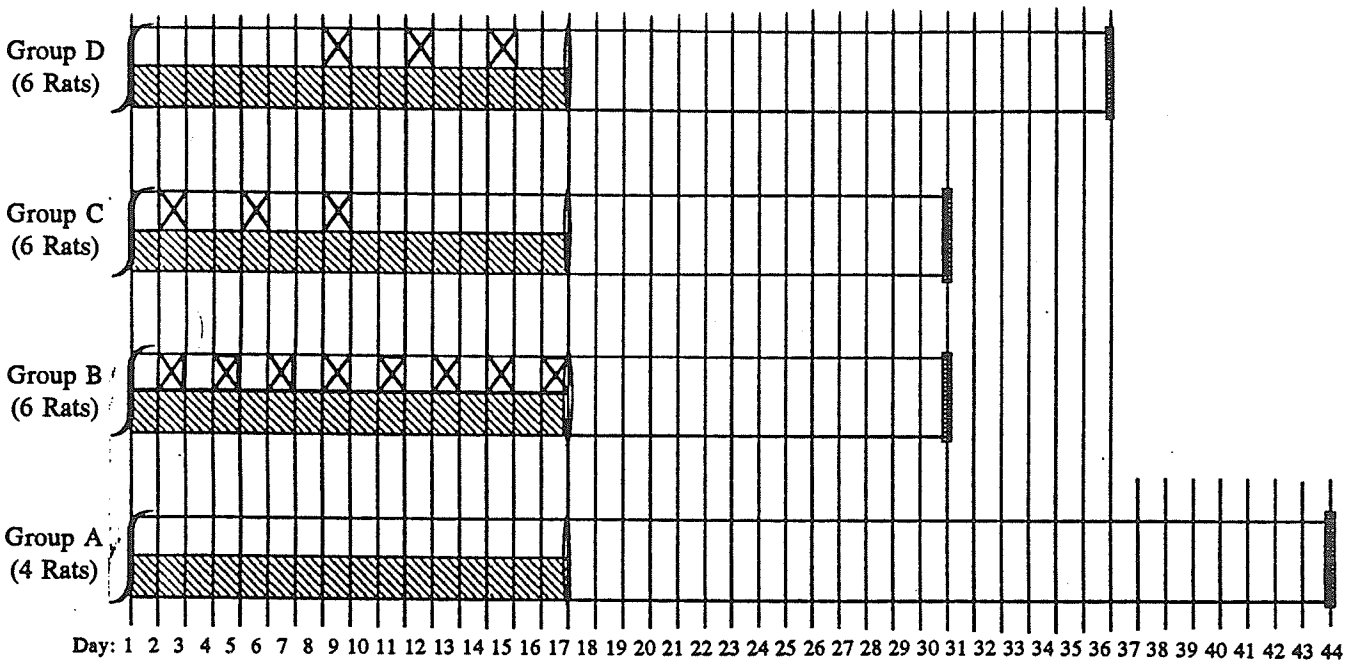
A manipulation session for a rat will involve the following procedure. The rat will be anaesthetized with methoxyflourine and the splint will be removed. With the splint removed the experimenter will manually apply passive mobilization to the immobilized knee of the rat. The passive mobilization will involve repeated flexion and extension of the knee (at a frequency of 0.5 Hz as paced by a metronome) until a full range of joint motion is achieved. Great care will be taken to not induce a femoral or tibial fracture during these sessions. Once a full range of joint motion is achieved the splints will be replaced and the animals will be allowed to recover from the anaesthesia.

Experimental Design

The rats will be divided into groups A-D. Group A will be composed of the 4 rats used in the pre-study. These rats will remain splinted for an additional 8 days after the pre-study phase, after which the splints will be removed. These rats will therefore have had their knees immobilized for a total of 16 days but will receive no manipulation sessions during this time. Groups B-D will consist of 6 rats per group and all of these rats will have their right knee immobilized in the same manner as those of group A. These rats also will be splinted for a total of 16 days. The period in which the animals are splinted will be referred to as the immobilization period.

During the immobilization period the rats of group A will receive no manipulation sessions. The rats of group B will receive 8 manipulation sessions at 48 hour intervals which commence 24 hours following the initial application of the splints. The rats of group C will receive 4 manipulation sessions at 48 hour intervals which will begin 10 days following the initial splint application. The rats of group D will receive 2 manipulation sessions, one 10 days and the other 16 days following the initial application of the splints. The final removal of the splints will initiate the remobilization period (Figure 3.1).

The remobilization period will continue until the rats are killed and no manipulations will be performed during this time. The remobilization periods for groups A, B, C and D will be 28, 14, 22 and 22 days respectively. The varied remobilization periods will maintain



Legend:

- Initial Splint Application
- Splint Immobilization
- 1 Manipulation Session
- Splint Removal
- Animals Killed

Figure 3.1 Experimental Schedule.

a constant length of the time from the initial manipulation session to the killing of the rats in all groups (Figure 3.1). This time period is 28 days, which according to the literature regarding other species (humans and rabbits), should be a sufficient amount of time for soft tissue calcification to appear radiographically. The remobilization period will be terminated upon euthanasia of the animals via an anaesthetic overdose (methoxyflourine). After the animals have been killed they will be prepared for radiographic and histological analysis.

Analytical Procedures

Immediately after the killing of the rats both thighs of all the rats will be disjointed at the hips and knees. The thighs will then be skinned and fixed by placing them in individually labelled containers of buffered formalin for a period of 1 week. The specimens will then be washed in water and placed onto a radiolucent plate opposite to their contralateral thigh. Lateral view radiographs will be taken of the thighs on each plate and the presence of soft tissue calcification (STC) of each section will be digitized and calculated.

Thighs which display STC will be analyzed histologically for the presence of EB with the zonal phenomena as described by Ackerman (1958). These thighs will be decalcified by soaking them in ethylenediaminetetraacetic acid (EDTA) for 2 weeks, fixed in formalin and embedded with chloroform. A 5 um cross-sectional slice will be taken at the location where the STC was most dense and it will be stained with haematoxylin and eosin. This section will be observed microscopically for the presence of the 3 distinct histological zones as described by Ackerman (1958). If all of these zones are present the specimen will be declared to contain traumatically induced EB.

Statistical Procedures

The following statistical procedures will be performed:

A. An two-way Anova will be performed to determine if there are any significant differences in the incidence of STC (as evidenced by radiographs) between the manipulated (right) thighs of each group and the non-manipulated (left) thighs of each group (6 group comparison) at alpha levels of 0.05 and 0.01.

B. An Anova will be performed to determine if there is a significant difference in the incidence of EB between the 3 groups manipulated (right) thighs at alpha levels of 0.05 and 0.01.

The following experiments were conducted to develop the procedures that would be necessary for the induction of EB via PM in the thighs of rats:

EXPERIMENT 1

Introduction

The purpose of Experiment 1 was to develop and refine a technique to externally immobilize the right knee of rats in an extended position with an apparatus that could be easily removed and replaced. This procedure, if successful, was to be subsequently used for application of PM to induce EB as described in Appendix A. A technique that was considered to be effective for at least 16 days was to be subsequently used. Experiment 1 attempted to immobilize a single knee in a manner which did not restrict movement of the other 3 limbs so the animals could ambulate with the unrestricted limbs. Only 2 rats were used initially, with the expectation that an additional 2 rats would be employed once the immobilization procedures appeared successful, in order to complete the 'pre-study' group.

Rats

Two male Sprague-Dawley rats with an initial body mass of 350 grams were used. The rats were group housed in wire cages with indirect bedding. The rats were fed stock rodent chow and water ad libitum throughout the experimental trials.

Immobilization Procedures

Prior to any attempt to immobilize the knee of a rat, the rats were anaesthetized in a dessicator jar with methoxyflourine. Attempts to immobilize the right knee of these rats

were as follows:

a) A splint made of malleable metal with dense foam lining ('Sam Splint' manufactured by The Seaberg Company, South Beach, Oregon) was wrapped over both the thigh and leg. These splints formed a conical shape with the opening of the base near the hip joint and the opening of the apex near the ankle joint.

b) A splint similar to that of 'a' made of plaster.

c) The splint of 'a' in conjunction with a plaster ring placed around the ankle joint.

d) The splint of 'a' in conjunction with copper pipe elbows (approx 1.5 cm internal diameter) and hose clamps. The pipe elbows were cut mid-sagitally into 2 equal halves, the internal surface was padded with adhesive cloth. The two halves were then placed over the ankle joint (with the leg segment at a 90 degree angle to the foot segment) and the two pipe halves held together with hose clamps placed at each pipe end.

Each of these apparatus systems were applied while the animals were under general anaesthesia and then monitored after the rats recovered from the anaesthesia.

EXPERIMENT 2

Introduction

Experiment 2 was necessitated by the results of Experiment 1 which determined that the rats needed to be restrained in a manner that prevented them from chewing or clawing the immobilization device with the contralateral hind limb. The same objectives of Experiment 1 were present in Experiment 2 with the exception that bilateral knee immobilization was attempted. The fact that bilateral immobilization could allow for bilateral control for the effect of immobilization only, led to a slight modification to the originally proposed experimental design wherein a separate "immobilization only" group was no longer deemed necessary. This modified experimental design which was intended to be utilized with this immobilization procedure is described in Appendix A.

The procedures of experiment 2 were considered to be too dissimilar from the methods and procedures of Experiment 1 to be employed under the initial animal care utilization application (Appendices A & B). An addendum to the previously approved proposal was

compiled and submitted (Appendix C). This addendum was initially rejected, however a written rationale for this addendum was submitted and was orally defended before a special animal care committee (Appendix D). The proposal was subsequently approved under an invasiveness classification of "D" (Appendix E). The procedures of Experiment 2 were as follows:

Rats

Three male Sprague-Dawley rats which had a body mass of 400-425 grams at the time their hind limbs were restrained were used. The rats were individually housed in plastic cages with direct bedding. The rats were fed stock rodent chow and water ad libitum throughout the experimental trials.

Immobilization Procedures

All procedures were performed while the rat was under general anaesthesia induced with intraperitoneal injections of a "ketamine cocktail" (0.8 ml ketamine and 0.6 ml xylazine per 1.0 kg body mass). The method of anaesthesia in Experiment 2 was altered from that of Experiment 1 because it was found that methoxyflourine gas was slow to take effect (10-15 minutes per rat) and caused excessive anxiety in the rats. Injection of the ketamine cocktail induced unconsciousness in a rat within 1-2 minutes and kept it unconscious for 30-45 minutes.

The theoretical design basis of Experiment 2 was that if the abdomen and both hind limbs of a rat were suspended above the cage floor, with both hind limbs immobilized, the rat would be unable to chew or claw the splints. This objective was to be met by placing the rat in a denim harness that was suspended from the top of the cage via a ball chain. The rat could move within the cage with its fore limbs which were not suspended above the cage floor.

The harnesses were frequently refined and adjusted over a period of 6 weeks until they fit the rats well enough that the rats were unable to wriggle free. A harness ran the full length of the rat's torso to encircle the fore limbs as well as its hind limbs. As a result, a rat could not use its fore limbs to pull the rest of its body away from the harness (Figure 3.2). The harnesses were fitted closely to the rats by using a lace closure across the back with a cloth tongue underneath the laces to prevent the laces from chafing the skin (Figure 3.3).

A ball chain end was passed under the harness laces where they crossed over the lumbar region (Figure 3.3). The ball chain end was then attached to the middle of the chain with a ball chain clip, forming a closed loop containing the lace cross (Figure 3.3). The other end of the ball chain was connected to the cage lid in the following manner: two steel washers were placed above and beneath the cage lid wire and were squeezed toward each other with a bicycle chainring nut and bolt (which have hollow centers). The nut and bolt created a narrow tunnel for the ball chain to pass from beneath to above the cage lid (Figure 3.4). Once the ball chain was threaded through the tunnel, a clip (which was too large to pass through the tunnel) was placed on the ball chain above the cage lid. The chain clip was placed on the ball chain at a location that suspended the hind limbs of the rat off the cage floor at a position in which the torso was approximately horizontal (Figure 3.4). Once the harnessing system alone was refined and deemed effective, the splint attachments were added. The splints were attached to the harness in the following manner:

Receptor velcro was glued to the exterior surface of the denim harnesses along the periphery of the holes through which the lower limbs passed (Figure 3.5). The splint consisted of a piece of the malleable metal material (described in Experiment 1) which was attached to hook velcro of the same shape. A central hole was placed through both the metal and the velcro and the two pieces were pinned to each other with a rivet at the cephalic and caudal sides of the hole (Figure 3.5).

The lower limbs of the rats were placed through the central holes of these splint pieces and the hook velcro surface of the splint was pressed against the receptor velcro of the harness. The metal aspect of the splint was wrapped over the thigh and leg with the knee in an extended position and held in this position with two narrow velcro strips (Figure 3.6). Both the hip and knee were placed in an extended position in order to keep the hamstring muscles in as short a position as is possible with the knee extended. The purpose of this was to minimize any knee flexion moment that could be created by the hamstring muscles either actively or passively. The splints were applied bilaterally on all of the rats and were then suspended and monitored over the following 16 days (Figure 3.7).

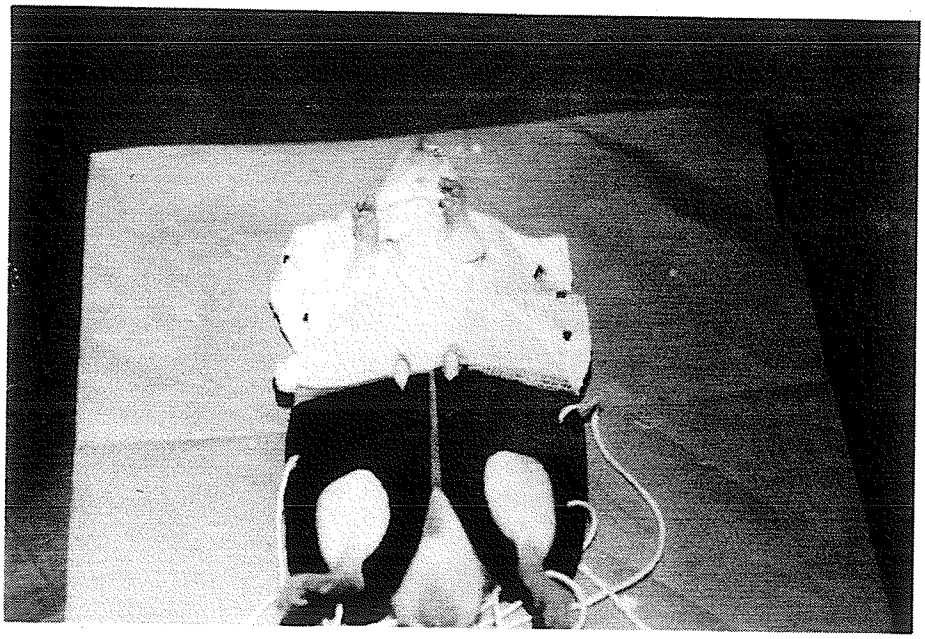


Figure 3.2 Initial installation of the harness (note the holes to allow access for the injection of the anaesthetic).

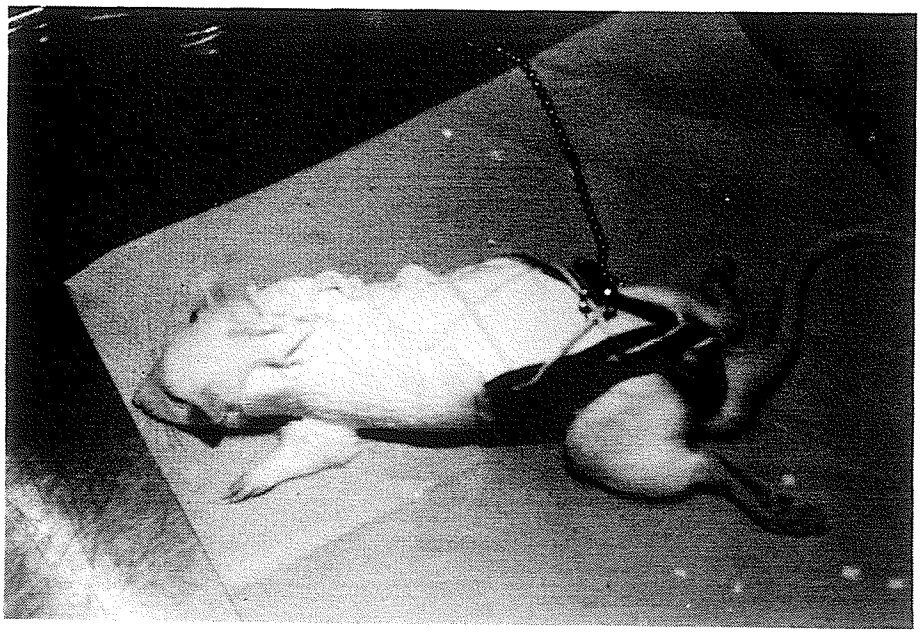


Figure 3.3 Lace closure of the harness.

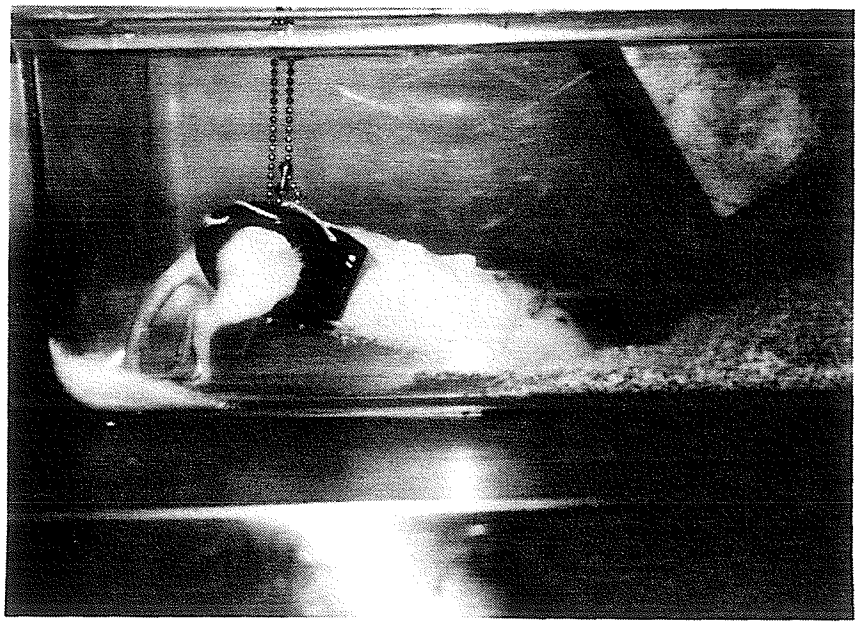


Figure 3.4 Suspension of the harnessed rat from the cage lid via ball chain.

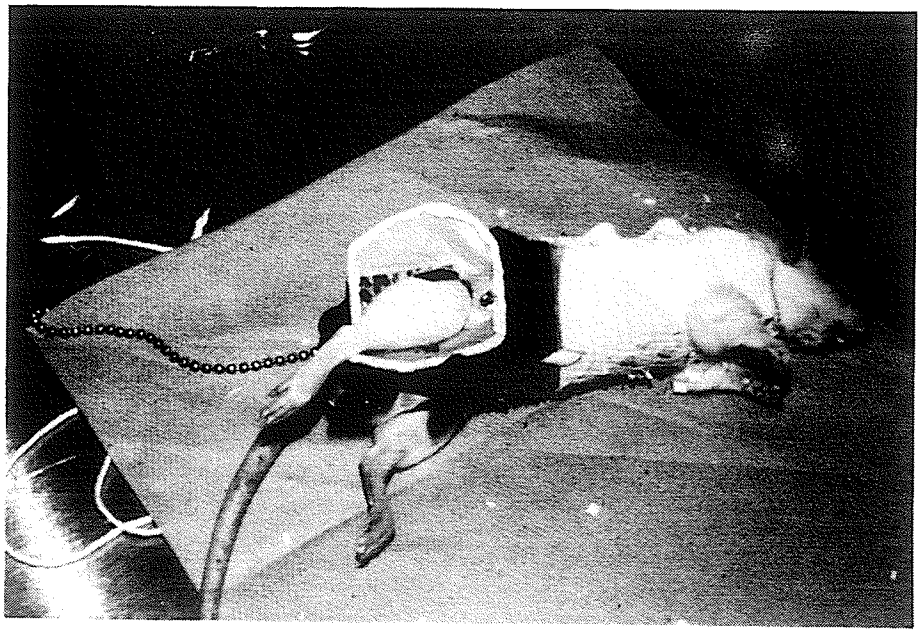


Figure 3.5 Installation of the splint apparatus.

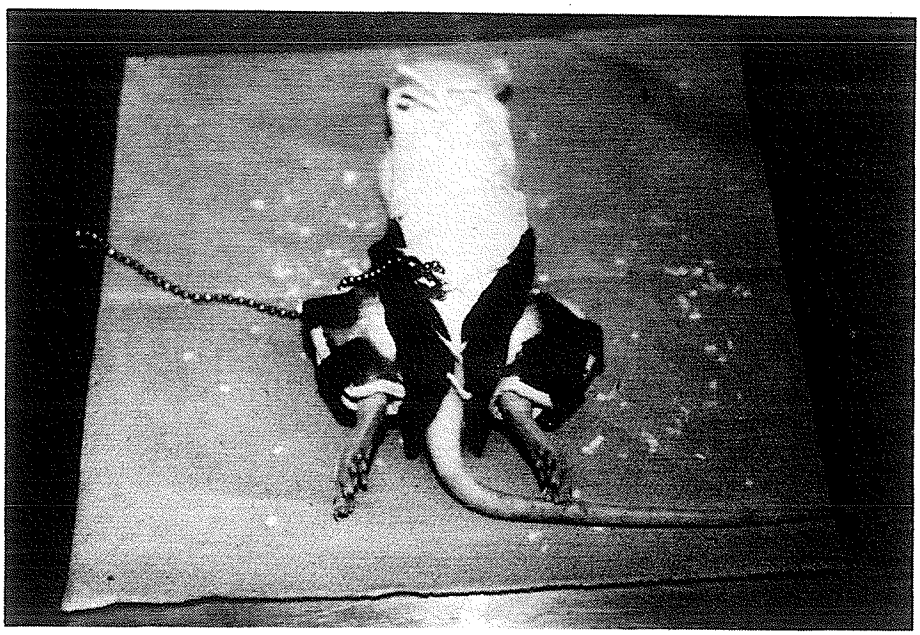


Figure 3.6 Rat with harness and splints completely installed.

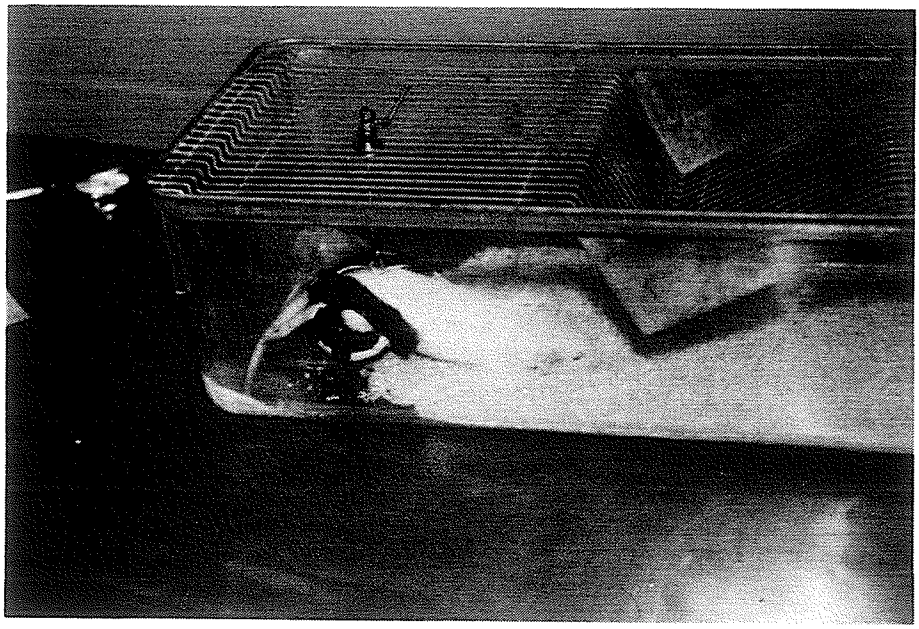


Figure 3.7 Suspension of the immobilized rat.

EXPERIMENT 3

Introduction

The purpose of Experiment 3 was to continue the Immobilization Procedures of Experiment 2 with smaller rats and employ the Modified Experimental Design using the smaller rats (200 grams). Smaller rats were chosen in order to reduce the amount of anaesthetic consumed and to reduce initial purchasing expenses of these animals. The procedures of Experiment 3 were as follows:

Rats

Six male Sprague-Dawley rats with an initial body mass of 200 grams were used. The rats were individually housed in plastic cages with direct bedding. The rats were fed stock rodent chow and water ad libitum throughout the experimental trials.

Immobilization Procedures

The immobilization procedures that were used in Experiment 3 are as described in the Immobilization Procedures of Experiment 2.

Chapter 4
RESULTS AND DISCUSSION
EXPERIMENT 1

Results

Experiment 1 was executed shortly after approval for the use of laboratory rats was obtained from the animal care authorities (Appendices A-C). The cone shaped splints of conditions 'a' (metal splints) and 'b' (plaster splints) each had a narrow opening at the apex which was located at the ankle, and a much wider opening at the base near the hip. The rats would plantar flex their ankle so that the long axis of the foot was aligned with the long axis of their leg. From this position, they could pull the splints over their foot by clawing at the splint with the opposite hind limb. Once the apical opening of the splint was pulled over their foot the splint fell off their leg.

In condition 'c' a ring was placed around the ankle which had an inner diameter narrow enough to prevent the ring from sliding over the forefoot (which was wider than the ankle). The outer diameter of the ring was wide enough to prevent the apex of the metal splint from sliding past the ring. This resulted in one of 3 situations. The first would be that the rat could simply pull the ring over the end of its foot if the inner diameter of the ring was too wide. The second situation was that if the ring could not be pulled over the forefoot, the rat would keep pulling the ring so tightly against its forefoot that circulation of blood distal to the ring was prevented. The third situation was that both the ring and the splint were pulled distally over the ankle, placing the ankle into a fully plantar flexed position. In rats, this results in occlusion of the plantar vessels because they become crimped in this position, and inadequate blood circulation was usually evident within 12 hours following the splint application.

Condition 'd' attempted to eliminate the problems encountered in condition 'c' by placing a copper tube bent at a 90 degree angle over the ankle joint in an attempt to prevent the ankle from plantar flexing or allowing the tube to slide distally on the forefoot. This was effective in keeping the malleable metal splint in place, however the rats were able to pull at the copper pipes with their other hind limb until the pipe was overly tight and cut off circulation distally.

Discussion

Despite the lack of success in attempting to unilaterally immobilize the knees of rats, personl communication with Michelsson (1992) indicated that it is possible to perform this feat. Michelsson (1992) claimed (in unpublished works) to have immobilized the knees of rats in extension with externally applied splints, though he admitted it was very awkward to accomplish and that rabbits were much easier to work with. Furthermore, Michelsson (1992) did not describe how these splints were designed or applied. No published reports have discussed either successful or unsuccessful attempts to unilaterally immobilize the knee joints of rats with externally applied splints.

It was clear from these trials that both hind limbs of the rats needed to be restrained if external splints were to be effective. The concept of placing the animals in a harness and suspending their hind limbs was suggested by Kriellaars (1991). Also, similar procedures had been described in the literature (Corley, Kowalchuk & McComas 1984; Fell, Gladden, Steffen & Musacchia, 1985; Alward, Roy, Hodgson & Edgerton, 1987; Elder & McComas 1987; Bonen, Elder & Meng, 1988).

EXPERIMENT 2

Results

The first goal of Experiment 2 was to design a denim harness that would effectively contain the rats. Initial harness designs used velcro to adjust the fit. This system was too imprecise and the rats were able to wriggle free of the harness shortly after regaining consciousness. The design, shape and size of the harnesses (accounting for growth of the rats) were continually modified over a period of 6 weeks until effective harnesses were produced (as described in Chapter 3). At this time the body mass of the 3 rats was 400 - 425 grams. The rats were observed in these harnesses over 4 days during which time no signs of undue stress were apparent with the exception of slight iron deposition around the eyes. The iron deposition was attributed to the anaesthetic agent causing drying of the lenses. An eye ointment was used to reduce this effect.

Once the harnesses were deemed effective the metal splint attachments were installed and monitored for effectiveness. Both right and left knees of the rats were effectively immobilized by the splints for 16 consecutive days with 2 rats, and 12 consecutive days with the other rat. Slight chafing of the skin over the thigh was seen in all of the rats. The rats displayed very minimal signs of distress during this immobilization period and this procedure was judged to be appropriate.

Discussion

No reports of previous external immobilization of the knees of rats have been published. Knee immobilization that is externally applied is desirable because it does not produce the trauma that may be caused by the surgery required for internal joint fixation. Surgical retraction of musculature is thought to cause EO in post THR patients (Ahrengart & Lindgren, 1984). External joint fixation allows for differentiation between the effect of surgical trauma and the immobilization itself. Surgical trauma may also be a confounding factor with the cellular induction technique when DBM is surgically implanted in a muscle pouch.

The success of this immobilization system indicates potential for additional studies on the effects of knee joint immobilization. This may include the study of EB induction, joint degeneration, muscle atrophy and other effects of immobilization disuse. Fournier, Roy, Perham, Simard and Edgerton (1983) however warn that limb immobilization may not be a good model of muscle disuse because a muscle that remains innervated can still contract. The fact that the knees of rats can be effectively immobilized externally with hind limb suspension leads to the next logical progression: the addition of PM of the immobilized knees.

EXPERIMENT 3

Results

Six rats (175-200 grams body mass) were anaesthetized (1 died during anaesthesia) 5 of which were successfully placed in the harnesses and splints. All of the rats appeared healthy and did not show signs of undue stress, however one rat died on the evening of the fourth day and another died the morning of the fifth day of immobilization. Autopsies were performed on

both rats by the facility veterinarian and no infectious or other disease conditions were found. It was hypothesized that the denim harnesses were installed too tightly and may have restricted chest expansion causing respiratory failure.

The response to this hypothesis was to replace the harnesses of the 3 surviving rats with harnesses that had elastic fabric (shown in Figures 3.3-3.8) around the chest instead of denim. This elastic fabric was intended to allow easier chest expansion while maintaining a close fit of the harnesses. These rats showed no signs of undue stress, however on the 7th day of immobilization 2 more rats died.

The surviving rat was anaesthetized and the splints and harness were removed. The skin over the thigh was chafed to a much greater degree than the rats of Experiment 2. During this time, the mobility of the knees of this rat was checked. The rat exhibited very little resistance to passive mobilization in either knee despite the fact that both knees appeared to have been immobilized in extension for 8 consecutive days. At this time the study was discontinued due to the unexpectedly high death rate.

Discussion

The fact that the larger, older rats of Experiment 2 (400-425 g) were able to withstand the stress of immobilization yet the younger rats of the Experiment 3 could not cope may indicate a greater resilience on the part of older rats, or they may have simply struggled less while in the splint and therefore sustained less stress. Another consideration is that many attempts to harness the rats of experiment 2 were made before effective harnesses and splints were developed. The rats of experiment 3 however, were harnessed and splinted very abruptly. It is possible that the rats of experiment 2 benefited from an adaptation period which was not provided to the rats of Experiment 3, and were therefore better able to cope with the immobilization procedure. Signs of stress that should be monitored include sores, lack of appetite, silvering of a normally white fur and a reduced body mass and food consumption (Thomson & Booth, 1990; Elder, 1992). Elder (1992) stated that a mortality rate of 10% was expected in his experiments with sling suspended rats.

The cause of death of the first 2 rats of the Experiment 3 was initially thought to be due to restriction of chest movement by the harness, however the modified harness apparently made

no difference. Elder (1992) also stated that pressure on the abdomen may cause renal failure and death. It is probable that this was the reason for the sudden deaths of the rats of Experiment 3. The weight of the lower bodies of the rats would tend to tighten the harnesses even further as they hung from the ball chains.

This abdominal pressure might be eliminated with the use of a tail suspension protocol such as those described by Fitts, Metzger, Riley and Unsworth (1986); Roy, Bello, Boussiou and Edgerton (1987) and McNulty, Otto, Kasper and Thomas (1992). With this technique, the rat is suspended by the proximal 2/3 of the tail which is cleaned thoroughly and wrapped with elastoplast. The elastoplast is connected by a wire to a swivel chain or cord that is hung from the cage lid.

The discovery that the surviving rat displayed very minimal resistance to knee flexion after being immobilized for 8 consecutive days indicates that the knees of this rat might not have been immobilized very effectively. If its knees were in fact immobilized effectively, then the expected amount of knee joint stiffness simply did not occur in spite of immobilization.

A basic premise of the rationale of this thesis was based on the assumption that knee joint stiffness would develop with knee joint immobilization in the rat. It may be possible that the quadriceps muscles of rats are fairly resistant to the effects of immobilization unless some type of trauma such as PM is added. This is unlikely however in view of the findings of other studies which have immobilized ankle joints of rats and found a markedly increased joint stiffness with immobilization (Booth, 1977). This issue was not discussed by Michelsson (1992) who claims to have immobilized the knees of rats and induced EB via PM.

The second and more likely possibility was the splint apparatus as it was applied to this particular rat had limited effectiveness in immobilizing the knee joint. As this was the only rat of Experiment 3 to have survived for 8 days in the harness and splint it is not known whether the other rats of this group were also effectively immobilized. The experimenter assumed that because the hind limbs of the other rats did not appear to move based on visual observation, that the joints had been immobilized. It is quite possible that the joints were immobilized, but also possible that they were not and that further modification of the splint apparatus is required.

GENERAL DISCUSSION

It is important to appreciate that the purpose of this study was to determine whether an animal model could be developed to induce ectopic bone non-invasively that was consistent, simple, labour efficient and easily reproduced. The rationale was that once such a protocol was developed, the ease of further application would offset any adversity encountered in developing the procedure. This may have been unrealistic because of the unpredictability of how animals react to certain procedures.

Rats are less expensive to purchase and maintain than rabbits and have been shown to be susceptible to EO via implantation techniques. However, they present some practical problems with the PM technique. Rats, particularly younger ones, are unwilling to accept any kind of restraint, and are very agile, strong and persistent. As a result, they are able to escape from many different types of immobilization devices and will do so to their own detriment, as was described in the results of Experiment 1. Michelsson (1992) stated that the PM induction technique is possible with rats but that it is much easier to use rabbits and therefore worth the extra expense.

The PM induction technique may be appropriate for mature rats but not young active ones. The problem of using older rats to study MOT is that with humans, MOT is most common during adolescence. Furthermore, many kinematic variables cannnot easily be accounted for with this technique.

Feasibility of Other Induction Techniques

Even if only 2 manipulation sessions were sufficient to induce EB via PM, the labour involved to accomplish this would still be much greater than that required to induce EB via DBM implantation. The process of making the harnesses and splints, installing them and applying manipulation sessions is extremely time consuming. Furthermore, it is very difficult to control or measure the kinematic variables that are involved during the application of PM. In contrast, a large quantity of DBM can be prepared at one time, stored indefinitely and implanted in a single operation. BMP is currently produced synthetically although it is difficult to acquire (Wozney, Rosen, Celeste, Mistock, Whitters, Kriz, Hewick & Wang, 1988).

If the objective of a study is simply to derive an EB tumour for the study of experimental management techniques, implantation of DBM or BMP is a feasible approach. Many very recent studies have implanted DBM, BMP or subcomponents of BMP such as bone matrix gelatin (BMG) to induce EB (Bagi & Miller, 1991; Okamato et, al. 1991). Research with DBM and BMP implantation has value not only for EB treatment testing but also for other orthopaedic applications such as bone grafts, fracture repair and prostheses.

If the objective of a study is to examine the process of EO, induction via PM will provide a model which will be free of 2 major problems of the implantation technique. The first is that the trauma is induced non-surgically, therefore the effect of surgical retraction of musculature is eliminated. The second is that the process of cell differentiation and the tissue source of these cells can be examined and discerned without the disorder caused by the presence of implanted tissue.

Suggested Modifications to the PM Induction Method

The following modification suggestions are based upon the assertion that the PM induction technique is preferable to implantation induction for the study of the EO process. The fact that rabbits have been successfully used with this technique on more than one occasion (Michelsson et al, 1980; Michelsson & Rauschning, 1983; Ahrengart et al, 1988) clearly indicates that this procedure is effective, despite its awkwardness. Ahrenagart et. al. (1988) were able to significantly reduce the amount of labour needed to employ this technique, however it is still plausible that this can be further reduced. This is based upon the rationale that allowing sufficient time for immobilization changes to occur prior to the initiation of manipulation sessions will intensify the trauma applied during each manipulation session. As a result, fewer manipulation sessions may be needed to induce a given amount of ectopic bone in these animals.

It is quite possible that rats could still provide a worthwhile model for use with the PM technique given that 2 modifications to the harness procedure were made. The first modification is to use older, larger rats (+400 g), which apparently deal with suspension immobilization much better than younger rats. The fact that in humans, MOT most commonly occurs in adolescents, warrants consideration of the appropriateness of the use of non-adolescent rats for

MOT related research. The second modification is to use tail suspension instead of torso harness suspension. This could eliminate the problem of pressure on the kidneys that may have occurred with the torso harness. A torso harness would still be necessary to provide a base for the attachment of the metal splints, however it would not need to bear the weight of the animal. Furthermore, a non-weight bearing torso harness might not need to reach the thorax and could be applied less tightly using an expandable elastic fabric.

Tail suspension would require the use of a small rodent such as a rat and would not be feasible with a rabbit. Several benefits could be derived with a combination of tail suspension with a harness that would not be available with the use of rabbits. The fact that both hind limbs can be immobilized with suspension would allow for bilateral comparison of thigh specimens or could provide twice as many EB specimens for a given number of animals.

This technique might also be less labour intensive for 2 reasons. The first is that if both thighs are to receive PM, the anaesthetic agent would be applied half as often as it would be with unilateral PM in order to derive the same number of EB specimens. Secondly, the malleable metal splints that were previously designed and developed would be much simpler and quicker to release and reattach than the plastic splints and bandages previously used with rabbits. The effeciveness splints require closer examination and possibly additional modification based upon the findings of further analysis.

The problem of uncontrolled kinematic variables encountered with previous PM induction protocols could be eliminated by using a small isovelocity dynamometer designed for rodents that is capable of producing an actively applied moment (therefore able to measure eccentric work). Such a device could control and vary both the magnitude of the applied moment and the angular velocity at which the moment is applied. The use of this type of dynamometer would allow investigation of the mechanism of tissue damage via PM as compared to eccentric muscle contraction.

Other issues which need to be examined are the effect of PM on BMP levels in the tissues being traumatized and whether there is any similarity between the mechanism of tissue damage from the PM induction technique and the mechanism of tissue damage from intense eccentric muscle contraction. Eccentric muscle contraction has been shown to produce forced detachment of the cross bridges between myofilaments, which results in the elastic components

(connective tissue) of muscle recoiling with subsequent damage to the sarcomeres (Flitney & Hirst, 1978).

The views that repeated PM result in EB because of extensive connective tissue damage and that eccentric muscle contraction also causes extensive damage to the connective tissue components of an actively working muscle, would suggest that intense eccentric muscle contraction could induce EB. Only one case of EB thought to be induced through intense resistance exercise has been reported (Jones & Ward, 1980). This indicates that there are differences between tissue damage via PM and tissue damage via intense eccentric muscle contraction. These differences may be related to the degree of connective tissue damage that is induced or to the fact that different connective tissue components of the muscle may be damaged in each situation.

The differences between eccentrically induced tissue damage and that induced by PM can be examined by analyzing urinary metabolites and through histological analysis of thigh cross sections. Urinary hydroxyproline release is well accepted as a good indication of connective tissue degradation, whereas elevated urinary 3-methylhistidine is a strong indicator of myofilament damage. These metabolic markers could be further substantiated with histological analysis of the thigh musculature. Furthermore histological analysis during different stages of the PM induction process could provide additional information about the tissue source of cells which are responsible for the process of EO.

It would be of value to compare these measures in animals subjected to different protocols. A group of animals subjected to the basic PM induction technique could be compared to a group of unrestrained animals that would be anaesthetized and have their quadriceps stimulated electrically (while anaesthetized) and subjected to an equal number of sessions of forced active mobilization (FAM). An isovelocity dynamometer used in conjunction with an EMS machine stimulating at the appropriate intensity would allow the eccentrically working animals (FAM group) to be subjected to the same angular velocity and moment as the rats receiving PM. Additional comparison could be made with a group of animals that group would combine the regular PM protocol with FAM.

Chapter 5
SUMMARY

Traditional management procedures of EB are limited in their potency. Most conventional approaches attempt to minimize the inflammatory process and avoid contraindicated therapeutic modalities. Other experimental management techniques tested have had limited success and associated risk factors. Research of new management techniques has been limited by the difficulty of EB induction in laboratory animals.

Two major approaches to experimental EB induction have included implantation of DBM or BMP into a muscle pouch or passive mobilization (PM) of an immobilized joint. Each of these systems has its own problems. The implantation technique involves at least 2 osteoinductive factors which cannot be isolated from one another. The PM method, as it has been previously employed, is extremely labourious and it is difficult to control the magnitude of the osteoinductive stimulus.

The purpose of this study was to develop an EB induction protocol which used PM that would be relatively simple, economical and reproducible. This was based on the concept of modifying a procedure previously established in rabbits (Michelsson et al, 1988) and applying it to rats. The concept was to allow a period of joint immobilization time to occur in order to increase stiffness about the joint and in turn increase the trauma to soft tissue when this joint was forcibly mobilized. This would theoretically increase the amount of trauma incurred from each manipulation session and therefore require fewer manipulation sessions for the same degree of stimulus for EB production. Rats were used because they were less expensive to purchase and house than rabbits.

Experiment 1 of this study attempted to duplicate the rabbit procedure of Michelsson et al, (1980) as closely as possible with rats. This required that the right knees of rats be immobilized with an externally applied splint that was easily removed and replaced. This was attempted several times with 4 different splint systems, none of which were satisfactory because of the ability of the rats to claw at the splints with their left hind limb. These results indicated a need to immobilize both hind limbs.

Experiment 2 attempted bilateral knee immobilization. The abdomen of the rats was kept off the cage floor by suspending the abdomen in a denim sling from the cage lid. The harness

was designed, refined and repeatedly adjusted until it effectively contained the rats. The knee splints were made from a malleable metal material and they were attached to the denim harness with velcro. Once the splints were installed the rats were observed for up to 16 days after the installation to establish the effectiveness of the procedure. The apparatus appeared to immobilize the knees successfully and all of the rats appeared healthy at the end of the experimental period.

Experiment 3 used the immobilization system developed in Experiment 2 but with younger rats with an initial body mass of approximately 200 grams. The execution of Experiment 3 resulted in 4 of 5 rats dying in the first 7 days of immobilization. The experiment was halted at this time because of the high mortality rate. It was clear that the larger rats of Experiment 2 were much better able to deal with immobilization than those of Experiment 3. The results of these 3 experiments led to several conclusions and recommendations.

CONCLUSIONS

1. Unilateral knee immobilization with an external splint is extremely difficult to achieve with rats.

2. The methods and procedures required to externally immobilize the knee joints of rats in a manner that may lead to the application of intermittent PM are labour intensive and rigorous.

3. The rats of Experiment 3 likely died of renal failure caused by excessive pressure of the denim harness on the abdomen.

4. Older, larger rats (400 g or more) appear to be better able to cope with restraint procedures than younger and smaller rats (200 g).

5. Immobilization of the knees of rats via externally applied splints appeared to have occured in Experiment 2. This has not been reported in previously published studies.

6. Bilateral immobilization of the knees of rats appeared to have occured in Experiment 2. This has not been reported in previously published reports.

RECOMMENDATIONS

1. The Modified Experimental Design (Figure 3.2) should be reattempted with larger rats (350-400 grams).

2. The Immobilization Procedures of Experiment 2 should be altered so that the rats are suspended by their tails instead of being suspended by their harnesses. The role of the harnesses should be to provide an anchor from which to attach the splints.

3. Additional testing and refinement of the splints used in Experiments 2 and 3 may be required to ensure that the knee joints are immobilized.

4. The mechanism of trauma from PM, that is which tissues are damaged, which damaged tissues are responsible for the process of EO and how these issues relate to eccentrically induced muscle damage all need additional study.

5. The above mechanisms of damage should be monitored through sequential histological analysis of the tissue as well as analysis of the levels of urinary hydroxyproline and 3-methylhistidine.

6. An isovelocity dynamometer should be used to apply PM in order to control kinetic variables.

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APPENDICES

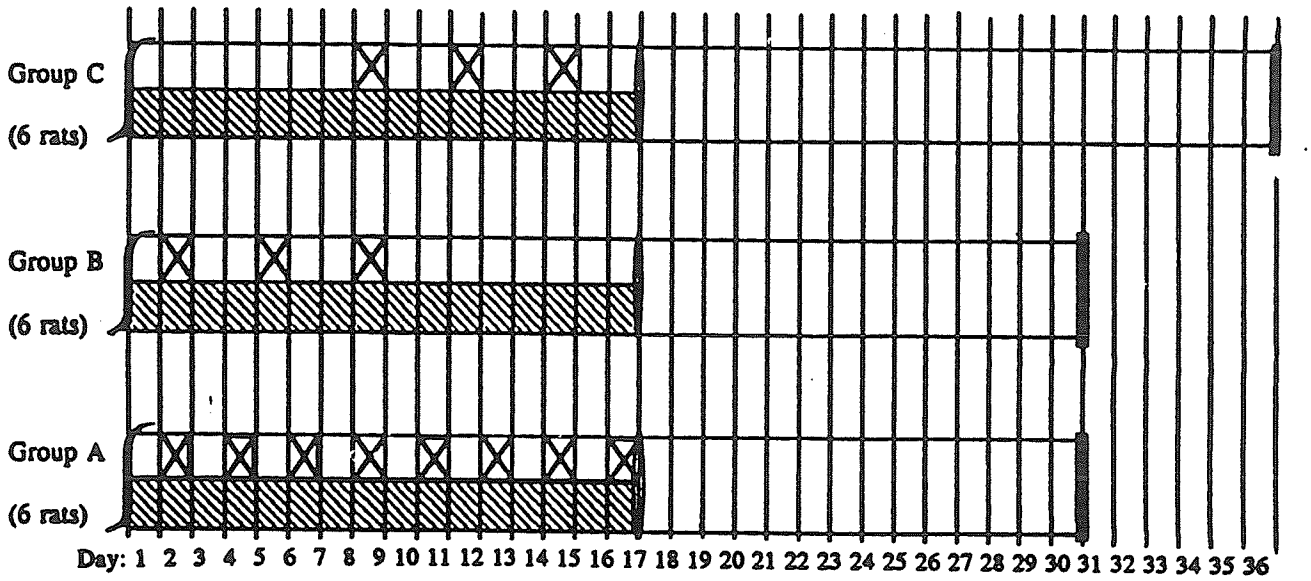
APPENDIX A

Modified Experimental Design

The rats should be divided into groups A-C with 6 rats per group. Both the right and left knees should be immobilized for a total of 16 days. The period in which the animals are splinted should be referred to as the immobilization period. The left knee of each rat should receive no manipulation sessions and should serve as a bilateral control for the effect of immobilization of the knee. The right knees of all rats should be mobilized in the manner previously described.

During the immobilization period the right knees of the rats of group A should receive 8 manipulation sessions at 48 hour intervals which should commence 24 hours following the initial application of the splints. The rats of group A should thus follow the same experimental schedule as the rabbits used by Ahrengart et al, (1988). The right knees of the rats of groups B and C should receive 3 manipulation sessions with 72 hour intervals between these sessions. For group B these would occur on the 2nd, 5th and 8th day of immobilization. For group C they should take place on the 8th, 11th and 14th day of immobilization.

A comparison of the results of group B and group C should indicate whether an initial immobilization period prior to the manipulation sessions is of significance since there is an equal number of manipulation sessions. The final removal of the splints should initiate the remobilization period (Figure 3.2). The remobilization period should continue until the rats are killed and no manipulations should be performed during this time. The remobilization periods for groups A, B, and C should be 14, 22 and 22 days respectively. The varied remobilization periods should maintain a constant length of the time from the initial manipulation session to the killing of the rats in all groups (Figure 3.2). This time period is 28 days, which according to the literature about other species (humans and rabbits), should be a sufficient amount of time for soft tissue calcification to appear radiographically (Molloy & McGuirk, 1976, Ahrengart et al, 1988). The remobilization period will be terminated upon euthanasia of the animals via an anaesthetic overdose.



Legend:

- Initial Splint Application
- Splint Immobilization
- 1 Manipulation Session
- Splint Removal
- Animals Killed

Modified Experimental Schedule.

APPENDIX B

UNIVERSITY OF MANITOBA
University Animal Care Utilization Application

Date March 8, 1991 Reference # 91-63
Act. date _____
Review date _____

PERSONNEL:

Principal Investigator Dr. Wendy Dahlgren
Department Physical Education
Academic rank Professor
Building University Center Room no. 500
Telephone 474-8089
Associated scientists: 1. _____
2. _____
3. _____

Research staff:

	Name	Dept.	Tech	Postdoc	Grad	Under
1.	Mark Reisdorf	Physical Education			X	
2.						
3.						

All personnel involved in direct care/treatment of animals have received appropriate technical training:
Yes X No ___
If "Yes", indicate date/type of training: Have attended courses conducted by Dr. McCutcheon February 11 & 25, 1991.
If "No", indicate steps to be taken: _____

All personnel involved in this project have attended courses concerning political/ethical issues of animal-based research: Yes X No ___
If "Yes", indicate date(s): February 11, 1991
If "No", indicate names of personnel who have not yet attended: _____

PROJECT TITLE: Experimental Induction of Ectopic Bone in Rats

Does this work relate to any disease category? _____
Provide index term(s): _____

FUNDING:

Agency Faculty Funds
Amount requested/approved $ 750.00
Grant supported/proposed? Yes
Contract work? Yes - Thesis
If "yes", will it be peer-reviewed? Yes
If "no" to either question, please suggest names/addresses of two appropriate reviewers:
1. _____
2. _____
Has an identical protocol been approved? No
If "yes", indicate protocol number: _____

HAZARDS:

Have the appropriate safety committees been notified? ____
a. Biohazard ___ Approval no. _____
b. Chemical ___
c. Radiation ___ Permit no. _____

ANIMALS:

If more than one species, check here and complete a "SCHEDULE A" for each additional species: ____
Species Rats
Strain (if applicable) Sprague-Dawley
No. of animals 22
Max. housed at one time 22
Housing location Central Animal Care, Basic Medical Sciences
Source Central Animal Care

USE:

CCAC Classification 10C
CCAC Category of Invasiveness (Max.) C
Check only appropriate boxes and provide requested info

1. Teaching ____
   Course No. and Title _____
   a. Class demo ____
      Are suitable A/V resources available? ____
      If "yes", why are they not in use?
      _____
      If "no", could this demo be taped for future reference? ____
   b. Naturalistic observation ___
   * c. Surgical procedures ___
   * d. Behavioural procedures ___

* Give details under EXPERIMENTAL DESIGN heading and indicate the tasks the students will be performing and the level/type of supervision.

2. Field Study ____
   a. Observation ___
   b. Capture/Release ___
      -- Specify technique used _____
      _____
   c. Other ___
      -- Specify: _____
3. Feeding Trial ___
   -- Specify diet and duration: _____
   _____
4. Behavioural
   Location of expt. _____
   a. Restraint ___
      Method of restraint: _____
      Number of days: _____
      Duration (days): _____

b. Deprivation ___
Type: _____
Duration: _____
Method of implementation: _____
c. Electric shock ___
Type: _____
Intensity: _____
Frequency: _____
Number: _____
Duration: _____
d. Other stressor (specify): _____
e. Social interaction
i. courtship ___
ii. maternal ___
iii. territorial ___
iv. fighting ___
v. predator-prey ___
f. Environmental parameters (e.g. temp.)
-- Specify: _____

5. Surgical ___
Location: _____
Type of surgery: Acute ___ Chronic ___
a. ___ Premedication agents: _____
Administration route: _____
Dose: _____
b. ___ Local anesthesia(agent): _____
Administration route: _____
Dose: _____
c. ___ Gen. anesthesia(agent): _____
Administration route: _____
Dose: _____
d. ___ Neuromuscular blocking agent: _____
_____
Administration route: _____
Dose: _____
How will level of anesthesia be maintained?
_____
_____
e. ___ Post operative care
Location: _____
Provided by: _____

6. Medical(animal models) X
Acute ___ Chronic X
Special treatment(dietary/drugs): _____
_____
Expected side effects: _____
_____

7. Tissue/Fluid ___
a. Bleeding ___
Method and Amount _____
Frequency _____
By whom _____
b. Other ___
-- Specify _____

EXPERIMENTAL DESIGN:

a. Describe primary objective of study in terms understandable to lay reviewer: The purpose of this study is to establish a simple technique to induce a benign ectopic bone tumor in a laboratory rat.

b. Provide a detailed statement of procedure such that experimental procedures to which the animals will be subjected and their duration can be evaluated. Justify number of animals used.

See attached methods and procedures.

c. Disposition of animals at termination of study [euthanasia(specify method), return to herd(location), other approved project(specify)]: Euthenasia via anaesthetic overdose (methoxyflourine)

DECLARATION:

All animals used in this project will be cared for in accordance with the guidelines of the Canadian Council on Animal Care and the U. of M. Animal Care Protocol. Consideration has been given to the use of alternative procedures, species and techniques that do not require the use of animals.

Principal Investigator — 91/03/07 Date

Chair, Local A C C — May 13/91 Date

Chair, U A C C — 14 May/91 Date

METHODS AND PROCEDURES

This study will be preceded by a brief pre-study with 4 rats to monitor and refine the knee splintation technique which will be used in the thesis study. This will take place over a 8 day period after which the animals will be used as part of the thesis study. During the 8-day pre-study phase the effectiveness of the splints will be closely monitored and any problems with these splints will be addressed. After these 8 days the rats will continue to be splinted as part of the thesis study.

Rats

Twenty-two male Sprague-Dawley rats (including the 4 from the pre-study) with an initial body mass of approximately 350 grams will be used. The rats will be group housed in wire cages with indirect bedding. The rats will be fed stock rodent chow and water ad libitum throughout the experiment.

Immobilization

At the commencement of the study all of the rats will be anaesthetized with pentobarbital and an external splint will be installed over their right knees. These splints will immobilize the knees in a fully extended position, but will not restrict hip or ankle motion. The splints will be made of a malleable aluminum exterior with a thin dense foam interior and will be secured with metallized automobile body tape. These splints will be easily adjustable, removable and replaceable and will be resistant to the gnawing of the rats.

Manipulation Sessions

A manipulation session for a rat will involve the following procedure. The rat will be anaesthetized with methoxyflourine and the splint will be removed. With the splint removed the experimenter will manually apply forced passive mobilization to the immobilized knee of the rat. The forced passive mobilization will involve repeated flexion and extension of the knee until a full range of joint motion is achieved. Great care will be taken to not induce a femoral or tibial fracture during these sessions. Once a full range of joint motion is achieved the splints will be replaced and the animals will be allowed to recover from the anaesthesia.

Experimental Design

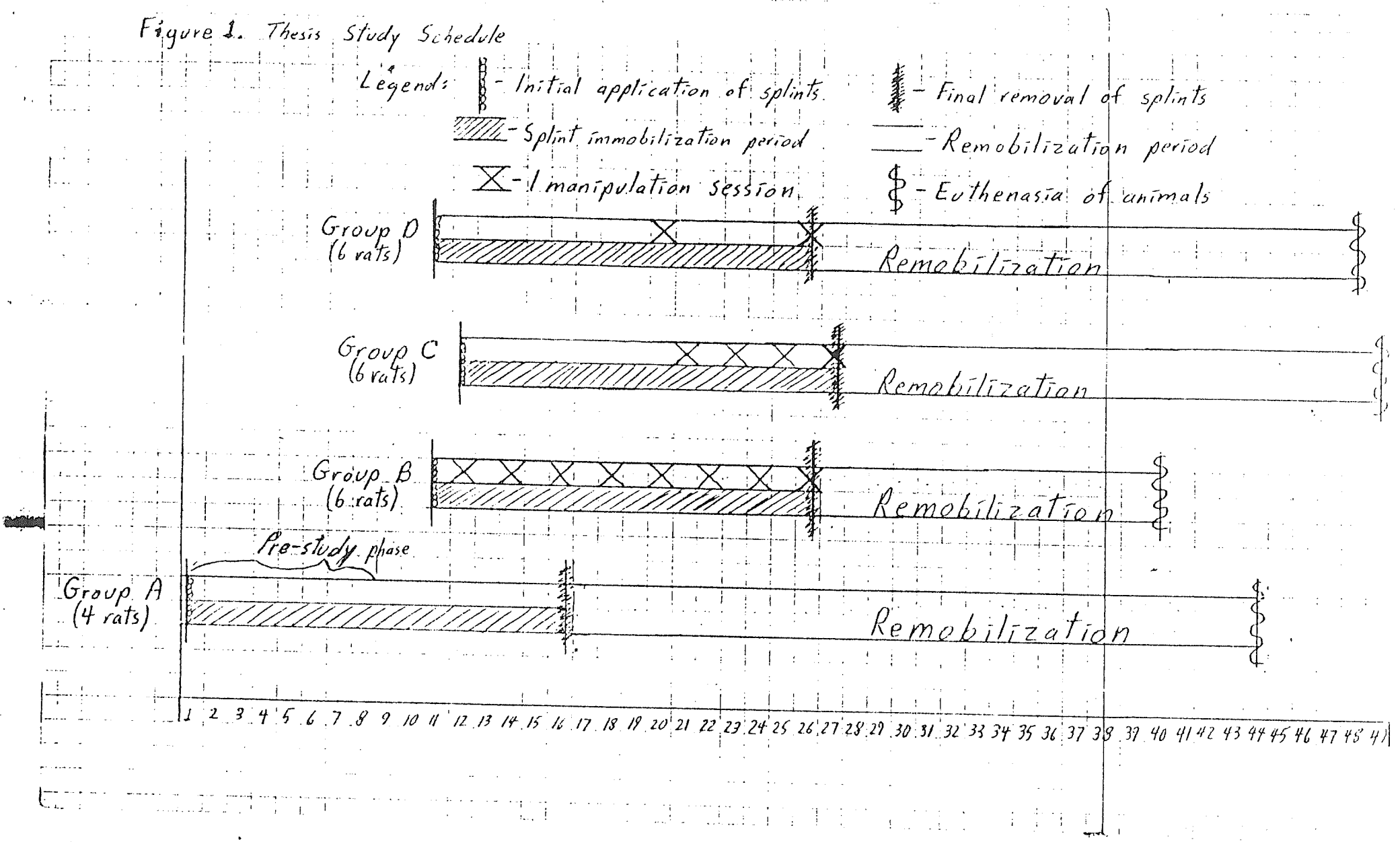
The rats will be divided into groups A-D. Group A will be composed of the 4 rats used in the pre-study. These rats will remain splinted for an additional 8 days after the pre-study phase, after which the splints will be removed. These rats will therefore have had their knees immobilized for a total of 16 days but will receive no manipulation sessions during this time. Groups B-D will consist of 6 rats per group and all of these rats will have their right knee immobilized in the same manner as those of group A. These rats will also be splinted for a total of 16 days. The period in which the animals are splinted will be referred to as the immobilization period.

During the immobilization period the rats of group A will receive no manipulation sessions. The rats of group B will receive 8 manipulation sessions at 48 hour intervals which will commence 24 hours following the initial application of the splints. The rats of group C will receive 4 manipulation sessions at 48 hour intervals which will begin 10 days following the

initial splint application. The rats of group D will receive 2 manipulation sessions, one 10 days and the other 16 days following the initial application of the splints. The final removal of the splints will initiate the remobilization period (See Figure 1).

The remobilization period will continue until the rats are killed and no manipulations will be performed during this time. The remobilization periods for groups A, B, C and D will be 28, 14, 22 and 22 days respectively. The varied remobilization periods will maintain a constant length of the time from the initial manipulation session to the killing of the rats in all groups (see Figure 1). This time period is 28 days, which according to reviewed literature about other species (humans and rabbits), should be a sufficient amount of time for soft tissue calcification to appear radiographically. The remobilization period will be terminated upon euthanasia of the animals via an anaesthetic overdose (methoxyflourine). After the animals have been killed they will be prepared for radiographic and histological analysis.

Figure 1. Thesis Study Schedule



THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
Office of the Dean

500 University Centre
Winnipeg, Manitoba
Canada R3T 2N2

(204) 474-9573
(204) 474-9887
FAX: (204) 275-6488

MEMORANDUM

DATE: May 8, 1991

TO: Dr. Arnold Froese, Chair, LACC, Immunology

FROM: Dr. Wendy Dahlgren, Associate Dean, Faculty of Graduate Studies

91-63

SUBJECT: **Animal protocol entitled "Experimental Induction of Ectopic Bone in Rats".**

The above project will form the basis for Mark Reisdorf's M.Sc. thesis. In order to proceed with this research, the proposal was presented in an open forum which included the Chair of the M.Sc. programme in the Faculty of Physical Education and Recreation Studies as well as members of Mr. Reisdorf's advisory committee, other members of the Graduate Programme Committee, and faculty members of the Faculty of Graduate Studies. The proposal was approved without revision.

The following individuals were directly responsible for approval of the proposal:

Dr. J. Butcher, Chair, M.Sc. Programme
Dr. K. Lindner, Associate Professor and member of the Advisory Committee.
Dr. D. Kriellaars, Assistant Professor and member of the Advisory Committee.

As I noted in our telephone conversation, the Faculty of Physical Education and Recreation Studies has very rigorous guidelines for the approval of graduate students' research and will not allow a project to proceed until approval is given by the Graduate Programme Committee/Thesis Advisory Committee.

cc: Dr. J. Butcher
Dr. K. Lindner
Dr. D. Kriellers
M. Riesdorf

APPENDIX C

THE UNIVERSITY OF MANITOBA

FACULTY OF MEDICINE
Office of the Dean

753 McDermot Avenue
Winnipeg, Manitoba
Canada R3E 0W3

(204) 788-6557

MAY 16 1991

Reference No: 91-63

Date: May 15, 1991

TO: Dr. W. Dahlgren, Physical Education

RE: Your project entitled:

"Experimental induction of ectopic bone in rats."

has been approved by the Animal Care Committee for the period May 15, 1991 to May 31, 1992.

Yours sincerely,

E. A. Kroeger, Ph.D.
Associate Dean (Research)

EK/ep

cc. Mr. Bob Madziak

APPENDIX D

Appended as a "D"

Sept 9/91

To: Dr. A. Froese
Dr. K. McCutcheon
Dr. E. Kroeger

From: Dr. W. Dahlgren & Mark Reisdorf

Subject: Addendum to Protocol ~~63-91~~ 91-63

Protocol ~~63-91~~ proposed immobilization of extended right knees of rats via a malleable metal splint material. Unilateral immobilization was proposed to allow ambulation with the unaffected hindlimb. Attempts to unilaterally immobilize rats knees have been attempted several times (unsuccessfully) since the approval of this protocol. The rats have been able to either chew or claw (with their unaffected hindlimb) the splint device off their leg, or force it into such a tight position that circulation to the foot was cut off at the ankle.

Several researchers have been able to suspend the hindlimbs of rats in a manner that prevents the rats from chewing and clawing their hindlimbs yet does not restrict access to food and water (see references). Fell, Gladden, Steffen & Musacchia (1985), fitted rats with full body length denim-Velcro harnesses which were suspended at the rear aspect by a steel wire connected to an overhead rod. The rest of the rat's body weight was supported by the forelimbs which were in contact with the cage floor. The rats were able to ambulate within the cage by propelling themselves with their forelimbs while their hindlimbs could not contact any supportive surfaces. The suspension period for these rats was 7 days, however the other studies referenced used similar suspension methods for 28 days without reported

complications.

The proposed addendum to protocol 63-91 would utilize the technique of hindlimb suspension as described above and diagrammed in Figures 1 & 2. The denim harnesses would also cover both thighs of the suspended rats. The malleable metal splint material described in the initial protocol would be placed over the thighs (adhered with Velcro) in an attempt to immobilize both knees in extension. This system would require that the rats be singly housed in plastic cages, but would not require permanent alteration to the cages. The suspension/immobilization period would be 16 days. During this 16 day period the right knees would be passively mobilized in the same manner and schedule described in the original proposal. This suspension/immobilization method would be initially attempted with 2 pilot study rats.

In addition, we propose to replace methoxyflurane with ether NC as an anaesthetic agent.

I trust that this revised protocol meets with your approval.

Yours Sincerely,

W Dahlgren

Figure 1.
Superior View

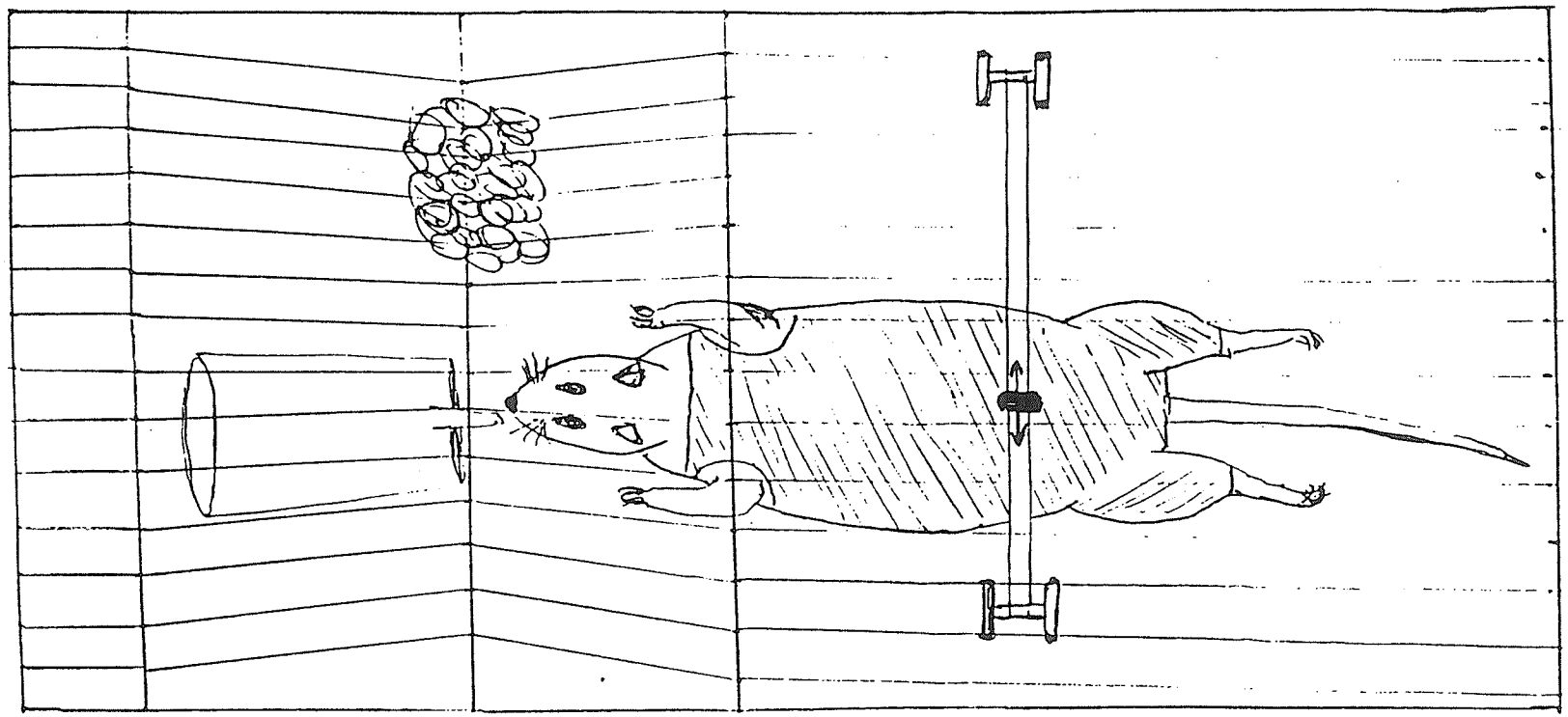
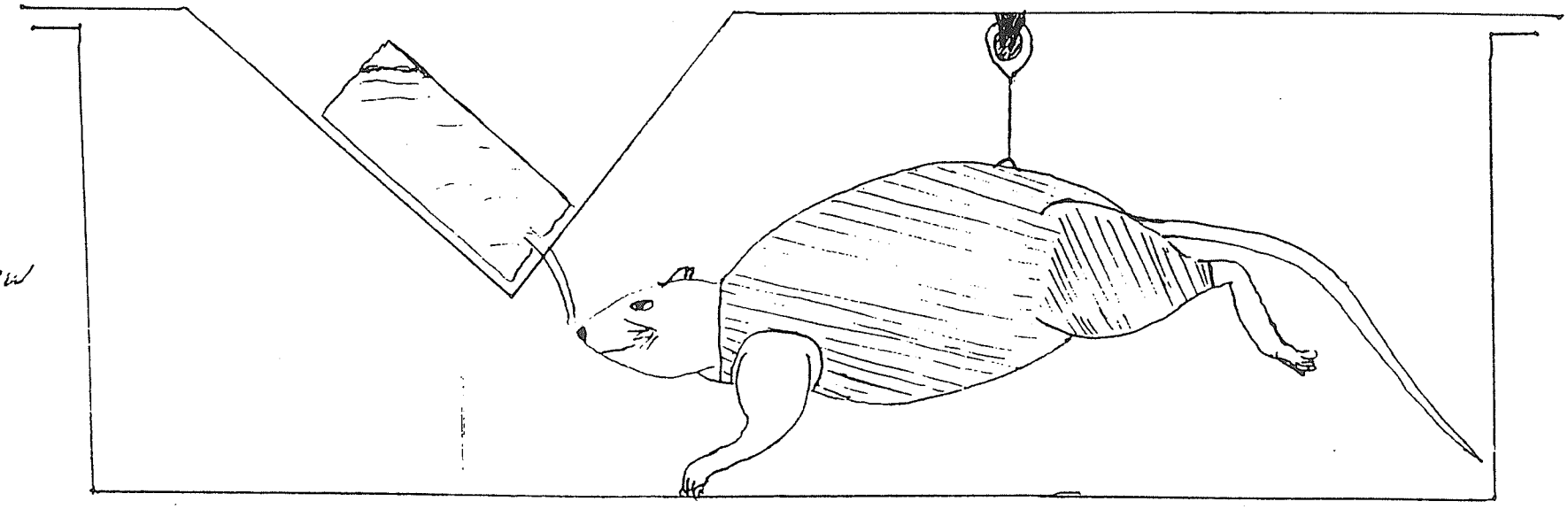


Figure 2.
Lateral view



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APPENDIX E

RATIONALE FOR THE PROPOSED METHODS AND PROCEDURES OF PROTOCOL 91-63 AND ADDENDUM

The purpose of this study is to develop an animal model to induce ectopic bone (EB) which is effective, easily duplicated and economically feasible. Currently, 2 methods of experimental EB induction have been shown to be consistently effective. These 2 methods are injection of decalcified bone matrix or bone morphogenetic protein into a muscle compartment and systematic forced passive mobilization of an immobilized joint. These two techniques produce EB tumors which have different histological characteristics. The EB tumors produced by forced passive mobilization resemble those found in humans more closely than the injection technique. A model which utilizes forced passive mobilization of immobilized joints has useful orthopaedic therapy application as little is understood about the effect of different mobilization schedules of previously immobilized joints.

The phenomena of EB formation due to forced passive mobilization has been well documented however no studies of this technique have been reported with other species. Rats are much more economical to buy, house and anaesthetize than rabbits and therefore increase the feasibility of research in this area.

Hindlimb suspension should provide an effective technique to immobilize the knees of rats externally. There does not appear to be any such technique currently developed. Joint immobilization of small rodents is typically accomplished by internal fixation which may produce undesired trauma to the

surrounding soft tissue being studied. Externally applied splints do not require the specialized personnel and equipment that internal fixation requires. Finally, hindlimb suspension techniques have been used by researchers for periods of up to 206 consecutive days with no reported undo stress to the animals.

APPENDIX F

THE UNIVERSITY OF MANITOBA

FACULTY OF MEDICINE
Office of the Dean

753 McDermot Avenue
Winnipeg, Manitoba
Canada R3E 0W3
(204) 788-6557

DEC 05 1991

Reference No: ***91-63***

Date: December 3/91

TO: Dr. W. Dahlgren, Department of Physical Education

RE: Your project entitled:

"Experimental induction of ectopic bone in rats."

has been approved (as per addendum dated November 21, 1991) for the ***Category of Invasiveness*** of ***"D"*** by the Animal Care Committee for the period ending December 31, 1992.

Yours sincerely,

E. A. Kroeger, Ph.D.
Associate Dean (Research)

EK/ep
cc. Mr. Bob Madziak

APPENDIX G

Sept. 30, 1992

Mr. Steve Tilton,
Journal of Bone and Joint Surgery,
10 Shattuck st.
Boston, Massachusets
102115

OCT 13 1992

Dear Mr Tilton,

I am writing to you to obtain permission to use photographs of figures from JBJS as part of my MSc. thesis, which are figures 12-15 inclusively from:

Ackerman, L.V. (1958). Clinical and pathological confusion with malignant neoplasm, The Journal of Bone and Joint Surgery, 40A: 279-298.

These figures will be fully referenced in the APA style as above. If you have any concerns about this please let me know what must be done to rectify the problem. If this is satisfactory as is, please forward your permission to me at your earliest convenience care of:

Mark Reisdorf
Rm. 102 Frank Kennedy Center
University of Manitoba
Winnipeg, Manitoba, Canada
R3T 2N2

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[illegible] ET
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Permission granted.
Stephen Tilton
Managing Editor