

# The Role of Actovegin in Muscle Injuries

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Thesis

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## Abbreviations

$\Delta C_t$	Change of threshold cycle compared to control gene
$\Delta\Delta C_t$	Change of threshold cycle compared to control gene to sample
ACS	Autologous Conditioned Serum
ATP	Adenosine triphosphate
BNF	British National Formulary
BSE	Bovine Spongiform Encephalopathy
$Ca^{2+}$	Calcium ion
CCES	Canadian Centre for Ethics in Sport
CD163 <sup>+</sup>	Cluster of Differentiation 163
CD68 <sup>+</sup>	Cluster of Differentiation 68
CI	Confidence interval
$C_t$	Threshold cycle
DNA	Deoxyribonucleic acid
ECGF	Endothelial Cell Growth Factor
EGF	Epidermal Growth Factor
FA	Football Association
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlUTs	Glucose transporters
GMTJ	Musculotendinous junction
HGF	Hepatocyte growth factor
HPLC	High-performance liquid chromatography



IGF-1	Insulin-like growth factor 1
IL-1	Interleukin-1
IL10	Interleukin-10
IL8	Interleukin-8
IOC	International Olympic Committee
IPO	Inositol phosphate oligosaccharides
MALDI-TOF	Matrix-assisted laser desorption/ionization Time Of Flight Mass Spectrometry
MCP-1	Monocyte chemo-attractant protein 1
MDC	Macrophage-derived chemokine
MHRA	Medicines and Healthcare products Regulatory Agency
MRI	Magnetic Resonance Imaging
MyHC	Myosin heavy chain isoforms
NIS-LL	Neuropathy Impairment Score of the Lower Limbs
NSAIDs	Non-steroidal anti-inflammatory drugs
PCR	Polymerised Chain Reaction
PMA	Phorbol 12-myristate 13-acetate
PRP	Platelet rich plasma
qPCR	Real time Polymerised Chain Reaction
RICE	Rest, ice, compression, elevation
RNA	Ribonucleic acid
RQ	Relative quantity
S.D.	Standard deviation
S.E.M	Standard Error Mean
SF-36	Short form 36 quality of life questionnaire

SLPI	Secretory leukocyte protease inhibitor,
TGF-A	Transforming growth factor A
TGF- $\beta$	Transforming growth factor $\beta$
THP-1	Human acute monocyte leukemia monocyte cell line
TNF $\alpha$	Tumor necrosis factor-alpha
TSE	Transmissible Spongiform Encephalopathies
TSS	Total Symptom Score
UKAD	United Kingdom Anti-Doping Agency
USADA	United States Anti-Doping Agency
VEGF	Vascular endothelial growth factor,
VPT	Vibration perception threshold
WADA	World Anti-Doping Agency
WOAH	World Organization for Animal Health

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## **Published paper**

## Abstract

Muscle injuries are one of the most common sports related injuries. An audit published by The Football Association (FA) in 2004, suggested that 12% of all injuries were hamstring injuries, which are 2.5 times more common than quadriceps injuries (Woods et al., 2004). Recent figures published by Ekstrand et al suggested that, in a professional male football team of 25 players, about 5 hamstring injuries occur each season, equivalent to more than 80 lost football days (Ekstrand et al., 2011). In terms of professional elite athletes, shortened recovery time could mean continuing with training, increased game play and benefit to the team and club. Therefore, further research is needed to analyse the new techniques in treating muscle injuries.

In 2008, an article in the British Journal of Sports Medicine titled "The early management of muscle strains in the elite athlete: Best practice in the world with a limited evidence", summarised that currently almost all our so-called knowledge has a basis of level 4 or level 5 (Orchard et al., 2008a). The panel of experts continued to highlight the importance of Dr. Mueller-Wohlfahrt's injection treatment regimen for treating muscle injuries. In brief, the treatment protocol involves multiple local injections and associated back injections with a mixture of a homeopathic and pharmacological cocktail (Wohlfahrt, 2008). Therefore, the biochemical property, pharmacodynamics and pharmacokinetics of each drug are altered and unpredictable. The only potential "active" substance in Dr. Mueller-Wohlfahrt's cocktail could be a drug called "Actovegin" which is a licenced clinically used drug with a track record of over 60 years. Therefore in this PhD thesis, in order to avoid the unpredictable nature of poly-pharmacy as discussed above, only Actovegin will be investigated.

In order to investigate the potential therapeutic effect or efficacy of Actovegin on muscle injury, basic muscle structures, histology and pathophysiology of the healing process were discussed. The biochemical

events following skeletal muscle injuries and repair are driven by cytokines, monocytes and leukocytes. The speed and quality of muscle healing are dependent on the inflammatory process. In order to alter the speed or quality of muscle repair, Actovegin must be able to modulate the inflammatory process.

The in-vitro study in this PhD thesis was the first study to investigate the role of Actovegin in the inflammatory process and demonstrated significant results. It confirmed that Actovegin could modulate the inflammatory process by influencing the CD68<sup>+</sup> and CD163<sup>+</sup> macrophages and CD163<sup>+</sup> THP-1 cells, which could influence the muscle healing process. Based on the findings from the *in vitro* studies and data from previous literature, a stand-alone single drug intramuscular Actovegin injection therapy regimen was developed to treat acute muscle injuries. The first clinical study using this stand-alone Actovegin treatment regimen was conducted in this PhD in professional footballer players and translated the *in vitro* findings to clinical practice, which confirmed that Actovegin could influence clinical outcome in treating acute muscle injuries.

This thesis summarises the current evidence on Actovegin. Compared with conventional conservative RICE and NSAID therapy, Actovegin proposes an exciting and legal alternative for high performance athletes. From the studies, Actovegin injection therapy seems safe and well tolerated. Overall, this PhD has suggested that Actovegin has an active role in the treatment of muscle strain injuries biochemically and clinically.

# Chapter One

## Introduction

### 1.1 Introduction

Muscle injuries are one of the most common sports related injuries. Their incidence varies from 30-55% in all sports related injuries per year (Noonan and Garrett, 1999, Verrall et al., 2001, Jarvinen et al., 2000). According to the World Health Organization, musculoskeletal injuries are the most common cause of long term pain and physical disability, which affects hundreds of millions of people in the world (Woolf and Pfleger, 2003). An audit published by The Football Association (FA) in 2004 suggested that 12% of all injuries were hamstring injuries, which are 2.5 times more common than quadriceps injuries (Woods et al., 2004). In the 2 seasons from 1997 to 1999, 749 hamstring injuries were reported across the 91 British football clubs (Woods et al., 2004). This accounted for 15 matches loss per club per season (Woods et al., 2004). Recent figures published by Ekstrand et al suggested that in a professional male football team of 25 players, about 5 hamstring injuries occur each season, equivalent to more than 80 lost football days (Ekstrand et al., 2011).

Muscle injuries are often assessed and diagnosed by clinical examination. Ultrasonography and Magnetic Resonance Imaging (MRI) can be useful in confirming clinical diagnosis. Muscle strains can be classified into three categories according to severity: Grade 1 (mild), a tear of few muscle fibres, minor swelling and discomfort with no or minimal loss of strength and restriction of movement. Grade 2 (moderate), a greater damage of muscle with clear loss of strength. Grade 3 (severe), a tear extending across the whole muscle belly, resulting in total loss of function (Jarvinen et al., 2000). There has not been any reliable method of predicting return to play or lay-off



time following hamstring injuries in high level athletes, as they are often multifactorial. Functional assessments such as isokinetic testing have been tried and used as a prognostication tool with mixed results (Ayala et al., 2013, Clark et al., 2006, Freckleton and Pizzari, 2013, Maffiuletti et al., 2007, McCleary and Andersen, 1992, Steiner et al., 1993).

Rest, immobilization (Jarvinen et al., 2000), physical therapy (Cibulka et al., 1986) and non-steroidal anti-inflammatory drugs (NSAIDs) (Reynolds et al., 1995) have been the mainstay of therapy for grade 1 and 2 muscle injuries (Kasemkijwattana et al., 2000). For Grade 3 muscle injuries, surgical repair may be possible depending on the location and anatomy (Jarvinen et al., 2000, Taimela et al., 1997). Immobilization can lead to improved granulation of the injured muscle and promote healing, but it will cause significant atrophy of healthy myofibres and joint stiffness (Jarvinen et al., 2000). Although some studies have shown that the administration of NSAIDs promotes muscle healing by reducing degeneration and inflammation (Abramson and Weissmann, 1989, Cheung and Tidball, 2003), other research has demonstrated that NSAIDs are detrimental to the entire healing process (Obremsky et al., 1994, Mishra et al., 1995, Almekinders and Gilbert, 1986). Recently, new treatment options such as growth factor injection therapy have shown good therapeutic results. However, due to their performance enhancing properties, growth factors and anabolic hormones are prohibited by the World Anti-Doping Agency (Lee et al., 2011).

In terms of professional elite athletes, shortened recovery time could mean continuing with training, increased game play and benefit to the team and club. Therefore further research is needed to analyse the new techniques in treating muscle injuries. In 2008, an article in the British Journal of Sports Medicine titled "The early management of muscle strains in the elite athlete: Best practice in the world with a limited evidence", summarised that currently almost all our so-called knowledge has a basis of level 4 or level 5 (Orchard et al., 2008a). The panel continued to highlight the importance of Dr. Mueller-Wohlfahrt's Traumeel and Actovegin injection treatment regimen for treating muscle injuries. This treatment regimen was considered by the panel of

experts to potentially be the current best practice with further research required (Orchard et al., 2008a).

Dr. Mueller-Wohlfahrt's is a sports medicine specialist whose treatment is based on his conviction and experience over the years. His treatment method was popular with athletes, but there has not been any publication in regards to his injection therapy regime. I have obtained an unpublished injection therapy protocol from Dr. Mueller-Wohlfahrt to further understand his method. In brief, his treatment protocol for muscle injuries involves multiple local injection and associated back injection with a mixture of homeopathic and pharmacological substances(Wohlfahrt, 2008). In this cocktail, Actovegin is mixed with Traumeel and local anaesthetics for injection, therefore the biochemical property, pharmacodynamics and pharmacokinetics of each drug are altered and unpredictable. On the other hand, its desirable therapeutic effects could be due to the specific quantity and mixture of these drugs.

As there have been no pharmacological and toxicology studies concerning Dr. Mueller-Wohlfahrt's cocktail to predict its lethal dose and half-life, it is not practical to investigate the therapeutic effect of this mixture in muscle injury scientifically without knowing the specific effect of how these three substances interact with each other. The only potential “active” substance in Dr. Mueller-Wohlfahrt’s cocktail may be a drug called “Actovegin” which is a licenced clinically used drug with a track record of over 60 years in treating ischaemic events and wound healing. Therefore in this PhD thesis, in order to avoid the unpredictable nature of poly-pharmacy as discussed above, only Actovegin will be investigated.

## **1.2 Scope of this PhD**

In order to investigate the potential therapeutic effect or efficacy of Actovegin on muscle injury, basic muscle structures, histology and its healing process will be discussed. The biochemical events following skeletal muscle injuries and repair are driven by cytokines, monocytes and leukocytes

(Gulevskii et al., 2011, Gates and Huard, 2005, Ciciliot and Schiaffino, 2010, Chazaud et al., 2009). The speed and quality of muscle healing are dependent on the inflammatory process. In order to alter the speed or quality of muscle repair, the substance must be able to modulate the inflammatory process. Therefore, *in vitro* experiments will be performed in this PhD to investigate any potential role of Actovegin in the inflammatory process, hence muscle healing. If the in-vitro study in this PhD establishes significant results, ethical approval will be sought and a clinical study will be performed to investigate any potential translation effects of the in-vitro findings. Actovegin is a licenced drug in Europe, however it does not have marketing authority in the UK for human use, therefore specialist approval will be sought for the clinical study. Furthermore, the clinical study will be performed in high level athletes as this is the patient group that would potentially gain the most potential benefit from this method of treatment if it was successful. Due to this specific regulation of Actovegin and this patient group, a method will be development to batch test and standardise the Actovegin to assure quality control. Although the specific rules and regulations on Actovegin do not apply with regards to the in-vitro study, to ensure standardisation between the biochemistry and clinical studies all Actovegin used in this PhD will be batch tested. With regards to the clinical study, the role of isokinetic testing will be explored as a functional assessment in this group of patients.

### **1.3 Aim of this PhD**

This PhD thesis is based on the call for future research from the current best practice paper from the British Journal of Sports Medicine “The early management of muscle strains in the elite athlete: Best practice in a world with a limited evidence basis” (Orchard et al., 2008a) on the treatment of muscle injuries by a panel of experts. The aim of the experiments and studies in this PhD is to provide further information on the role of Actovegin in muscle injuries and its clinical efficacy as an injection therapy in the treatment of hamstring muscular injuries in professional footballers. The structure of this PhD thesis is outlined in the flow diagram below.

#### 1.4 Work flow diagram of the experiment in this PhD



# Chapter Two

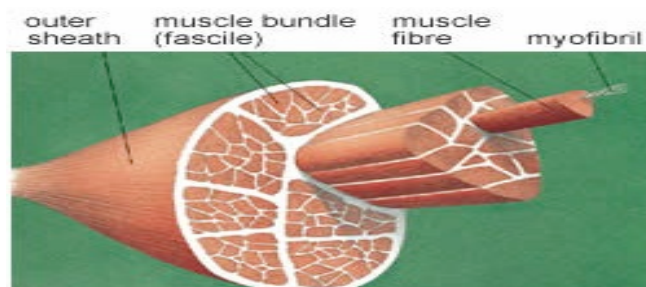
## Background and Review of literature

### 2.1 Introduction

In order to investigate any potential therapeutic effect of any substance to muscle injury, the basic muscle structures, histology and its healing process would need to be explored to identify a possible role of action for such substance. Basic Structure of Skeletal Muscle and the pathophysiology of muscle injury and healing will be discussed in this chapter. Its aetiology and evidence on treatment will be analysed. Background information and the evidence of the use of Actovegin in the treatment of muscle injury will be discussed. After reviewing all the evidence on muscle injuries and Actovegin, research question and hypothesis will be raised.

### 2.2 Basic Structure of Skeletal Muscle

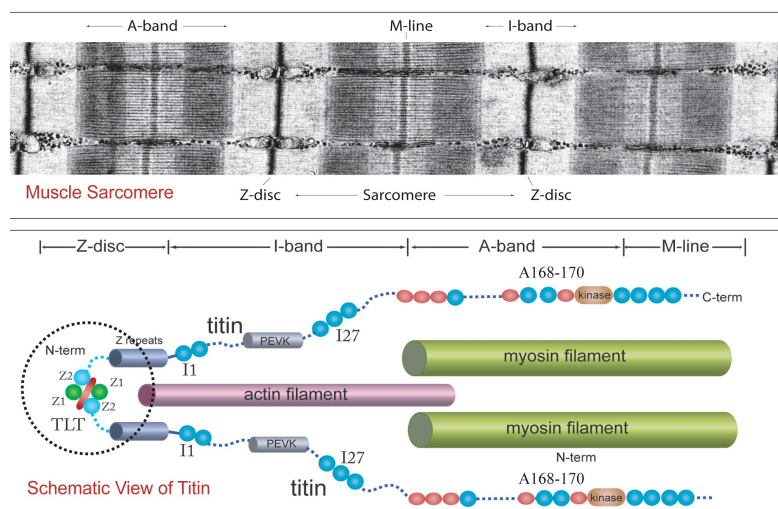
Skeletal muscle is a mixture of muscle cells, nerves, blood vessels, and a complex extracellular connective-tissue matrix. A single unit of skeletal muscle cell is known as a myofibre, which is a highly organized, multinucleated structure consisting of microfilaments arranged to optimize contraction. (Figure 2.2)



(Figure 2.2) Basic Structure of Skeletal Muscle (Gregorio and Antin, 2000)

## 2.2.1 Histology of muscle

Myofibrils are composed of repeating contractile units known as sarcomeres, perhaps the most highly ordered macromolecular structures in eucaryotic cells (Gregorio and Antin, 2000). Each sarcomere consists of thick and thin filaments whose arrangement is largely responsible for the cross-striated banding pattern observed under light and electron microscopy (Figure 2.2.1). Sarcomeres are delineated at their ends to Z-lines where thin actin filaments of opposite directions are linked together by actinin dimers (Luther, 2000). Z-lines are located in the middle of the I-band, which appears lighter in a light microscope and contains mainly actin filaments. Polymers of myosin molecules form the darker A-band. The A band is bisected by a light region called the H band, the major component of which is creatinine kinase. Running through the midline of H band is the M line in which the thick filaments are anchored by several myosin-binding proteins. Thick filaments are connected to giant titin molecules expanding to half of a sarcomere, from the Z-line to M-line (Figure 2.2). Titin is thought to function as a spring and a ruler defining sarcomere length after muscle contraction (Linke et al., 1999), which happens when actin filaments interact with the myosin filaments so that the thin filaments move past the thick filaments towards the centre of the sarcomere thus shortening it.



(Figure 2.2.1) Basic histological structure of Skeletal Muscle (Linke et al., 1999)

### 2.2.2 Muscle type and structure

Although all skeletal myofibres have the same basic sarcomeric organization, a number of distinct types have been described according to structural, physiological and biochemical criteria. Skeletal muscle fibres can be generally classified as fast or slow twitch, based on their contractile and metabolic properties and associated patterns of gene expression (Hughes et al., 1998). These properties of skeletal muscle fibres are dependent on the pattern of motor neuron stimulation, so that tonic motor neuron activity promotes the slow fibre phenotype while infrequent motor neuron firing results in fast fibres; the muscle phenotype according to neuron activity (Wu et al., 2000).

There are 2 types of myofibres:

Type I            Myofibres are slow and fatigue-resistant,

Red slow twitch oxidative fibres (type I), are involved in sustained, tonic contractile events and maintain intracellular  $\text{Ca}^{2+}$  concentrations at relatively high levels (100-300nM).

Type II            Myofibres are fast and have poor fatigue resistance  
(e.g. hamstring muscle)

White fast twitch glycolytic fibres (type II) are used for sudden bursts of contraction and are characterized by brief, high-amplitude  $\text{Ca}^{2+}$  transients and lower ambient  $\text{Ca}^{2+}$  levels (<50nM).

Sub Type A: Fast twitch and have good fatigue resistance

Used more during sustained power activities such as sprinting 400 meters or doing repeated lifts with a weight below maximum. Type IIA fibres have large amounts of myoglobin, and high capacity for generating ATP by oxidative metabolic

processes, and break down ATP at a very rapid rate. They have a fast contraction velocity and are resistant to fatigue. Such fibres are infrequently found in humans.

Sub Type B: Fast twitch and have poor fatigue resistance

Used for very short-duration high-intensity bursts of power such as maximal and near-maximal lifts and 100 metre sprints. Type IIB fibres contain a low content of myoglobin, relatively few mitochondria, relatively few blood capillaries and large amounts glycogen.

Type IIB fibres can generate ATP by anaerobic metabolic processes, but are not able to supply skeletal muscle fibres continuously with sufficient ATP, and fatigue easily. Such fibres are found in large numbers in the muscles of the arms in humans.

Structurally, when compared to type II myofibres, type I myofibres tend to be narrower, have thicker Z and M bands, have more glycogen, and their sarcoplasm is rich in mitochondria. The molecular basis for the functional diversity of myofibres is the expression of specific isoforms of most of the proteins involved in muscle contraction and relaxation. Myofibre classification is based on contraction speed and other physiological properties but predominantly according to specific myosin heavy chain (MyHC) isoforms.

Morphological details vary in different muscle fibres. Skeletal muscles respond to changes in physiological demands by remodelling the architecture of individual fibres. Sarcomeres are added or removed when muscles are held at abnormally long or short lengths, and myofilaments are added or removed when muscle fibres function against abnormally heavy or light loads (Trotter, 2002). This leads to changes in overall mass of the tissue. Also the spatial relationship among muscle cells and other components of muscle tissue can



change and gene expression can be reprogrammed to alter specialized metabolic and contractile properties of myofibres (Wu et al., 2000).

The plasma membrane encasing the myofibre is called the sarcolemma. External to the sarcolemma is the basal lamina (basement membrane), which is a 100- to 200-nm thick external connective tissue layer that contains a number of proteins including collagen, fibronectin, laminin, and many glycoproteins. Normally, the region between the sarcolemma and the basal lamina is occupied by satellite cells that lie quiescent. Acute injury to skeletal muscle stimulates satellite cell proliferation and differentiation, producing myogenic precursors such as myoblasts (Gates and Huard, 2005). This process is discussed in detail in section 2.3.2

## **2.3 Muscle injuries**

Muscle injuries are one of the most common sports related injuries; their incidence varies from 30-55% in all sports related injuries per year (Noonan and Garrett, 1999, Verrall et al., 2001, Jarvinen et al., 2000). More than 90% are caused either by excessive strain or contusions. Strain injuries are common in high power sports that are associated with sprinting or jumping (Jarvinen et al., 2000, Garrett, 1996b). These injuries are typically present at the myotendinous junction or the mid substance of muscles working across two joints, such as hamstrings and calf muscles (Garrett, 1996b). On the other hand, contusion injuries are typically associated with contact sports and could present at any site. A strain injury frequently occurs in the setting of eccentric contraction. Forces generated within eccentrically activated muscle are higher than in a concentrically activated muscle, thus increasing susceptibility to injury (Jarvinen et al., 2000, Garrett, 1996b).

### **2.3.1 Classification of muscle injuries**

The clinical manifestation of a muscle strain could depend on the severity of the injury. Jarvinen *et al* classified it in three categories according

to their severity (Jarvinen et al., 2000). This classification is widely used in sports medicine and it is purely a clinical diagnosis, based on patient's symptoms and physical signs from clinical examination. Although radiological imaging techniques such as MRI and ultra sound scanning could be a useful adjuncts to enforce clinical findings, the diagnosis and classification of muscle injuries only depend on the clinical factors.

Clinical Classification of muscle strains by Jarvinen *et al* (Jarvinen et al., 2000) : (Figure 2.3.1)

Mild (Grade 1) strain

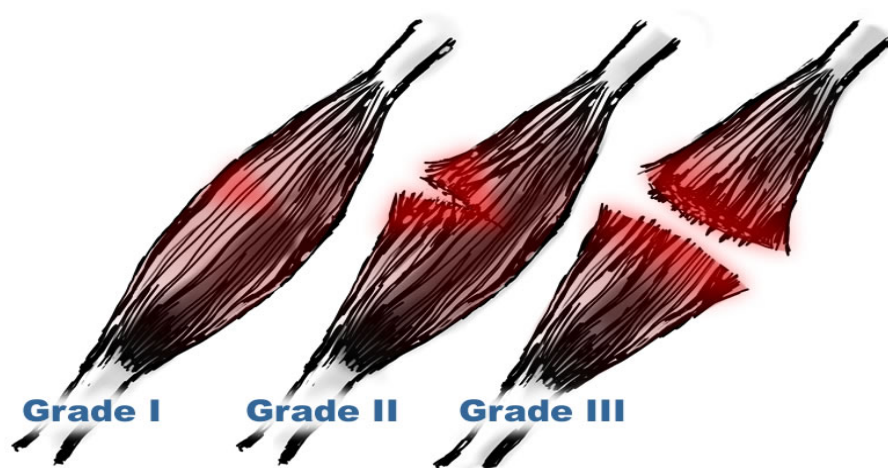
Tear of a few muscle fibres; minor swelling, minimal loss of function

Moderate (Grade 2) strain

Greater damage to muscle, palpable gap, clear loss of strength

Severe (Grade 3) Strain

Tear across the whole muscle belly, palpable gap, total loss of function

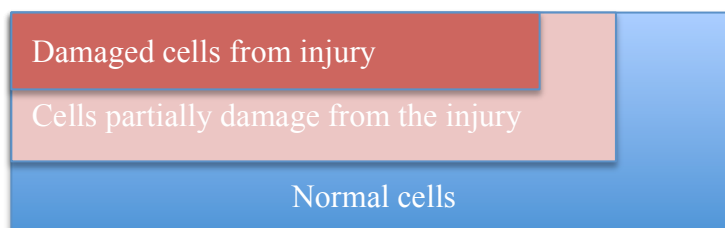


(Figure 2.3.1) Classification of muscle strain injuries (Jarvinen et al., 2000)

### 2.3.2 Pathophysiology of muscle strain injuries

The damage caused by the strain of the skeletal muscle is defined as a shearing injury; this would lead to mechanical and ischaemic damage to the muscle cells. The mechanical force tears the entire myofibre, damages its plasma membrane and leaves the sarcoplasm open at the end of the stumps and initiates necrosis. (Further details on muscle structure please see section 2.2.1) It will extend along the whole length of the ruptured myofibre causing inflammation and further cell damage (Gates and Huard, 2005). At the same time the injury has disrupted normal blood supply to the muscle cells and leads to ischaemia; this event will further enhance muscle necrosis and exacerbate the cellular damage cause by the acute inflammatory responds (Blaisdell, 2002). These ischaemic effects would depend on the muscle type as they have different cellular characteristics. (Further information on muscle type in section 2.2.2)

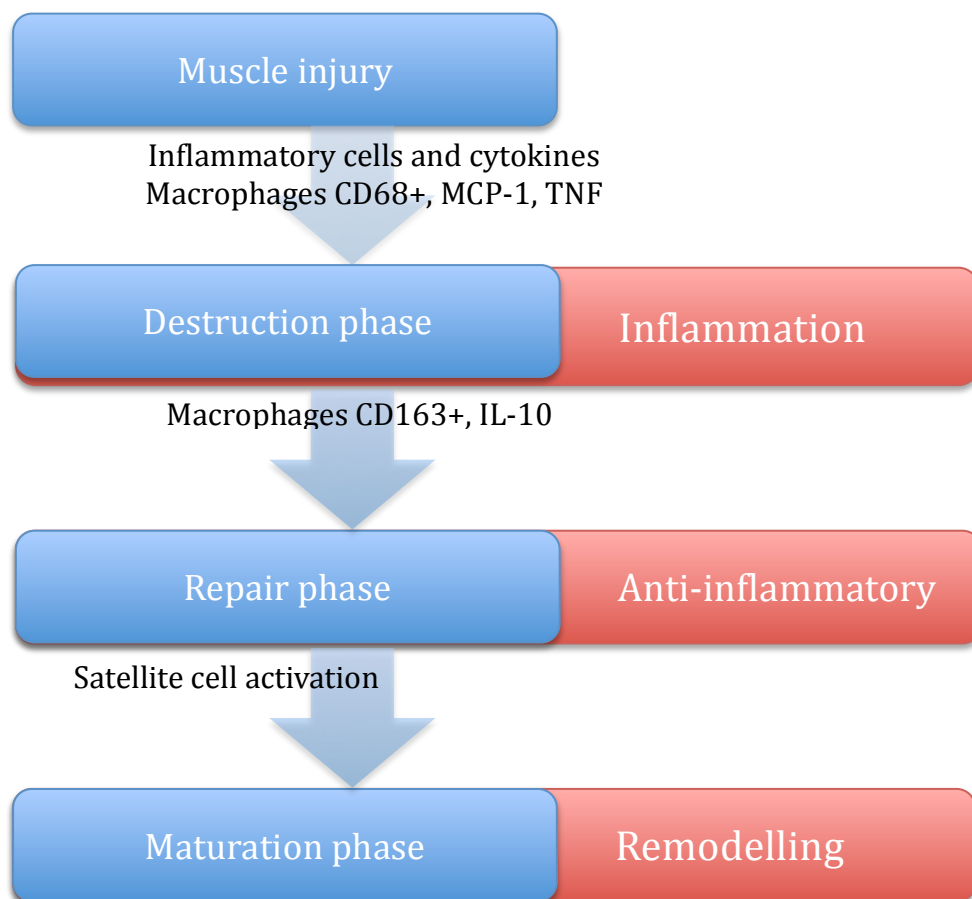
During the ischaemic event, initially myocyte glycogen and creatine phosphate are depleted in preference of ATP, and little muscle necrosis occurs(Blaisdell, 2002). However once ATP levels start to diminish, muscle necrosis sets in rapidly. Therefore the speed and extent of muscle necrosis are dependent on the amount of myocyte glycogen and ATP availability hence the muscle type. The extent of the mechanically induced inflammatory response and ischaemic damage to the muscle cells following muscle strain injuries are dependent on the severity of injury and muscle type –(type IIB muscle are more dependent on ATP, hence are more susceptible to ischemic insult), therefore treatment targeted to modulate the inflammatory response or the ischaemic effects can potentially improve overall outcome (Figure 2.3.2).



**(Figure 2.3.2)** illustration to show the cellular pathophysiology of muscle strain injuries. Red area = cells in damage zone, Pink area = cells in ischemic zone and Blue area = cells with normal tissue perfusion.

### 2.3.3 Pathophysiology of the healing process in muscle strain injuries

Muscle injury typically initiates a rapid inflammatory response as discussed in 2.3.2. The sequential invasion of inflammatory cells to the injury site persists from days to weeks, during muscle repair and regeneration. Recent studies have distinguished between inflammatory processes that disrupt muscle homeostasis and processes that promote muscle repair after muscle injury (Tidball and Wehling-Henricks, 2007). The repair process after muscle strain injuries can be divided into three phases, (Figure 2.3.3).



**(Figure 2.3.3)** A schematic presentation of the different phases of muscle strain injuries: Inflammation destruction phase, Anti-inflammatory repair phase, Remodelling maturation phase. (Garrett, 1996a, Grefte et al., 2010, Jarvinen et al., 2000, Gates and Huard, 2005)

## 1) Inflammatory destruction phase

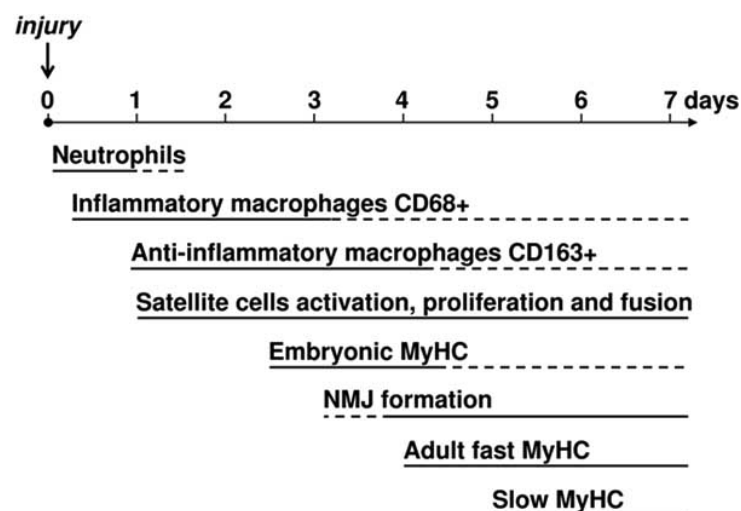
This is characterized by haematoma formation, myofibre necrosis and phagocytosis of dead tissue, initiated by inflammatory cells and cytokines (Garrett, 1996b, Grefte et al., 2010, Jarvinen et al., 2000).

Mechanical disruption of the sarcolemma significantly increases the permeability of the membrane to calcium, leading to an influx of extracellular calcium into the myofibre. This influx of calcium triggers a chain of events beginning with the stimulation of the complement cascade and culminating with the activation of intrinsic proteases that digest the myofibre, leading to myofibre necrosis. The necrotic myofibres naturally retract, creating a gap (Orchard and Best, 2002, Grefte et al., 2010, Gates and Huard, 2005).

Blood vessels are torn and capillaries damaged during muscle injuries leading to haematoma formation, which quickly fills this gap. It contains multiple growth factors, cytokines, macrophage, naive myogenic cells, and infiltrating inflammatory cells. Inflammation can be conceptualized as the response of the immune system to the injury. The interactions between these cells, proteins, and molecules strongly influence the remainder of the healing process (Gates and Huard, 2005).

The inflammatory process begins within the first hour after injury and peaks at 24 hours. Pro-inflammatory cytokines such as interleukin IL-1, IL-8, and tumour necrosis factor  $\alpha$  (TNF  $\alpha$ ) are secreted by a variety of cell types, attract and activate macrophages, and native myogenic cells (Gates and Huard, 2005).

Macrophages have been traditionally viewed as scavengers only involved in the removal of necrotic debris, however recent studies have suggested that they may also have an active role in promoting muscle regeneration (Garrett, 1996b, Grefte et al., 2010, Jarvinen et al., 2000). Recent studies suggest that there are two distinct subpopulations of macrophages sequentially involved in this process (Garrett, 1996b, Grefte et al., 2010, Jarvinen et al., 2000). The early invading “phagocytic” macrophages (CD68<sup>+</sup>), characterized by the expression of the CD68<sup>+</sup> cell surface markers and lacking the CD163<sup>+</sup> marker, reach a highest concentration in damaged muscle at about 24 hours after the onset of injury and then rapidly decline (Figure 2.5). These CD68<sup>+</sup> macrophages are pro-inflammatory phagocytes that secrete inflammatory cytokines such as TNF  $\alpha$  and IL-1 and are responsible for the removal of necrotic debris. After 48 hours, a second subpopulation of “non-phagocytic” macrophages (CD163<sup>+</sup>), characterized by the expression of the CD163<sup>+</sup> surface marker and lacking the CD68<sup>+</sup> marker are then found and reach a peak at day 4 after the initial injury (Figure 2.3.4). This CD163<sup>+</sup> subgroup displays an anti-inflammatory property and secretes cytokines, such as IL-10, which contributes to the termination of inflammation (Garrett, 1996b, Grefte et al., 2010, Jarvinen et al., 2000)



**(Figure 2.3.4)** Schematic presentation of current theoretical sequence of events following muscle injury: inflammation, anti-inflammation and regenerative (Grefte et al., 2010).

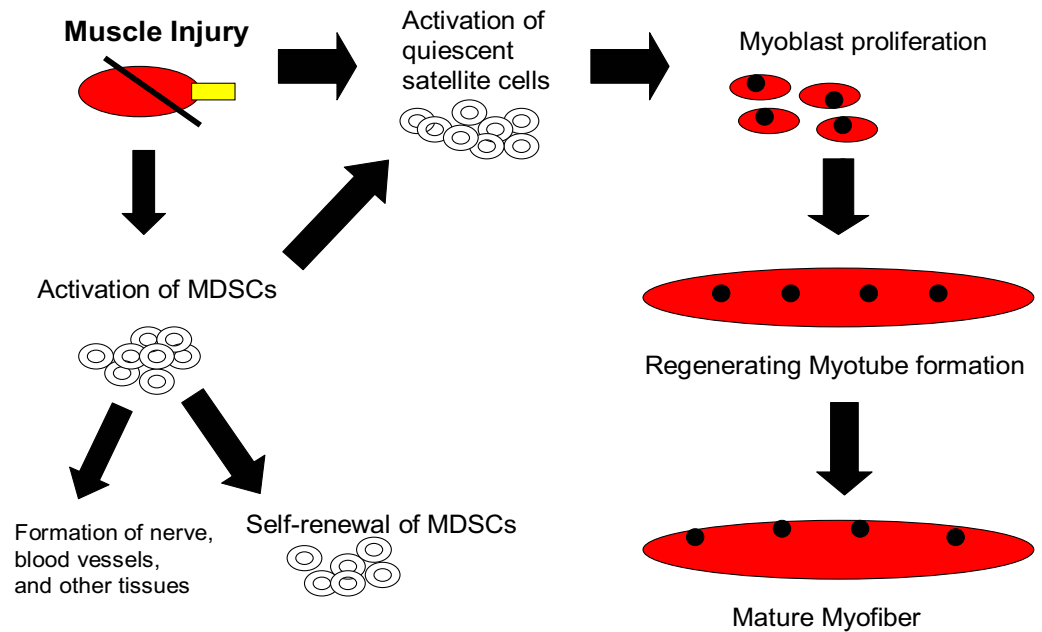
## 2) Anti-inflammatory, repair phase

Several findings suggest that macrophages may play a more direct role in muscle repair and re-modelling than merely removing tissue debris. After 48 hours, a second subpopulation of “non-phagocytic” macrophages (CD163<sup>+</sup>), characterized by the expression of the CD163<sup>+</sup> surface marker and lacking the CD68<sup>+</sup> marker are then found and reach a peak at day 4 after the initial injury. This CD163<sup>+</sup> subgroup displays an anti-inflammatory property and secretes cytokines, such as IL-10, which contributes to the termination of inflammation (Garrett, 1996b, Grefte et al., 2010, Jarvinen et al., 2000, Orchard and Best, 2002, Gates and Huard, 2005). Thus macrophages have a central regulatory role in the muscle response to injury, not only by removing necrotic tissue but also by promoting muscle regeneration.

## 3) Remodelling, maturation phase

Satellite cells located under the basal lamina of muscle fibres are responsible for muscle regeneration following the inflammatory process. There are several postulated mechanisms explaining satellite cell activation after trauma. Some researchers have posited that disruption of the integrity of the sarcolemma and basal lamina activates satellite cells (Orchard and Best, 2002, Grefte et al., 2010, Gates and Huard, 2005). Others maintain that cytokines released by infiltrating inflammatory cells result in satellite cell activation. Activated satellite cells proliferate and differentiate for as long as 10 days. The progeny of satellite cell differentiation are myogenic precursors such as myoblasts, which proliferate and begin to differentiate and fuse into multinucleated myotubes, immature myofibres, and eventually mature myofibres (Orchard and Best,

2002, Grefte et al., 2010, Gates and Huard, 2005). Figure 2.3.5 summarizes these events.



**(Figure 2.3.5)** A schematic presentation of the remodelling / regeneration phase after muscle injury (Gates and Huard, 2005).

Several growth factors released at the injury site play key roles in enhancing myoblast proliferation and differentiation. These growth factors include insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), Epidermal Growth Factor (EGF) and hepatocyte growth factor (HGF). This leads to the maturation of regenerated myofibres, contraction and re-organisation of scar tissue. (Orchard and Best, 2002, Grefte et al., 2010, Gates and Huard, 2005)



#### **2.3.4 Areas identified for further investigation in this PhD**

The pathophysiology of muscle injuries and its repair process are complex as described in 2.3.2 and 2.3.3. The extent of the tissue damage and myofibre necrosis depends on the inflammatory response and ischaemic damage, which are dictated by the mechanical strain at the time of the injury. As discussed in 2.3.2, myofibre necrosis depends on the available ATP, hence ischemia time. It would be logical to assume that, at the zone of injury, cell necrosis sets in rapidly due to the initial tissue trauma; little can be done to mitigate cell death at the zone of injury. However, at the peripheral zone around the zone of injury, the blood supply is interrupted and muscle homeostasis is imbalanced. Therefore the cells in the perhiperal zone are susceptible in developing cellular necrosis if they sustain further insult (Blaisdell, 2002). As the secondary inflammatory response from the zone of injury progresses, the cytotoxic effects from its metabolites increase the hostility of the surrounding cells. It will have a negative effect on the cells in the perhial zone and cause further cell necrosis; hence exacerbate the initial damage caused by the injury. As discussed in 2.3.2, this secondary onset of myofibre necrosis depends on muscle cell type and its metabolic rate. Therefore, the ability of the cells in the peripheral zone to withstand further insult can influence the overall effect of the injury. With regards to the healing process, the biochemical events involved in the muscle healing process are better understood, as discussed in 2.3.3. Macrophages play an important role in the inflammatory and anti-inflammatory process (Gulevskii et al., 2011, Gates and Huard, 2005, Ciciliot and Schiaffino, 2010, Chazaud et al., 2009); therefore modulation of specific macrophages could also potentially influence the muscle repair process.

## **2.4 The hamstrings**

The Hamstrings is a muscle group consisting of three muscles - semitendinosus, semimembranosus and biceps femoris. These posterior leg muscles originate at the bottom of the pelvis from the ischial tuberosity. Two of the hamstrings insert into the top of the tibia (medial); the other inserts into the fibula (lateral). Its main function is hip extension; semitendinosus and semimembranosus also flex the knee and medially rotate the lower leg when the knee is bent. Whereas the biceps femoris (both short and long heads) laterally rotates the lower leg during flexion (Bennell et al., 1998, Slavotinek et al., 2002, Woods et al., 2004, Maffiuletti et al., 2007, Ekstrand et al., 2009, Goldman and Jones, 2009). The hamstrings play a crucial role in many daily activities, such as, walking, running, jumping, and controlling some movement in the trunk. In walking and running they are most important as an antagonist to the quadriceps in the deceleration of knee extension.

The hamstrings muscles are skeletal muscles; their histological structures have been discussed in 2.2.1. This group of muscles contain type II fast twitch glycolytic fibres, its ratio of A and B type fibres varies between individuals and depends on training (Jarvinen et al., 2000). Further details of muscle sub types and structure were discussed in 2.2.2. Type II muscle fibres are highly energy dependent and therefore its function is sensitive to the availability of ATP. During muscle injuries, the blood supply to the muscle is disrupted; hence the oxidative metabolic processes are compromised and reduce the amount of available ATP. As the hamstring muscles are highly energy dependent, tissue damage alongside with this ischaemic effect could cause further damage to the surrounding tissue after the initial injury. For further details of the pathophysiology of muscle injury please see 2.3.2

### **2.4.1 Hamstring injuries**

Hamstring injuries are very common in sport with high demands on speed and power such as football (soccer), Australian-rules football, rugby,

basketball and American football. They are 2.5 times more common than quadriceps injuries (Woods et al., 2004, Askling C, 2003).

Hamstring injuries occur during the last part of the swing phase or at foot strike (Liemohn, 1978, Paton et al., 1989, Burkett, 1970). At both of these phases of running, the hamstrings generate peak torque values and work maximally eccentrically to decelerate the leg (Reurink et al., 2012, Paton et al., 1989, Liemohn, 1978, Herzog, 1988). Therefore most hamstring tears are typically partial and commonly take place during the eccentric phase of muscle usage when the muscle develops tension while lengthening (Kujala et al., 1997, Liemohn, 1978, Paton et al., 1989). The most commonly injured hamstring muscle is the biceps femoris (Slavotinek et al., 2002). Muscle strength deficiency has been proposed as one of the risk factors for such injuries (Kujala et al., 1997, Jarvinen et al., 2000, Clark et al., 2006, Goldman and Jones, 2009). Some studies suggest that a greater than 10% bilateral deficit in isometric hamstring strength is predictive of hamstring injury (Burkett, 1970, Orchard et al., 1997). Strength imbalances between the quadriceps and hamstring muscle groups have been reported as the mechanism behind the hamstring injuries (Askling C, 2003, Orchard et al., 1997, Woods et al., 2004, Gabbe B, 2006). It was associated with significantly lower concentric isokinetic hamstring to quadriceps muscle peak torque ratio (Bennell et al., 1998, Askling C, 2003, Woods et al., 2004). The injured hamstring muscles were weaker than in the opposite leg in absolute values and hamstring-to-quadriceps muscle ratios (Herzog, 1988, Garrett, 1996a, Orchard et al., 1997, Funk D, 2001, Woods et al., 2004). It was significantly associated with a low hamstring-to-quadriceps muscle peak torque ratio at 60 deg/sec on the injured side and a low hamstring muscle side-to-side peak torque ratio at 60 deg/sec (Orchard et al., 1997). However, there are also retrospective and prospective observational studies suggesting that isometric (Liemohn, 1978) and isokinetic (Paton et al., 1989) quadriceps to hamstring ratios cannot reliably predict the likelihood of hamstring injuries. Further discussion and detail on isokinetic testing will be discussed in section 4.3.4.

## **2.4.2 Literature review of the treatment of hamstring injuries**

Although hamstring muscle injuries are one of the most common muscle injuries sustained by athletes, the evidence of therapeutic intervention is limited (Orchard et al., 2008a). There is currently no consensus on the best management options. Rest, ice, compression, elevation (RICE), non-steroidal anti-inflammatory drugs (NSAIDs) and injection therapy are used among sports physicians to treat varying types of hamstring injuries (Orchard et al., 2008a, Reurink et al., 2012).

### **2.4.2.1 RICE**

Rest, ice, compression, elevation (RICE) treatment is the most traditional and commonly used method to treat muscle sprains and strains. It has been described in textbooks and practiced by many professional athletes, coaches and members of the healthcare team. These four procedures have the same objective, to minimize bleeding from ruptured blood vessels at the rupture site. It was assumed that it would prevent the formation of a large haematoma, which could impact upon the size of scar tissue at the end of the regeneration. A small haematoma and the limitation of interstitial oedema accumulation at the rupture site was believed to shorten the ischaemic period in the granulation tissue, which may in turn accelerate regeneration (Jarvinen et al., 2000).

#### **Rest and Elevation**

Although it is the most commonly used method of treatment in acute hamstring injuries, there is no consensus on the duration and technique used in the practical aspect (Reurink et al., 2012). The initial resting period could range from 2 -72 hours and some protocols describe complete rest of the limb as compared to mobilising the limb as pain allows (Reurink et al., 2012, Rask and Lattig, 1972, MacAuley, 2001). Furthermore, resting methods vary between protocols and

patient perception. It ranges from complete splinting(Lipscomb et al., 1976), passive range of movement to minimal active resistance exercise(Reurink et al., 2012, Freckleton and Pizzari, 2013).

In terms of elevation, the general principle is to raise the injured site above heart level to facilitate lymphatic drainage and venous return. There is no evidence suggesting that elevation alone can reduce soft tissue swelling. In facial and shoulder injuries, although there is assumed anatomical advantage of the injured site being above the heart, the amount of swelling is not slighter compared to lower limb injuries. Unless the patient is supervised 24 hourly with a team nursing staff support, it is impossible to completely rest and elevate the injured limb, therefore compliance to such protocols may be difficult. Due to these logistical factors, there are currently no control trials to demonstrate the effect of rest and elevation (Thorsson et al., 1997, Reurink et al., 2012). As there is such large variation in the understanding and practice of rest and elevation, there are no common consensus of best practice.

## **Ice**

With regards to ice treatment (cryotherapy), it is perhaps the simplest and oldest therapeutic modality in the treatment of acute soft-tissue injuries. It is assumed that by decreasing tissue temperature, cryotherapy can reduce pain, slow down metabolism and minimise the inflammatory process (K, 1989, KL, 1976). The majority of research studies and reviews to date have used healthy human subjects to investigate these proposed physiological effects. Although there is evidence that cryotherapy can reduce deep-tissue temperature in both animal (Barlas et al., 1996) and human subjects, (Draper et al., 1995, Merrick et al., 1993, Zemke et al., 1998) the degree of cooling seems to depend on the method and duration of application, the initial temperature of the ice, and even the depth of subcutaneous fat (MacAuley, 2001).

Current recommendations in standard textbooks on the clinical use of ice also have many shortcomings, and most physicians rely on empirical evidence (MacAuley, 2001). The selection of parameters in a clinical environment continues to be made pragmatically, and recommendations in review articles range from 10 to 20 minutes, 2 to 4 times per day, up to 20 to 30 minutes, or 30 to 45 minutes every 2 hours (Bleakley et al., 2004). The most recent surveys of clinical practice have identified variations on the optimal mode, duration, and frequency of ice application, yet such factors dictate the degree of cooling and the potential effectiveness of treatment (MacAuley, 2001).

There is no consensus on the duration of time the ice pack is applied and the frequency of use. However, there seems to be a common agreement that ice packs should not be used more than 48 hours post injury. There are no randomized studies assessing the efficacy of ice in the treatment of muscle contusions; only five studies have assessed the effect of ice on acute ankle sprains. Single applications of combined ice and compression seem to be as effective as no treatment after an acute sprain.

Given the popularity of cryotherapy treatment with the layman, it may be difficult to randomize a subject to a “no ice” group. Within clinical practice, ice is commonly combined with compression and elevation, making it difficult to determine the value of cryotherapy alone (Meeusen and Lievens, 1986, Thorsson, 2001). There have been some side effects of cryotherapy reported such as skin burns (O'Toole and Rayatt, 1999) and nerve damage (Moeller et al., 1997) after as little as 20 minutes of cooling.

Cryotherapy is a versatile modality and may be used in the immediate and rehabilitative phases of injury management. Immediately ice application is advocated post injury, it is assumed that cryotherapy can reduce tissue metabolism and may reduce secondary

hypoxic injury, cell debris, and oedema. Therefore cryotherapy initiated between 24 and 48 hours after injury may not have optimized this positive physiological effect.

The majority of studies in current literature have not fully considered the pathophysiological basis of cryotherapy and may not have used it to its full potential. Recommendations on ice dosage remain anecdotal and usually do not change based on the circumstances of injury. The depth of muscle injury and the surrounding adipose tissue must be considered and collate with the clinical effectiveness of cooling (Bleakley et al., 2012). Future research into the effectiveness of ice and muscle injury is needed. Treatment dosage should potentially be developed based on injury depth and should be factored into future clinical trial design.

### **Compression**

The principle of acute compression to treat soft tissue injury is widely based on bleeding control and reduction of haematoma; it was believed to shorten recovery time and prevent myositis ossificans (Rask and Lattig, 1972, Lipscomb et al., 1976, Thorsson et al., 1997). Although there is an extensive amount of literature on acute soft tissue injuries, there is no stand-alone controlled clinical study comparing compression to no treatment. The majority of publications regarding the use of compression to treat acute muscle injuries are based on post eccentric training rather than traumatic muscle injuries. For the remaining studies, ice or cryotherapy are usually combined with compression post injury and duration and frequency are also different, therefore it is impossible to draw meaningful conclusions from these studies. Furthermore, due to different body habitus, the type of compression bandage used, and the technique of its application, the compressive force across the injured site cannot be standardised.

Thorsson et al 1997 demonstrated that immediate treatment (within 5 min of the occurrence of an acute muscle injury) with a maximum compression bandage did not reduce the formation of a haematoma and did not shorten the recovery time of the injured players in this investigation compared with treatment of minimal compression, rest and elevation only (Thorsson et al., 1997).

#### **2.4.2.2 Non-steroidal anti-inflammatory drugs (NSAIDs)**

NSAIDs are primarily used for their analgesic, anti-inflammatory, and antipyretic properties, although their in-vivo effects in treating musculoskeletal injuries in humans remain largely unknown (Paoloni et al., 2009). Non-steroidal anti-inflammatory medications have become popular in the treatment of sports injuries. Conventional wisdom has dictated that inflammation is bad and needs to be reduced after acute injuries. Although there is little evidence to promote the use of NSAIDs in acute muscle injuries and some studies even suggests that NSAIDs could be counterproductive to the healing process, their use is still remains common (Reurink et al., 2012).

NSAIDs' analgesic action is not significantly greater than paracetamol for musculoskeletal injuries but they have a higher risk profile, with side effects including asthma exacerbation, gastrointestinal and renal side effects, hypertension and other cardiovascular diseases. A number of studies and reviews found that NSAIDs are no more effective than placebo in the management of acute soft tissue injuries (Mehallo et al., 2006, Ziltener et al., 2010). Furthermore, concerns have been raised about the possible harmful effect of NSAIDs on muscle healing after acute injury, because of delayed muscle regeneration and promotion of fibrosis (Ziltener et al., 2010, Shen et al., 2005). Reynolds et al found no statistically significant effect of treatment with NSAIDs on pain score and isokinetic hamstring testing compared with placebo. Pain scores measured with a visual analogue scale after 1 week were  $7.9 \pm 6.6$ ,  $8.8 \pm 7.7$  and  $3.9 \pm 3.3$  for the meclofenamate, diclofenac and



placebo group, respectively (Reynolds et al., 1995). Adverse effects such as gastrointestinal upset and headache were reported in 38% of the NSAIDs group compared to 14% of the placebo group (Reynolds et al., 1995).

Inflammatory cells play an important role in the healing process of an injured muscle. Therefore, the use of drugs that inhibit these cells, such as NSAIDs, is contradictory. On the other hand, NSAIDs could also play a beneficial role in the acute injury setting. The inflammatory process can be excessive and cause oedema, resulting in anoxia and further cell death. This can be prevented by the administration of low-dose NSAIDs (Paoloni et al., 2009).

There have been many review papers on the use of NSAIDs, but none have advocated their use as a first line treatment or routine management to treat acute muscle injuries (Paoloni et al., 2009, Reurink et al., 2012, Ziltener et al., 2010, Orchard et al., 2008b). NSAIDs may be used but no sooner than 48 hours following exercise-induced muscle injuries to provide analgesia and to reduce the early inflammatory response. Earlier use can interfere with the cell chemotaxis that is necessary for the repair and remodeling of regenerating muscle. Prolonged use of NSAIDs (over 7 days) is not recommended; it may delay muscle regeneration by inactivating the proliferation and differentiation of satellite cells and inhibiting the production of growth factors (Reynolds et al., 1995, Shen et al., 2005, Reurink et al., 2012, Paoloni et al., 2009).

There is limited evidence that the use of NSAIDs has no effect on pain scores and isokinetic strength testing in acute hamstring injuries. Furthermore, concerns have been raised about the possible harmful effects of NSAIDs on the cardiovascular system and muscle healing after acute injury. Despite the widespread use of NSAIDs in acute muscle injuries, there is no evidence for their efficacy for hamstring injuries (Orchard et al., 2008b).

#### **2.4.2.3 Injection therapy**

In recent years, injection therapies have gained popularity for the treatment of muscle injuries. Many pharmaceutical or homeopathic substances have been described in the literature to treat acute muscle injuries. In the past, corticosteroids such as dexamethasone sodium phosphate, triamcinolone hexacetonide, or triamcinolone acetonide were used in conjunction with either lidocaine hydrochloride or bupivacaine hydrochloride to treat such injuries (Levine et al., 2000). Prolotherapy such as hypertonic saline, dextrose, phenol, glycerine, and cod liver oil extract have also been tried (Reurink et al., 2012). There have been limited and yet contradictory results in the literature regarding these substances for intramuscular injections. Local anaesthetic drugs are commonly used and mixed with these substances for intramuscular injections, in the aim of reducing discomfort and providing immediate pain relief (Zink and Graf, 2004). Although it is an uncommon side effect, local anaesthetic intramuscular injections can result in reversible myonecrosis (Zink and Graf, 2004). (Figure 2.4.2.3) Furthermore, when the “therapeutic substance” is mixed with local anaesthetic drugs, the pharmacodynamic and pharmacokinetic properties of each substance is altered and unpredictable.

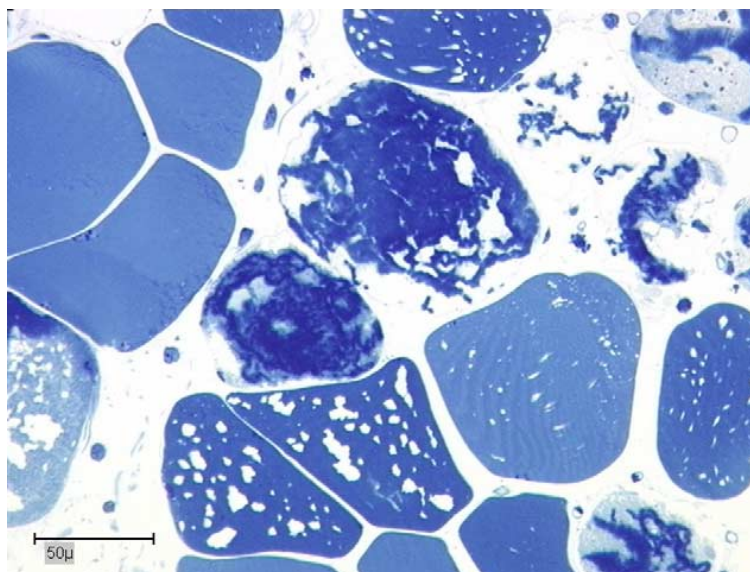


Figure 2.4.2.3 Skeletal muscle cross-section with characteristic histologic changes after continuous exposure to bupivacaine for 6 hours. A whole spectrum of necrobiotic changes can be encountered, ranging from slightly damaged vacuolated fibres and fibres with condensed myofibrils to entirely disintegrated and necrotic cells. (Zink and Graf, 2004).

As the understanding of human biochemistry has increased over the past decade, human growth hormone and insulin like growth factors have

been used to accelerate the recovery of muscle injuries. However, due to the anabolic nature of these growth factors, their use may lead to over growth or even malignant cell transformation (Falk et al., 1979, Socas et al., 2005, Gorayski et al., 2008). It is classified as doping in section S1 of the prohibited list by WADA. Recently, platelet rich plasma (PRP) has become popular among professional athletes as of 2011 WADA relaxing its rule and it is no longer prohibited under certain circumstances. Recently, the International Olympic Committee (IOC) released a consensus statement, which further promoted the use of PRP (Engebretsen et al., 2010). The consensus paper highlighted and summarised the current facts and published reports on PRP. Although this paper did not find any clinical noticeable or significant beneficial effect with PRP, it heavily focused on reporting no negative effects of PRP and therefore recommended its use. It is rather unusual for an international governing body to release such a publication to support the use of PRP based on very poor evidence (Kjaer and Bayer, 2011). On the other hand, the IOC, in the past, issued a ban on a drug in similar circumstances based on speculation and rumours. Therefore, any statement, evidence or publication produced by the IOC should be treated with caution, as with PRP.

Besides corticosteroids, prolotherapy, growth factors and PRP, there is another alternative regarding muscle injection therapy. In professional elite-level athletes, Orchard et al (2008) summarised that currently almost all the so-called knowledge in the treatment of muscle injuries was based on very poor scientific evidence (Orchard et al., 2008b). Dr. Mueller-Wohlfahrt's Actovegin / Traumeel injection treatment regime for treating muscle injuries was considered by a panel of experts to be the current best practice (Orchard et al., 2008b). In 2006, an article from the Times newspaper reported a comment from Dr. Hans-Wilhelm Müller-Wohlfahrt, team doctor for the German National Football Team and Bayern Munich Football Club regarding the use of this regimen.

In this report Dr. Müller-Wohlfahrt discussed his experience with Actovegin/Traumeel and the success of its treatment with high profile sports people such as Maurice Green, Asafa Powell, Diego Maradona, Darren

Gough and Paula Radcliffe (Crompton, 2006) “I am an empirical doctor and, over 30 years, I have treated so many that nobody can tell me it doesn’t work. Nobody I have seen has had an adverse effect, or an allergic or other reaction.” (Crompton, 2006) A best practice statement mentioned Dr. Mueller-Wohlfahrt’s Actovegin and Traumeel injection therapy, although it is not a published clinical study but his thirty years specialist experience with muscle injuries in elite athlete should not be overlooked (Orchard et al., 2008a).

#### **2.4.2.4 Areas identified for further investigation in this PhD**

In terms of the professional elite athletes, shortened recovery time could mean continuing training, increased game play and benefit to the team and club. As the review in 2.4.2 suggested, traditional treatment of muscle injuries such as RICE, NSAIDs and injection therapies are based on “fashion” and poor evidence. Dr. Mueller-Wohlfahrt’s injection treatment regimen seems to produce internationally recognised anecdotal results among elite athletes. Therefore, this PhD will further explore this regimen.

In terms of Dr. Mueller-Wohlfahrt’s injection regime, Actovegin is mixed with Traumeel and local anaesthetics for injection, therefore the biochemical property, pharmacodynamics and pharmacokinetics of each drug are altered and unpredictable. Local anaesthetics can lead to myonecrosis and Traumeel is a homeopathic substance which contains 99% sterile isotonic saline (Schneider, 2011); the only potential “active” substance in Dr. Mueller-Wohlfahrt’s regimen may be a drug called “Actovegin”. For further details, please see 2.4.2.3 In order to reduce the complex pharmacodynamics and pharmacokinetics of drugs / substance interaction, this PhD thesis will focus on Actovegin only.

## 2.5 Background information on Actovegin

Through the background research and literature review summarised in 2.4.2.4, Actovegin was identified to be a drug that is potentially useful in the treatment of acute hamstring injuries. Therefore in this PhD Actovegin will be further analysed.

Actovegin® is a deproteinised haemodialysate of ultra-filtered calf serum under 8 months of age, produced by Nycomed GmbH, Austria. Austria is officially categorized as a Bovine Spongiform Encephalopathy (BSE), Transmissible Spongiform Encephalopathies (TSE) and Scrapie free country by the World Organization for Animal Health (WOAH) and the Scientific Steering Committee of the European Union. The manufacturing process of Actovegin® is BSE validated, thus proven to be capable of removing hypothetically present TSE agents (Nycomed). According to the manufacturer, it is ultra filtered to 6000 Daltons, therefore it does not contain protein, growth factors or hormone-like substances. Physiological components, such as amino acids, nucleosides, intermediary products of carbohydrate and fat metabolites constitute approximately 30% of the organic components in Actovegin (Nycomed). The active ingredients in this mixture have yet to be identified. Actovegin can be administered as tablets, topical formulations, injections or infusion via intramuscular, intravenous or intra-arterial routes (Nycomed). Within the scope of this PhD, only the injectable form of Actovegin will be discussed.

Actovegin is a licensed drug in Europe, China and Russia and has been used by clinicians for over 60 years (Nycomed, Pforringer et al., 1994, Wright-Carpenter et al., 2004, Beetz et al., 1996). Clinically, it has been used as an intravenous infusion to treat acute stroke (Derev'yannykh et al., 2008, Boyarinov et al., 1998) and as a topical form to treat skin and mouth ulcers (Biland et al., 1985). It has also been reported to be used as an intra-arterial infusion to treat long bone fractures (Khomutov et al., 1999) and postpartum haemorrhage (Appiah, 2002).

### 2.5.1 Manufacturing process

Actovegin® is a deproteinised, pyrogen- and antigen-free haemodialysate of calf blood. It is manufactured from calf blood in several steps by ultrafiltration (Buchmayer et al., 2011):

- 1) Ultrafiltration cut off of 6 kD
- 2) Vacuum distillation to remove the precipitate by filtration (0.45 µm)
- 3) Titration to pH 6.8
- 4) Sterile filtration with pre-filters of 0.2 µm and 0.45 µm
- 5) Stored at 2 – 6°C for 14 days
- 6) Sterile filtration (0.45 µm) and titrated to pH 6.8
- 7) Ultrafiltration step with a 10 kD cut off
- 8) Sterile filtration a pre-filter of 0.45 µm and 0.2 µm
- 9) Storage at 2 – 6°C for 56 days
- 10) Final precipitate is removed by filtration (0.45 µm) and diluted to a nominal concentration to 200 mg/ml dry weight.
- 11) Finally, deproteinization is completed by sterile filtration with prefilters of 0.2 µm and 0.45 µm.

The analysis of the final product shows that it contain a mixture of substances (Buchmayer et al., 2011):

<b>Inorganic components</b>	<b>Organic components</b>
Chloride	Amino acids
Phosphate	Oligopeptides (<20 amino acid)
Sodium	Nucleosides
Potassium	Glycosphingolipids
Calcium	
Magnesium	
Several sources for nitrogen	
Amino acids	
Peptides	
Glucose	
Acetate	

## Quality control by the manufacturer

Since Actovegin® is being produced from a biological source it undergoes two bio-assay tests to ensure homogenous activity of the product (Buchmayer et al., 2011).

- 1) Determination of the oxygen consumption ( $\mu\text{l}_{\text{oxygen}}/\text{mg}_{\text{tissue}}/\text{hour}$ ) in the homogenate of guinea pig liver homogenates (measured with a Warburg micro spirometer). The target value for Actovegin® is oxygen consumption of 1.0  $\mu\text{l}$  per 2 mg per hour. (0.5  $\mu\text{l}/\text{mg}/\text{hr}$ )
- 2) The uptake of tritium labelled glucose into the lipid fraction of adipocytes, prepared from epididymic rat adipose tissue. Actovegin® enhances this uptake in an Insulin-like fashion and exhibits at 1 mg/ml incubation medium an Insulin-like activity (ILA) of more than 20  $\mu\text{U}$ .

## Toxicity

Toxicity tests in mice and other relevant species reveal that Actovegin®, after intravenous application, has an acute toxicity of more than 50 times the maximum therapeutic dose. Negative subchronic toxicity testing over a period of three months also lends support that Actovegin® is non-toxic and no chronic pathological organic changes have been observed either macro or microscopically (Buchmayer et al., 2011).

### 2.5.2 potential active ingredients in Actovegin

Actovegin® was believed to have a broad range of activities, it can influence cell metabolism and leads to increased oxygen uptake and utility as well as glucose uptake by the cells (Boyarinov et al., 1998, Hoyer and Betz, 1989, Peronnet and Massicotte, 1991). It promotes oxidative metabolism and shifts the redox-balance of the cells into the direction of

oxidised substrates(K.W et al., 1992). This may also lead to an increased availability of energy-rich phosphates like ATP and creatine phosphate. The pre-clinical studies leading to the assumption of these positive effects have been reported from a broad repertoire of testing strategies conducted in both mainly rat's and guinea pig's cell cultures(Haring et al., 1981, Hoyer and Betz, 1989, Kuninaka et al., 1991, Villalba et al., 1988).

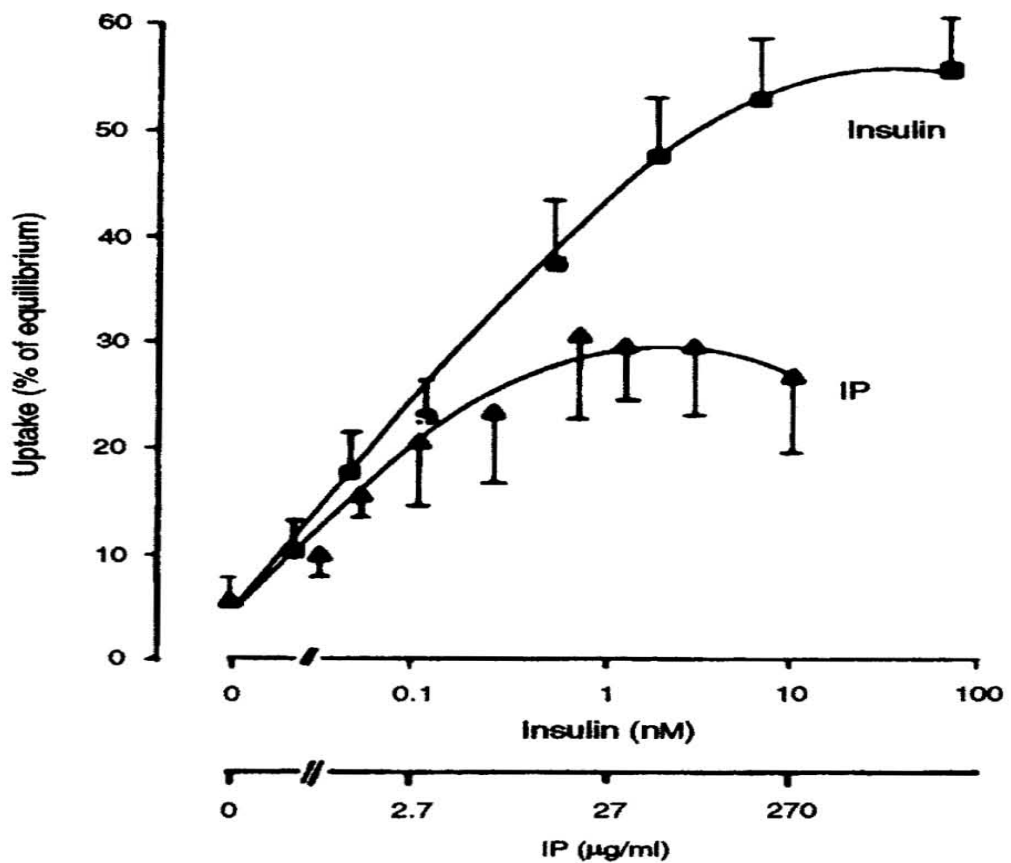
Many studies have tried to identify the active ingredients from this mixture, but have been unsuccessful. Studies *in vitro* have suggested that Actovegin promotes oxidative metabolism and shifts the redox-balance of the cells into the direction of oxidised substrates, which may protect against hypoxic cell injury (Schoenwald et al., 1991). Hoyer *et al* suggested that Actovegin does not directly influence cells during the ischaemic period as the cells' glucose and lactate levels are similar to the untreated animal cells during the ischaemic period (Hoyer and Betz, 1989). One of the most important goals of any post-ischaemic therapeutic strategy should be the early interruption of the process of cell-damaging events to avoid cell death. Because of the properties of Actovegin to promote oxidation and energy production, the efficacy of Actovegin was assumed to benefit post-ischaemic metabolic events although its active constituent has not yet been elucidated (Hoyer and Betz, 1989).

Obermaier-Kusser et al (1989) isolated Inositol phosphate oligosaccharides (IPO) from Actovegin, its composition were (Obermaier-Kusser et al., 1989):

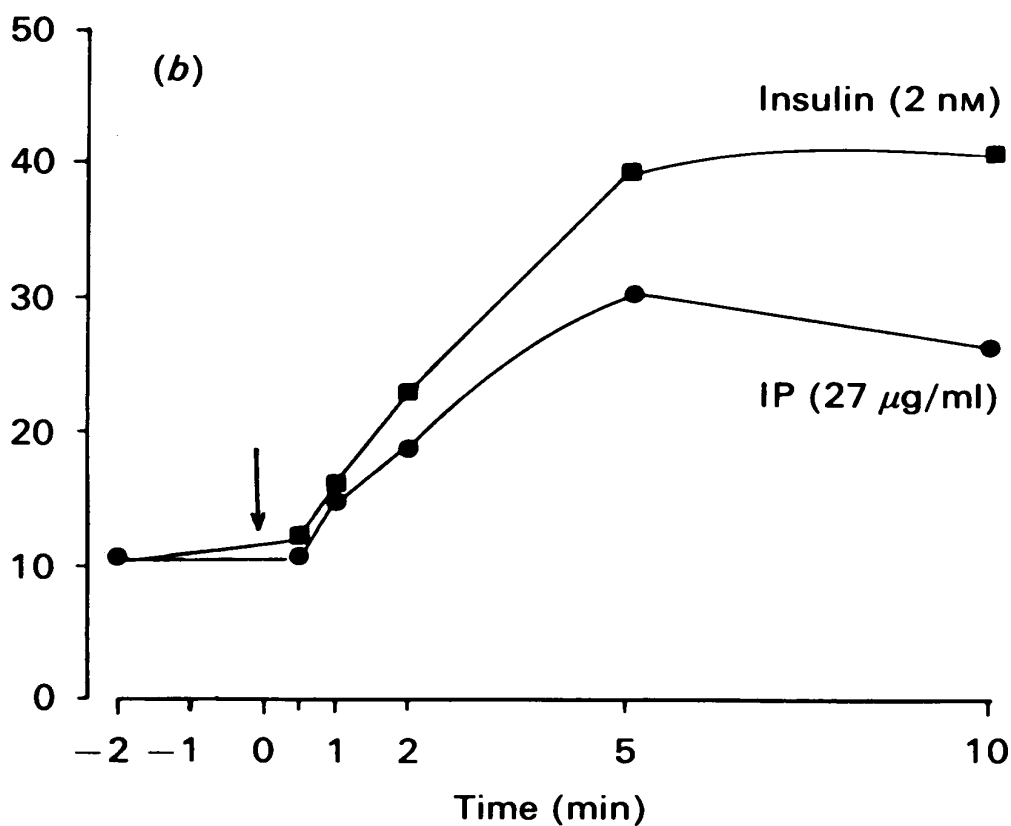
- Erythrose 2.54mM (0.305 mg/ml)
- Ribose 1.49mM (0.166 mg/ml)
- Arabinose 0.5mM (0.150 mg/ml)
- Xylose 1.26mM (0.189 mg/ml)
- Mannose 0.60mM (0.108 mg/ml)
- Galactose 0.30 mM (0.053 mg/ml)
- Glucose 1.22 mM (0.220 mg/ml)
- Inositol 0.56mM (0.100 mg/ml)
- Saccharides were complexed as phosphates or sulphates



In the same study, they also showed partial insulin-like dose dependent effects on glucose transport activity of fat cells (Obermaier-Kusser et al., 1989) (Figure 2.5.2.1). Furthermore, in another experiment performed by the same group with isolated rat adipocytes and 3-O-methylglucose transport measurement, after 20 minutes of incubation, the IPO fraction from Actovegin achieved approx. 70% of glucose transport compared to Insulin. (Figure 2.5.2.2)



(Figure 2.5.2.1) Graph to show insulin like activity from IPO extract from Actovegin against insulin in isolated rat adipocytes. The curves show mean values  $\pm$  SEM. IPO fraction from Actovegin have similar dose dependent effects to glucose uptake compared to insulin. (Obermaier-Kusser et al., 1989)



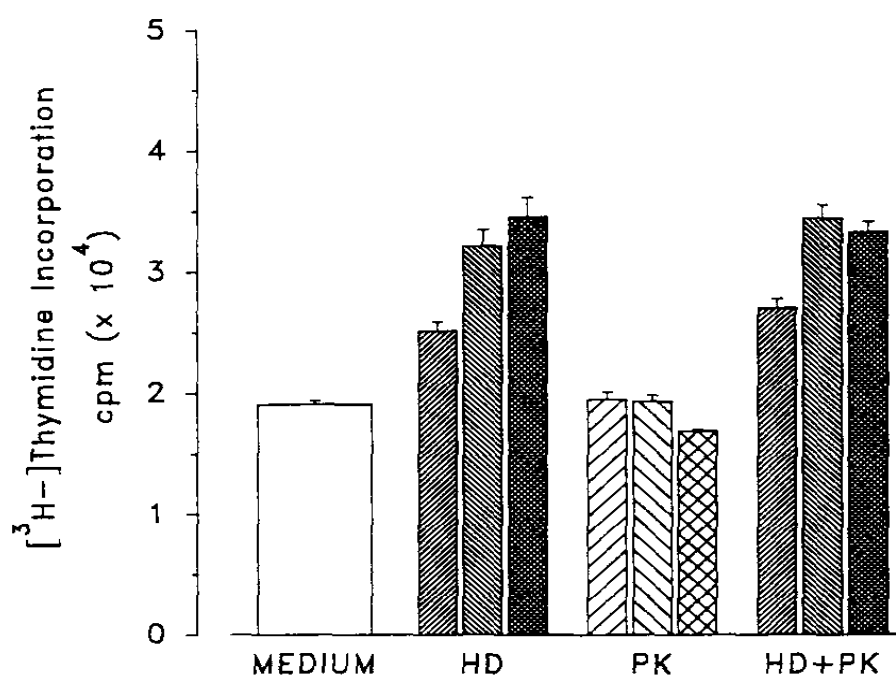
**(Figure 2.5.2.2)** Graph to show 3-O-methylglucose transport activity from IPO extract from Actovegin and insulin in isolated rat adipocytes over time. The curves show mean values  $\pm$  SEM. IPO fraction from Actovegin can achieve approx. 70% of Insulin -0-Methylglucose uptake. (Obermaier-Kusser et al., 1989)

Furthermore, IPOs do not induce carrier translocation or stimulate insulin-receptor kinase *in vitro* or *in vivo* (Obermaier-Kusser et al., 1989). It activates glucose transporters (GluTs) in the plasma membrane (Kelly et al., 1987) and improves glucose uptake by cells (de Groot et al., 1990). IPOs can contribute up to 70% of the maximum insulin effect of glucose transport and also stimulate the activity of certain enzymes including pyruvate dehydrogenase, the key enzymes of the citric acid cycle (Obermaier-Kusser et al., 1989), hence improve the efficiency of the respiration process via an independent route.

Schoenwald et al (1991) suggested that the active fractions in Actovegin were strongly negatively charged and were thought to be phosphorylated and/or sulfated oligosaccharides of approximately 3K Dalton

molecular weight and different to the IPO fraction reported by other studies (Schoenwald et al., 1991).

Actovegin has a synergistic effect on cell proliferation *in vivo* with Epidermal growth factor (EGF), Basic fibroblast growth factor (bFGF), and Endothelial Cell Growth Factor (ECGF), causing an increase in cell numbers, increased activity of acid phosphatase and an improved level of thymidine incorporation compared to controls, as demonstrated in figure 2.5.2.3 (Schoenwald et al., 1991). As shown in Figure 2.5.2.3, these effects are resistant to proteinase K digestion, therefore the active compounds of Actovegin are unlikely to be growth factors or their derived fragments (Schoenwald et al., 1991). The trivial nutritive effects of Actovegin were excluded as a mixture of the same level of amino acids and substrates did not stimulate proliferation or have insulin like activity *in vivo* (Schoenwald et al., 1991).



(Figure 2.5.2.3) HD – Actovegin group, PK – proteinase K group, HD+PK – proteinase K digested IPO group. ▨ 0.1 mg/ml; ▩ 0.25 mg/ml; ▤ 0.5 mg/ml. There was no significant difference in the thymidine incorporation between the Actovegin and Actovegin with proteinase group in all conditions (Schoenwald et al., 1991).

From the results of the pre-clinical studies, the properties of the active compounds in Actovegin are summarized in Table below:

<b>Property</b>	<b>Determined by</b>
Molecular weight approx. 3KD	Gel filtration
Strong negative charge, pKa < 2	Anion exchange
Bound phosphate and/or sulphate	Analysis prior to and post hydrolysis
Insensitive to protease	Proteinase K digestion
Un-extractable by organic solvents	Chloroform/methanol/HCl-extraction
contains mainly oligosaccharides	Analysis prior to and post hydrolysis

### 2.5.3 Legality

Actovegin® has received a great deal of media attention in recent years, especially surrounding its use in sports medicine. In 2009, a sports physician was arrested with this "performance-enhancing drug", while an editorial in a sports medicine journal strongly questioned the evidence base for using this drug in the treatment of acute muscle injuries (Franklyn-Miller et al., 2011). There is also a report suggesting that Actovegin might have induced anaphylactic shock in a cyclist (Maillo, 2008). However, as discussed in 2.6.3, there are also good safety results from a large multi-centre randomized control trial (Ziegler et al., 2009). Nevertheless, Actovegin has received much publicity and there are also many anecdotal beliefs surrounding this drug (Franklyn-Miller et al., 2011). Currently, Actovegin is not on the World Anti-Doping Agency (WADA) prohibited substance list (WADA, Accessed 21st April, 2011). However, many athletes, coaches and doctors still have reservations about its use in sports medicine. In this section, the evidence of the potential role of Actovegin in sports medicine is discussed.

Besides its clinical properties, there are anecdotal beliefs among athletes that Actovegin® possesses an oxygen carrying capacity and has the potential to enhance oxygen uptake which leads to better performance. Although these claims are not based on any objective scientific evidence or published clinical reports, the IOC announced in December 2000 that Actovegin was banned under the classification of blood-doping agents. Two months later, however, the IOC lifted the ban as there was no evidence that

Actovegin actually enhances performance (Tsitsimpikou et al., 2009). So far, there have not been any sports related studies or performance testing with this drug on healthy individuals. Currently, the intramuscular use of Actovegin is not prohibited in or out of competition for any given sport according to the latest search (2010) of Global Drug Reference Online (Global DRO), which is approved by UK Anti-Doping (UKAD), the Canadian Centre for Ethics in Sport (CCES), the United States Anti-Doping Agency (USADA) and WADA. According to the latest 2011 WADA prohibited list, Actovegin is not prohibited in any sport. Furthermore, WADA has issued a specific guidance on Actovegin on their website (WADA, Accessed 21st April, 2011). According to Section M2 of the WADA code, the volume of intravenous injection of any non-prohibited substance must not exceed 50 ml with a simple syringe and further serial injections must be at least 6 hours apart. Therefore, it should be stated clearly here that Actovegin® cannot be administered by intravenous infusion or single intravenous injection with a volume exceeding 50 ml (WADA, Accessed 21st April, 2011). Further more write evidence was obtained from the FA in the UK to confirm that the use of Actovegin is not prohibited in or out of competition. (see appendix A)

#### **2.5.4 Areas identified for further investigation in this PhD**

Wound healing is heavily dependent on oxygen, glucose and ATP. It relies on the capacity of single cells to migrate into the area of the wound and replication of these cells. Actovegin is likely to have membrane stabilising effects in ischaemic cells. This may be due to the presence of negatively charged IPO, shifting the cells to the direction of oxidized substrates. In the immediate post ischemic period, these factors may help cellular recovery. Its insulin and growth factor synergistic properties could also be beneficial and make the initial recovery period more efficient. Therefore, Actovegin is assumed to be useful in circulatory disturbances and post ischaemic events. With regards to muscle injuries, the features of Actovegin listed above could be potentially beneficial. In the next section, *in vitro* and *in vivo* published studies will be explored and discussed.

## 2.6 Literature review of Actovegin

### 2.6.1 *in vitro* and *in vivo* studies

Over the past 60 years, researchers have endeavoured to identify the active ingredients in Actovegin, but have been unsuccessful. Studies *in vitro* have suggested that it promotes oxidative metabolism and shifts the redox-balance of cells into the direction of oxidised substrates. Actovegin was therefore initially thought to have protective effects against hypoxic cell injury (Schoenwald et al., 1991). Hoyer *et al* demonstrated that Actovegin does not directly influence cells during the ischaemic period as the intracellular levels of glucose and lactate are similar to untreated animal cells during this period (Hoyer and Betz, 1989). Therefore, its mechanism of action is thought to improve the efficacy of cell's energy balance in the post-ischaemic metabolic events and interrupt the process of cell damage to avoid further cell death (Hoyer and Betz, 1989).

Studies on oxygen uptake, metabolism and hypoxia have been performed in different animal models like rat, guinea pig and dog. Jager et al, published a study on the first evidence that Actovegin<sup>®</sup> exerts effects on cellular respiration of purified rat liver mitochondria: oxygen consumption was significantly enhanced (Malaker and Sellwood, 1970). De Groot et al, examined the effect of hypoxia on isolated rat hepatocytes using a trypan blue survival test: hypoxic conditions in the presence of Actovegin<sup>®</sup> led to a significantly higher survival (de Groot et al., 1990). In line with these observations, Schäfer analysed the oxygen uptake in rat liver parenchymal cells under the influence of Actovegin<sup>®</sup> in comparison to placebo and described an enhancing effect by Actovegin<sup>®</sup> on cell respiration (Schreier et al., 1993). Reichel et al, showed that the augmenting effect of Actovegin<sup>®</sup> on oxygen intake and glucose uptake in cellular systems relies on an enhancement of the phosphorylating properties of the cells in that oxygen uptake increases by up to 40% in the presence of Actovegin<sup>®</sup> (Malaker and Sellwood, 1970). Kuninaka et al assessed mitochondrial respiration by

polarography in a rat liver mitochondria preparation and suggested that oxidative phosphorylation is significantly augmented by the addition of Actovegin (Kuninaka et al., 1991).

Rammler et al, photometrically determined whether Actovegin<sup>®</sup> exerts an effect on the ATP-concentrations of the brain over 240 min (Charlesworth et al., 1975). The results show that Actovegin<sup>®</sup> possesses the potency to increase the ATP in tissue. This result was one of the first observations to clearly show that Actovegin<sup>®</sup> has a direct bearing on metabolism in the brain. In later years, Chanh et al, demonstrated that Actovegin can also be a beneficial support to oxygen consumption and metabolism of heart tissue (Chanh et al., 1980).

Actovegin<sup>®</sup> has the capability to stimulate cellular metabolism, increase oxygen uptake and performance of energy production. Therefore, it is assumed to be a stimulant agent to support energy-deprived and starved tissues. It should have a positive effect on wound healing.

Inositol phosphate oligosaccharides (IPO) are one of the ingredients in Actovegin (Obermaier-Kusser et al., 1989). The IPOs have been shown to have a partial insulin-like effect on the glucose transport activity of adipocytes but do not induce carrier translocation or stimulate insulin-receptor kinase *in vitro* or *in vivo* (Obermaier-Kusser et al., 1989). It has been reported that IPOs activate glucose transporters (GluTs) hence promoting glucose uptake by cells (de Groot et al., 1990). IPOs can contribute up to 50% of the maximum insulin effect on glucose transport and can also stimulate the activity of certain enzymes including pyruvate dehydrogenase, the key enzymes of the citric acid cycle (Obermaier-Kusser et al., 1989). It has a synergistic effect with insulin and promotes glucose activity when insulin levels are suboptimal, but does not alter the peak effect (Obermaier-Kusser et al., 1989). A strongly negatively charged sulphated oligosaccharide of approximately 3000 Daltons molecular weight has also been isolated from Actovegin, which is different to the IPO fraction reported by the other studies

(Schoenwald et al., 1991). This fraction has a similar effect to IPO but with less effectiveness.

Actovegin has a synergistic effect on cell proliferation *in vivo* with Epidermal growth factor (EGF), Basic fibroblast growth factor (bFGF), and Endothelial Cell Growth Factor (ECGF), causing an increase in cell numbers, increased activity of acid phosphatase and an improved level of thymidine incorporation compared to controls (Schoenwald et al., 1991). These effects are resistant to proteinase K digestion, therefore the active compounds of Actovegin are unlikely to be growth factors or their derived fragments (Schoenwald et al., 1991). The trivial nutritive effects of Actovegin were excluded as a mixture of the same level of amino acids and substrates did not stimulate proliferation or have insulin like activity *in vivo* (Schoenwald et al., 1991).

### **2.6.2 Actovegin in Wound healing**

Neinhardt et al, observed that Actovegin<sup>®</sup> treatment leads to wound closure approximately 2 days earlier as compared to controls. Mochida et al tested the influence of Actovegin<sup>®</sup> on the tensile strength of an incised abdominal muscle in animals; it concludes that Actovegin<sup>®</sup> significantly supports wound healing (Mochida et al., 1989).

Wound healing heavily relies on the capacity of single cells to migrate into the area of the wound and replication of these cells. Therefore, several studies investigated the migration-stimulative effects of Actovegin<sup>®</sup> in cell culture assays. Miltenburger et al, assessed the influence of Actovegin<sup>®</sup> on the functions of fibroblasts and keratinocytes and found that cell migration was highly significantly affected by the addition of Actovegin<sup>®</sup> alone, and even more stimulated by the co-administration of Actovegin<sup>®</sup> and TGF- $\beta$  (Miltenburger et al., 1994). Furthermore, the keratinocyte outgrowth was distinctly enhanced in the presence of Actovegin<sup>®</sup>, hence the trophic possibilities of tissue to actively rebuild tissue integrity.



Actovegin also affects the functional integrity and activity of immune cells that enhance tissue remodelling and thereby wound healing. It exerts significant effects on human monocytes cultured *in vitro* in the presence of human serum. Spessotto et al, prepared monocytes from buffer coated and assayed monolayer cultures under a microscope for morphology and cell density, as well as protein content (Spessotto et al., 1993). Cell density was increased in all concentrations of applied Actovegin<sup>®</sup>, as well as the cell protein content. Actovegin<sup>®</sup> partly acted like a substitute for blood serum favours survival and differentiation of monocytes in culture (Spessotto et al., 1993).

Radiation induced damage in cells and tissues has been regarded as a crucial test case for the protective and supportive action of many different pharmacologically active substances. Actovegin<sup>®</sup> has been studied regarding the effects of radiation on animals and exerted positive effects. Bauer and Locker investigated the effects of Actovegin<sup>®</sup> on the survival of mice that have been irradiated by a lethal dose of gamma radiation (Bauer and Locker, 1974). The application of Actovegin<sup>®</sup> led to a concentration-dependent better survival of the Actovegin<sup>®</sup>-treated animals after 30 days following irradiation. Barth and colleagues, examined the time-dependence of the injection of Actovegin<sup>®</sup> in relation to the damage produced by irradiation. The results revealed an optimal time point for the injection of three hours after the irradiation had taken place. Prophylactic treatment with Actovegin<sup>®</sup> on 6 consecutive days before the radiation did not reveal any preventive effects of Actovegin<sup>®</sup>. In a similar study, Basu et al, examined the radio-protective effects of Actovegin<sup>®</sup> in adult rats (Basu et al., 1985) ; while control animals died within 30 days, the rats treated with Actovegin<sup>®</sup> 1 hour before irradiation had a statistically significant higher survival rate, as already described in the study of Bauer and Locker, thereby confirming their results (Bauer and Locker, 1974). In contrast, Tamou and Trott, described findings on the occurrence of radiation-induced ulcers in the rectum of rats where the results in the Actovegin<sup>®</sup> group did not differ from control conditions (Tamou and Trott, 1994).

Sigdestad and colleagues, demonstrated the intracellular effects of Actovegin® in irradiated cells in culture (Sigdestad et al., 1988). Although, there was no difference in cell survival *in vitro*, the cells treated with Actovegin® exhibited much less single-strand DNA breaks as compared to the control cells. This also supported the effects described earlier that pointed to a beneficial effect of Actovegin® on cell survival, also on the level of the cell nucleus.

Actovegin is likely to have membrane stabilising effects in ischaemic cells. This may be due to the presence of negatively charged oligosaccharides, shifting the cells to the direction of oxidized substrates. In the immediate post ischaemic period, these factors may help cellular recovery. Its insulin and growth factor synergistic properties could also be beneficial and make the initial recovery period more efficient. Therefore, Actovegin is assumed to be useful in circulatory disturbances and post ischaemic events.

### **2.6.3 Clinical reports on Actovegin indirectly related to muscle injuries**

Although the active ingredient(s) in Actovegin are yet to be identified, many clinical studies have indicated its safety and effectiveness. There has been one case report of a possible anaphylactic reaction related to the use of intravenous Actovegin injections by an amateur cyclist. In this report, the diagnosis of “anaphylactic reaction” was not confirmed with any biochemical testing and the patient improved with broad spectrum antibiotics. The author later stated in the communication letter that this patient had used intravenous Actovegin once before with no adverse reaction, thus an anaphylactic response is unlikely. Although it is possible that the first use of Actovegin had predispose or prime the immune cells to react severely following the second or subsequent administrations(Maillo, 2008). As the patient improved on broad spectrum antibiotics, the most likely cause for this acute shock was due to bacterial contamination during injection, not anaphylactic reaction to the drug (Maillo, 2008).

Pfrringer et al, in a double-blinded placebo controlled single centre study with 60 recreational athletes, demonstrated that ultrasound guided para-tendon injection of Actovegin was effective in the treatment of Achilles tendinitis (Pfrringer et al., 1994). The tendon cross section measurement was reduced significantly ( $p<0.0001$ ), patients' physical activity and perception of pain was also improved ( $p<0.002$ ) in the treatment group (Pfrringer et al., 1994). The overall clinical outcome, which is measured by patient satisfactory score was significantly better in the Actovegin group ( $p<0.0001$ ) and no adverse events were reported in this study (Pfrringer et al., 1994). Although it is a relatively small-scale study with limited power, it is a well-conducted study that was featured in a Cochrane review.

Ziegler et al 2009, reported a double-blinded multi-centre randomized control study with 567 patients with type 2 diabetes, in which 281 patients treated with 20 daily high dose intravenous Actovegin infusions followed by 1800mg of Actovegin daily for 120 days in the treatment of symptomatic diabetic polyneuropathy compared to placebo (Ziegler et al., 2009). Total Symptom Score (TSS) of the lower limbs, vibration perception threshold (VPT), Neuropathy Impairment Score of the Lower Limbs (NIS-LL), and quality of life (SF-36) were used as patient reported outcome measures, which all show statistically significant differences compared to placebo and baseline scores ( $p<0.05$ ). Furthermore, no anaphylactic reactions were reported with this study after 5,620 infusions and the adverse effect profile was no different compared to placebo (Ziegler et al., 2009).

#### **2.6.4 Evidence in muscle injuries**

The treatment of muscle tears with intramuscular Actovegin was first published by Pfister and Koller in 1990 (Pfister and Koller, 1990). Their partially blinded case control study with 103 patients, showed a reduction in recovery time with the treatment group of 5.5 weeks compared with 8.3 weeks for the control group (Pfister and Koller, 1990). However, in this study, patients were recruited from various sports and competitive levels and the treatment regimen and the rehabilitation protocol were not standardised. The

diagnosis of muscle injury was only based on clinical findings and was not graded according to MRI. Actovegin was mixed with local anaesthetics before injection, therefore its pharmacodynamics and pharmacokinetics were altered. The treatment regimen in this study was not standardised; the number of injections ranged from 3-8 and the final outcomes were based on patients and various clinicians' subjective observations and there was no pre-injury data to compare outcomes. Despite the limitations of the study, it is the first published study regarding its use as an intra-muscular injection in the treatment of muscle injuries and no adverse events were reported in this paper. Since Pfister and Koller (1990), there has not been any published report in the medical literature regarding the use of Actovegin in muscle injuries until Wright-Carpenter et al in 2004. In this small non-randomised study, Autologous Conditioned Serum (ACS) was compared to Actovegin. The Actovegin group in this study was created by the retrospective analysis of the Pfister and Koller study; therefore it should not be seen as new evidence.

Ziegler et al (2009) reported a double-blinded multicentre randomized control study with 567 diabetic patients treated with Actovegin for diabetic neuropathies which was discussed above (Ziegler et al., 2009). Although muscle assessments were not the primary objective for this study, it was measured as a secondary objective to monitor the progression of treatment. There was no significant difference in the adverse event rate compared to placebo; Actovegin did not improve muscle strength ( $p = 0.731$ ) or muscle reflex ( $p=0.571$ ) (Ziegler et al., 2009). Therefore, it is reasonable to conclude that Actovegin is a safe drug and does not have anabolic or ergogenic effects on muscles.

### **2.6.5 Summary**

There are many studies confirming the safety and effectiveness of Actovegin. It is evident that Actovegin is beneficial to ischaemic cells (2.6.1) and wound healing (2.6.2.). Furthermore Actovegin has a good clinical track record and good safety profile as discussed in 2.6.3. There has only been a single clinical study published in the literature with regards to the use of Actovegin in the muscle injuries, however it is a single drug study (2.6.4). There are currently no clinical studies reporting the use of Actovegin as a single agent in the treatment of muscle injuries.

## 2.7 Research question and Hypothesis

As discussed in sections 2.3 and 2.4, muscle injuries are very common sports related injuries. They result in significant morbidity and time lost from training and competitions. As discussed in section 2.4.2.3, there have been many new potential treatment options available on the market. In 2008, a current best practice paper from the British Journal of Sports Medicine “The early management of muscle strains in the elite athlete: Best practice in a world with a limited evidence basis” (Orchard et al., 2008a) on the treatment of muscle injuries by a panel of experts, suggested that injection therapy could be potentially useful in treating muscle injuries. For further details, please see section 2.4.2.3. With regards to injection therapy, platelet-rich plasma (PRP) and Actovegin have attracted significant interest in sports medicine. Although the evidence is limited for both of these substances, PRP has received many so-called expert opinions and the IOC has encouraged its use (Engebretsen et al., 2010). On the other hand, Actovegin, a licenced drug that has a good clinical track record for 60 years (further details in section 2.5) has caused a lot of controversy and negative media attention.

Section 2.4.2.3 has discussed the limitations with the studies regarding PRP; furthermore there are many methods of producing so-called “PRP”. The concentration of platelets and growth factors in PRP alter between methods of preparation and the brand of equipment. It also differs between individual’s blood biochemistry and hydration status. Therefore it is impossible to standardise treatment and compare results between studies. Actovegin is a deproteinised haemodialysate of ultra-filtered calf serum, although it is originally from a different species, it shares a similar principle to the PRP (see section 2.5). Due to the issues discussed above, the quality and quantity of PRP varies between injections; currently there is no consensus on the molecular weight cut off for PRP. On the other hand, Actovegin is manufactured through a more robust method with a molecular weight cut of point of 6 kD and strict quality control as a licenced drug (For further details please see 2.5.1 and 2.5.2). One could view that Actovegin is a fine filtered “PRP” from Bovine serum, which is standardised and well regulated by the

manufacturer. Through the review of literature in section 2.5, there is good *in vitro*, and clinical evidence suggesting the efficacy of Actovegin in the treatment of various conditions. There has only been one published study reporting the use of Actovegin in the treatment of muscle injuries, its limitations have been discussed in section 2.6.4. Furthermore, there have been anecdotal reports from high profile athletes suggesting that Dr. Mueller-Wohlfahrt's injection regime produces good clinical results in treating muscle injuries. In this injection regime, Actovegin is mixed with Traumeel and local anaesthetics for injection, therefore the biochemical properties, pharmacodynamics and pharmacokinetics of each drug are altered and unpredictable. Although it is possible that a specific quantity mixture of these drugs produce the desirable therapeutic effects, it is not practical to investigate this effect scientifically as the effect of each of the substances are unknown. As demonstrated in the literature review in section 2.6, Actovegin is a drug with 60 years of track record and it is beneficial to ischaemic cells and wound healing. Therefore Actovegin is the most likely "active" substance in Dr. Mueller-Wohlfahrt's regimen. In this PhD thesis, in order to avoid the unpredictable nature of poly-pharmacy, only Actovegin will be investigated.

As discussed in section 2.6, Actovegin is a drug that has good published results in the treatment of some medical conditions, but its use in the treatment of muscle injuries is limited. No studies have reported its use as a single agent to treat muscle injuries. Actovegin contains physiological components, electrolytes and essential trace elements (Nycomed). The active ingredients in this mixture have yet to be identified (see section 2.5.1). Although Actovegin is a licenced drug in Europe, it does not have marketing authority in the UK. Therefore it is not possible to obtain this drug in a regular pharmacy in the UK. In order to progress with this PhD, the supply of Actovegin will have to be established via a specialist route, which will be discussed in the method section in 3.2.

The biochemical events following skeletal muscle injuries and repair are driven by cytokines, monocytes and leukocytes. The speed and quality of muscle healing are dependent on the inflammatory process. As demonstrated

in the section 2.3.3, in order to alter the speed or quality of muscle repair, the substance must be able to modulate the inflammatory process. Recent studies suggest that there are two distinct subpopulations of macrophages sequentially involved in the inflammatory process (Garrett, 1996b, Grefte et al., 2010, Jarvinen et al., 2000). The balance between the pro-inflammatory macrophages (CD68<sup>+</sup>) and the anti-inflammatory macrophage (CD163<sup>+</sup>) will dictate the progression from the destruction phase to the repair phase of the muscle healing process as detailed in section 2.3.3. Therefore, this PhD will focus on the role of Actovegin in the inflammatory process, especially in the acute phase with the CD163<sup>+</sup>, CD68<sup>+</sup> sub group of macrophages. *In vitro* studies will be performed to establish the appropriate type of cell culture and method of analysis, as detailed in section 3.4. Depending on the results of the *in vitro* studies, a treatment regimen will be established and a clinical study will be performed to investigate the clinical efficacy of Actovegin. In this PhD, a clinical study will only be performed when there are sufficient findings to support the use of Actovegin in muscle injury from the *in vitro* study. Clinical assessment, treatment and rehabilitation regimen will be established and ethical approval will be sought prior to the commencement of the clinical study. The clinical study is detailed in chapter 4.



**Research question:**

There are two elements to this PhD thesis; the objective is to investigate the role of Actovegin in the acute muscle injuries. There have not been any study investigate the effect of Actovegin to the inflammatory process. The *in vitro* study section in this PhD is to investigate the biochemical role of Actovegin in the acute inflammatory process following muscle injuries. Further more there as not been any clinical studies report the use of Actovegin as a single agent in the treatment of acute muscle injuries. The clinical section in this PhD is to investigate the clinical efficacy of the Actovegin single agent treatment protocol developed based on the *in vitro* study from this PhD.

**Hypothesis:**

- 1) Actovegin has a biochemical role in the acute inflammatory process following muscle injuries.
- 2) Actovegin treatment regimen developed from this PhD can influence clinical outcome in treating acute muscle injuries.

# Chapter Three

## *in vitro* study

### 3.1 Introduction

In section 2.7 the research question and hypothesis is clearly stated. The pathophysiology and biochemical events following skeletal muscle injuries and repair is complex as discussed in section 2.3.3. This chapter will describe the *in vitro* element of the PhD to investigate the role of Actovegin in the inflammatory process. Furthermore, standardisation and acquisition of Actovegin would be discussed in this chapter.

Hypothesis for the *in vitro* studies in this PhD:

Actovegin has a biochemical role in the acute inflammatory process following muscle injuries.

### 3.2 Actovegin acquisition

In section 2.4, the background information of Actovegin is discussed. Although Actovegin is a licenced drug in Europe, it does not have marketing authority in the UK. Therefore it is not possible to import Actovegin directly from the manufacturer or purchase it from a standard UK pharmacy. In order to progress with this PhD, the supply of Actovegin must first be established.

The Medicines and Healthcare Products Regulatory Agency (MHRA) is an executive agency of the Department of Health for the UK government, responsible for the regulation of any medicinal product. The MHRA operates a system of licensing before the marketing of medicines. Medicines that meet the standards of safety, quality and efficacy are granted a marketing

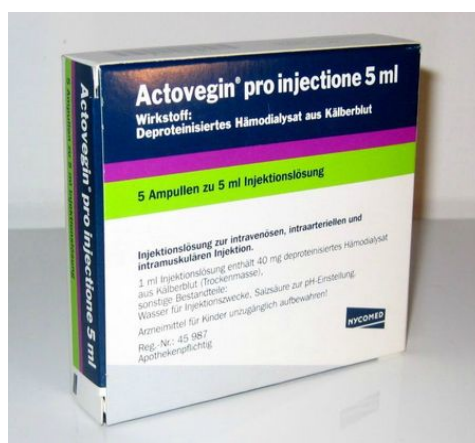
authorisation. Under regulation 46 of The Human Medicines Regulations 2012 (SI 2012/1916), all medicines must have a marketing authorisation before they can be prescribed or sold. In addition, the companies that are involved in all stages of the manufacture and distribution of the product need to have licensed. Applications for marketing authorisation are assessed by medical, pharmaceutical and scientific staff at the MHRA, working in multidisciplinary functional teams. Usually applications for marketing authorisation come mainly from the pharmaceutical industry but anyone with the necessary supporting data may apply. The task of evaluating the beneficial effects against the possible harmful effects of any medicine is complex. Evaluation takes into account the nature of the active ingredients, the dosage form (for example, tablet or liquid), the nature of the disease or condition to be treated, the effective dose that needs to be given, the type of patient and the duration of treatment.

In order to use Actovegin in the UK, I contacted the MHRA and requested the drug to be imported via a specialist prescription route from a fully UK GMC registered medical doctor. However, this only partially fulfilled the MHRA's requirement. As Actovegin is a bovine animal product, further documentation of terms were needed to demonstrate minimal risk of transmitting animal spongiform encephalopathy agents (BSE) via Actovegin administered to humans before it could be imported into the UK. Therefore, I contacted the manufacturer, Nycomed, and arranged a meeting in their headquarters in Zürich with a team of clinicians and scientists involved in the development of Actovegin. Through that meeting I was able to obtain a copy of the Actovegin European drug license and a copy of the certificate to prove that Actovegin is free from BSE (appendix B). After further discussion with the MHRA with these documents, the agency gave their approval to import Actovegin to the UK through a specialist prescription. In order to comply with the regulations and the supply chain, a specialist pharmacy was used to import the drug. All the Actovegin was checked by a Royal Pharmaceutical Society approved Qualified Person (QP) to ensure quality and compliance. It is a legal requirement for a QP to batch certify medicinal products prior to clinical use. The involvement of the MHRA, Nycomed, specialist Pharmacy

and the QP have ensure the legality and authenticity of the source of Actovegin used in this PhD study.

In section 2.5.1, the manufacturing process and background information of Actovegin have been discussed. In this PhD, the clinical study could potentially involve high-level professional footballers, therefore further in-house testing will be performed to ensure quality and that the Actovegin is free from contamination. Further details are described in section 3.3. Furthermore, approval from official governing bodies such as WADA, UK sports and the FA will be confirmed prior to the clinical aspect of this PhD.(see appendix A).

### 3.3 Standardisation of Actovegin



(figure 3.3) 5x5ml packaging of Actovegin for injection

The packaging of injectable Actovegin may vary worldwide. In Western Europe, there only 2 sizes and quantities officially produced by Nycomed. Actovegin is a light yellow-brown colour clear aqueous solution, its concentration is 40 mg/ml, and can be packaged as a box of twenty five 2ml ampules or a box of five 5ml ampules. In this PhD, only sealed boxed 2ml ampules will be imported to ensure standardisation.

### 3.3.1 MALDI ToF spectrometry

In order to perform standardisation for the Actovegin used in this PhD, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF) was used to try to establish a drug profile.

Matrix assisted laser desorption/ionization (MALDI) is a soft ionisation technique used in mass spectrometry, allowing the analysis of biomolecules such as DNA, proteins, peptides and sugars and large organic molecules such as polymers, dendrimers and other macromolecules, which tend to be fragile and fragment when ionised by more conventional ionisation methods. It is similar in character to electrospray ionisation in that both techniques are relatively soft ways of obtaining large ions in the gas phase, though MALDI produces many fewer multiply charged ions.

Samples (Actovegin) for analysis by MALDI are embedded in matrix crystals (3,5-dimethoxy-4-hydroxycinnamic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid) deposited on the surface of a sample plate that comprises one electrode of an ion accelerator. Desorption is triggered by a UV laser pulse. Matrix material heavily absorbs UV laser light, leading to the ablation of upper layer of the matrix material. The hot plume produced during ablation contains many species: neutral and ionized matrix molecules, protonated and deprotonated matrix molecules, matrix clusters and nanodroplets. Then the analyte molecules are protonated or deprotonated in the hot plume. Ablated species may participate in the ionisation of analyte, though the mechanism of MALDI is still debated. Pulsed and static electrical fields may be applied to accelerate and focus the ions in both space and time. The ideal ion source produces a narrow, nearly parallel beam with all ions of each  $m/z$  arriving at the detector with a flight time that is nearly independent of the initial position and velocity of the ions. The initial velocities used are less than 1000m/s and are independent of the mass of the ion. The velocity depends on the properties of the matrix and the laser fluence.

The time of flight (ToF) is measured relative to the time that the extraction pulse is applied to the source electrode. The extraction delay,  $\Delta t$ , is the time between the application of the laser pulse to the source and the extraction pulse. The linear analyser with pulsed acceleration provides excellent sensitivity for high-mass ions and can provide nearly constant low resolving power over a broad mass range, but an ion reflector is required for higher resolving power. This allows the effective length to be increased without increasing the other contributions to peak width. The time of flight can be calculated exactly depending on the distance and applied voltages.

3 ampules of Actovegin were given to the Cardiff CBS laboratory for MALDI-ToF analysis using the ABI 4800 MALDI-TOF/TOF Analyser with standard matrix (3,5-dimethoxy-4-hydroxycinnamic acid). A graph of ToF was plotted against the molecular mass. In the graphic analysis of all 3 Actovegin samples only Matrix (3,5-dimethoxy-4-hydroxycinnamic acid) peaks were identified. Therefore, MALDI-Tof mass spectrometry is not an appropriate method for drug profiling for Actovegin, as it did not provide any meaningful data regarding its components. However, this has also confirmed that there are no protein molecules in the Actovegin mixture.

MALDI-TOF is not a useful tool to standardise and batch test Actovegin for its composition. Therefore, an alternative method is needed to quantify the components in Actovegin. High Performance Liquid Chromatography (HPLC) is a separation technique used to separate components of a mixture; it is commonly used in pharmaceutical and toxicology analysis. This could be a useful method to create a drug profile and standardise Actovegin.

### 3.3.2 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying or purifying the individual components of the mixture. Chromatography can be described as a mass transfer process involving adsorption. A mixture of various components enters a chromatography process, and the different components travel through the system at different rates. These differential rates of migration occur as the mixture moves over absorptive materials providing separation. The smaller the affinities a molecule has for the stationary phase, the faster it travels, hence it travels further in the given time.

HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with a sorbent, leading to the separation of the sample components. The active component of the column, the sorbent, is typically a granular material made of solid particles (e.g. silica, polymers), 2-50 micrometres in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the sorbent particles.

The pressurised liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as "mobile phase". Its composition and temperature plays a major role in the separation process by influencing the interactions taking place between sample components and sorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole-dipole and ionic, most often a combination thereof.

HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50-350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC typical column dimensions are 2.1 - 4.6 mm diameter, and 30 - 250 mm length. Also HPLC columns are made with

smaller sorbent particles (2- 5 micrometre in average particle size). This gives HPLC superior resolving power when separating mixtures, which is why it is a popular chromatographic technique.

The sampler brings the sample mixture into the mobile phase stream, which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. The HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface areas are less retained as they are better integrated into water. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. Such surface hindrance typically results in less retention.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond.

Another important factor is the mobile phase pH, since it can change the hydrophobic character of the analyte. For this reason, most methods use

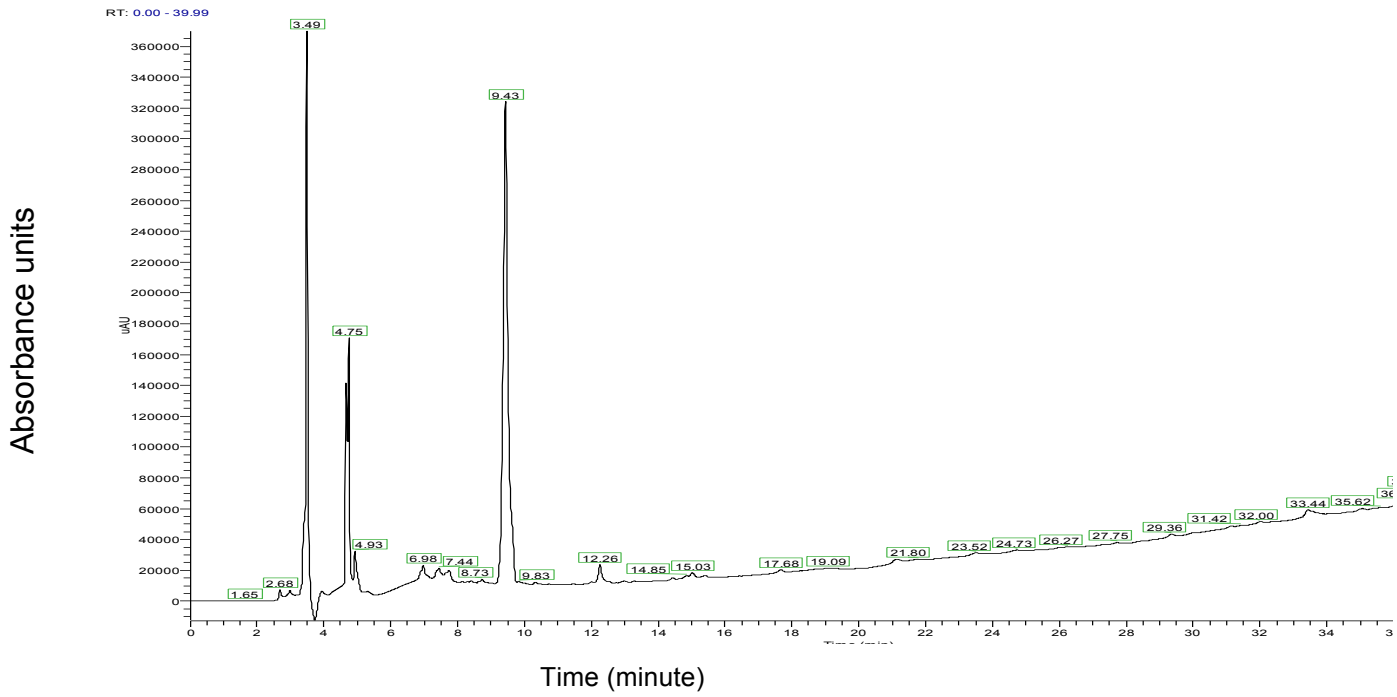


a buffering agent, such as sodium phosphate, to control the pH. Buffers serve multiple purposes: control of pH, neutralize the charge on the silica surface of the stationary phase and act as ion pairing agents to neutralize analyte charge. The effects of acids and buffers vary by application but generally improve chromatographic resolution. This chromatography approach separates components depending on their molecular weight and solubility; it does not require the use of a matrix. Therefore, HPLC could be useful in producing a drug prolife for Actovegin and will be used in this PhD.

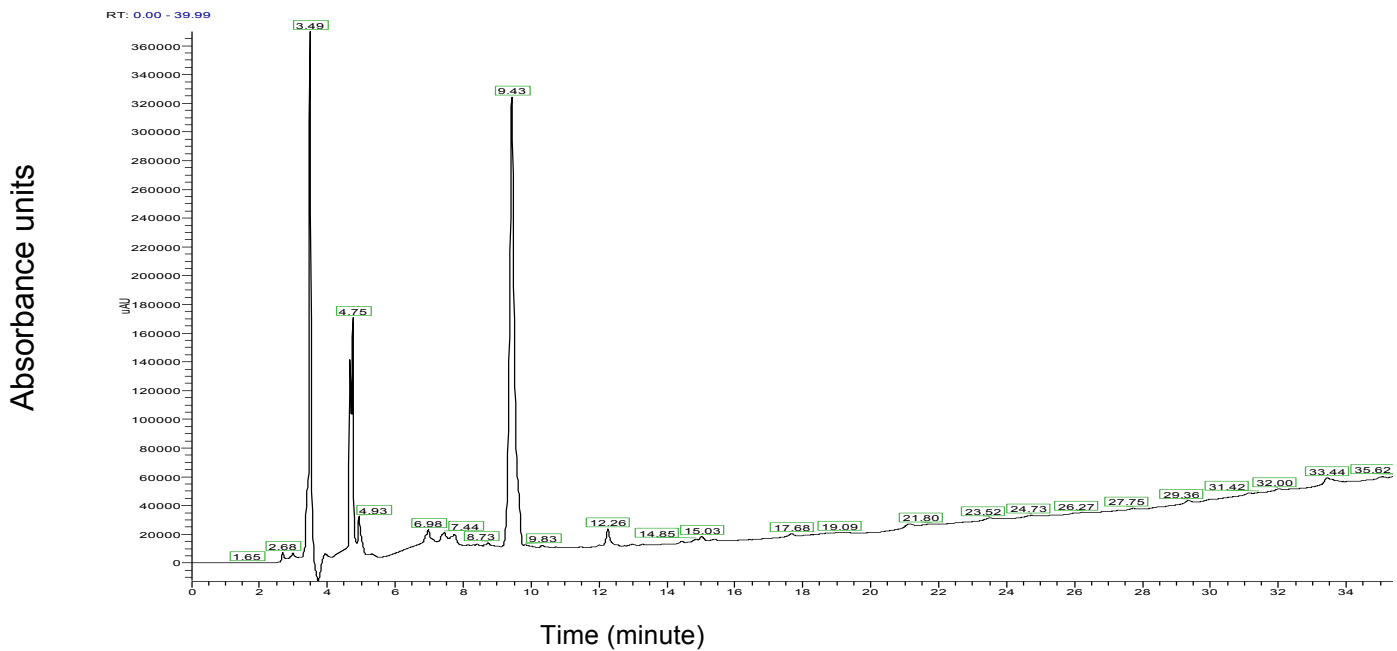


**(Figure 3.3.2a)** HPLC system used in Cardiff University to analyse Actovegin  
HPLC method:

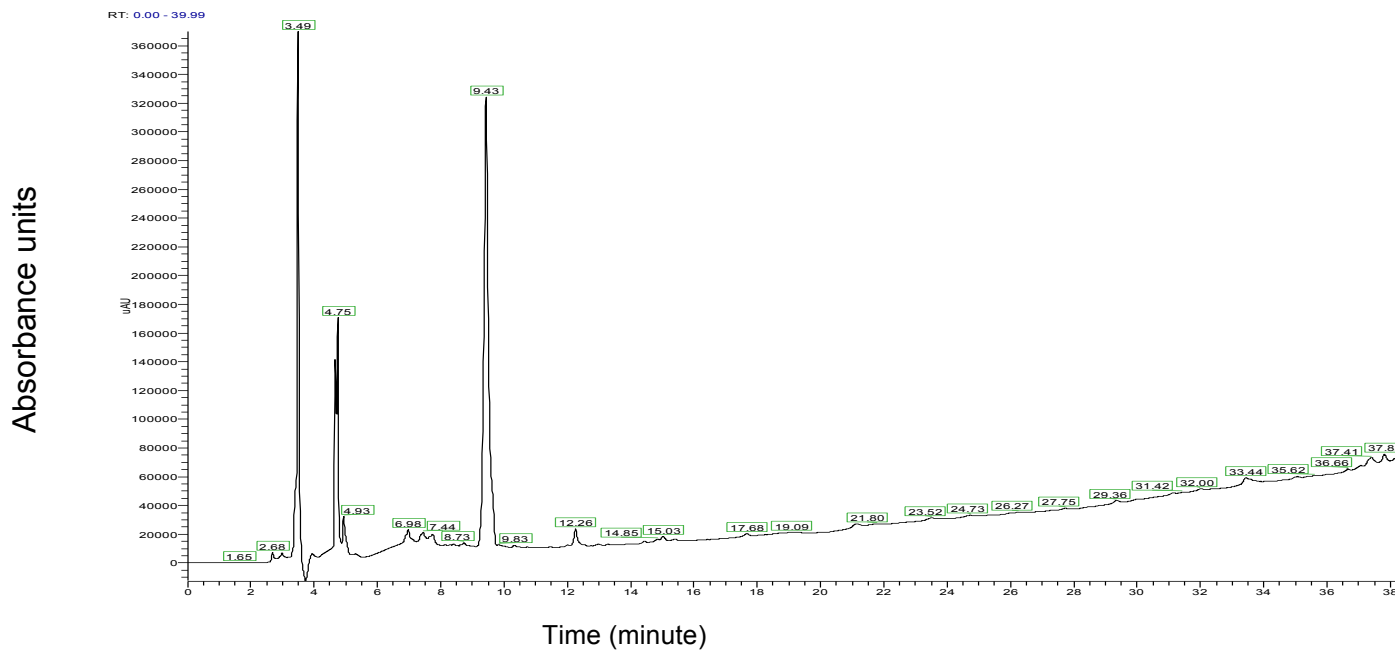
BLC20G Plus HPLC system (Buck-Chrom, Germany) was used to perform HPLC on Actovegin (figure 3.3.2a). A 2500mm x 4.6mm C 18 column (Phenomenex Ltd, Germany) was used for the HPLC. 100% Elga UHP water and 0.3% formic acid is mixed with 100% Acetonitrile and 0.3% formic acid. The mixed solvent ratio is used as per manufacturer recommendation. 20 ul of Actovegin (40mg/ml) was injected to the C18 column by an Auto-sampler with a flow rate of 1ml/min at 25°C. The peaks were detected using a thermo finnigan detector (uv6000LP, spectra system, Germany) at wavelength of 200-800nm. The results were interpreted via Xcalibur software (ver. 1.2, Thermo Scientific, USA). 3 independent samples were tested per ampule of Actovegin to ensure the consistency of the results.



(Figure.3.3.2.1) HPLC graph time against absorption of Actovegin sample – Test 1  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes



(Figure.3.3.2.2) HPLC graph time against absorption of Actovegin sample – Test 2  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes



**(Figure.3.3.2.3)** HPLC graph time against absorption of Actovegin sample – Test 3  
 3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes

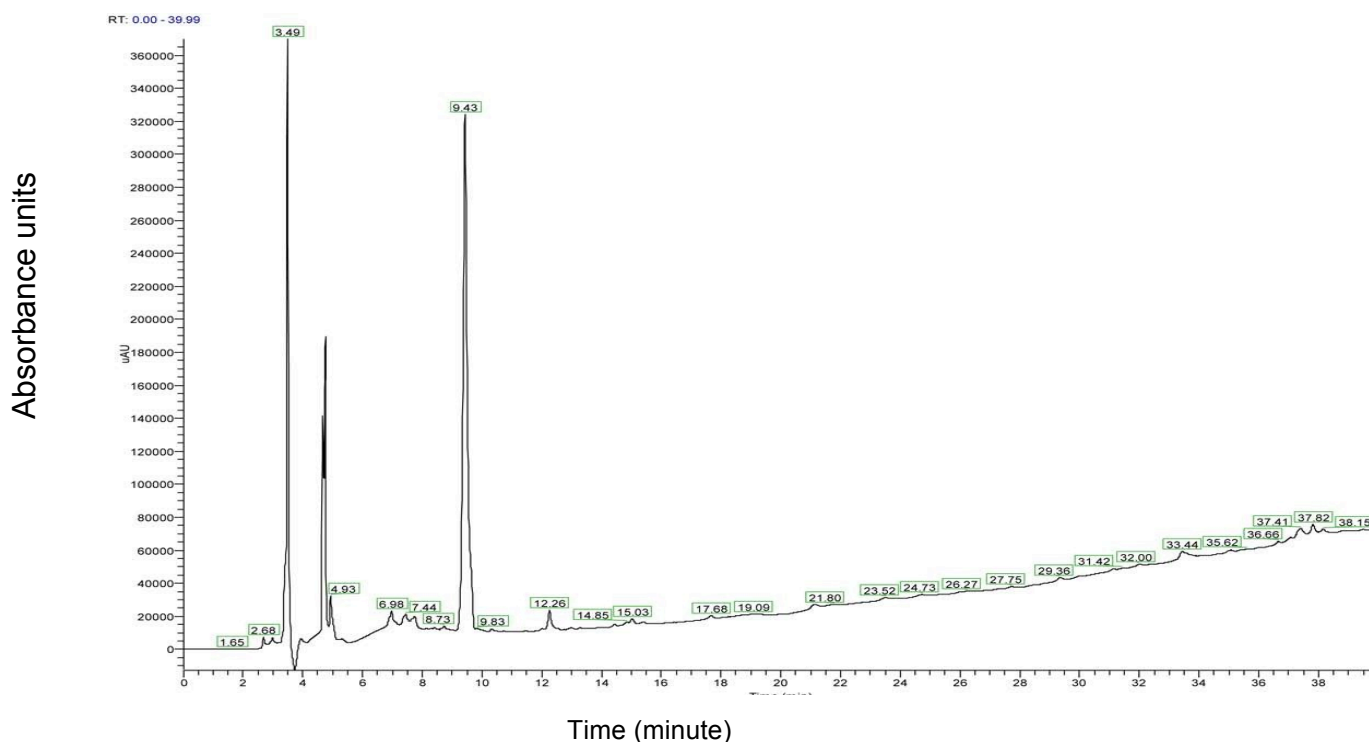
Results:

The peaks on the graphs represent separation of Actovegin against time (figure 3.3.2.1, 3.3.2.2, 3.3.2.3). There are consistent peaks at 3 minutes, 5 minutes and 10 minutes with all the Actovegin samples. The amplitude of the peaks was also within 10% of each other. Therefore, a HPLC “footprint” for Actovegin is formulated and can be used to standardise all future Actovegin batches.

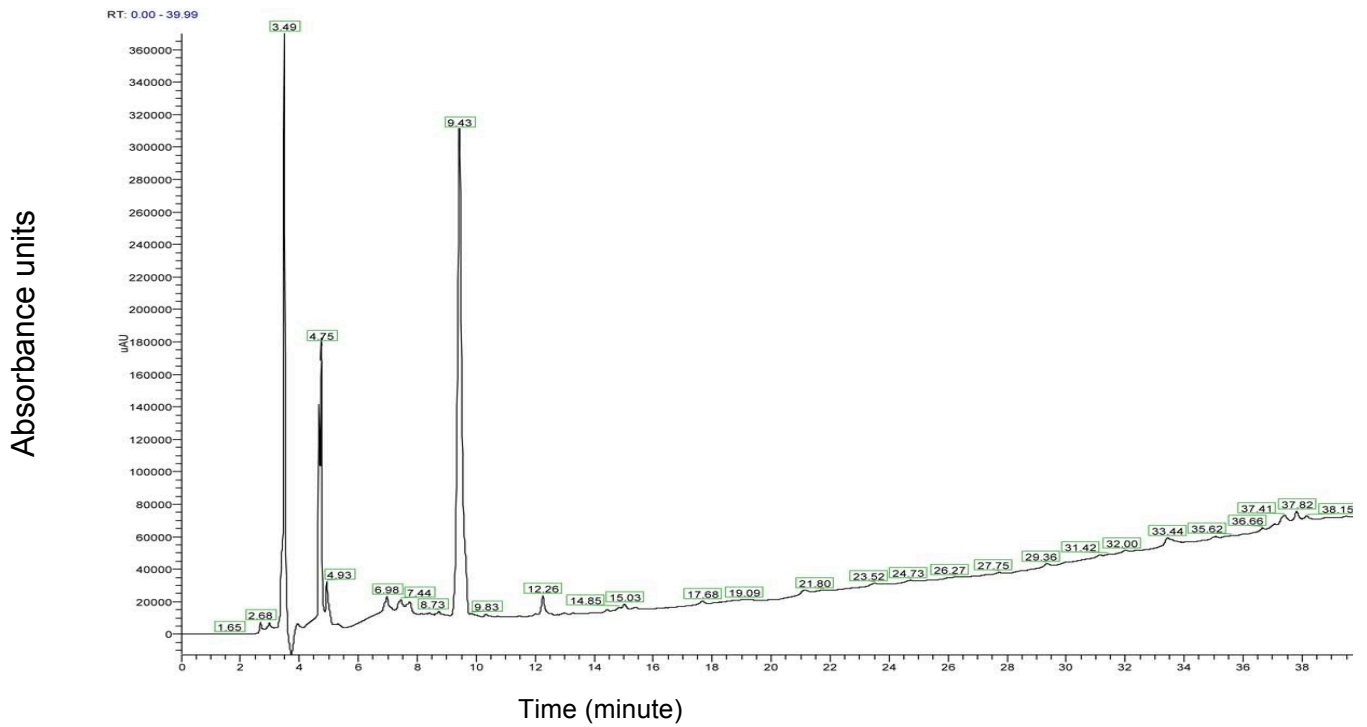
### 3.3.3 Batch testing

In order to standardise the quality of Actovegin, due to the reason discussed in 3.2, all imported Actovegin was batch tested against the “footprint” drug profile obtained from the HPLC analysis above, with the same equipment and solvent ratio. Only Actovegin matched with all 3 peaks within 10% in amplitude to the “footprint” will be used in this PhD.

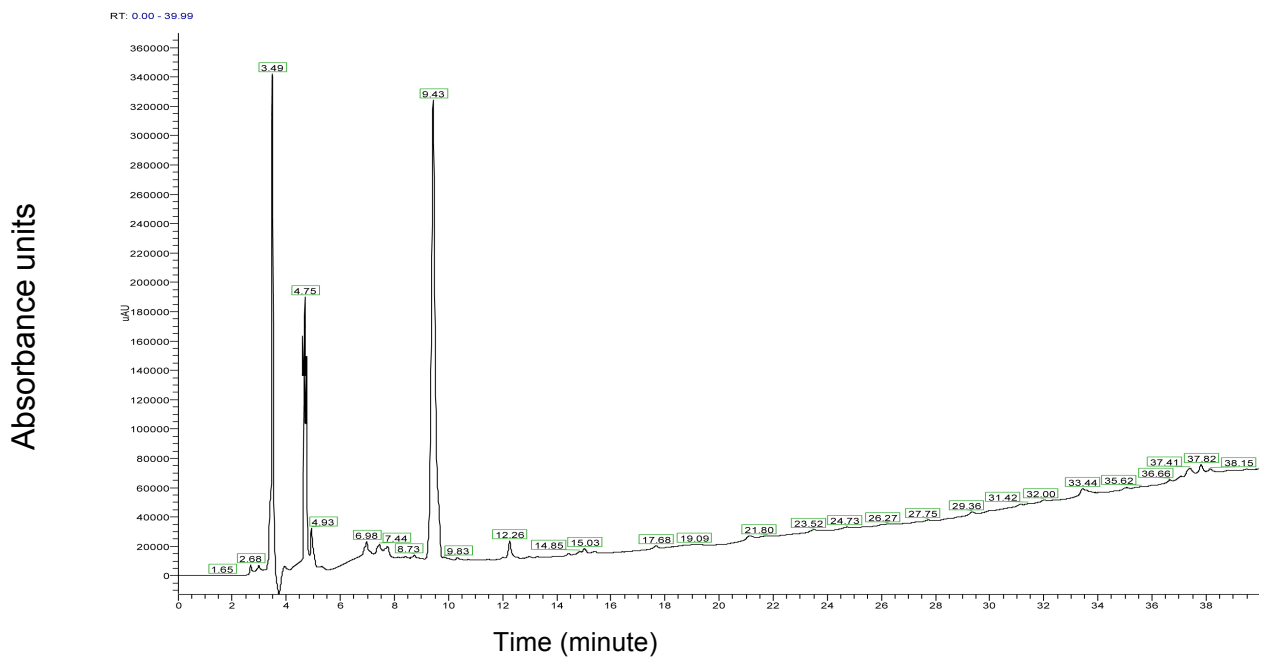
HPLC is a reliable method to separate and identify Actovegin, it can provide a graphical profile of Actovegin; therefore it can be used to compare different batches of Actovegin to assure quality and minimise the risk of contamination. All the Actovegin used in this PhD will be batch tested with HPLC. Batch 13608343, 1608753, 18128753, 02233453 and 18233453 were tested as per HPLC method described; all batches demonstrated similar results and no contaminants were found.



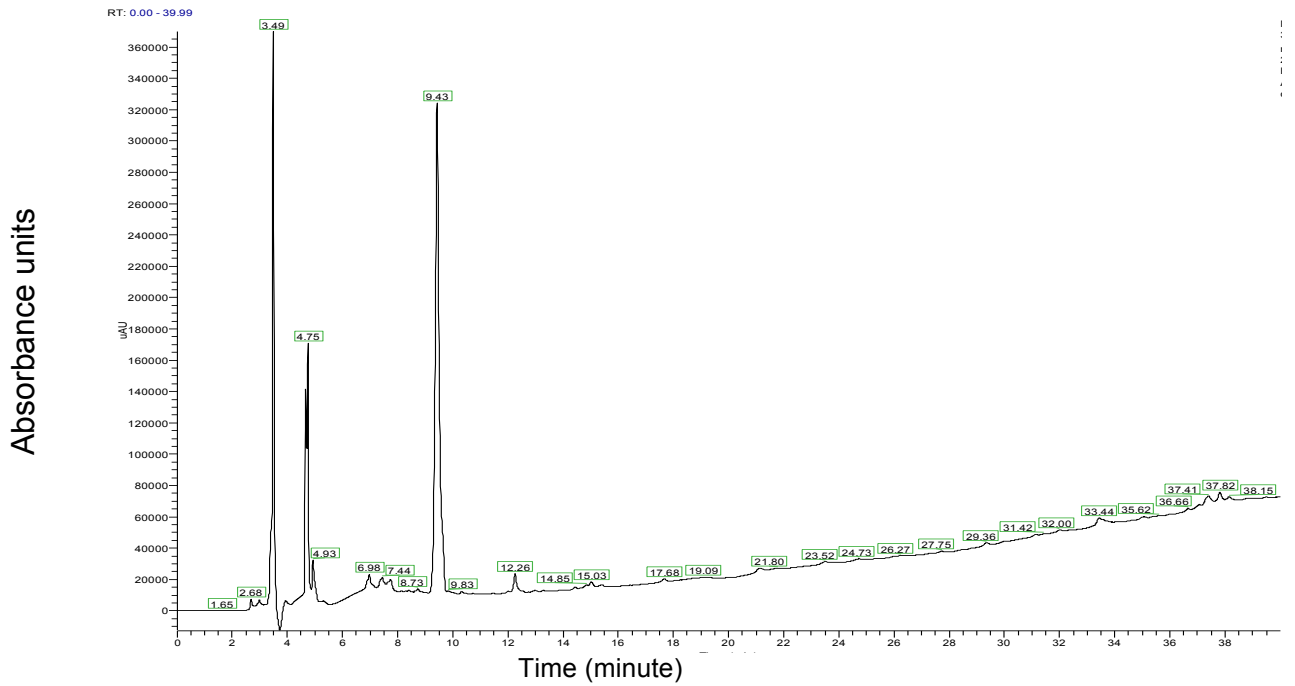
Graph to demonstrate the composition of Actovegin batch **10608753** using HPLC. The peaks on the graph represent separation of Actovegin against time.  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes



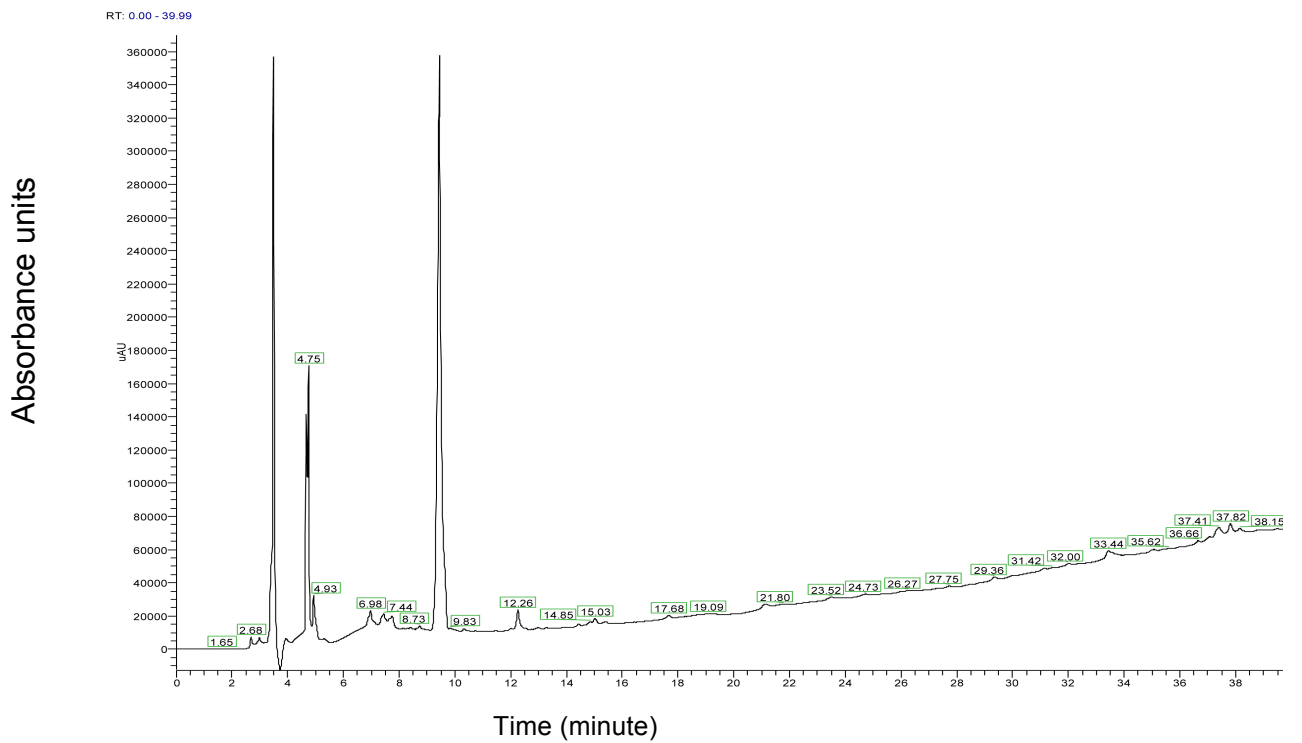
Graph to demonstrate the composition of Actovegin batch **18128753** using HPLC. The peaks on the graph represent separation of Actovegin against time.  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes



Graph to demonstrate the composition of Actovegin batch **02233453** using HPLC. The peaks on the graph represent separation of Actovegin against time.  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes



Graph to demonstrate the composition of Actovegin batch **18233453** using HPLC. The peaks on the graph represent separation of Actovegin against time.  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes



Graph to demonstrate the composition of Actovegin **13608343** batch using HPLC. The peaks on the graph represent separation of Actovegin against time.  
3 large peaks, 3.49 minutes, 4.75 minutes, 9.43 minutes

### **3.4 *In vitro* study:**

The biochemical events following skeletal muscle injuries and repair is driven by cytokines, monocytes and leukocytes. The speed and quality of muscle healing are dependent on the inflammatory process as discussed in section 2.3.3. Therefore, this PhD is focused on the role of Actovegin in the inflammatory process. In this section, an in-vitro cell culture model will be used to explore the role the hypothesis stated in section 2.7.

In order to investigate the role of Actovegin in muscle injuries, a cell culture model must first be established. To identify the type of cells can be used for cell culture, preliminary cell culture experiments were performed. As detailed in section 2.6, Actovegin is a biological drug, manufactured from Bovine Serum. Therefore, the cell culture in this PhD would ideally be cultured under a serum free environment. Many cell culture protocols involve the use of Fetal Bovine Serum (FBS), which could potentially invalidate the study.

FBS is produced from blood collected at commercial slaughterhouses from cattle bred via needle cardiac puncture to the calf right atrium. The collected blood was allowed to clot and separate under gravity. The centrifugation process was then performed to remove the red blood cells and fibrin clot; the serum was then passed through a series of filters dependent on the manufacturer. The manufacturing process of FBS is similar to the manufacturing process of Actovegin as detailed in 2.5.1. Depending on the batch and manufacturer of the FBS, its effect on the cell culture is unpredictable, therefore a serum free cell culture is needed for the biochemical investigation in this PhD.

Serum free skeletal muscle cell culture of chick embryos was attempted, however without FBS the cell culture failed to mature and was not viable for further experiments. Primary human muscle cell line culture (Promo Cell) was also considered for this PhD, however after detailed discussion with the manufacturer, it was not recommended to be used as a FBS free culture.

It is not possible to culture eukaryotic muscle cells without the presence of FBS; therefore alternative cell types must be identified for serum free culture in this PhD.

Section 2.3.3 in this thesis detailed that the speed and quality of muscle healing is dependent on the inflammatory process. Serum free monocytes cell culture was attempted and the culture results were encouraging. Therefore, the cell culture studies in this PhD will be based on Monocyte cell lines.

### **3.4.1 Serum free cell culture**

THP-1 cell-line was established as the cell line for *in vitro* cell cultures (European collection of Animal Cell Culture, Salisbury). The cells were donated by Dr. Dipak Ramji, Reader, Cardiff School of Biosciences. They are derived from the peripheral blood of a 1-year-old human male with acute monocytic leukemia. THP-1 cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulins. These cells also stain positive for alpha-naphthyl butyrate esterase, produce lysozymes and are phagocytic. They can be differentiated into macrophage cells using TrypLE. Cells were supplied at -80°C with the cell ampule immersed in glycerol as a cryoprotectant.

#### Resuscitation of Frozen Cell Lines

In a microbiological safety cabinet, the frozen ampoule was cleaned with a tissue soaked in 70% alcohol. The cap was turned a quarter turn to release any residual liquid nitrogen trapped during the storage process. The cap was then retightened, and the ampoule quickly (minimising the damage to the cell membrane) transferred to a 37°C waterbath. In order to reduce the risk of contamination, the ampoule was not totally immersed into the waterbath. The cell suspension was then centrifuged at low speed for 5 minutes. The suspenstant was removed and cells were re-suspended in fresh medium with 2mM Glutamine and 20% of Fetal Bovine Serum. It was then incubated at 37 °C with 5% CO<sub>2</sub>. Cells were checked daily and the medium



was refreshed when required according to the colour change to the medium. Cell counts were performed after each medium change to ensure that growth was monitored. The culture flasks were kept at a vertical position until the cells reached  $6 \times 10^5$  cells/ml. Once the culture was established, cells were incubated in a serum free environment with RPMI-1640 medium.

### Establishing THP-1 culture

The THP-1 cells were then washed twice with complete medium RPMI-1640, supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, 25 mM HEPES and 1% L-glutamine, using a 50 ml Flacon tube and 20 ml medium to wash, spun down for 5 min at 1500 rpm. Cells were then placed in T<sub>75</sub> flasks to expand cell numbers with 10 ml RPMI-1640 and later in T<sub>150</sub> flasks in 50 ml RPMI -1640. Sub-cultures were established by centrifugation with subsequent re-suspension at  $2-5 \times 10^5$  viable cells/ml, every 2-3 day. Cells reached a density of  $8 \times 10^5$  cells/ml within this period of time. In order to ensure quality control, cells did not exceed  $1 \times 10^6$  cells/ml before sub-culturing. Cell cultures were incubated at 37°C and 5% CO<sub>2</sub> in a certified human tissue laboratory.

### Establishing Macrophage culture

The macrophages used in this PhD study were differentiated from THP-1 cells. The differentiation process was triggered by the addition of phorbol 12-myristate 13-acetate (PMA) in Dimethyl Sulfoxide (DSMO). In order to differentiate  $1.6 \times 10^5$  cells, a 0.16µM solution of (PMA) was added. Cells were then incubated for another 16 hours (overnight) for the THP-1 to fully differentiate into macrophages. After incubation at 37°C and 5% CO<sub>2</sub> in a certified human tissue laboratory, non-attached cells were removed by aspiration. The macrophages had readily attached to the bottom of the dishes during the incubation process. The culture flask was then washed 3 times with Phosphate buffered saline.

1 ml of TrypLE Express solution was then added to the dish, and incubated for 10min at 37 °C in an atmosphere of 5% CO<sub>2</sub>- 95% air. 9 ml of the growth medium RPMI-1640 was then added to the culture dish and the macrophages were then gently scraped off with a cell scraper. The cells were then transferred into a 15 ml conical tube and centrifuged at 180 x g for 5 min at 25°C. The supernatant was then discarded and the cells were dissociated into single cell pellets by vigorous pipetting. The cell number was confirmed by haemocytometer-counting. THP-1 cells in the same passage with no PMA were used as control cells during the differentiation process.

### Culture medium

Roswell Park Memorial Institute medium (RPMI-1640) was developed by Roswell Park Memorial Institute. The formulation is based on the RPMI-1630 series of media utilizing a bicarbonate buffering system and alterations in the amounts of amino acids and vitamins. RPMI-1640 medium has been used in many published *in vitro* studies and described by many textbooks as a standard method of cell culture medium.

Penicillin and streptomycin (100ug/ml) were also used in the culture medium to minimise the risk of bacterial contamination during the incubation process. Fetal bovine serum (FBS) was used in the quantity of 20% at the initial resuscitation of frozen THP-1 cell-lines. FBS was an essential ingredient for the resuscitation process as previous experience demonstrated that initial cell line failure to survive in a serum free environment. As discussed in previous chapters, Actovegin is manufactured from fetal bovine serum, therefore in order to reduced the potential error and noise from the final outcome, all sub-cultures were performed in a serum free environment. Once the THP-1 culture density was established, cells were washed 3 times with PBS and transferred to a serum free culture environment with RMPI-1640 culture medium only for sub-culture.

## Cell culture experiment

THP-1 and macrophage cultures were established with the method described above. For the main experiment, after centrifugation, cells were re-suspended in 5ml of medium. 0.5 ml was then pipetted from the cell mixture into one of the wells in a 12-well cell culture plate (BD falcon, California, USA). This process was repeated 10 times in order to established 10 sets of cell cultures. 0.05ml of Actovegin (40mg/ml) was added to 5 independent wells to establish culture with 10% Actovegin. 0.05ml of RPMI 1640 was added to each of the other 5 independent wells to establish the control culture. All cultures were mixed independently via repeated pipetting with independent sterile tips. A sample of 100ul of each cell mixture was collected into a 96 well plate in order to perform MTS cell proliferation assays (cellTiter 96, Promega, USA) and manual haemocytometer counts (further details of MTS cell Proliferation assay will be explained in following sections). The 12-well cell culture plate was incubated at 37.5°C in a humidified cell incubator in an atmosphere of 95% air and 5% CO<sub>2</sub>, for 24 hours. The MTS cell proliferation assays and manual haemocytometer counts were then repeated.

### **3.4.2 Cell proliferation assay**

#### **3.4.2.1 Method**

The CellTiter 96 Aqueous non-radioactive cell proliferation assay (Promega,USA) is a colourimetric method for determining the number of viable cells in proliferation assays. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), is a tetrazolium compound with an electron coupling reagent which can be uptaken and bio-reduced by living cells into a bluish-green coloured formazan product that is soluble in tissue culture medium. This assay measures cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes (cell proliferation). The quantity of formazan product is measured by the amount of 490nm absorbance and is directly proportional to the metabolic rate and hence the number of living cells in culture.

The MTS assay was prepared according to the manufacturer's protocol from Promega. MTS Solution and the PMS Solution were defrosted in a water bath for 10 minutes at 37°C. The entire content of the 1ml PMS solution was transferred into the 20ml bottle of MTS Solution. 2.0ml of the MTS/PMS mixture was pipetted into a test tube and mixed with a further 1ml of PMS Solution. 20 ul of the final mixture was pipetted into each well of a transparent 96 well assay plate (Invitrogen). Cell culture experiments were established as described above. 100 ul of cell mixture was pipetted into one of the wells in a 96 well plate from each cell culture. The cells and MTS/PMS mixture was mixed with a repeated pipetting technique with new sterile pipette tips. The 96 well plate was when incubated for 2 hours at 37oC in a 5%CO2 incubator. BMG labtech FLUOstar optima (Germany) Fluorescence plate reader was used to determine 490nm absorbance in each well. The results were then calibrated and confirmed with the haemocytometer count.

#### **3.4.2.2 Results**

Five sets of THP-1 and Macrophage cell cultures were completed in triplicate (in total 15 samples) according to the study protocol. The mean cell density was  $0.156 \times 10^4$  cells/100  $\mu$ l/well. According to the manufacture guidance MTS cell assay produced normal destitution data, therefore parametric data analysis such as t test were used for data analysis. There were no significant differences in cell count between the Actovegin treated cells compared to the control cells before incubation in both THP-1 (Table 3.4.2.2.nT.1) and macrophage (Table 3.4.2.2.nM.1) groups. The mean absorbance value of 1.829 and 1.825 from the MTS assays for both THP-1 groups (Table 3.4.2.2.aT.1). This is confirmed by the haemocytometer manual counting. The starting cell number of the macrophages would also be the same as the cells were differentiated from the THP-1 cell line confirmed by the MTS absorbance (Table 3.4.2.2.aM.1) and manual counting (Table 3.4.2.2.nM.1).

THP-1 cells before incubation (Absorbance)						
THP	control	control	control	Actovegin	Actovegin	Actovegin
1	1.83	1.85	1.87	1.87	1.78	1.83
2	1.87	1.84	1.83	1.84	1.94	1.69
3	1.80	1.86	1.77	1.89	1.84	1.74
4	1.76	1.84	1.90	1.81	1.89	1.64
5	1.81	1.98	1.64	1.83	1.99	1.81
<b>Mean</b>			<b>1.829</b>			<b>1.825</b>
<b>S.D.</b>			<b>0.075</b>			<b>0.090</b>
					<b>p value</b>	<b>0.905</b>

(Table 3.4.2.2.aT.1). Absorbance of the cell proliferation assay at 490nm before incubation in THP-1 cells. Absorbance assays show no difference between control and Actovegin group prior to incubation in THP-1 cell line. P = 0.905

THP-1 cells before incubation (cell count)						
THP	control	control	control	Actovegin	Actovegin	Actovegin
1	0.16	0.16	0.16	0.15	0.15	0.16
2	0.16	0.16	0.16	0.16	0.17	0.15
3	0.15	0.16	0.15	0.15	0.16	0.15
4	0.15	0.16	0.16	0.16	0.17	0.14
5	0.16	0.17	0.14	0.16	0.17	0.16
<b>Mean</b>			<b>0.156</b>			<b>0.155</b>
<b>S.D.</b>			<b>0.006</b>			<b>0.008</b>
<b>T- test</b>					<b>0.694</b>	

(Table 3.4.2.2.nT.1). Cell count before incubation in THP-1 cells (104 cells/100µl) Cell counts show no difference between control and Actovegin group prior to incubation THP-1 cell line. (p=0.694)

Macrophages cells before incubation (Absorbance)						
Mac	control	control	control	Actovegin	Actovegin	Actovegin
1	2.69	3.03	2.76	2.99	2.62	3.50
2	3.15	3.33	2.69	2.98	2.96	3.33
3	2.65	2.74	3.22	2.41	2.94	2.57
4	2.64	2.25	2.79	2.67	2.18	2.39
5	2.41	2.89	2.41	2.69	2.93	2.48
<b>Mean</b>			<b>2.777</b>			<b>2.776</b>
<b>S.D.</b>			<b>0.306</b>			<b>0.360</b>
<b>T- test</b>					<b>0.992</b>	

(Table 3.4.2.2.aM.1). Absorbance of the cell proliferation assay at 490nm before incubation in Macrophages. Absorbance assays show no difference between control and Actovegin group prior to incubation in differentiated macrophages cell line (p=0.992)

Macrophages cells before incubation (cell count)						
Mac	control	control	control	Actovegin	Actovegin	Actovegin
1	0.23	0.26	0.24	0.26	0.23	0.31
2	0.27	0.29	0.23	0.24	0.25	0.29
3	0.23	0.24	0.28	0.21	0.26	0.22
4	0.23	0.19	0.24	0.23	0.19	0.22
5	0.21	0.25	0.21	0.23	0.26	0.23
<b>Mean</b>			<b>0.241</b>			<b>0.241</b>
<b>S.D.</b>			<b>0.027</b>			<b>0.029</b>
<b>T- test</b>						<b>0.986</b>

(Table 3.4.2.2.nM.1). Cell count before incubation in **Macrophages** (104 cells / 100  $\mu$ l). Cell counts show no difference between control and Actovegin group prior to incubation in differentiated macrophage cell line ( $p=0.986$ )

After 24 hours of incubation, both the Actovegin and control groups showed increased numbers of cells in the THP-1 cultures. The mean absorbance value of 1.994 and 2.803 from the MTS assays between the Control and Actovegin groups respectively (Table 3.4.2.2.aT.2.AC). This is confirmed by haemocytometer manual counting. The mean cell count in the control group was  $0.168 \times 10^4$  and the Actovegin group was  $0.233 \times 10^4$  (Table 3.4.2.2.nT.2.AC). There was a significant increase ( $p=0.0001$ ) in the cell count between the groups compared to their pre incubation cell counts. In the control group, the cell count increased from 0.156 to 0.168. (Table 3.4.2.2.nT.12.c) In the Actovegin group, the cell count increased from 0.155 to 0.233 (Table 3.4.2.2.nT.12.c). The Actovegin group showed an additional 39% increase in THP-1 cell count compared to control after 24 hours of incubation ( $p=0.0001$ ).

THP-1 cells after incubation (Absorbance)						
THP	control	control	control	Actovegin	Actovegin	Actovegin
1	2.15	1.95	1.87	3.06	2.57	3.47
2	2.12	1.74	2.29	2.15	1.98	2.78
3	1.84	1.87	2.04	3.31	2.22	2.12
4	1.97	1.91	2.18	3.68	3.14	2.86
5	2.01	2.13	1.84	2.79	3.35	2.58
<b>Mean</b>			<b>1.994</b>			<b>2.803</b>
<b>S.D.</b>			<b>0.155</b>			<b>0.532</b>
					<b>p value</b>	<b>0.00004</b>

(Table 3.4.2.2.aT.2.AC) **Absorbance** of the cell proliferation assay at 490nm after 24 hours of incubation in **THP-1** cells. Absorbance assays show significant difference between control and Actovegin group after 24 hours of incubation in THP-1 cell line.  $p=0.00004$

THP-1 cells after incubation (cell count)						
THP	control	control	control	Actovegin	Actovegin	Actovegin
1	0.18	0.16	0.16	0.26	0.22	0.29
2	0.20	0.15	0.19	0.15	0.18	0.26
3	0.15	0.17	0.16	0.28	0.19	0.18
4	0.17	0.16	0.18	0.30	0.25	0.27
5	0.17	0.18	0.15	0.24	0.26	0.20
<b>Mean</b>			<b>0.168</b>			<b>0.233</b>
<b>S.D.</b>			<b>0.014</b>			<b>0.046</b>
					<b>p value</b>	<b>0.0001</b>

(Table 3.4.2.2.nT.2.AC) Cell count after 24 hours of incubation in THP-1 cells. Cell count shows significant difference between control and Actovegin group after 24 hours of incubation in THP-1 cell line. p=0.0001

THP-1 cell count in control group pre and post incubation						
	pre	pre	pre	Con 24 hr	Con 24 hr	Con 24 hr
1	0.16	0.16	0.16	0.18	0.16	0.16
2	0.16	0.16	0.16	0.20	0.15	0.19
3	0.15	0.16	0.15	0.15	0.17	0.16
4	0.15	0.16	0.16	0.17	0.16	0.18
5	0.16	0.17	0.14	0.17	0.18	0.15
<b>Mean</b>			<b>0.156</b>			<b>0.168</b>
<b>S.D.</b>			<b>0.006</b>			<b>0.014</b>
					<b>p value</b>	<b>0.005</b>

(Table 3.4.2.2.nT.12C) THP-1 cell count shows significant difference between pre and post 24 hours incubation in control group p=0.005

THP-1 cell count in Actovegin group pre and post incubation						
	pre	pre	pre	Acto24 hr	Acto24 hr	Acto24 hr
1	0.15	0.15	0.16	0.26	0.22	0.29
2	0.16	0.17	0.15	0.15	0.18	0.26
3	0.15	0.16	0.15	0.28	0.19	0.18
4	0.16	0.17	0.14	0.30	0.25	0.27
5	0.16	0.17	0.16	0.24	0.26	0.20
<b>Mean</b>			<b>0.155</b>			<b>0.233</b>
<b>S.D.</b>			<b>0.008</b>			<b>0.046</b>
					<b>p value</b>	<b>0.005</b>

(Table 3.4.2.2.nT.12A) THP-1 cell count shows significant difference between pre and post 24 hours incubation in Actovegin group p=0.005

## Macrophages

After 24 hours of incubation, both the Actovegin and control groups showed increased numbers of cells in the macrophage cultures. The mean absorbance value of 2.823 and 3.473 from the MTS assays between the Control and Actovegin groups respectively (Table 3.4.2.2.aM.2.AC). This is confirmed by the haemocytometer manual counting. The mean cell count in the control group is  $0.244 \times 10^4$  and the Actovegin group is  $0.320 \times 10^4$  (Table 3.4.2.2.nM.2.AC). There was a significant increase ( $p=0.00006$ ) in the cell count between the groups compared to its pre incubation cell count. In the control group the cell count has increased from 0.241 to 0.244. (Table 3.4.2.2.nM.12.A) In the Actovegin group the cell count has increased from 0.241 to 0.320 (Table 3.4.2.2.nM.12.A). The Actovegin group showed an additional 31% increase in macrophage cell count compared to control after 24 hours of incubation ( $p=0.00006$ ).

Macrophages cells absorbance between control and Actovegin post incubation						
	control	control	control	Actovegin	Actovegin	Actovegin
<b>1</b>	2.64	2.39	2.33	3.76	3.16	3.24
<b>2</b>	3.54	3.52	2.89	3.27	2.64	2.99
<b>3</b>	2.26	2.98	2.32	4.07	3.20	3.14
<b>4</b>	2.42	3.21	3.35	4.38	3.73	4.23
<b>5</b>	2.47	3.59	2.43	3.58	3.79	2.91
<b>Mean</b>	<b>2.823</b>					<b>3.473</b>
<b>S.D.</b>	<b>0.501</b>					<b>0.509</b>
<b>p value</b>						<b>0.004</b>

(Table 3.4.2.2.aM.2.AC) Absorbance of the cell proliferation assay at 490nm after 24 hours of incubation in **Macrophages** cells. Absorbance assays show significant difference between control and Actovegin groups after 24 hours of incubation in **Macrophage** cell line.  $p=0.004$



Macrophages cell count between control and Actovegin post incubation							
	Con24	Con24	Con24	Acto24	Acto24	Acto24	
1	0.24	0.22	0.22	0.35	0.29	0.30	
2	0.33	0.20	0.26	0.30	0.24	0.35	
3	0.21	0.23	0.21	0.37	0.25	0.29	
4	0.22	0.26	0.29	0.40	0.34	0.39	
5	0.23	0.33	0.22	0.33	0.35	0.27	
<b>Mean</b>			<b>0.244</b>			<b>0.320</b>	
<b>S.D.</b>			<b>0.041</b>			<b>0.049</b>	
						<b>p value</b>	<b>0.00006</b>

(Table 3.4.2.2.nM.2.AC) Cell count after 24 hours of incubation in **Macrophage** cells. Cell count shows significant difference between **control and Actovegin** groups after 24 hours of incubation in **Macrophage** cell line.  $p=0.00006$

Macrophages cell count in control group pre and post incubation							
Mac	pre	pre	pre	24 hr	24 hr	24 hr	
1	0.23	0.26	0.24	0.24	0.22	0.22	
2	0.27	0.29	0.23	0.33	0.20	0.26	
3	0.23	0.24	0.28	0.21	0.23	0.21	
4	0.23	0.19	0.24	0.22	0.26	0.29	
5	0.21	0.25	0.21	0.23	0.33	0.22	
<b>Mean</b>			<b>0.241</b>			<b>0.244</b>	
<b>S.D.</b>			<b>0.027</b>			<b>0.041</b>	
						<b>p value</b>	<b>0.720</b>

(Table 3.4.2.2.nM.12.C) Macrophage cell count shows **no** significant difference between **pre and post** 24 hours incubation in **control** group  $p=0.72$

Macrophages cell count in Actovegin group pre and post incubation							
	pre	pre	pre	24 hr	24 hr	24 hr	
1	0.26	0.23	0.31	0.35	0.29	0.30	
2	0.24	0.25	0.29	0.30	0.24	0.35	
3	0.21	0.26	0.22	0.37	0.25	0.29	
4	0.23	0.19	0.22	0.40	0.34	0.39	
5	0.23	0.26	0.23	0.33	0.35	0.27	
<b>Mean</b>			<b>0.241</b>			<b>0.320</b>	
<b>S.D.</b>			<b>0.029</b>			<b>0.049</b>	
						<b>p value</b>	<b>0.0002</b>

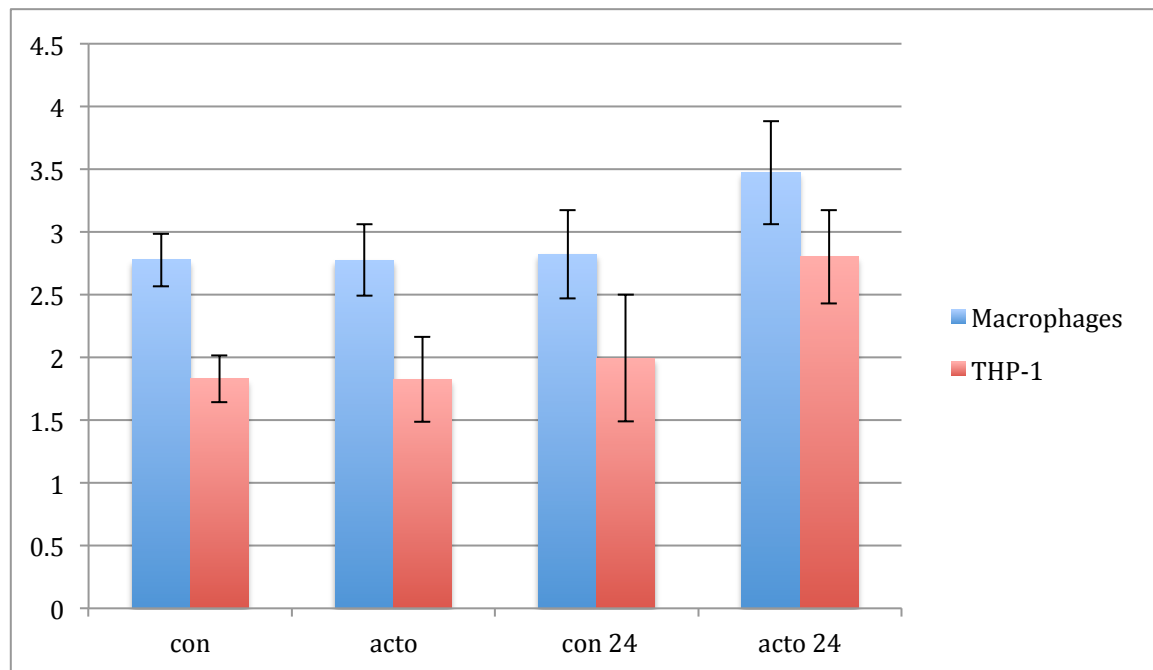
(Table 3.4.2.2.nM.12A) Macrophage cell count shows significant difference between **pre and post** 24 hours incubation in **Actovegin** group  $p=0.0002$

**Results Summary :**

After 24 hours of incubation, both Actovegin and Control groups in the THP-1 cell culture showed significant increases in cell counts. The Actovegin group showed 39% additional increase in THP 1 cell count compared to control,  $p= 0.0001$  (Table 3.4.2.2.nTM.2S). In comparison to the Macrophages, the control group failed to demonstrate a significant increase in cell count after 24 hours of culture. On the other hand, the Actovegin group showed significant increase in cell count. The Actovegin group showed 39% additional increase in macrophage cell count compared to control after 24 hours of culture,  $p=0.00006$  (Table 3.4.2.2.nTM.2S). Figure 3.4.2.2.nTM.S shows a graphical representation of the results discussed in this section.

		mean	S.D.	mean diff	t-test
<b>THP-1</b>	<b>Actovegin 24</b>	0.233	0.046	<b>+39%</b>	<b>0.0001*</b>
<b>Macrophages</b>	<b>Actovegin 24</b>	0.320	0.041	<b>+31%</b>	<b>0.00006*</b>

(Table 3.4.2.2.nTM.2S) Summaries of the cell count changes after 24 hours of cell culture in THP-1 and macrophage cells.



(Figure 3.4.2.2.nTM.S) con = control group pre-incubation, acto = Actovegin group pre-incubation, con 24 = control group after 24 hour of incubation, acto 24 = Actovegin group after 24 hours of incubation.

Graph to summarise the overall changes in cell counts between the Actovegin and control groups before and after 24 hours of cell cultures.

### **3.4.2.3 Discussion**

The MTS assay and cell count in the in-vitro study confirmed that Actovegin has significant proliferative effects on THP-1 and Macrophages. This findings are in keeping with other in-vitro studies reported in the literature with different cell types, detailed in Section 2.6.1. In-vitro cell cultures of human origin are uncommon in the published literature related to Actovegin; this could be due to the fact that most human cell types require the use of FBS. As discussed in Section 3.4.1, FBS will potentially affect the outcome and interpretation of the results when investigating the role of Actovegin in an in-vitro setting. FBS -free human THP-1 cell cultures are a well-established cell culture method used to investigate haematological and immunological diseases and malignancy as detailed in Section 3.4.1. This study is the first study reporting the proliferative effect of Actovegin in these cells of human origin.

Besides the proliferative effect of Actovegin demonstrated in this study, I have also identified a suitable serum free human cell in-vitro model to investigate the biochemical role of Actovegin in the inflammatory process. Section 2.3.3 has detailed the pathophysiology of the muscle healing process, further in vitro experiment will be discussed in Section 3.4.3, attempting to identify the role of Actovegin in muscle injuries.

### **3.4.3 Polymerase chain reaction (PCR)**

In Section 3.4.1, a serum free in-vitro human cell culture system was identified; Section 3.4.2 demonstrated the efficacy of this in-vitro model and also suggested that Actovegin has a significant proliferative effect on these inflammatory cells. As discussed in Sections 2.3.3 and 2.3.4, the speed and quality of muscle healing are dependent on the inflammatory process. Cytokines and inflammatory cells govern the inflammatory process. Although Section 3.4.2 demonstrated the proliferative effects of Actovegin on these cells, it is not possible to identify its subtype and function with the MTS assay. In this section, polymerase chain reaction will be used to identify the subtype

of these inflammatory cells and further explore the role of Actovegin, as stated in the hypothesis in Section 2.7.

PCR is a laboratory technique used to make many copies of a piece of DNA. The copies of DNA double with every cycle, hence it can reliably detect a very small amount of change in the total amount of DNA. The principle of PCR is based on the use of DNA polymerase to make a copy of a single stranded DNA. Therefore, double stranded DNA must first be melted to single stranded DNA via the denaturation process with a set temperature. A short piece of single stranded DNA (gene of interest), called the primer is added to the mixture. A forward primer binds to one strand and a reverse primer binds to the opposite strand. The DNA polymerase enzyme forms a double stranded DNA between the single stranded DNA and the primer. This annealing process is achieved by cooling the PCR mixture. After the annealing process, the mixture is then heated up again to a specific temperature depending on the DNA polymerase to facilitate the extension process. In this process, the DNA polymerase enzyme copies the single stranded molecule, starting at the bound primer region and makes a complementary copy of the single stranded DNA (cDNA). The only DNA that is copied is the region between the forward primer binding location and the reverse primer binding location. Both single strands of the original double-stranded DNA molecule are copied. Therefore, the result is two double stranded molecules each identical to the original double-stranded DNA fragment. The three steps of the PCR process, denaturation, annealing and extension are repeated. After 30 cycles,  $1 \times 10^9$  copies of double stranded cDNA are produced from a single double stranded DNA. If there were 2 double strands of DNA,  $1 \times 10^{18}$  copies of double stranded cDNA are produced. After the amplification process, agarose gel electrophoresis is employed for size separation of the PCR products, which can be quantified by Fluorescence light absorptiometry, similar to the MTS assay described in Section 3.4.2. This method is useful in determining the presence of a specific gene, however it will only give a semi-quantitative result as gel electrophoresis fluorescence light absorptiometry can only indirectly measure the end product of the PCR. As technology advanced, real time PCR was

developed over recent years to improve the accuracy and efficiency of PCR.

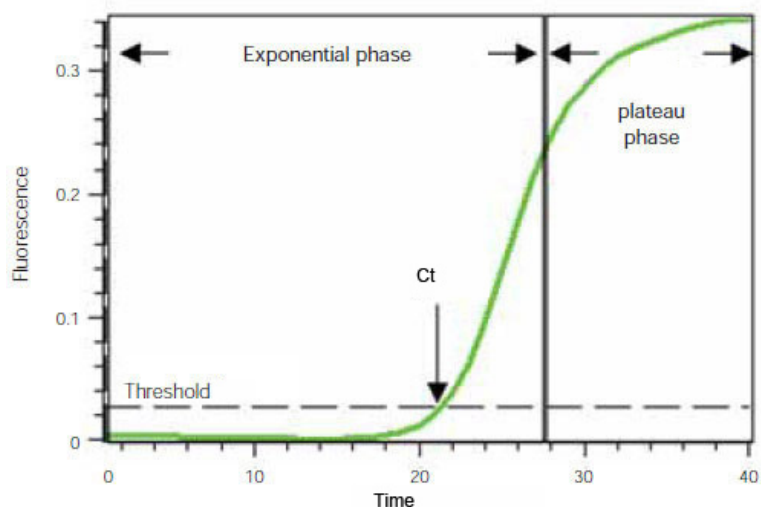
### Real time quantitative PCR (qPCR)

qPCR is based on the same principles as PCR, as discussed above. qPCR enables both detection and quantification of one or more specific sequences in a DNA sample. With this method, the quantity can be either an absolute number of copies or a relative amount when normalised to DNA input or additional normalising genes. This offers distinct advantages over traditional PCR, as it can detect the magnitude of changes in a specific gene real time. Estimation errors can arise from variations in the quantification method and can be the result of DNA integrity, enzyme efficiency and many other factors. Instead of quantifying the absolute amount of the total gene expression, the quantification of a specific gene being studied in relation to a housekeeping gene is used instead in qPCR. As the housekeeping gene and the specific gene of interest are measured at the same time, it reduces the estimation errors. Instead of the absolute quantity of a specific gene, a ratio for the expression of the gene of interest divided by the expression of the selected housekeeping gene is reported.

qPCR utilizes the same PCR steps, denaturation, annealing and extension process. In qPCR a fluorophore is added to this mixture in a thermal cycler that contains sensors for measuring the fluorescence of the fluorophore after it has been excited at the required wavelength, allowing the generation rate to be measured for one or more specific products. This allows the rate of fluorescence generation by the amplified product to be measured at each PCR cycle by a specially designed qPCR machine. The fluorescence generated by a sample of DNA during real-time PCR is plotted over time, rather than PCR cycle number. The middle of its curve represents the exponential phase of PCR (Figure 3.4.3.a), when the levels of generated fluorescence exceeds the background fluorescence, but reagents have not nearly begun to reach limiting factors. This specific value is referred to as the

threshold cycle ( $C_t$ ) or it could also be called the threshold time ( $C_q$ ) (Figure 3.4.3.a). Genes with the highest starting target amount will also have the highest values of amplified target in a given PCR cycle number. This means their fluorescence curves will exceed background earlier and cross the threshold at an earlier cycle number; thus, the more abundant the starting quantity of gene, the lower the  $C_t$  value of that sample. The plateau stage (Figure 3.4.3.a) of the qPCR curve represents the endpoint of that reaction as at one or more reaction components is depleted. At the plateau stage the amplification curves of qPCR are no longer exponential, which means that the association of a two-fold increase in quantity from one cycle number to the next has come to an end.

After the plateau stage, it is important to establish a melt graph for the qPCR. This is due to the fact that SYBR Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR products from mis-annealed primer. The melt graph analysis can identify contamination, as contaminating DNA or primer dimers will show up as an additional peak separate from the desired peak.



(Figure 3.4.3.a) demonstrates a typical graph for qPCR, fluorescence generated by a sample of DNA against time, the threshold time ( $C_t$ ) is when the levels of generated fluorescence exceed background fluorescence Reference: qPCR manual

Cells in all organisms regulate gene expression by turnover of gene transcripts via messenger RNA (mRNA). The amount of an expressed gene by a cell can be measured by the number of copies of its mRNA transcribed. qPCR can robustly detect and quantify gene expression, however it is limited to DNA detection. Therefore, mRNA in cell culture is needed to be reverse-transcribed to DNA with reverse transcriptase prior to qPCR. In order to analyse the cell's genetic expression, mRNA must first be extracted. Once the quality of extraction is confirmed, it can then be reverse-transcribed to complementary DNA (cDNA), which can then be used for qPCR. cDNA is a much more stable form of nucleic acid compared to mRNA, therefore mRNA should be reverse-transcribed to cDNA before storage.

As discussed in Section 2.3.4, the speed and quality of muscle healing are dependent on the inflammatory process. The inflammatory process is governed by cytokines and inflammatory cells. Although Section 3.4.2 has demonstrated the proliferative effect of Actovegin in these cells, it is not possible to identify its sub type and function with the MTS assay. Therefore, qPCR will be used to identify the role of Actovegin on the CD68<sup>+</sup> and CD163<sup>+</sup> subtypes of THP-1 and Macrophages, furthermore inflammatory cytokines MCP-1, TNF $\alpha$ , CD163<sup>+</sup>, CD68<sup>+</sup> and IL1 will also be analysed. The housekeeping gene used for this qPCR is GAPDH.

### **3.4.3.1 Method**

#### Cell culture

THP-1 and macrophage cell cultures in the same format as described in the cell proliferation experiment with control and 10% Actovegin. 6 biological replications of each group were performed at separate time points. After 24 hours incubation, cells were lysed with TRIZOL Reagent (Invitrogen, USA) and stored as cell pellets at -20°C until RNA extraction and cDNA conversion. Once all 24 individual culture samples were collected, RNA

extraction, cDNA conversion and qPCR for all experiments were performed on the same day.

#### RNA extraction:

Frozen TRIZOL cell pellets were put into a 37°C water bath for 2 minutes to rewarm. The samples were then incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. All work surfaces, equipment and sample bottles were cleaned with RNAaway solution prior to RNA extraction. 0.2 ml of chloroform was added and samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 minutes. The samples were centrifuged at 12,000 RPM for 15 minutes at 4°C. Following centrifugation, the mixture was separated into lower red, phenol- chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was transferred carefully without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5ml isopropyl alcohol. Samples were incubated at room temperature for 10 minutes and centrifuged at 12, 000 rpm for 10 minutes at 4°C. The RNA precipitate, formed a gel-like pellet on the side and bottom of the tube, the supernatant was then completely removed and washed with 1ml of 75% ethanol. The sample was mixed by vortexing and centrifuging at 7,500 RPM for 5 minutes at 4 °C. The RNA pellet was air-dried for 5 minutes and then dissolved in DEPC-treated water by passing the solution a few times through a pipette tip. The RNA quality control was performed immediately with RNA 6000 LabChip. Once quality was confirmed, cDNA synthesis was performed to minimise the RNA degradation.

#### RNA quality control:

The Agilent 2100 Bioanalyzer (Agilent Technologies) was used in conjunction with the RNA 6000 LabChip (Caliper Technologies Corporation) to provide detailed information about the condition of RNA samples including integrity, concentration and purity. This method of automated system RNA



analysis only required small amounts of RNA as compared to the traditional agarose gel method.

Agilent 2100 Bioanalyzer was switched on and set up, as per manufacturer's guidance, and 2100 expert software was loaded. RNA 6000 LabChip was removed from 4°C storage to the bench to equilibrate at room temperature for 30 minutes. The ladder aliquot (standardized manufacture control) was taken out of the freezer at -70 °C and thawed on ice. A dummy chip was introduced to the chamber and filled with 350ul of RNaseZAP and left for 1 minute to decontaminate the machine. A second dummy chip was introduced to the chamber and filled with 350ul of fresh Milli-Q water and left for 10 minutes with the lid closed. The dummy chip was then removed and the electrode was allowed to dry.

The RNA 6000 chip was placed onto the chip priming station and each well was primed with 9ul of Gel Dye Mix. 5ul of RNA 6000 Nano Marker was pipetted into each well and 1ul of each sample was also introduced to each well. 1ul of ladder was pipetted to the ladder well. The chip was then placed on an adapter and vortexed for 1 minute. The sample loaded chip was then introduced to the Agilent 2100 Bioanalyzer for RNA analysis. Once RNA quality was confirmed, it could be transcribed to more stable cDNA.

#### cDNA synthesis:

Complementary DNA (cDNA) was reverse transcribed from total RNA samples using High Capacity cDNA Reverse Transcription Kit with SuperScript® VILO™ Master Mix (Invitrogen, USA). 4 µl of SuperScript® VILO™ Master Mix was mixed with 2 µg of RNA generated from the RNA extraction process described above. All samples were then incubated at 42°C for 90 minutes. Then the temperature was increased to 85°C for 5 minutes to terminate to process. Once the cDNA was produced, it was stored in -80 °C until the qPCR process

## qPCR

Polymerase Chain Reaction (PCR) products were synthesized from cDNA samples using the PCR master mix, SYBR GREEN PCR Master Mix 2 X 10 $\mu$ l, dH<sub>2</sub>O 4.8 $\mu$ l, Forward primer (100 $\mu$ M) 0.6 $\mu$ l, Reverse primer (100 $\mu$ M) 0.6  $\mu$ l. Forward and reverse primer of GAPDH, TNF $\alpha$ , CD163<sup>+</sup>, CD68<sup>+</sup>, IL1 and MCP1 from e-Bioscience (UK) were used (Table 3.4.3.1). All the above steps were performed in a certified biotechnology laboratory, ABI 7900HT Real-Time PCR system was used for the Real time PCR. The reaction was carried out at 25°C for 5 minutes followed by another 60 minutes at 42°C and was terminated by the deactivation of the enzyme at 70 °C for 5 minutes. All samples were analyzed twice with and without reverse transcriptase, and no amplification was seen in the samples in the absence of reverse transcriptase. Comparisons of Actovegin treated cells with the control in the proliferation assay, qPCR gene expression, two tailed t-test was used to determine mean difference, using SPSS version 20 (IBM Corp.).

Gene	Forward primers	Reverse primers
GAPDH,	5' CGCTCTCTGCTCCTCCTGTT 3'	5' CCATGGTGTCTGAGCGATGT 3'
TNF $\alpha$ ,	5' CTCTTCTCCTTCCTGATCGTGGCA 3'	5' GAAAGCATGATCCGGGACGTGGA 3'
CD163 <sup>+</sup> ,	5' CCAGTCCCAAACACTGTCCT 3'	5' ATGCCAGTGAGCTTCCCGTTCAGC 3'
CD68 <sup>+</sup>	5' GGGAGAAGGGAGGGAGAGAA 3'	5' GGAAAACCCCGTCAAAGATT 3'
IL1	5' AGTAGCAACCAACGGGAAGG 3',	5' TGGTTGGTCTTCATCTTGGG 3'
MCP1	5' CTGGATCGGAACCAAATGAG 3'	5' CGGGTCAACTTCACATTCAA 3'

Table 3.4.3.1 for the gene of the primers

### Calculation:

All data were expressed as arbitrary units in relation to GAPDH mRNA (housekeeping gene). The number of mRNA “threshold cycle” difference of each gene compared to the control was expressed as “ $\Delta C_t$ ”, the “threshold cycle” of each gene compared to the Actovegin group was expressed as “ $\Delta\Delta C_t$ ”. As per international standards, relative quantity (RQ) values were calculated to express changes in the Actovegin group in relation to control.

Example for calculation for Macrophage CD68<sup>+</sup> gene expression:

Threshold cycle (ct) for GAPDH in Control = 16.63

Threshold cycle (ct) for CD68<sup>+</sup> in Control = 20.95

$\Delta C_t$  for CD68<sup>+</sup> in Control = 20.95 - 16.63 = 4.32

(table 3.4.3.2.h, page 106)

Threshold cycle (ct) for GAPDH in Actovegin = 16.93

Threshold cycle (ct) for CD68<sup>+</sup> in Actovegin = 20.61

$\Delta C_t$  for CD68<sup>+</sup> in Actovegin = 20.61 - 16.93 = 3.68

(table 3.4.3.2.h, page 106)

“ $\Delta\Delta C_t$ ” of CD68<sup>+</sup> between Actovegin and control = 3.68 - 4.32 = -0.64

(table 3.4.3.2.i, page 106)

RQ vale = 2 to the power of - ( - 0.64 ) = 1.56

(table 3.4.3.2.j, page 1067)

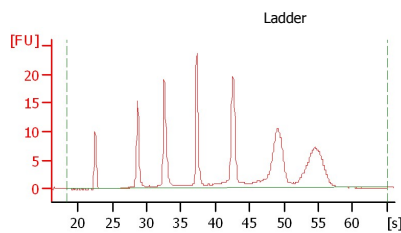
In this specific example, CD68<sup>+</sup> gene expression in the Actovegin group is 156% compare to control.

### 3.4.3.2 Results:

Six sets of independent samples were used for each gene of interest and qPCR was carried out as triplicate according to the protocol described in 4.3.4.1 of the method section. In total, 216 samples were analysed for each cell type. All samples were stored at -80 °C until qPCR, as per protocol. The quality of mRNA was excellent, the RNA integrity number ranged between 9.9 -10. According to the previous studies and text book(Acharyya et al., 2007, Shen et al., 2005, Sulahian et al., 2000, Cory et al., 1991, Tsuchiya et al., 1980), all qPCR data could be assumed to produce normal destitution data, therefore parametric data analysis such as t test were used for data analysis.

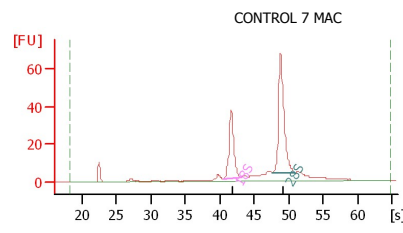
#### RNA quality

The graphs in this section demonstrate the extracted RNA ratio and the quality of the RNA, suggesting that the highest possible quality of RNA was extracted from all of the samples of Macrophages and THP-1 cells.



#### Overall Results for Ladder

RNA Area: 209.6  
 RNA Concentration: 150 ng/ul  
 Result Flagging Color:    
 Result Flagging Label: All Other Samples



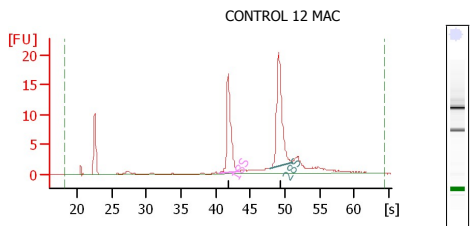
#### Overall Results for sample 1 : CONTROL 7 MAC

RNA Area: 317.3  
 RNA Concentration: 227 ng/ul  
 rRNA Ratio [28s / 18s]: 1.8  
 RNA Integrity Number (RIN): 9.9 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN: 9.90

#### Fragment table for sample 1 : CONTROL 7 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.58	43.12	67.1	21.2
28S	47.68	51.01	119.3	37.6

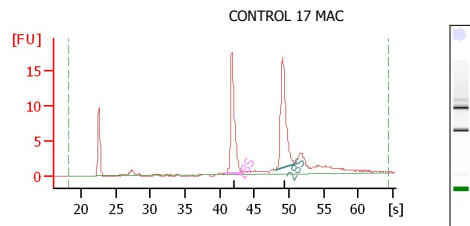




**Overall Results for sample 2 : CONTROL 12 MAC**  
 RNA Area: 96.8  
 RNA Concentration: 69 ng/µl  
 rRNA Ratio [28s / 18s]: 1.3  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 2 : CONTROL 12 MAC**

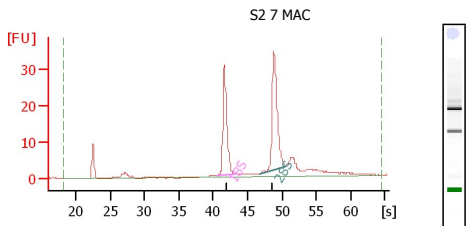
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.57	43.32	25.9	26.8
28S	47.80	51.07	33.4	34.5



**Overall Results for sample 3 : CONTROL 17 MAC**  
 RNA Area: 85.1  
 RNA Concentration: 61 ng/µl  
 rRNA Ratio [28s / 18s]: 1.0  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 3 : CONTROL 17 MAC**

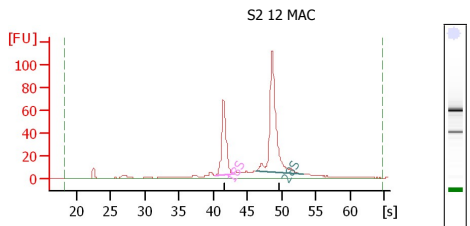
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.71	43.46	26.8	31.5
28S	48.08	50.98	27.5	32.3



**Overall Results for sample 4 : S2 7 MAC**  
 RNA Area: 160.0  
 RNA Concentration: 115 ng/µl  
 rRNA Ratio [28s / 18s]: 1.2  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 4 : S2 7 MAC**

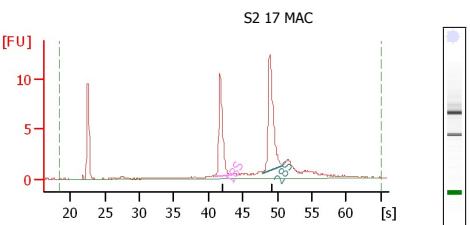
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.51	43.18	47.5	29.7
28S	46.73	50.57	56.9	35.6



**Overall Results for sample 5 : S2 12 MAC**  
 RNA Area: 548.3  
 RNA Concentration: 392 ng/µl  
 rRNA Ratio [28s / 18s]: 2.0  
 RNA Integrity Number (RIN): 9.9 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN: 9.90

**Fragment table for sample 5 : S2 12 MAC**

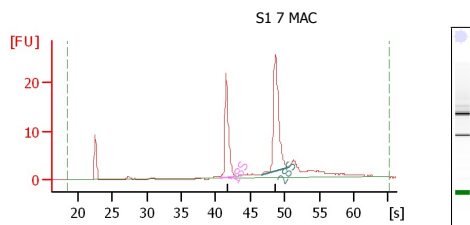
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.35	42.98	116.6	21.3
28S	46.32	53.13	236.0	43.0



**Overall Results for sample 6 : S2 17 MAC**  
 RNA Area: 67.2  
 RNA Concentration: 48 ng/µl  
 rRNA Ratio [28s / 18s]: 1.4  
 RNA Integrity Number (RIN): 9.9 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN: 9.90

**Fragment table for sample 6 : S2 17 MAC**

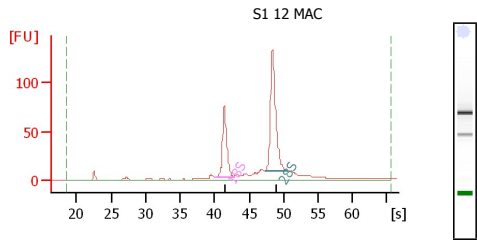
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.85	43.44	14.4	21.5
28S	47.83	50.80	19.6	29.2



**Overall Results for sample 7 : S1 7 MAC**  
 RNA Area: 112.0  
 RNA Concentration: 80 ng/µl  
 rRNA Ratio [28s / 18s]: 1.4  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 7 : S1 7 MAC**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.56	43.11	30.5	27.3
28S	46.65	50.53	42.4	37.9

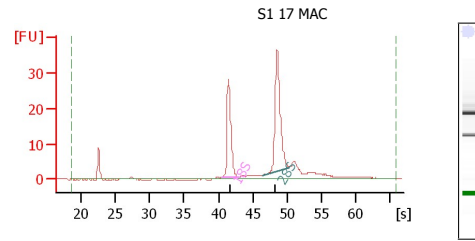


**Overall Results for sample 8 :** S1 12 MAC

RNA Area: 586.3  
 RNA Concentration: 420 ng/µl  
 rRNA Ratio [28s / 18s]: 1.9  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 8 :** S1 12 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.24	42.81	123.2	21.0
28S	47.28	50.42	229.4	39.1

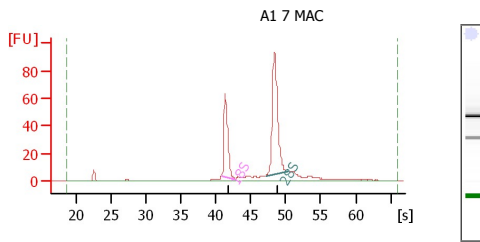


**Overall Results for sample 9 :** S1 17 MAC

RNA Area: 154.7  
 RNA Concentration: 111 ng/µl  
 rRNA Ratio [28s / 18s]: 1.5  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 9 :** S1 17 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.46	42.95	42.8	27.7
28S	46.48	50.25	62.2	40.2

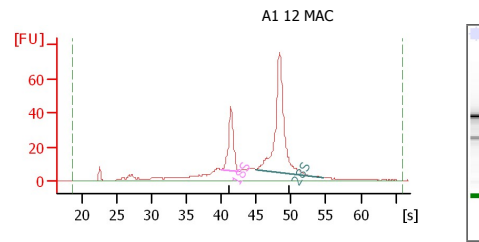


**Overall Results for sample 10 :** A1 7 MAC

RNA Area: 385.6  
 RNA Concentration: 276 ng/µl  
 rRNA Ratio [28s / 18s]: 1.8  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 10 :** A1 7 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.88	42.70	90.9	23.6
28S	47.20	50.36	161.6	41.9

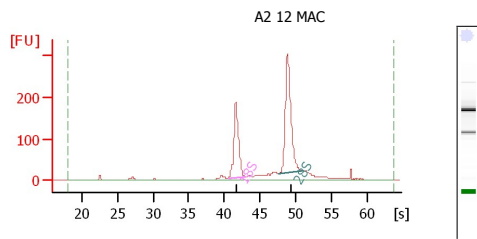


**Overall Results for sample 11 :** A1 12 MAC

RNA Area: 547.8  
 RNA Concentration: 392 ng/µl  
 rRNA Ratio [28s / 18s]: 2.9  
 RNA Integrity Number (RIN): 9 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:9

**Fragment table for sample 11 :** A1 12 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.02	42.51	71.7	13.1
28S	45.00	54.62	207.1	37.8

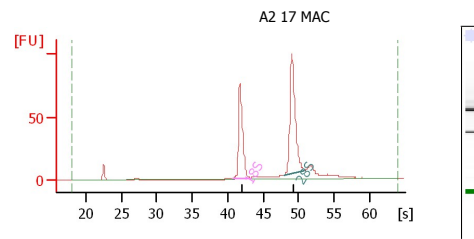


**Overall Results for sample 2 :** A2 12 MAC

RNA Area: 1,350.0  
 RNA Concentration: 730 ng/µl  
 rRNA Ratio [28s / 18s]: 1.6  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 2 :** A2 12 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.51	43.10	334.3	24.8
28S	47.64	51.20	538.3	39.9

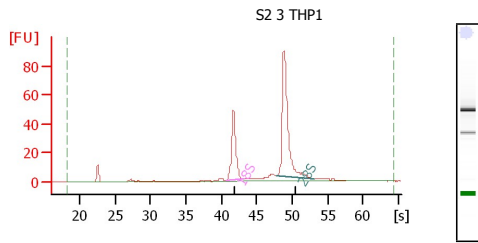


**Overall Results for sample 3 :** A2 17 MAC

RNA Area: 405.3  
 RNA Concentration: 219 ng/µl  
 rRNA Ratio [28s / 18s]: 1.4  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 3 :** A2 17 MAC

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.59	43.28	118.6	29.3
28S	47.88	50.85	167.4	41.3

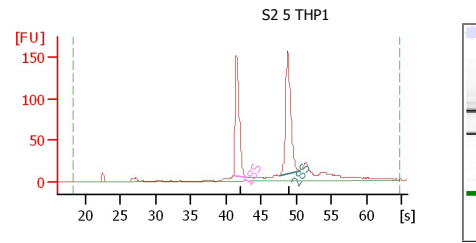


**Overall Results for sample 4 : S2 3 THP1**

RNA Area: 337.7  
 RNA Concentration: 183 ng/ $\mu$ l  
 rRNA Ratio [28s / 18s]: 2.1  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 4 : S2 3 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.71	43.18	80.6	23.9
28S	47.85	53.12	167.4	49.6

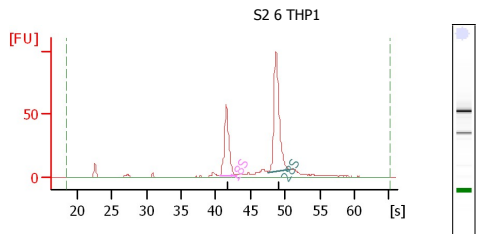


**Overall Results for sample 5 : S2 5 THP1**

RNA Area: 763.3  
 RNA Concentration: 413 ng/ $\mu$ l  
 rRNA Ratio [28s / 18s]: 1.1  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 5 : S2 5 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.10	43.03	228.1	29.9
28S	47.72	50.50	255.9	33.5

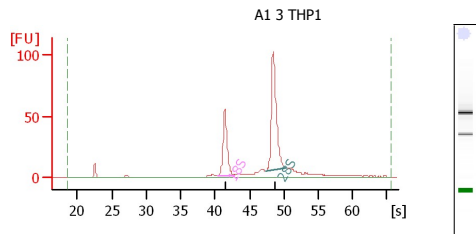


**Overall Results for sample 6 : S2 6 THP1**

RNA Area: 395.3  
 RNA Concentration: 214 ng/ $\mu$ l  
 rRNA Ratio [28s / 18s]: 1.7  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 6 : S2 6 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.51	43.01	99.7	25.2
28S	47.55	50.72	170.9	43.2

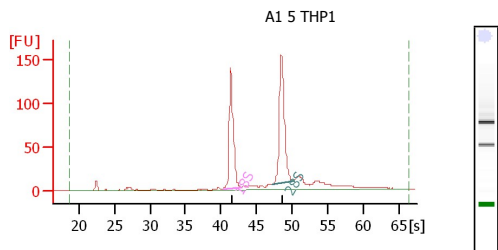


**Overall Results for sample 7 : A1 3 THP1**

RNA Area: 393.3  
 RNA Concentration: 213 ng/ $\mu$ l  
 rRNA Ratio [28s / 18s]: 2.0  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 7 : A1 3 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.29	42.86	87.7	22.3
28S	47.33	50.28	171.2	43.5

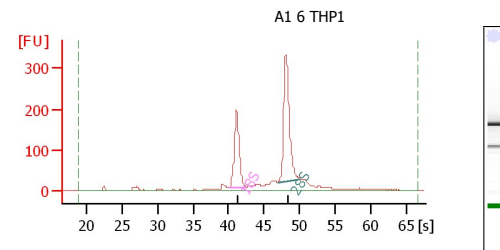


**Overall Results for sample 8 : A1 5 THP1**

RNA Area: 718.0  
 RNA Concentration: 388 ng/ $\mu$ l  
 rRNA Ratio [28s / 18s]: 1.2  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 8 : A1 5 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.24	42.79	213.2	29.7
28S	47.12	50.05	246.6	34.3

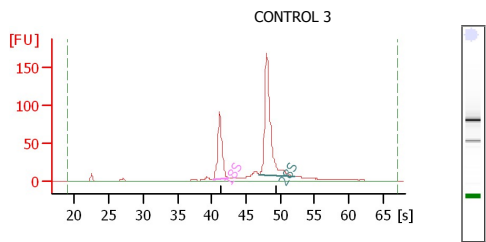


**Overall Results for sample 9 : A1 6 THP1**

RNA Area: 1,583.5  
 RNA Concentration: 856 ng/ $\mu$ l  
 rRNA Ratio [28s / 18s]: 1.6  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 9 : A1 6 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.06	42.53	367.9	23.2
28S	46.94	50.03	587.4	37.1

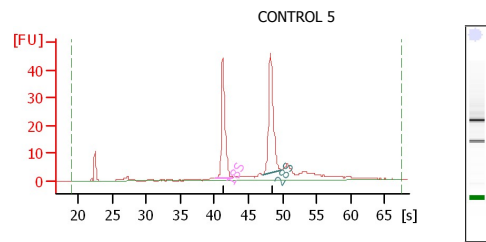


**Overall Results for sample 10 : CONTROL 3**

RNA Area: 722.7  
 RNA Concentration: 391 ng/µl  
 rRNA Ratio [28s / 18s]: 2.1  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 10 : CONTROL 3**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.08	42.56	152.1	21.0
28S	46.95	52.26	326.1	45.1

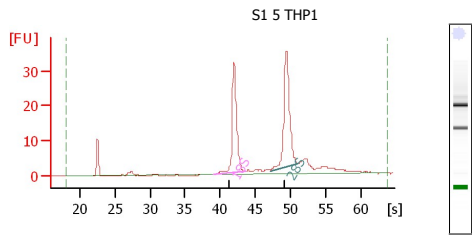


**Overall Results for sample 11 : CONTROL 5**

RNA Area: 222.3  
 RNA Concentration: 120 ng/µl  
 rRNA Ratio [28s / 18s]: 1.1  
 RNA Integrity Number (RIN): 9.9 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN: 9.90

**Fragment table for sample 11 : CONTROL 5**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.21	42.70	64.0	28.8
28S	47.15	49.84	71.8	32.3

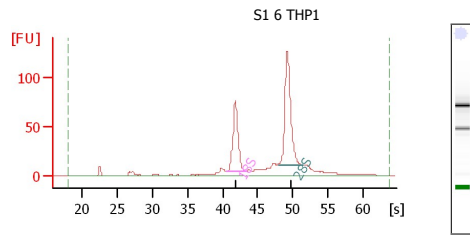


**Overall Results for sample 2 : S1 5 THP1**

RNA Area: 176.3  
 RNA Concentration: 278 ng/µl  
 rRNA Ratio [28s / 18s]: 1.1  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 2 : S1 5 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	38.98	43.61	55.4	31.4
28S	47.13	51.06	61.8	35.1

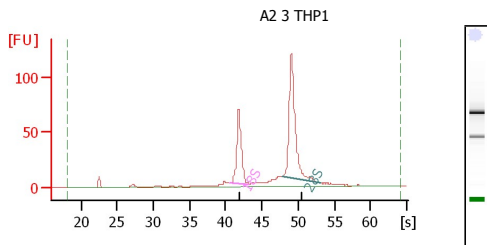


**Overall Results for sample 3 : S1 6 THP1**

RNA Area: 656.6  
 RNA Concentration: 1,036 ng/µl  
 rRNA Ratio [28s / 18s]: 1.5  
 RNA Integrity Number (RIN): 9.7 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN: 9.70

**Fragment table for sample 3 : S1 6 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.61	43.34	142.0	21.6
28S	47.86	51.42	219.0	33.4

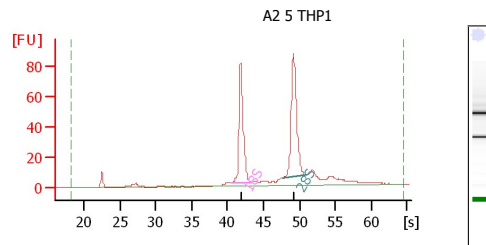


**Overall Results for sample 4 : A2 3 THP1**

RNA Area: 549.0  
 RNA Concentration: 866 ng/µl  
 rRNA Ratio [28s / 18s]: 1.9  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

**Fragment table for sample 4 : A2 3 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.49	43.23	119.5	21.8
28S	47.88	53.00	225.4	41.1



**Overall Results for sample 5 : A2 5 THP1**

RNA Area: 439.6  
 RNA Concentration: 694 ng/µl  
 rRNA Ratio [28s / 18s]: 1.1  
 RNA Integrity Number (RIN): 10 (B.02.07)  
 Result Flagging Color:    
 Result Flagging Label: RIN:10

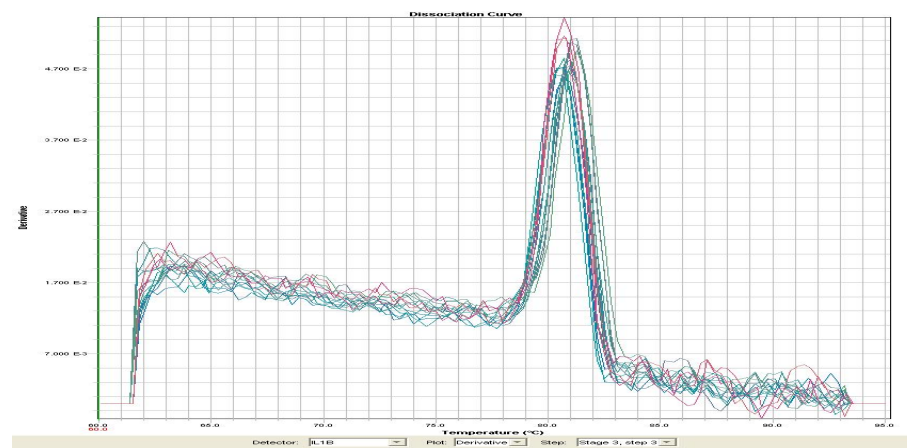
**Fragment table for sample 5 : A2 5 THP1**

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.56	43.32	129.5	29.5
28S	47.76	50.94	145.6	33.1

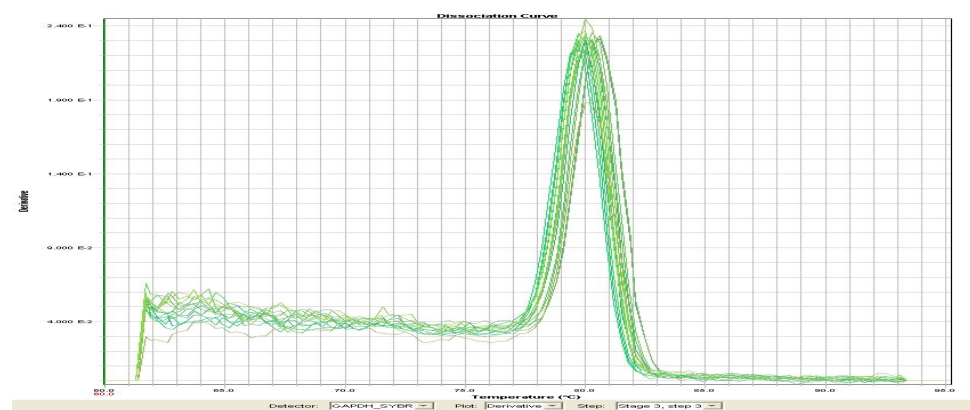


## Melt Curve

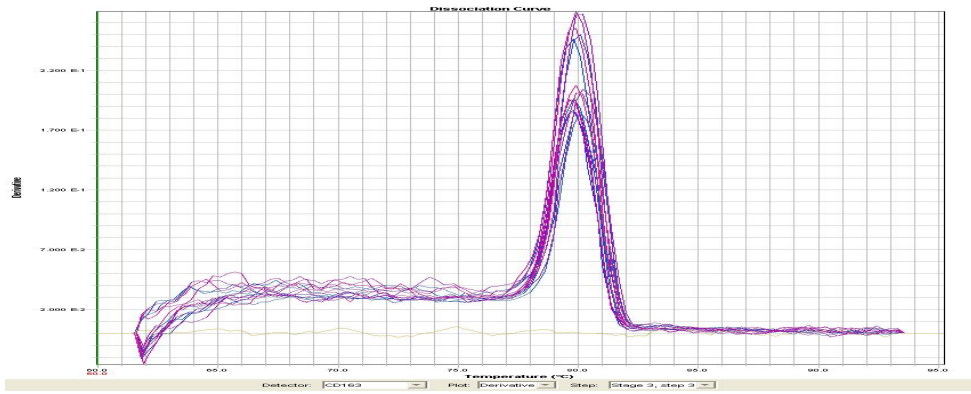
Melt curve analysis suggested that all the reaction was free from contamination; only single peaks were detected in each of the gene analysis.



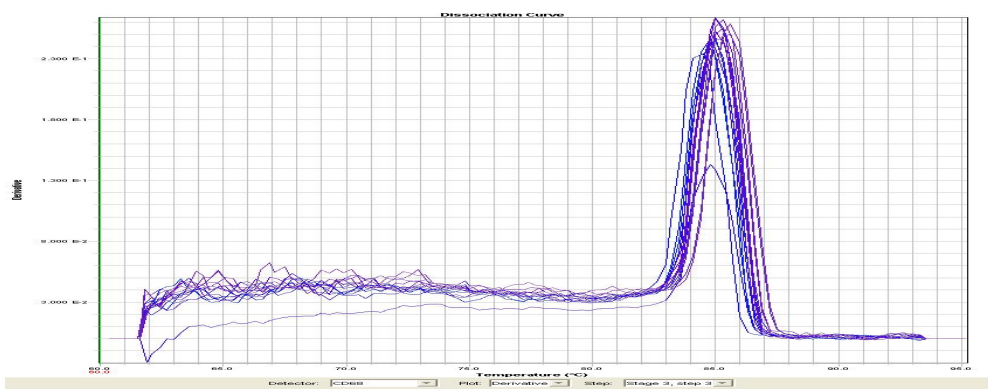
Melt curve analysis showing that the IL1 gene qPCR reactions are free from contamination for all of the samples.



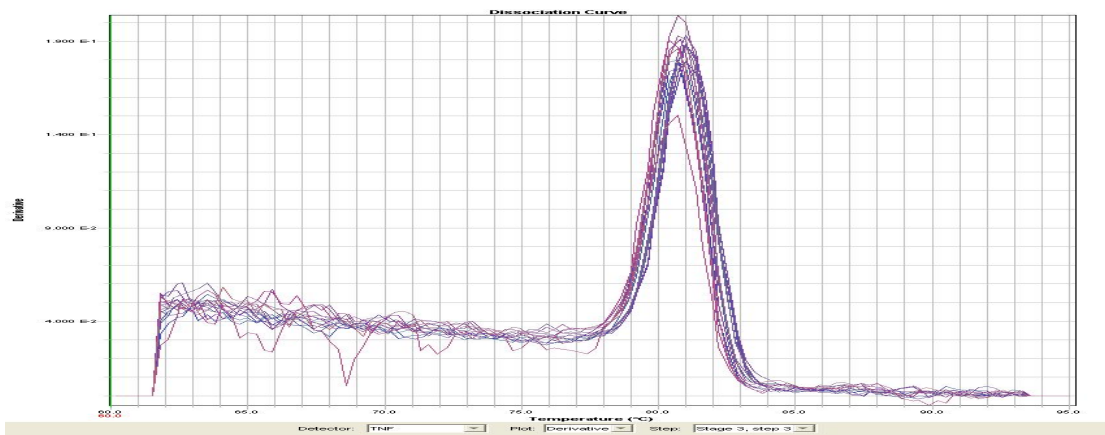
Melt curve analysis showing that the GAPDH gene qPCR reactions are free from contamination for all of the samples.



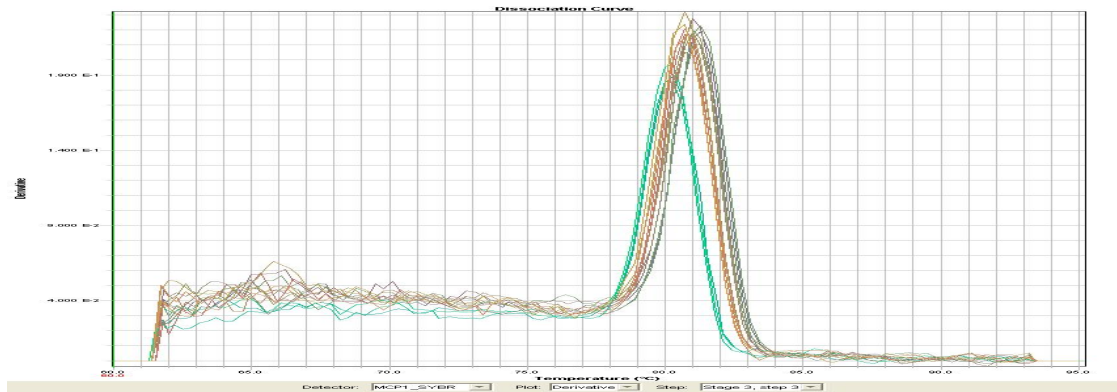
Melt curve analysis showing that the CD163<sup>+</sup> gene qPCR reactions are free from contamination for all of the samples.



Melt curve analysis showing that the CD68<sup>+</sup> gene qPCR reactions are free from contamination for all of the samples.



Melt curve analysis showing that the TNF  $\alpha$  gene qPCR reactions are free from contamination for all of the samples



Melt curve analysis showing that the MCP-1 gene qPCR reactions are free from contamination for all of the samples

### THP-1 Cell line

There were significant changes in RQ values for CD68<sup>+</sup>, CD163<sup>+</sup>, MCP-1 and TNF  $\alpha$  compared to control after 24 hours of incubation for THP-1 cells. Mean CD68<sup>+</sup> was 73% in the Actovegin group, CD163<sup>+</sup>, MCP-1 and TNF  $\alpha$  were significantly higher in the Actovegin group, 147%, 133% and 137% respectively (Table 3.4.3.2.a). There were no significant changes for IL1 gene expression compared to the control group. Two tailed t-test was used to compare the RQ values; there were significant changes ( $p < 0.05$ ) in the gene expression of CD68<sup>+</sup>, CD163<sup>+</sup>, MCP1 and TNF $\alpha$  in the Actovegin group compared to control (Table 3.4.3.2.e).

THP	CD68 <sup>+</sup> RQ	CD163 <sup>+</sup> RQ	IL1B RQ	MCP1 RQ	TNF $\alpha$ RQ
Actovegin	73%	147%	99%	133%	137%
Statistical sig.	Yes	Yes	No	Yes	Yes

(Table 3.4.3.2.a) Results summary for THP-1 cells after 24 hours of incubation. Actovegin group showed statistical difference in CD68<sup>+</sup>, CD163<sup>+</sup>, MCP-1 and TNF  $\alpha$

The threshold cycle ( $C_t$ ) is shown in Table 3.4.3.2.b and the  $\Delta\Delta C_t$  values are shown in Table 3.4.3.2.c. Table 3.4.3.2.d shows the relative quantity of each gene in the Actovegin group compared to control in THP-1 cell line after 24 hours of incubation.

THP	GAPDH Ct	CD68 <sup>+</sup> Ct	CD163 <sup>+</sup> Ct	IL1B Ct	MCP1 Ct	TNF α Ct
control 1	16.44	20.78	30.74	28.70	25.58	24.06
control 2	16.34	20.31	30.86	28.72	26.00	24.26
control 3	16.08	20.52	30.47	28.19	25.29	24.49
control 4	15.94	20.49	31.33	25.32	25.13	25.34
control 5	16.02	20.58	31.51	25.90	26.05	26.53
control 6	16.62	20.89	32.30	26.59	26.72	27.03
<b>mean</b>	<b>16.24</b>	<b>20.60</b>	<b>31.20</b>	<b>27.24</b>	<b>25.80</b>	<b>25.29</b>
acto 1	16.09	20.83	31.57	25.70	25.18	24.87
acto 2	16.50	21.15	31.37	26.66	25.92	26.06
acto 3	16.05	20.94	31.59	26.27	26.06	26.30
acto 4	16.71	21.20	30.23	28.66	25.31	23.90
acto5	16.37	21.39	30.31	28.54	25.51	23.92
acto 6	16.36	21.76	29.75	28.44	25.05	24.81
<b>mean</b>	<b>16.35</b>	<b>21.21</b>	<b>30.80</b>	<b>27.38</b>	<b>25.51</b>	<b>24.98</b>

(Table 3.4.3.2.b) Q- PCR C<sub>t</sub> of each gene in control vs Actovegin group in THP-1; the value of each cell represents the average of the triplet per experiment, in total 216 sets of pPCR were performed.

THP	CD68 <sup>+</sup> ΔΔ Ct	CD163 <sup>+</sup> ΔΔ Ct	IL1B ΔΔ Ct	MCP1 ΔΔ Ct	TNF α ΔΔ Ct
<b>acto 1</b>	0.19	0.08	0.23	-0.11	-0.63
<b>acto 2</b>	0.09	-0.62	0.27	-0.62	-0.95
<b>acto 3</b>	0.62	-0.16	0.24	-0.10	-0.17
<b>acto 4</b>	0.14	-0.79	-0.31	-0.54	-0.43
<b>acto5</b>	1.04	-0.58	-0.22	-0.52	-0.38
<b>acto 6</b>	0.96	-1.00	-0.03	-0.52	0.04
<b>mean</b>	0.51	-0.51	0.03	-0.40	-0.42
<b>s.d.</b>	0.43	0.40	0.26	0.23	0.35

(Table 3.4.3.2.c) ΔΔC<sub>t</sub> value for each gene of interest in qPCR in THP-1

THP	CD68 <sup>+</sup> RQ	CD163 <sup>+</sup> RQ	IL1B RQ	MCP1 RQ	TNF α RQ
acto 1	0.88	0.94	0.85	1.08	1.54
acto 2	0.94	1.54	0.83	1.53	1.94
acto 3	0.65	1.11	0.84	1.07	1.13
acto 4	0.91	1.73	1.24	1.46	1.35
acto5	0.49	1.50	1.16	1.43	1.30
acto 6	0.51	2.00	1.02	1.43	0.97
<b>mean</b>	0.73	1.47	0.99	1.33	1.37
<b>s.d.</b>	0.20	0.39	0.18	0.20	0.34

(Table 3.4.3.2.b) Relative quantity of each gene in the Actovegin group compared to control in THP-1. RQ values for each gene of interest in qPCR in reference to control group which is = 1.

THP	t-test for Equality of Means (Equal variances not assumed)						
	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% C.I of the Difference	
						Lower	Upper
CD68 <sup>+</sup>	3.22	5	<b>0.024*</b>	0.27	0.084	0.054	0.486
CD163 <sup>+</sup>	-2.94	5	<b>0.032*</b>	-0.47	0.160	-0.880	-0.060
IL1	0.14	5	<b>0.896</b>	0.01	0.073	-0.178	0.198
MCP-1	-4.01	5	<b>0.001*</b>	-0.33	0.083	-0.547	-0.120
TNF α	-2.68	5	<b>0.004*</b>	-0.37	0.139	-0.728	-0.015

(Table 3.4.3.2.e) t-test confirmed that there was statistical difference in RQ values between control and Actovegin treated cells in CD68<sup>+</sup>, CD163<sup>+</sup>, MCP-1 and TNF α. There was no statistical difference in RQ values found in IL1 between the groups \*= Statistically significant

### Macrophage Cell line

There were significant changes in RQ values for CD68<sup>+</sup>, CD163<sup>+</sup>, MCP-1 and TNF α compared to control after 24 hours of incubation of the Macrophage cells. Mean CD163<sup>+</sup> was 63% in the Actovegin group. CD68<sup>+</sup>,

MCP-1 and TNF  $\alpha$  were significantly higher in the Actovegin group, 177%, 310% and 410% respectively (Table 3.4.3.2.f). Student t-test confirmed statistical significance. There were no significant changes for IL1 gene expression in the parametric test compared to the control group. (Table 3.4.3.2.g)

Mac	CD68 <sup>+</sup> RQ	CD163 <sup>+</sup> RQ	IL1B RQ	MCP1 RQ	TNF $\alpha$ RQ
Actovegin	177%	63%	120%	310%	410%
Statistical significant	Yes	Yes	No	Yes	Yes

(Table 3.4.3.2.f) Results Summary for **macrophage** cells after 24 hours of incubation. Actovegin group showed statistical difference in CD68<sup>+</sup>, CD163<sup>+</sup>, MCP-1 and TNF  $\alpha$

mac	t-test for Equality of Means (Equal variances not assumed)						
	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% C.I. of the Difference	
						Lower	Upper
CD68 <sup>+</sup>	-2.91	5	0.034*	-0.77	0.266	-1.457	-0.089
CD163 <sup>+</sup>	2.73	5	0.041*	0.37	0.137	0.022	0.725
IL1	-1.18	5	0.291	-0.20	0.166	-0.625	0.231
MCP-1	-2.96	5	0.032*	-2.10	0.708	-3.917	-0.276
TNF $\alpha$	-2.59	5	0.049*	-3.13	1.209	-6.236	-0.020

(Table 3.4.3.2.g) t-test confirmed that there was statistical difference in RQ values between control and Actovegin treated cells in CD68<sup>+</sup>, CD163<sup>+</sup> MCP-1 and TNF  $\alpha$ . There was no statistical difference in RQ values found in IL1 between the groups. \*= Statistical significant

The threshold cycle ( $C_t$ ) is shown in Table 3.4.3.2.h and the  $\Delta\Delta C_t$  values are shown in Table 3.4.3.2.i. Table 3.4.3.2.j shows the relative quantity of each gene in the Actovegin group compared to control in the macrophage cell line after 24 hours of incubation.

<b>mac</b>	<b>GAPDH Ct</b>	<b>CD68<sup>+</sup>Ct</b>	<b>CD163<sup>+</sup>Ct</b>	<b>IL1B Ct</b>	<b>MCP1 Ct</b>	<b>TNF <math>\alpha</math> Ct</b>
<b>control 1</b>	16.63	20.95	20.54	17.50	20.32	22.93
<b>control 2</b>	17.88	21.12	20.95	18.00	21.35	22.91
<b>control 3</b>	15.97	20.92	20.68	18.07	21.13	22.93
<b>control 4</b>	15.39	19.66	20.56	14.92	19.33	21.64
<b>control 5</b>	15.80	19.08	21.30	15.02	19.65	21.93
<b>control 6</b>	16.07	20.08	21.62	15.26	19.59	22.17
<b>Mean</b>	<b>16.29</b>	<b>20.30</b>	<b>20.94</b>	<b>16.46</b>	<b>20.23</b>	<b>22.42</b>
<b>acto 1</b>	16.93	20.61	21.06	17.58	18.80	20.23
<b>acto 2</b>	16.63	20.00	21.60	17.56	19.02	20.23
<b>acto 3</b>	16.81	20.44	21.28	18.02	19.31	20.80
<b>acto 4</b>	16.72	19.62	22.70	16.09	19.04	21.81
<b>acto5</b>	15.87	18.39	22.57	14.78	18.62	20.89
<b>acto 6</b>	15.72	19.25	22.47	14.59	18.71	20.96
<b>Mean</b>	<b>16.45</b>	<b>19.72</b>	<b>21.95</b>	<b>16.44</b>	<b>18.92</b>	<b>20.82</b>

(Table 3.4.3.2.h) Q- PCR C<sub>t</sub> of each gene in control vs Actovegin group in the **Macrophage cell line**, the value of each cell represents the average of the triplet per experiment, in total 216 set of pPCR were performed.

<b>mac</b>	<b>CD68<sup>+</sup> <math>\Delta\Delta</math> Ct</b>	<b>CD163<sup>+</sup> <math>\Delta\Delta</math> Ct</b>	<b>IL1B <math>\Delta\Delta</math> Ct</b>	<b>MCP1 <math>\Delta\Delta</math> Ct</b>	<b>TNF <math>\alpha</math> <math>\Delta\Delta</math> Ct</b>
<b>acto 1</b>	-0.64	0.22	-0.23	-1.81	-3.00
<b>acto 2</b>	0.14	1.91	0.81	-1.07	-1.42
<b>acto 3</b>	-1.32	-0.24	-0.89	-2.66	-2.98
<b>acto 4</b>	-1.38	0.81	-0.15	-1.62	-1.16
<b>acto5</b>	-0.76	1.20	-0.32	-1.10	-1.11
<b>acto 6</b>	-0.47	1.20	-0.31	-0.53	-0.86
<b>mean</b>	-0.74	0.85	-0.18	-1.47	-1.76
<b>s.d.</b>	0.56	0.77	0.55	0.74	0.97

(Table 3.4.3.2.i) "Threshold cycle" difference in Actovegin group compared to control in Macrophages.  $\Delta\Delta C_t$  value for each gene of interest in qPCR in Macrophages

mac	CD68 <sup>+</sup> RQ	CD163 <sup>+</sup> RQ	IL1B RQ	MCP1 RQ	TNF α RQ
acto 1	1.56	0.86	1.17	3.51	8.00
acto 2	0.91	0.27	0.57	2.11	2.68
acto 3	2.49	1.18	1.85	6.30	7.87
acto 4	2.59	0.57	1.11	3.07	2.24
acto5	1.70	0.44	1.24	2.15	2.16
acto 6	1.39	0.44	1.24	1.44	1.82
<b>mean</b>	1.77	0.63	1.20	3.10	4.13
<b>s.d.</b>	0.65	0.34	0.41	1.74	2.96

(Table 3.4.3.2.j) Relative quantity of each gene in the Actovegin group compared to control in Macrophages. RQ value for each gene of interest in qPCR in reference to control group which is = 1.

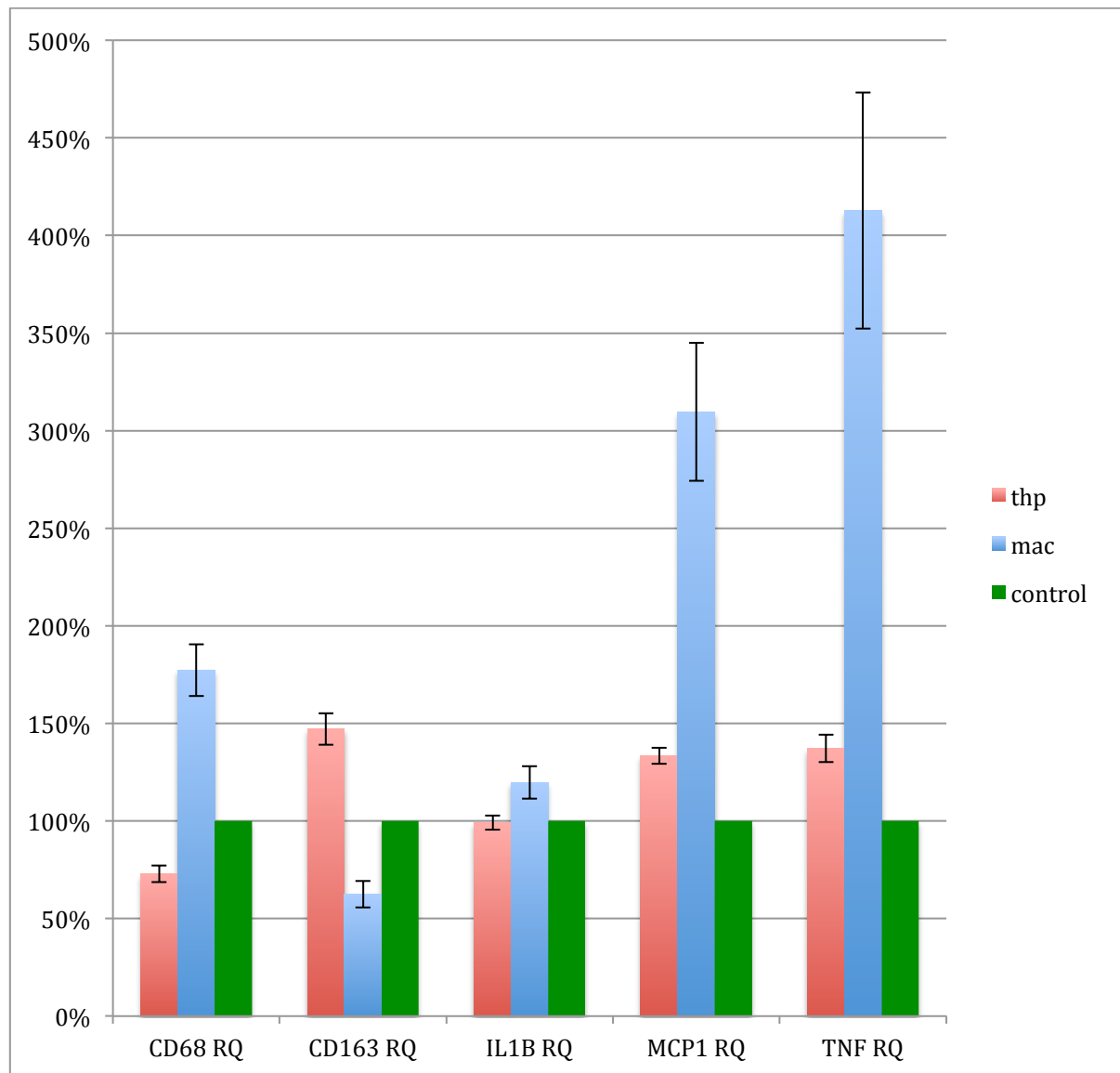
Overall results summary:

qPCR demonstrated Actovegin significantly up regulated the THP-1 cell subtype CD163<sup>+</sup> to 147% and suppressed the CD68<sup>+</sup> subtype to 73%. It also up regulated the gene expression of the inflammatory cytokine MCP-1 to 133% and TNF α to 137% (Table 3.4.3.2.k). In Macrophages, it significantly up regulated the CD68<sup>+</sup> subtype to 177% and suppressed the CD163<sup>+</sup> subtype to 63%. It also caused a 3 fold MCP-1 and 4-fold increase in TNF α gene expression (Table 3.4.3.2.k). Figure 3.4.3.2 summaries the results in graphical format.

	CD68 <sup>+</sup> RQ	CD163 <sup>+</sup> RQ	IL1B RQ	MCP1 RQ	TNF α RQ
<b>THP-1</b>	73%*	147%*	99%	133%*	137%*
<b>Macrophages</b>	177%*	63%*	120%	310%*	410%*

(Table 3.4.3.2.k) Results summary - qPCR Relative Quantity value (RQ) results summary, Blue = reduced, Red = increased \*= Statistical significance

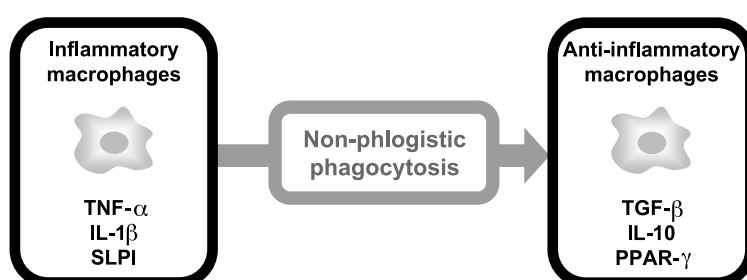




**(Figure 3.4.3.2)** Graph to show mean Relative Quantity (RQ) of gene expression from qPCR for THP-1 and Macrophage cells compared to control after 24 hours of incubation. There were significant changes in CD68<sup>+</sup>, CD163<sup>+</sup>, MCP-1 and TNF  $\alpha$  gene expression in the Actovegin Group compared to control.

### 3.4.3.3 Discussion

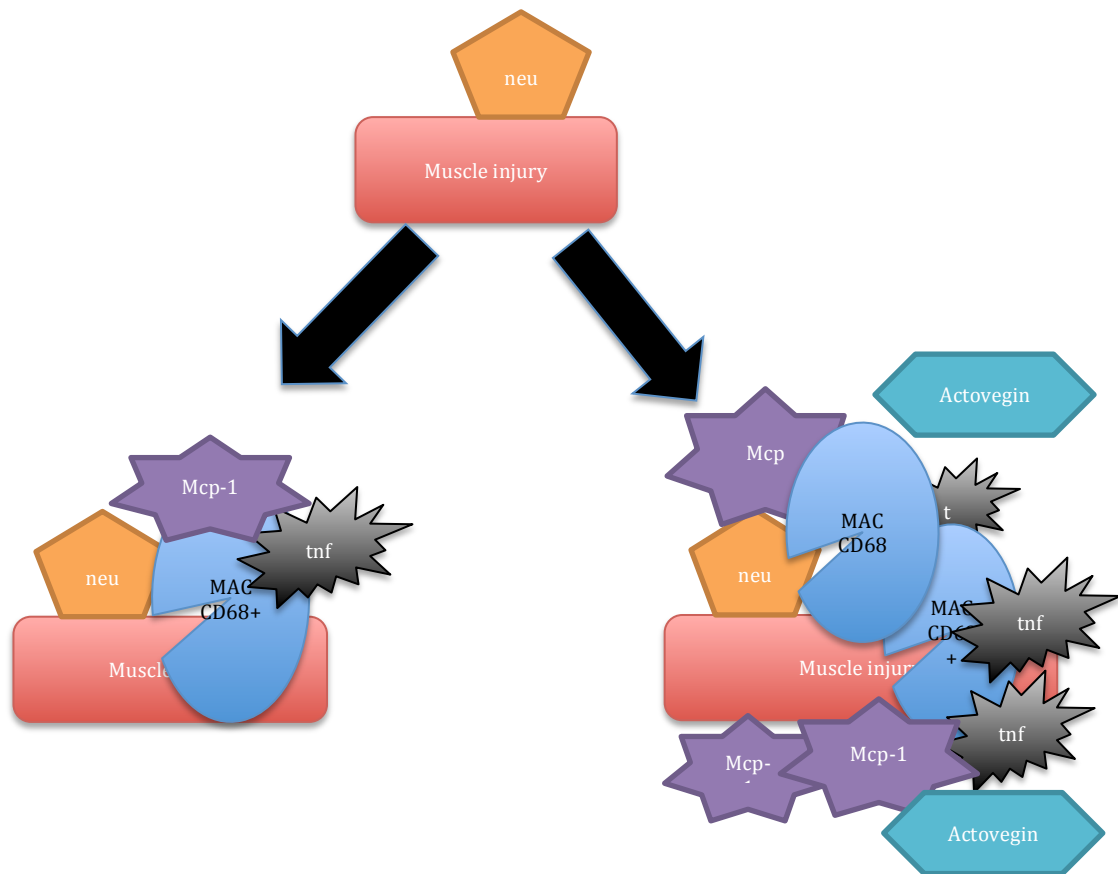
The *in vitro* study in this PhD confirmed that Actovegin has a role in the inflammatory process. It can significantly affect the cell genotype expression of the acute inflammatory cells such as THP-1 and macrophages. Actovegin up regulates the CD68<sup>+</sup> subtype of “phagocytic” macrophages, characterized by the expression of the CD68<sup>+</sup> cell surface marker and lacking the CD163<sup>+</sup> marker. These CD68<sup>+</sup> subtypes of macrophages are pro-inflammatory phagocytes and were thought to be responsible for the removal of necrotic debris during the initial phase of the inflammatory process following muscle injury. It also down regulates the CD163<sup>+</sup> subtype of “non-phagocytic” macrophages, which was thought to contribute to the termination of the destruction phase and initiate the repair phase of the muscle healing process (Ciciliot and Schiaffino, 2010, Chazaud et al., 2009) (Figure 3.4.3.3.a). Further details are explained in Section 2.3. Therefore, the change of macrophage subtype suggests that Actovegin could significantly increase the acute inflammatory process, which is also confirmed by the expression of the inflammatory cytokines MCP-1 and TNF  $\alpha$ . Figure 3.4.3.3.b explain the finding in a graphical format.



TNF  $\alpha$  = tumor necrosis factor, IL-1 =interleukin-1, SLPI = secretory leukocyte protease inhibitor, TGF-A=transforming growth factor, IL-10= interleukin 10, PPAR =peroxisome proliferator activated receptor

Figure 3.4.3.3.a Switch of macrophage phenotype upon phagocytosis. *In vitro*, phagocytosis of both apoptotic and necrotic debris switches the phenotype of pro-inflammatory macrophages into anti-inflammatory macrophages, which is associated with the resolution of inflammation and tissue repair (Arnold et al., 2007).

### Inflammatory Phase (destruction)



### Anti Inflammatory Phase (repair)



cd163 = CD163<sup>+</sup> anti-inflammatory macrophages neu = neutrophils, MAC CD68 = CD68<sup>+</sup> pro-inflammatory macrophages, TNF  $\alpha$  = Tumour Necrosis Factor, MCP-1 = Monocyte Chemotactic Protein -1

**(Figure 3.4.3.3.b)** explained the *in vitro* study findings. Actovegin up regulated the CD68<sup>+</sup> macrophages in the initial destruction phase, this leads to increase in cell number as well as increase the inflammatory responds by increase the inflammatory cytokines MCP-1 and TNF  $\alpha$ . In the repair phase Actoveign up regulated THP-1 cell, which matured to become CD163<sup>+</sup> macrophages and speed up repair.

The role of Actovegin in the acute inflammatory process following muscle injury is not limited to its effects on the CD68<sup>+</sup> macrophages. It also exhibits an effect on THP-1 cells and up regulates a specific sub group of THP-1 cells. Although THP-1 cells have a limited role in the inflammatory process, they differentiate into macrophages. The qPCR results demonstrated that Actovegin could significantly up regulate the CD163<sup>+</sup> subtype of THP-1 cells, which will differentiate into the CD163<sup>+</sup> subtype of macrophage. The differentiation process, which depends on other biochemical factors, was believed to take place approximately 48 hours after acute muscle injuries. Once the CD163<sup>+</sup> subtype of THP-1 cells differentiates into the CD163<sup>+</sup> subtype of “non-phagocytic” Macrophages, they will have an impact on the repair phase of the inflammatory process. (figure 3.4.3.3.b)

The qPCR study in this PhD is based on high quality RNA extracted from 216 samples for each cell type. As demonstrated by gene chip analysis, the RNA integrity is the highest possible grade (range between 9.9 -10). The cDNA transcription and the primer used were also free from contamination as the melt curve for all 432 samples only demonstrated a single peak. Therefore the qPCR study in this PhD is high quality and reliable.

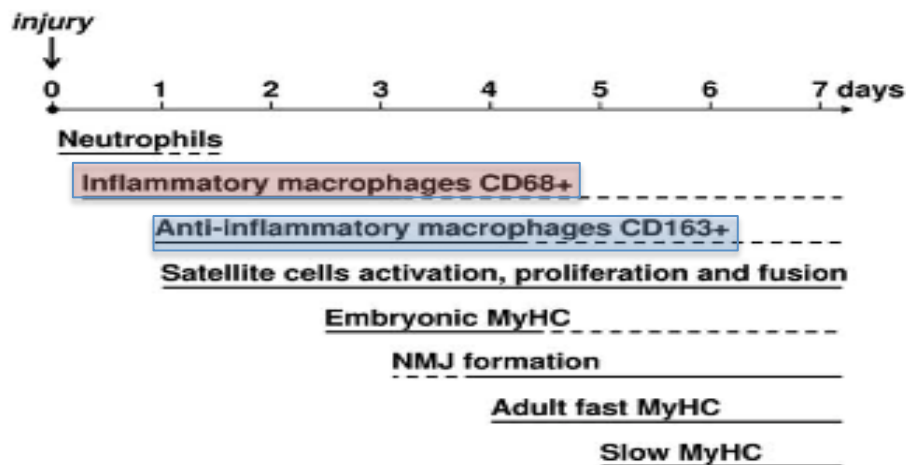
### 3.5 Summary for in vitro study

The aim of the *in vitro* study in this chapter was to investigate the biochemical role of Actovegin in the acute inflammatory process as stated in Section 2.7. The cell proliferative studies (Section 3.4.2) from this PhD suggested that Actovegin could significantly ( $p=0.00006$ ) increase macrophage proliferation (+33%) after 24 hours of incubation compared to control. This increase in cell number could theoretically increase the initial inflammatory response if this increase is specific to the pro-inflammatory subtype of macrophages. The qPCR gene analysis from this PhD (Section 3.4.3) confirmed that it specifically up regulated CD68<sup>+</sup> pro-inflammatory macrophages (+77%,  $p<0.05$ ) and down regulated CD163<sup>+</sup> anti-inflammatory macrophages (-47%,  $p<0.05$ ). At the beginning of the inflammatory phase of the muscle healing process, up regulation of CD68<sup>+</sup> pro-inflammatory macrophages could be beneficial as it could increase the chemokine signalling pathway and increase subsequent recruitment of circulating monocyte cells to the injury site (further details in Section 2.3.3). Furthermore, the qPCR gene analysis on the inflammatory mediators suggested a significant ( $p< 0.05$ ) 3 - 4 fold increase in Macrophage specific MCP-1 and TNF  $\alpha$  Chemo-Cytokines. These chemo-cytokines are known to be a potent chemo-attractant for circulating inflammatory cells (Arnold et al., 2007, Acharyya et al., 2007, Chazaud et al., 2009). This demonstrates that the up regulation of the pro inflammatory CD68<sup>+</sup> macrophage by Actovegin could translate to up regulation of the inflammatory process. Table 3.5 summarises the significant findings from the in-vitro studies.

Actovegin treated cells	Cell number	qPCR			
		CD68 <sup>+</sup>	CD163 <sup>+</sup>	MCP1	TNF $\alpha$
THP-1	+39%		+147%		
Macrophages	+31%	+77%	- 47%	+ 3-4 fold	+ 3-4 fold

(Table 3.5) Results summary – proliferative cell essay results, qPCR results on Actovegin treated THP-1 and Macrophages cell line. Actovegin treated cells show significant increase in cell number and up regulation of the pro inflammation macrophages CD68<sup>+</sup>, anti-inflammatory THP-1 CD163<sup>+</sup> and the pro inflammatory markers TNF  $\alpha$  and MCP-1. It down regulates the CD163<sup>+</sup> anti inflammatory macrophages.

This combination of findings demonstrates that Actovegin is an active agent, which causes significant up regulation of the initial pro-inflammatory response following injury. It may also have a role in the later anti-inflammatory phase as the CD163<sup>+</sup> THP-1 matures. Figure 3.5 summary the finding in a diagram modified form Ciciliot and Schiaffino 2010. This finding confirmed the hypothesis, stated in Section 2.7, suggesting that Actovegin is the most likely active substance in a pharmacological cocktail used in previous published literature. As discussed in Section 2.3.3, modulation of the inflammatory process could theoretically alter the speed and quality of the muscle healing process. The in-vitro studies confirmed that Actovegin could cause the up regulation of the pro inflammatory stage of the inflammatory process; therefore a clinical study is needed to investigate this clinical efficacy in the treatment of acute muscle injuries. The rationale and methodology of the clinical study will be discussed in Chapter 4.



**(Figure 3.5)** Diagram above illustrates the biochemical events of the muscle healing process including inflammatory, anti-inflammatory, proliferation and maturation phases. The *in vitro* study suggested that Actovegin can speed up stage 2 (red) and 3 (blue) of the muscle healing process.

# Chapter Four

## Clinical Study

### 4.1 Introduction

The in-vitro study in Chapter Three confirmed the role of Actovegin in the inflammatory process of acute muscle injuries. Actovegin can directly and indirectly influence the key players involved in the biochemical process of inflammation (CD68<sup>+</sup>) and repair (CD163<sup>+</sup>) of the acute inflammatory process. As discussed in Section 2.3.3, these subtypes of macrophages can influence the quality and speed of muscle healing. Section 2.7 outlined this PhD thesis research question, as there was convincing evidence from the in-vitro study suggesting a positive role for Actovegin in acute muscle injuries; a clinical study was performed to investigate the clinical impact of the in-vitro findings. There has been little published evidence on the use of Actovegin in the treatment of muscle injuries. There has only been 1 study reporting the use of IM Actovegin in the treatment of acute muscle injuries. Besides the fact that it was not a single drug study, there were also many limiting factors, which have been discussed in Section 2.6.4. There have not been any studies reporting the use of Actovegin as a single intramuscular agent in treating muscle injuries. Therefore, after ethical approval from Cardiff University Ethics Committee (see appendix C), the clinical element of this PhD, as stated in Section 2.7, was commenced.

In this Chapter, the clinical efficacy of Actovegin in the treatment of acute muscle injuries will be explored. The rationale for patient selection, method selected for diagnosis of muscle injuries, Actovegin treatment regimen, rehabilitation protocol and functional assessment will be discussed. The clinical study protocol and its results will be reported and discussed.

Hypothesis for the clinical studies in this PhD:

Actovegin treatment regimen developed from this PhD can influence clinical outcome in treating acute muscle injuries.

## **4.2 Patient Selection**

Muscle injuries are common in sports, as discussed in Section 2.4.2; its treatment regimen and recovery are multifactorial. In the general population, muscle injuries may cause some pain and discomfort; they do not usually lead to any significant mortality or morbidity and the patient is usually able to carry on with daily activities. In contrast to the professional elite athlete, muscle injuries are a serious condition as they could significantly affect their performance, which could potentially change their career. Shortened recovery time from muscle injuries in professional athletes would enable training to be continued, competition demand to be fulfilled and increased game played. These consequences have direct influence on the athlete and their sponsors, therefore the effect of muscle injuries is much more profound in professional athletes. Section 2.4.1 explained the aetiology of muscle injuries in professional athletes, it accounted for 15 matches and 80 training days lost per club per season in British professional football (Woods et al., 2004, Ekstrand et al., 2011). Therefore, high level professional football players are an ideal group for the clinical study in this PhD. In order to limit other confounding factors in the study, only a single club of high-level football players was used. All the players in the club would follow the same pre-season training, diagnostic method, rehabilitation regimen, physiotherapy treatment and return to play assessment.



## **4.3 Method of Clinical Measurement**

### **4.3.1 Diagnosis**

As discussed in Section 2.3.1, muscle injuries can be classified into 3 grades. Traditionally, the assessment of muscle injuries is only based on clinical symptoms. Modern imaging techniques such as MRI scanning are a useful adjunct for helping the clinician to confirm the diagnosis.

#### **4.3.1.1 Clinical Assessment**

Clinical assessment is the most common form of assessment to diagnose muscle injuries. Although it is a subjective assessment that could vary between clinicians, it can be a powerful tool for identification and grading when used appropriately by experienced clinicians. To ensure the reliability and reproducibility of this study, 3 independent clinicians, the team doctor and the physiotherapists will carry out independent assessments on each injured subject to assess the severity of the muscle injuries.

The mechanism of injury was reviewed prior to clinical assessment, either by direct questioning of the injured player or from the recorded video footage of the event leading to the injury. Each individual clinician performed clinical examination on the injured player, including observation, palpation, strength testing and evaluation of motion. The clinicians recorded their findings independently and summarised the injury to clinical grading according to Section 2.2.1.

#### **4.3.1.2 MRI**

The classification of muscle injuries should be based on clinical assessment by the clinician, however MRI scanning is a useful adjunct to confirm clinical findings. Depending on the power of the MRI scanner and its selected sequence of signal interpretation, the image quality and its sensitivity

to acute muscle injuries varies (Kujala et al., 1997, Ekstrand et al., 2012, Elliott et al., 2008, Jarvinen et al., 2007). Although there have been reports suggesting that MRI scans could accurately grade muscle injuries and predict recovery time, it requires high power MRI machines with a specific sequences, which are not commonly available (Ekstrand et al., 2012, Elliott et al., 2008, Jarvinen et al., 2007). Therefore MRI scans can only be used as an adjunct for diagnosis and grading of muscle injuries. The professional football club policy dictated that all suspected injured player should undergo MRI scanning and its result to be reported by a consultant radiologist.

Although the MRI scan data is not an essential part for the diagnosis and classification of the muscle injuries, it will further validate the clinical assessment. However if the clinical assessment and MRI scan do not reveal the same diagnosis and grading, further consultation with the radiologist will be sought.

#### **4.3.2 Treatment**

Section 2.7 has discussed the potential problems with the polypharmacy of Dr. Mueller-Wohlfahrt's injection treatment regimen of Actovegin, local anaesthetic and Traumeel mixture. Local anaesthetics can lead to myonecrosis and Traumeel is a homeopathic substance which contains 99% sterile isotonic saline (Schneider, 2011); the only potential "active" substance in Dr. Mueller-Wohlfahrt's regimen may be a drug called "Actovegin". For further details, please see 2.4.2.3. There have not been any studies investigating the role of stand-alone Actovegin intramuscular injections in acute muscular injuries. In order to reduce the complex pharmacodynamics and pharmacokinetics of drug / substance interaction, the aim of this study is to investigate the role of Actovegin as a stand-alone agent in treating muscle injuries.

#### 4.3.2.1 Actovegin regimen

Actovegin is a drug that is believed to have positive effects on ischaemic cells and wound healing. Its clinical evidence is discussed in Section 2.6. The pathophysiological insult on cells following muscle strain injuries is a mechanical and physiological process as detailed in 2.3.2. Cells that are damaged during the injury need to be debrided by the inflammatory process during muscle healing; these “dead” cells do not have an active role in muscle healing. On the other hand, the cells in the nearby ischaemic zone will influence the pathophysiology of healing. (Figure 2.3.2) Therefore, in order to target these cells, treatment agents should be directly delivered to this area. As these cells are in the ischaemic zone, the only reliable way of delivery is via direct intramuscular injection instead of intravenous injection.

The pathophysiology of muscle healing is via the inflammatory process; it is energy/ATP dependent as discussed in Section 2.3.3. Actovegin is believed to be able to enhance oxidative phosphorylation to improve the cell's ischaemic process and also exhibit a cell membrane stabilising effect, therefore could potentially benefit the cells in the ischaemic zone, further details can be found in Section 2.3.4. Furthermore, from the in-vitro study described in Chapter 3, Actovegin has a direct and indirect effect on the key players involved in the biochemical process of the inflammation (CD68<sup>+</sup>) and repair (CD163<sup>+</sup>) of the muscle healing process. Therefore, in order for Actovegin to exhibit the local effect, it has to be injected directly to the nearby area of the injury site.

There were two common methods of delivering injection therapy described in the published literature - injection to the injury site under direct palpation and the multiple needle penetration injection technique (Speed, 2007, Reurink et al., 2012). One of the major criticisms of the direct palpation technique is the accuracy of the injections; however, there have been many studies suggesting that accuracy does not correlate with efficacy (Eustace et al., 1997, Jaeger and Reeves, 1986, Reeves et al., 1986). With regards to the multiple needle penetration injection technique, it was originated from

prolotherapy techniques, assuming fresh trauma to the injury site would “stimulate” recovery (Speed, 2007, Refai et al., 2011, Topol et al., 2005, Reeves and Hassanein, 2000). However, there have been case reports suggesting that multiple needle penetration injections could further weaken the injured structure and lead to muscle rupture. (Aydemir et al., 2010, Chen et al., 2009, Kim et al., 2010, Fitzgerald et al., 2005) There have been no studies comparing the efficacy of these two injection techniques. In this clinical study, due to the acute nature of the injuries and diagnosis, it is fruitless to induce unnecessary trauma by multiple needle penetration injection techniques. In order to minimize the potential hazard of sharps related injuries and infections, the single direct palpation injection technique was used.

According to the manufacturer’s recommendations from Nycomed, Actovegin solution is acidic and the intramuscular injection should not exceed 5ml (400mg) to a single site. Pfister et al 1990, described a five injection site technique with a mixture of 3ml (120mg) Actovegin and 2 ml of Traumeel around the injured area (Pfister and Koller, 1990). The volume of intramuscular injection was based on muscle size, large volume (>5ml) was associated with increased complication rate and discomfort (Workman, 1999, Wynaden et al., 2005). According to published research from intramuscular vaccination programmes, a volume of less than 4 ml is well tolerated in the hamstring (Diggle and Deeks, 2000, Wynaden et al., 2005). Only a single injection site was used in this clinical study. In order to achieve maximum penetration with minimum discomfort, 2ml was chosen to be the most appropriate volume for injection.

With regards to the timing of the treatment regimens, initial injection would be performed at clinical diagnosis of muscle injury. Two more injections would be given 24 hours apart. Therefore, the last dose of Actovegin injection would be 48 hours after muscle injury, this regimen is based on the pathophysiological response of muscle healing and the in-vitro study results. Initial injection of Actovegin could limit the extent of the nearby cells’ ischaemic damage from the injury as well as its effects on the inflammatory

process. The treatment regimen is terminated at 48 hours, as the inflammatory process will start to enter the repair phase and the CD163<sup>+</sup> macrophages will start to have a more dominant role. The in-vitro study suggested that Actovegin can significantly down regulate CD163<sup>+</sup> macrophages (Section 3.4.3.2), therefore treatment beyond 48 hours does not provide any biochemical advantage in the pathophysiological process of muscle repair. In order to comply with logistical constraints within professional football, injections are 24 hours apart. Figure 4.3.2.1 illustrates the regimen.

Day 0	Day 1	Day 2	Rehabilitation	
1 <sup>st</sup> Injection	2 <sup>nd</sup> Injection	3 <sup>rd</sup> Injections		Return to play
Injury confirmed				

Figure 4.3.2.1 Actovegin treatment regimen for the study

The use of ultrasound guidance is unnecessary for Actovegin intramuscular injections, the targeted site of drug delivery are cells near to the injured site instead of directly to the injured tissue. The site of injury can easily be identified following clinical examination and the patient can usually pin point the location of the injury site. Ultrasound scanning does not add extra value to the Actovegin intramuscular injection technique but may introduce potential variables. Therefore, ultrasound scans are not used in this clinical study.

#### 4.3.2.2 Control group

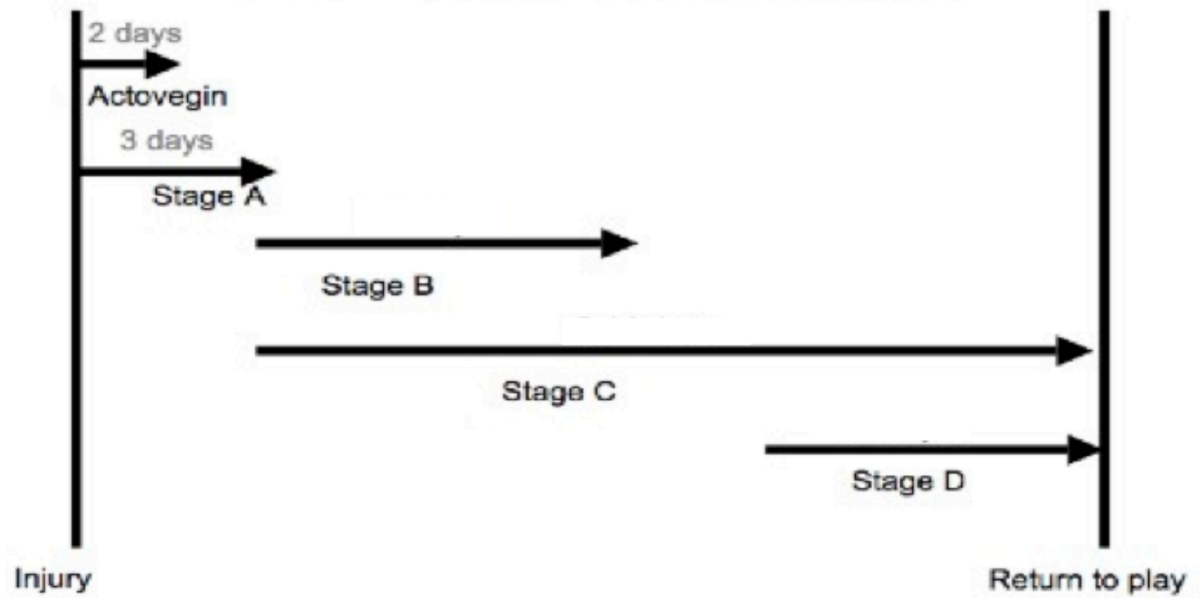
As discussed in Section 2.4.2, there is no consensus on the best treatment options for muscle injuries. Ideally a placebo injection should be used to compare against Actovegin, to minimise the psychological effect of the needle and volumetric effect of injection. As the treatment of muscle injuries is based on low-level evidence, there has not been any report or evidence on intramuscular isotonic saline injection therapy. Therefore, it is unethical to assume that intramuscular isotonic saline is a placebo and can be used as a control group.

This is the first clinical pilot study using Actovegin as a single agent to treat muscle injuries; the target group of participants are high-level professional footballers. After consultation with the club, it was decided that the best option for the control group was for the injured players to undergo the same physiotherapy treatment and rehabilitation protocol without any injections. Although this method of selecting control has its limitations, it was the safest option for the players and the club medical-legally. Furthermore, in order to fulfil the medial-legal obligations of the football club to the professional football players, this pilot study has been carried out in a non-randomised and non-blinded fashion.

#### **4.3.3 Rehabilitation protocol**

The same doctor and physiotherapist will treat all injured players and follow the same rehabilitation protocol set out below in Figure 4.3.2. This has 3 days set for the RICE period for the initial stage of the rehabilitation protocol. Following Stage A treatment, Stages B and C are carried out simultaneously depending on the progression of the patient. Once Stage B is completed, Stage D can begin depending on the patient's recovery alongside with Stage C. Actovegin treatment regimen, as discussed in 4.3.2.1, would be used in Stage A of the rehabilitation program.

### Time line on the rehabilitation program



(Figure 4.3.2) Rehabilitation regimen for the study

Stage A: RICE, gentle movement,

Stage B: Mobilize hamstring to full range, core stability exercise,

Stage C: Strengthening exercise, core stability exercise, proprioceptive,

Stage D: Sudden movement, stop start, cutting

The rehabilitation protocol is designed and set out by the professional football club, each of the stages of the rehabilitation protocol are explained below:

Stage A: RICE, gentle movement (3 days)

Complete rest from routine training

Compression garments, cold therapy machine, advice on elevation

Low- to moderate-intensity sidestepping, grapevine stepping

Single-leg stand progressing from eyes open to eyes closed

Prone abdominal body bridge

Stage B: Mobilize hamstring to full range, core stability exercise

Moderate- to high-intensity sidestepping

Grapevine stepping (flat surface)

Single-leg stand windmill touches

Push-up stabilization with trunk rotation

Fast feet in place

Proprioceptive neuromuscular facilitation trunk pull-downs with Thera-Band,

Symptom-free practice without high-speed manoeuvres

Stage C: Strengthening exercise, core stability exercise, proprioceptive

High-intensity sidestepping

Grapevine stepping (with elevation)

Single-leg stand windmill touches



Swiss ball stabilization with trunk rotation

Proprioceptive neuromuscular facilitation trunk pull-downs with Thera-Band,

Running with ball

Stage D: Sudden movement, stop start, cutting

Swiss ball stabilization with trunk rotation

Nordic Hamstring curl

Cutting exercise with ball

Sudden stop and start running with ball

#### **4.3.4 Functional assessment**

Muscle contractions are classified by how the load is applied or how the velocity changes and the directional change in length of the muscle or a combination of the two (Herzog, 1988, De Ste Croix et al., 2003, Maffiuletti et al., 2007). There are three classifications of muscle contraction, isotonic, isometric and isokinetic. Isotonic contraction refers to when the muscle tries to move a constant load, thereby having a constant value of tension with the length of muscle changing. Isometric testing refers to muscle contraction where there is no change in muscle length regardless of the force applied. The final type of muscle contraction is isokinetic. For this type, the muscle contracts at a constant velocity. Due to the contraction being kept at a constant velocity it means the force applied to the muscle will vary as the tension the muscle creates varies (Herzog, 1988, De Ste Croix et al., 2003). Therefore, the length of the muscles and tension it creates change throughout the tested range of motion. This type of muscle testing was therefore used, as it best resembles how the muscles are acting during sporting activities (Durand et al., 1991).

Assessment of muscle strength is an important component of diagnosis and treatment in muscle injuries. A variety of methods have been used to test quadriceps strength. The two most common methods used in clinical testing are manual muscle testing and Isokinetic testing. Manual muscle testing is easier to use, however results are subjective (Herzog, 1988, Durand et al., 1991). On the other hand, Isokinetic testing offers the benefit of objective

measurement, but there is controversy about which is the most clinically significant testing speed (Askling C, 2003, Clark et al., 2006).

Isokinetic dynamometry claims to be able to provide objective measurement of concentric and eccentric dynamic strength. It provides optimal and efficient loading of muscles and joints through range, therefore strength evaluation is not limited to the weakest point in the range (Tredinnick and Duncan, 1988, Paton et al., 1989, Durand et al., 1991, Garrett, 1996a). It can also identify muscle weakness at certain points in the range, which may help to pin point injuries. There have been many published studies suggesting that hamstring to quadriceps ratio can be used as a measure of muscle balance and hence are linked to increased stress and injury susceptibility. Therefore, this ratio may be a useful objective measuring tool to monitor the progress of treatment.

Previous studies using isokinetic data to try and predict hamstring injuries have all followed similar patterns. They have used prospective cohort studies in order to determine if isokinetic parameters can identify previous and predict future injuries (Paton et al., 1989). Previous literature all appears to indicate that a hamstring to opposite hamstring ratio of less than 0.9 and a hamstring to quadriceps ratio of less than 0.6 may influence injury (Kujala et al., 1997, Woods et al., 2004, Gabbe Bj, 2006). However, there appears to be a lot of varied results as to whether the ratio can predict injuries across different studies (Durand et al., 1991, Li et al., 1996, Orchard et al., 1997,

Funk D, 2001, Maffioletti et al., 2007, Howatson et al., 2009, Yeung and Yeung, 2009).

Owing to the anatomical and histological arrangement of the muscle fibres, the lengths of each fibre vary in each muscle (further details in Section 2.2.). Therefore, the forces provided by the muscle across its working length are not constant even at identical joint angles(Kawakami et al., 2002). The peak torque has been shown to shift upward in more extended positions with increasing angular velocity(Kawakami et al., 2002). There also reports suggested that with increased angular velocity, the quality of the results are be affected as the participant's high stress levels could lead to muscle inhibition(Aagaard et al., 1997, Baltzopoulos, 1995, Sahin et al., 2011). There have been reports suggesting that the uni-planar movement during isokinetic testing can cause significant muscle tears during high speed maximal effort movements(Orchard et al., 2001). Furthermore, there have been concerns that the angular velocity of the isokinetic testing cannot represent the normal physiological range of movement in sports especially in high performance sports such as football(Newman et al., 2004, Housh et al., 1988). Therefore, its role as an overall objective screening tool in high performance football players is questionable.

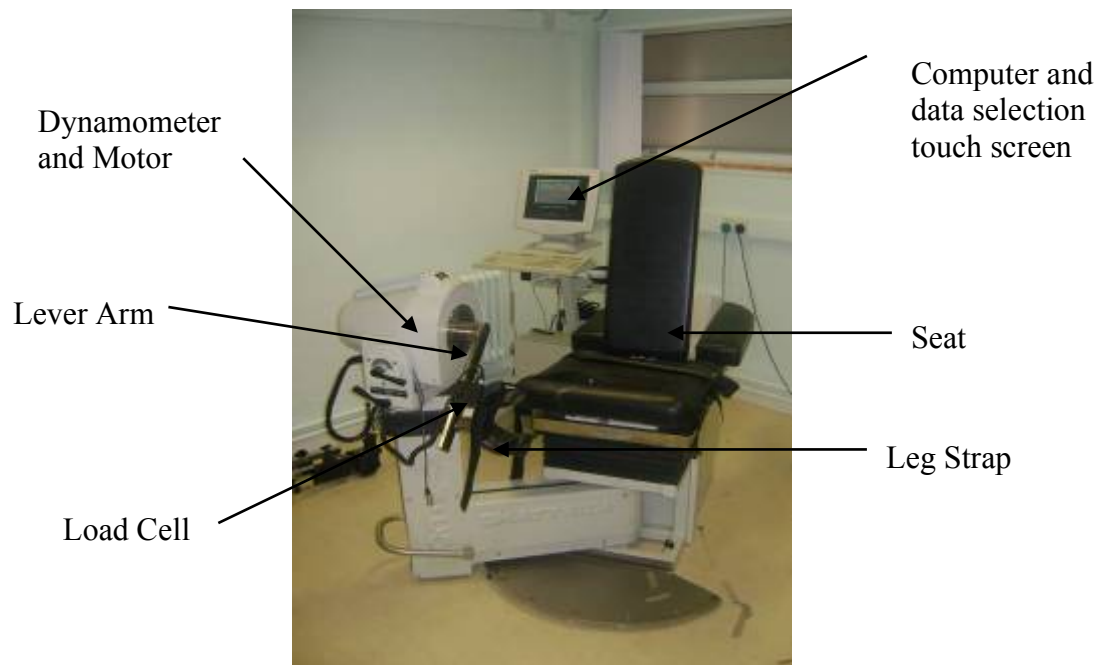
Although there is much uncertainty regarding the use of isokinetic testing for muscle function in high performance athletes, it may be able to provide some objective method of assessing hamstring function, which may help the medical team to decide when a player is ready to return to play.

Therefore, after discussion with the football club, pre-season hamstring isokinetic testing of all players were preformed in order to obtain baseline data. Additionally, all players were encouraged to return for further isokinetic testing during the season.

The following study is designed to analyse the suitability of the use of isokinetic testing as an objective method of measuring the hamstring function in elite footballer players. As suggested by previous literature, Hamstring eccentric torque / Quadriceps concentric ratio will be analysed with the isokinetic data. Furthermore, total work and torque ratios will be analysed to determine the relationship between pre-season and mid season injury data. The physiological movement and the angular velocity on the knee joint exerted by the hamstring during a football match for professional players would be much higher than the upper limit (360 degree/sec) of the isokinetic dynamometry. The reliability of the test reduces and the risk of injury increases when the angular velocity increases (Newman et al., 2004, Housh et al., 1988). In order to minimise injury risk and obtain reliable data, angular velocity was chosen to be 60 degrees per second for this study.

#### 4.3.4.1 Equipment and method

The machine used to test the subjects was a Kin-Com 125E Isokinetic Dynamometer (Chattex Corporation, Chattanooga Group Inc, Tennessee, USA). ) (Figure 4.3.4.1.a)



(Figure 4.3.4.1.a) KinCom 125E Dynamometer

In order to determine whether isokinetic data can be used as an objective marker for hamstring injury, a baseline test was performed pre-season. The pre-season testing took place in the physiotherapy laboratory at St.Davids House at the University of Wales Heath Hospital Cardiff, under the guidance of Dr. Nicola Phillips, Cardiff University. 19 players were tested on the Kin-Com Dynamometer machine where the isokinetic variables measured were for the movements of hamstrings concentrically, hamstrings

eccentrically, quadriceps eccentrically and quadriceps concentrically for each leg. For this testing, the machine was set at 60 degrees per second.

## **Method**

Prior to the 1st subject's arrival, the machine was turned on and the positioning adjustments were set to the correct location. This included moving the lever arm and height adjustments to allow for the knee to be tested. The seat was positioned vertically so that the subject would be in an upright position. The right leg was tested first for all the subjects. All subjects had 5 minutes warm up on the treadmill prior to testing.

The subject was then asked to sit in a comfortable position with the knee joint aligned with the centre of rotation of the lever arm. (Figure 4.3.4.1.b) This involved adjusting the height and orientation of the dynamometer so that the two were aligned. This was done via the control buttons on the top of the dynamometer. Once aligned, the back rest for the subject was positioned to give something to rest against. The subject was strapped in via the seat belts around the waist and thigh so that movement during testing was reduced.

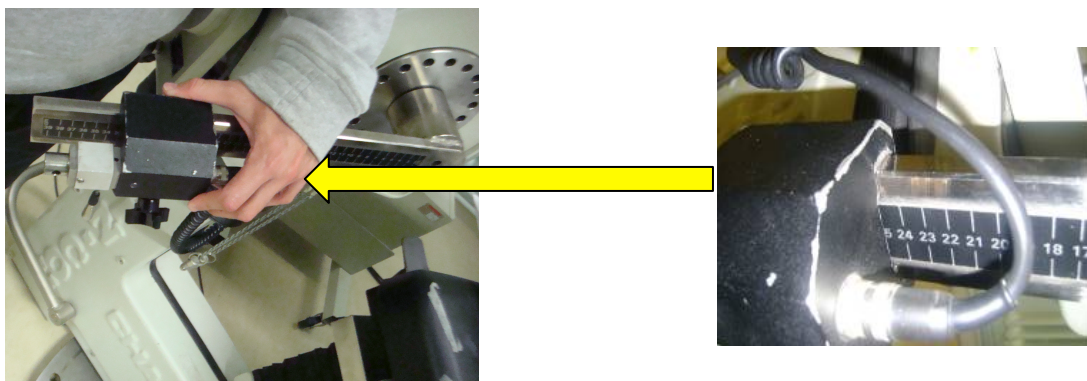


**(Figure 4.3.4.1.b)** Knee position to the centre of rotation to the centre lever arm

The position of the load cell on the lever arm was then adjusted on the distal region of the subject's leg just above the medial malleolus. It was positioned until the subject was happy with the level of comfort. It was then tightly secured to the leg using the strap.

The appropriate setting was selected from the Kin-com machine. The initial selection was evaluation and then overlay. Subjects' demographics were then entered into the computer. Knee isokinetic protocol and laterality was selected. The protocol was designed to test extension then flexion as the knee was already in a flexed position prior to testing.

The distance of the load cell on the lever arm (Figure 4.3.4.1.c) was then entered into the computer. The position of the load cell on the lever arm was gained from reading the scale on the lever arm.



(Figure 4.3.4.1.c) Distance of the load cell on the lever arm



The anatomical zero position was then set in full knee extension and the thigh and leg were parallel. Therefore, the subject was asked to extend his leg until at this position. (Figure 4.3.4.1.d)

The lever arm then needed to be situated in the stop position for extension. In the case of extension, this position was the same as the anatomical zero position. The machine displayed an angle, which needed to be 0. When the lever arm was at this position, enter was selected from the machine. The subject was then asked to flex their leg 90 deg. At this location the start position on the machine was selected. The angle velocity of 60deg / s was then selected and the subject was allowed to warm up and get used to the movement while the leg was attached to the machine. The subject was informed that the machine would maintain a constant angular velocity, so no matter how hard they pushed, it would not result in their leg moving at a faster speed. The subject was asked if all the strapping felt comfortable and that the movement felt natural before proceed to the next stage of the test.

The subject returned their leg to the flexed position. The start measure button was selected from the test screen. The subject was informed to extend the leg with maximum force until it was stopped by the machine at the stop angle. Once at the stop angle, the subject was informed to resist the machine as it was going to apply force in order to return it to the start position. Once returned to the start position, the machine displayed the results. Provided the subject was happy that they had performed to their maximum ability, the results were accepted. The movement of extension gave the quadriceps

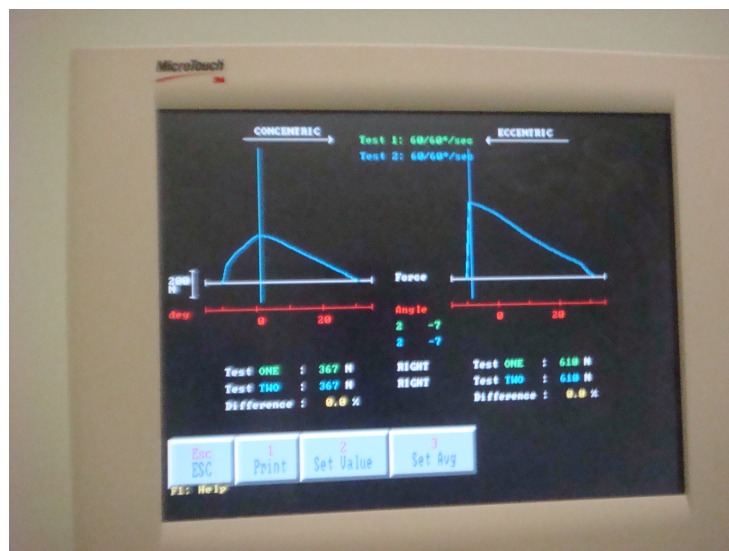
concentric values for the right leg, and the resistance from an extended position to a flexed position gave the quadriceps eccentric values.

The next stage of the testing involved measuring the performance of the hamstrings. Whilst the leg was in a flexed position, the load cell strap was removed and turned around. This was for safety reasons because if the strapping had remained in the same position as for quadriceps testing, then the leg would have loosened resulting in possible injury to the subject or surrounding people. After the load cell pad was turned around, it was re-attached to the subject's leg.

The subject was informed to flex their leg until at approximately 90 degrees of flexion. At this time, the stop angle was selected from the machine. The machine then displayed the stop angle, which needed to be at approximately 90 degrees. The subject was informed to move their leg to the maximum extended position so that the start angle could be chosen. New test was then selected from the machine. On the test page, an angle velocity of 60 degrees per second was selected. From the test page, warm up was selected so that the subject could get used to flexing the leg whilst being attached to the machine before data gathering begins.

The subject moved their leg to a fully extended position after being given the instruction to do so. Start measure was selected from test page, the subject was asked to flex their knee with a maximum force until stopped at the stop angle. Whilst the knee was at the stop angle, the subject was informed to

resist the machine whilst it applied force, in order to return the leg to start angle. At the point where the subject's leg had been returned to the start position, the machine displayed the results. Provided the subject was happy that they had performed to their maximum ability, the results were accepted. The movement of flexion gave hamstring concentric results and the resistance to the machine from a flexed position to an extended position gave the hamstring eccentric results. (Figure 4.3.4.1.d)



(Figure 4.3.4.1.d) Isokinetic results display on screen of the hamstring following flexion and extension movements

The results for the right leg of the machine were now complete, thus the subject was unstrapped from the machine and asked to move away from the machine. At this point the machine set up was changed. The lever arm and dynamometer were moved to the left side of the machine and positioned to be facing inward. The procedure for the left leg follows the same procedure as for the right. The KinCom Protocol used in this study can be found in appendix D

#### **4.3.4.2 Data processing**

After the data collection on a Kin-com Isokinetic Dynamometer, the results needed to be processed to allow for further analysis of the data. The data for the 19 players was exported into 76 individual files as ASCII text format to a USB storage device and was transferred to a computer.

Each ASCII file contained each individual test record containing concentric and eccentric movements for a specific action of each limb. The data collected by the machine was angle (deg), angular velocity (deg/s) and force (N) in 0.5-degree interval. ASCII files were labelled with the subjects' initials, action and side to avoid confusion. For example, JB (initial of subject)\_R(right)\_Flex(flexion). A complete test for each subject should yield 4 ASCII files with approximately 1600 data points in total. The ASCII data was manually transferred to an XLS spreadsheet (Excel 2003) to create an overall profile for each subject. Separate files were created for each limb.

Hamstring eccentric, hamstring concentric, quadriceps eccentric and quadriceps concentric were recorded according to angle and angular velocity. The torque was calculated through multiplying the force by the length of the lever arm. Data was then summarised into one spreadsheet for further processing.

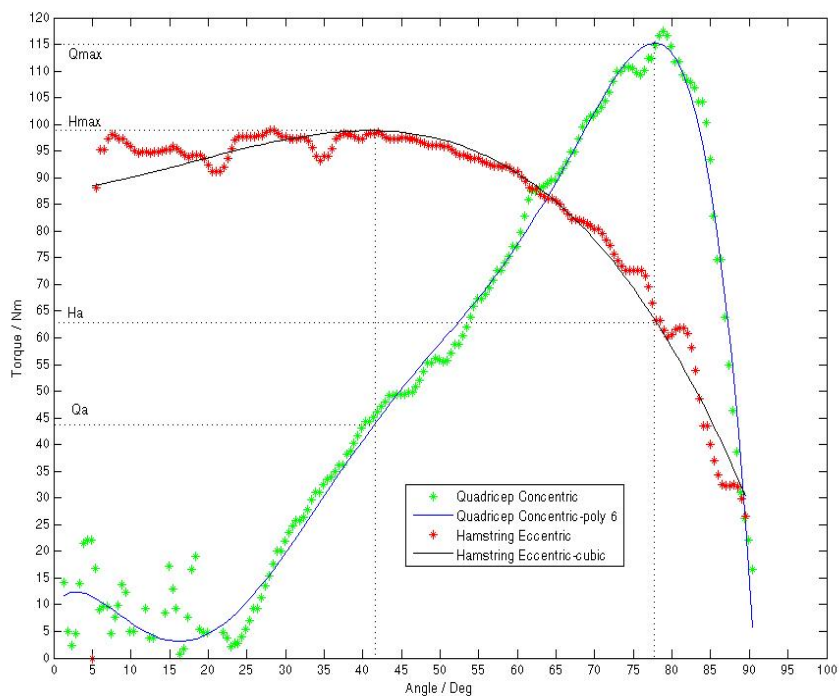
Matlab (matrix laboratory, MathWorks 2008) was used to simulate a mathematical model and produce a line best fit for each set of data points. After curves were fitted, data could be extracted from the graph. The data

extracted and ratios calculated in pre-season testing for each subject can be seen in the table below.

### Example Calculations

Comparison of the quadriceps concentric and hamstring eccentric movements of an individual left leg of a subject, when the knee is moved from 90 degree flex to a complete extend position.

For the data in Figure 4.3.4.2.a the quadriceps concentric data was fitted with a polynomial curve of order 6 and the hamstring eccentric data was fitted with polynomial curve of order 3.



**(Figure 4.3.4.2.a)** Torque versus angle for the quadriceps concentric and hamstring eccentric movement of the left leg of a subject.

## Data extraction from curve fitting

The peak quadriceps concentric torque ( $Q_{con}max$ ) = 115Nm

The peak hamstrings eccentric torque ( $H_{ecc}max$ ) = 98Nm

The hamstring eccentric torque at the angle of  $Q_{con}max$  ( $H_{ecc}a$ )=63Nm

The quadriceps concentric torque at the angle of  $H_{ecc}max$  ( $Q_{con}a$ ) = 44Nm

The equation for the line for quadriceps concentric was:

(Where Y refers to the variable on y axis and x the variable on the x axis)

$$Y = -2.7294e-008x^6 + 6.5664e-006x^5 - 5.9302e-004x^4 + 0.0243x^3 - 0.3966x^2 + 1.7202x + 10.1108$$

To calculate the work done by the muscles over a range of motion, the area under the curve needed to be calculated.

$$Q_{work} = \int_0^{90} Y \, dx = 4550.2 \text{ Nmdeg}$$

Convert to over all work done –  $\omega$

Convert from degrees to radians multiply by  $\pi/180$ .

$$= 4550.2 \times (\pi / 180) = 79.4 \text{ Joules } (Q_w)$$

The equation for the line for hamstring eccentric was:

$$Y = -2.4552e-004x^3 + 0.0132x^2 + 0.1485x + 87.4752$$

$$H_{work} = \int_0^{90} Y dx = 7654.675616 \text{ Nmdeg}$$

Convert to over all work done -  $\omega$

$$= 7654.7 \times (\pi / 180) = 133.6 \text{ Joules (Hw)}$$

### **Further analysis based on the curve fitting**

Peak hamstring eccentric torque / peak quadriceps concentric torque

$$H_{eccmax} / Q_{conmax} = 98 / 115 = 0.85 (H/Q_{max})$$

Hamstring eccentric torque at angle of peak quadriceps torque / Peak quadriceps torque.

$$H_{ecc_a} / Q_{conmax} = 63 / 115 = 0.55 (H_a/Q_{max})$$

Quadriceps concentric torque at angle of peak hamstrings eccentric torque / peak hamstrings eccentric torque.

$$Q_{con_a} / H_{eccmax} = 44 / 98 = 0.45 (Q_a/H_{max})$$

Hamstrings eccentric work done / Quadriceps concentric work done.

$$H_{eccwork} / Q_{conwork} = 79.4 / 133.6 = 1.68 (H_w/Q_w)$$

#### **4.3.4.3 results**

##### Pre-season testing

Table 4.3.4.3.a, presents the data from the pre-season testing, peak quadriceps concentric torque ( $Q_{conmax}$ ), peak hamstrings eccentric torque ( $H_{eccmax}$ ), hamstring eccentric torque at the angle of  $Q_{conmax}$  ( $H_{ecc\alpha}$ ), quadriceps concentric torque at the angle of  $H_{eccmax}$  ( $Q_{con\alpha}$ ), work done by quadriceps muscle ( $Q_w$ ) and work done by hamstring muscle ( $H_w$ ) was calculated according to the method described in 4.3.4.2. All 19 players from the club attended and completed isokinetic testing. Due to computer data corruption it is not possible to retrieve the right leg data on subject 17. Table 4.3.4.3.b presented the data calculated from Table 4.3.4.3.a. Peak hamstring eccentric torque / peak quadriceps concentric torque ( $H/Q_{max}$ ), Hamstring eccentric torque at angle of peak quadriceps torque / Peak quadriceps torque ( $H_a/Q_{max}$ ), Quadriceps concentric torque at angle of peak hamstrings eccentric torque / peak hamstrings eccentric torque ( $Q_a/H_{max}$ ) and Hamstrings eccentric work done / Quadriceps concentric work done ( $H_w/Q_w$ ) were calculated.



Subject	Leg	$Q_{conmax}$ (Nm)	$H_{eccmax}$ (Nm)	$H_{ecc a}$ (Nm)	$Q_{con a}$ (Nm)	$Q_w$ (joules)	$H_w$ (Joules)
1	Left	115	98	63	44	79	134
1	Right	142	84	61	32	102	115
2	Left	214	119	76	100	215	142
2	Right	220	124	70	27	170	161
3	Left	101	85	67	10	82	105
3	Right	117.5	74	59.5	8	109	97
4	Left	143	75	51	28	107	101
4	Right	134	107	77.5	46	116	144
5	Left	127.5	70	43	16	107	94
5	Right	130	74	55	53	107	102
6	Left	157	103	74.5	34	131	143
6	Right	113	96	81	30	101	130
7	Left	180	86	44	86	156	105
7	Right	169	115.5	65	109	178	143
8	Left	102	79	54	49	99	104
8	Right	130	80	57	58	110	104
9	Left	145	125	95	94	130	163
9	Right	123	135	95.5	27.5	114	180
10	Left	200	120	69	52	124	159
10	Right	186	117	84	80	143	149
11	Left	117	78	54	76	106	101
11	Right	222	130	85	89	183	170
12	Left	120	78	59	56	96	104
12	Right	128	66	60	35	107	99
13	Left	215	124	77	42	156	165
13	Right	224	121	64	43	158	159
14	Left	128	75.5	40	40	116	92
14	Right	155	108	65	30	149	133
15	Left	156	89	48	45	133	112
15	Right	175	98	45	7	156	96
16	Left	187	80	48	58	157	100
16	Right	182.5	107	69	45	156	143
17	Left	151	98	50	114.5	155	104
18	Left	202	84	75	137	191	117
18	Right	163	88	60	20	151	110
19	Left	150	81	87.5	95	120	96
19	Right	119	89	61	73	111	106

**(Table 4.3.4.2.a)** Isokinetic data from pre-season testing.

Peak quadriceps concentric torque ( $Q_{conmax}$ ), peak hamstrings eccentric torque ( $H_{eccmax}$ ), hamstring eccentric torque at the angle of  $Q_{conmax}$  ( $H_{ecc a}$ ), quadriceps concentric torque at the angle of  $H_{eccmax}$  ( $Q_{con a}$ ), work done by quadriceps muscle ( $Q_w$ ), work done by hamstring muscle ( $H_w$ )

NB: Subject 17 Right leg data corrupted and not possible to recover from KinCom source

Subject	Leg	H/Q <sub>max</sub>	H <sub>a</sub> /Q <sub>max</sub>	Q <sub>a</sub> /H <sub>max</sub>	H <sub>w</sub> /Q <sub>w</sub>
1	Left	0.85	0.55	0.45	1.68
1	Right	0.59	0.43	0.38	1.13
2	Left	0.56	0.36	0.84	0.66
2	Right	0.56	0.32	0.22	0.95
3	Left	0.84	0.66	0.12	1.28
3	Right	0.63	0.51	0.11	0.89
4	Left	0.52	0.36	0.37	0.94
4	Right	0.80	0.58	0.43	1.24
5	Left	0.55	0.34	0.23	0.88
5	Right	0.57	0.42	0.72	0.96
6	Left	0.66	0.47	0.33	1.09
6	Right	0.85	0.72	0.31	1.29
7	Left	0.48	0.24	1.00	0.67
7	Right	0.68	0.38	0.94	0.81
8	Left	0.77	0.53	0.62	1.05
8	Right	0.62	0.44	0.73	0.94
9	Left	0.86	0.66	0.75	1.26
9	Right	1.10	0.78	0.20	1.58
10	Left	0.60	0.35	0.43	1.28
10	Right	0.63	0.45	0.68	1.04
11	Left	0.67	0.46	0.97	0.96
11	Right	0.59	0.38	0.68	0.93
12	Left	0.65	0.49	0.72	1.08
12	Right	0.52	0.47	0.53	0.92
13	Left	0.58	0.36	0.34	1.06
13	Right	0.54	0.29	0.36	1.01
14	Left	0.59	0.31	0.53	0.79
14	Right	0.70	0.42	0.28	0.90
15	Left	0.57	0.31	0.51	0.84
15	Right	0.56	0.26	0.07	0.62
16	Left	0.43	0.26	0.73	0.64
16	Right	0.59	0.38	0.42	0.91
17	Left	0.65	0.33	1.17	0.67
18	Left	0.42	0.37	1.63	0.61
18	Right	0.54	0.37	0.23	0.73
19	Left	0.54	0.58	1.17	0.80
19	Right	0.75	0.51	0.82	0.96

**(Table 4.3.4.2.b)** Isokinetic data ratio from pre-season testing

Peak hamstring eccentric torque / peak quadriceps concentric torque (H/Q<sub>max</sub>), Hamstring eccentric torque at angle of peak quadriceps torque / Peak quadriceps torque (H<sub>a</sub>/Q<sub>max</sub>), Quadriceps concentric torque at angle of peak hamstrings eccentric torque / peak hamstrings eccentric torque (Q<sub>a</sub>/H<sub>max</sub>), Hamstrings eccentric work done / Quadriceps concentric work done (H<sub>w</sub>/Q<sub>w</sub>)

## Mid-season testing

All subjects were encouraged to attend re-testing 4 months after initial pre-season testing. Due to logistic and players' professional commitments, only 5 subjects returned for 2<sup>nd</sup> isokinetic testing following the same testing protocol by the same operator. Furthermore, the isokinetic load cell and motor was malfunctioning towards the end of the season. This machine was not repaired until the season had finished. The same data processing methods described in section 4.3.4.2 were used. The results of mid season testing for all 5 players is summarised in Table 4.3.4.2.c and the isokinetic data ratio is summarised in Table 4.3.4.2.d.

Subject	Leg	$Q_{conmax}$ (Nm)	$H_{eccmax}$ (Nm)	$H_{ecc a}$ (Nm)	$Q_{con a}$ (Nm)	$Q_w$ (joules)	$H_w$ (Joules)
2	Left	219	182	126	40	214	217
2	Right	255	157	120	142	262	207
5	Left	184.5	97.5	71	99	156	131
5	Right	137	105	26.5	47	116	105
7	Left	226	109	65	48	200	120
7	Right	235	126	71	85	213	148
18	Left	195	104	77.5	89	164	140
18	Right	185	142	88	78	170	184
19	Left	180	48	39	124	163	54
19	Right	150.5	78	70	130	81	90

**(Table 4.3.4.2.c)** 5 subjects returned for retesting during the mid season. 3 subjects were treated for grade 2 hamstring injuries with Actovegin injections, these are highlighted in grey.

Peak quadriceps concentric torque ( $Q_{conmax}$ ), peak hamstrings eccentric torque ( $H_{eccmax}$ ), hamstring eccentric torque at the angle of  $Q_{conmax}$  ( $H_{ecc a}$ ), quadriceps concentric torque at the angle of  $H_{eccmax}$  ( $Q_{con a}$ ), work done by quadriceps muscle ( $Q_w$ ), work done by hamstring muscle ( $H_w$ )

Player	Leg	H/Q <sub>max</sub>	H <sub>a</sub> /Q <sub>max</sub>	Q <sub>a</sub> /H <sub>max</sub>	H <sub>w</sub> /Q <sub>w</sub>
2	Left	0.83	0.58	0.22	1.01
2	Right	0.62	0.47	0.90	0.79
5	Left	0.53	0.38	1.02	0.84
5	Right	0.77	0.19	0.45	0.91
7	Left	0.48	0.29	0.44	0.60
7	Right	0.54	0.30	0.67	0.69
18	Left	0.53	0.40	0.86	0.85
18	Right	0.77	0.48	0.55	1.08
19	Left	0.27	0.22	2.58	0.33
19	Right	0.52	0.47	1.67	1.11

(Table 4.3.4.2.d) 5 subjects returned for retesting during the mid season. 3 subjects were treated for grade 2 hamstring injuries with Actovegin injections, they are highlighted in grey.

Peak hamstring eccentric torque / peak quadriceps concentric torque (H/Q<sub>max</sub>), Hamstring eccentric torque at angle of peak quadriceps torque / Peak quadriceps torque (H<sub>a</sub>/Q<sub>max</sub>), Quadriceps concentric torque at angle of peak hamstrings eccentric torque / peak hamstrings eccentric torque (Q<sub>a</sub>/H<sub>max</sub>), Hamstrings eccentric work done / Quadriceps concentric work done (H<sub>w</sub>/Q<sub>w</sub>)

With the 5 subjects that returned for re-testing, 3 had sustained grade 2 hamstring injuries and were treated with 3x Actovegin injections as per clinical study protocol (see Section 4.4). These subjects recovered from injury and returned to play at least 34 days prior to mid-season testing. The average number of days between pre-season and mid season testing in this sub group of players was 267 days. Table 4.3.4.2.c summarises the number of days between retest, injury and return to play.

Subject	Number of days between tests	Number of days between Injury to re-test	Number of days between return to play to re-test
2	254	131	109
7	262	47	34
19	285	84	62
Avg.	267		

(Table 4.3.4.2.c) Number of days between pre and mid season isokinetic testing in the injury subgroup.

Pre season isokinetic data and ratios are compared to mid season testing for the injured limb in Table 4.3.4.2.d and Table 4.3.4.2.e respectively. The uninjured limb of the injured players was also analysed, Tables 4.3.4.2.f and 4.3.4.2.g. The isokinetic data of the uninjured players is shown in Tables 4.3.4.2.h and 4.3.4.2.i. Due to the limited data set it is not possible to identify any meaningful trend in the results.

There have not been any specific trends observed in this limited data set.

Subject		Leg	$Q_{conmax}$ (Nm)	$H_{eccmax}$ (Nm)	$H_{ecc a}$ (Nm)	$Q_{con a}$ (Nm)	$Q_w$ (joules)	$H_w$ (Joules)
2	pre	Right	220	124	70	27	170	161
2	mid	Right	255	157	120	142	262	207
7	pre	Left	180	86	44	86	156	105
7	mid	Left	226	109	65	48	200	120
19	pre	Left	150	81	87.5	95	120	96
19	mid	Left	180	48	39	124	163	54

(Table 4.3.4.2.d) Pre and Mid season isokinetic Data for injured limbs. The mid season results are highlighted in grey.

Subject		Leg	$H/Q_{max}$	$H_a/Q_{max}$	$Q_a/H_{max}$	$H_w/Q_w$
2	pre	Right	0.56	0.32	0.22	0.95
2	mid	Right	<b>0.62</b>	<b>0.47</b>	<b>0.90</b>	<b>0.79</b>
7	pre	Left	0.48	0.24	1.00	0.67
7	mid	Left	<b>0.48</b>	<b>0.29</b>	<b>0.44</b>	<b>0.60</b>
19	pre	Left	0.54	0.58	1.17	0.80
19	mid	Left	<b>0.27</b>	<b>0.22</b>	<b>2.58</b>	<b>0.33</b>

(Table 4.3.4.2.e) Pre and Mid season isokinetic Data ratio for the injured limbs. Mid season results are highlighted in grey.

Subject		Leg	$Q_{conmax}$ (Nm)	$H_{eccmax}$ (Nm)	$H_{ecc a}$ (Nm)	$Q_{con a}$ (Nm)	$Q_w$ (joules)	$H_w$ (Joules)
2	pre	Left	214	119	76	100	215	142
2	mid	Left	219	182	126	40	214	217
7	pre	Right	169	115.5	65	109	178	143
7	mid	Right	235	126	71	85	213	148
19	pre	Right	119	89	61	73	111	106
19	mid	Right	150.5	78	70	130	81	90

(Table 4.3.4.2.f) Pre and Mid season isokinetic Data for uninjured limbs in the injured subjects. Mid season results are highlighted in grey.

Subject		Leg	H/Q <sub>max</sub>	H <sub>a</sub> /Q <sub>max</sub>	Q <sub>a</sub> /H <sub>max</sub>	H <sub>w</sub> /Q <sub>w</sub>
2	pre	Right	0.56	0.36	0.84	0.66
2	mid	Right	0.83	0.58	0.22	1.01
7	pre	Left	0.68	0.38	0.94	0.81
7	mid	Left	0.54	0.30	0.67	0.69
19	pre	Left	0.75	0.51	0.82	0.96
19	mid	Left	0.52	0.47	1.67	1.11

(Table 4.3.4.2.g) Pre and Mid season isokinetic ratios for the injured limbs. Mid season results are highlighted in grey.

### Pre and Mid season KinCom Data for uninjured subjects

Subject		Leg	Q <sub>con</sub> max (Nm)	H <sub>ecc</sub> max (Nm)	H <sub>ecca</sub> (Nm)	Q <sub>con</sub> a (Nm)	Q <sub>con</sub> max (joules)	H <sub>ecc</sub> max (Joules)
18	pre	Left	202	84	75	137	191	117
18	mid	Left	195	104	77.5	89	164	140
18	pre	Right	163	88	60	20	151	110
18	mid	Right	185	142	88	78	170	184
5	pre	Left	127.5	70	43	16	107	94
5	mid	Left	184.5	97.5	71	99	156	131
5	pre	Right	130	74	55	53	107	102
5	mid	Right	137	105	26.5	47	116	105

(Table 4.3.4.2.h) Pre and Mid season isokinetic ratios for the uninjured subjects. Mid season results are highlighted in grey.

### Pre and Mid season Data ratio for the uninjured subjects

Subject		Leg	H/Q <sub>max</sub>	H <sub>a</sub> /Q <sub>max</sub>	Q <sub>a</sub> /H <sub>max</sub>	H <sub>w</sub> /Q <sub>w</sub>
18	pre	Left	0.42	0.37	1.63	0.61
18	mid	Left	0.53	0.40	0.86	0.85
18	pre	Right	0.54	0.37	0.23	0.73
18	mid	Right	0.77	0.48	0.55	1.08
5	pre	Left	0.55	0.34	0.23	0.88
5	mid	Left	0.53	0.38	1.02	0.84
5	pre	Right	0.57	0.42	0.72	0.96
5	mid	Right	0.77	0.19	0.45	0.91

(Table 4.3.4.2.i) Pre and Mid season isokinetic ratios for the uninjured subjects. Mid season results are highlighted in grey.

**Table to show total hamstring eccentric power difference between injured and uninjured limbs and between un-injured subjects**

			Mid season - pre season	
	Subject	Leg	Difference in $H_w$ (Joules)	%
Injured limb	2	Right	45.3	28.0
	7	Left	15.3	14.6
	19	Left	-42.4	-44.2
Injured subject uninjured limb	2	Left	74.5	52.5
	7	Right	4.4	3.1
	19	Right	-16.6	-15.7
Uninjured subject	18	Left	23.4	20.1
	18	Right	73.8	66.9
	5	Left	36.9	39.4
	5	Right	3.0	3.0

**(Table 4.3.4.2.j)** Hamstring eccentric power ( $H_w$ ) difference between injured and uninjured limbs on injured subjects and between un-injured subjects.

#### **4.3.4.4 Discussion**

Detailed analysis is demonstrated in Table 4.3.4.2.j on  $H_w$  with the pre and mid season data between the injured and uninjured limb of the injured players and also the data from the uninjured players. There were large discrepancies between pre and mid season hamstring eccentric power data on the uninjured players between each leg. Due to the limited data set, no meaningful trends can be identified with the isokinetic data.

Due to the logistical constraints and the poor compliance for re-testing by the players, isokinetic testing is not a practical method for functional assessment in a professional football club. Furthermore, from the limited data collected in this study there were large discrepancies between pre and mid season testing data. Therefore, isokinetic testing was not a suitable method for me to use as a functional assessment tool to predict recovery and was not used in the clinical study in this PhD.

#### **4.3.5 Return to play**

The decision to return to play following a muscle injury is a multifactorial process involving both physical and psychological parameters. There are many functional tests and scoring systems claiming to be able to help clinicians to determine this process. As discussed in Section 4.3.4, functional assessment in the form of isokinetic testing is not a reliable tool in this setting with this specific group of patients. Besides the compliance issue with the professional football players, introducing a new form of functional outcome measure to the professional football club would expose the club and the medical team to unnecessary potential medico-legal issues. After discussing with the football club, it was decided that the only form of outcome measure for the players to return to play was clinical assessment by the team doctor and physiotherapist. Therefore, in order to make the subjective clinical assessment for return to play more robust and more objective, each player would need to be assessed by 3 clinicians independently before they returned to play.



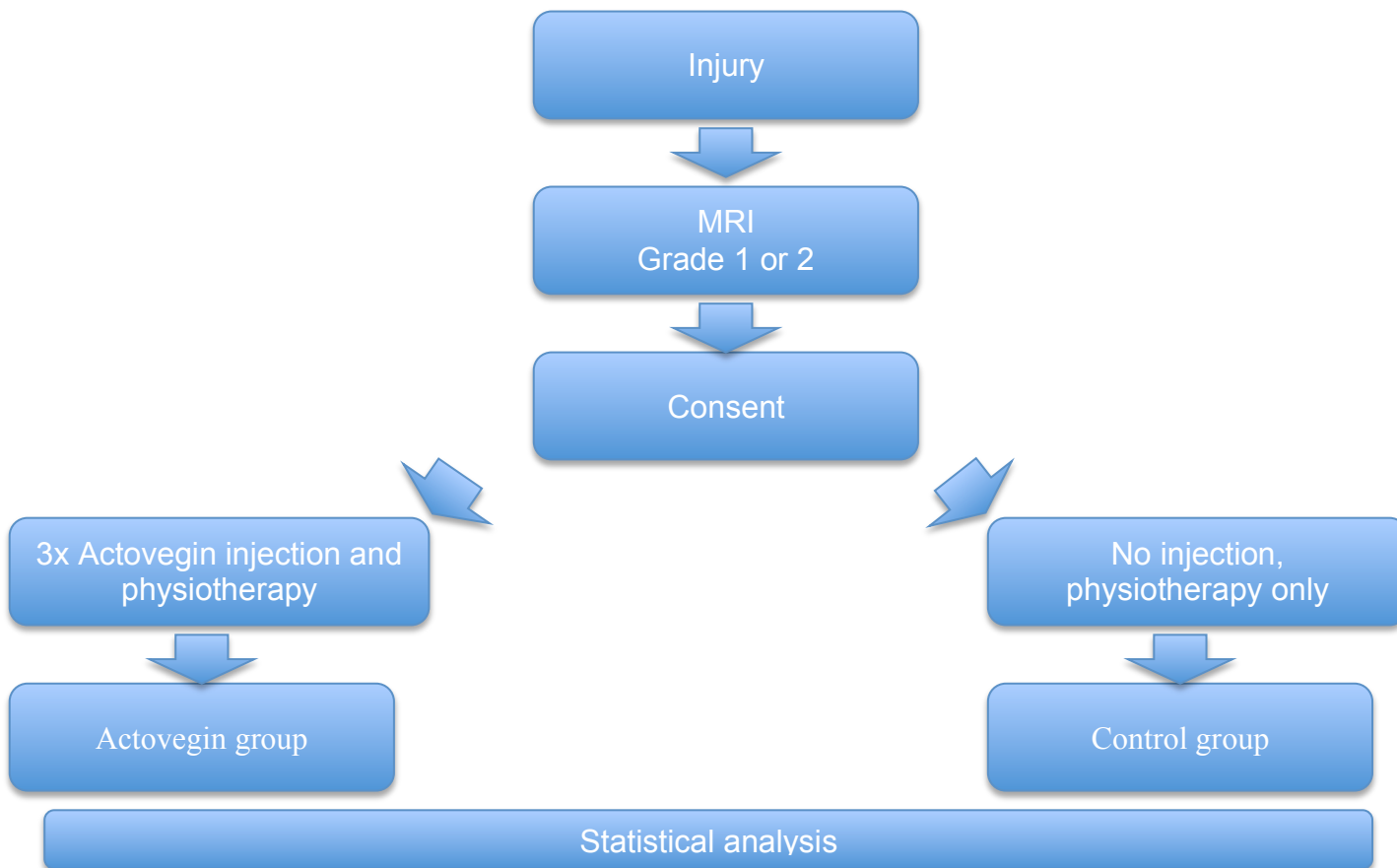
## 4.4 Clinical Study Protocol

The rationale for the clinical study, based on the in-vitro findings are discussed in Section 4.1. Patient selection, diagnosis, treatment regimens, rehabilitation protocol and return to play assessment were discussed in Section 4.3. This section focuses on describing the procedure of the clinical study.

After initial assessment and diagnosis by the team physician and team physiotherapists, all injured players underwent MRI to confirm the diagnosis. Actovegin injection therapy was initiated immediately once muscle tears were defined and concurred clinically by 3 independent practitioners. The muscle injuries were then graded as per Jarvinen et al 2000 detailed in Section 2.3.1. Players with normal clinical examination and normal MRI findings or grade III tears were excluded from this study. All players were given a patient information sheet (see appendix E) Players who refused Actovegin treatment were allocated to the control group. All players in the Actovegin treatment group followed the same injection regimen of 3 intramuscular injections as described in Section 4.3.2.1. There was no blinding or randomization in this study. 2ml of Actovegin (80mg) was injected into the injury site under direct palpation. The injection process was repeated after 24 hours. Players who opted-out of the injection therapy followed the same rehabilitation protocol. All players were discouraged from taking any anti-inflammatory (NSAIDs) or oral supplements. All players followed the same rehabilitation protocol as described in 4.3.3. Players were able return to train with the first team once they had completed the rehabilitation program and passed the assessment by the team physiotherapist as per stage D in the rehabilitation program and return to play assessment as described in Section 4.3.5. Figure 4.4.a and Figure 4.4.b summarized the clinical study protocol in a graphical format. The study has been ethically reviewed and approved by Cardiff University (see appendix C). For further study protocol, please see appendix F.

Day 0	Day 1	Day 2	Rehab		
1 <sup>st</sup> Injection	2 <sup>nd</sup> Injections	3 <sup>rd</sup> Injections		Deemed fit to return by the team physiotherapist	Return to play
Injury confirmed by MRI				Return to play assessment	
Stage A			B, C, D		

**(Figure.4.4a)** Injection and rehabilitation regimen, 1st injection at diagnosis, 2nd injection at 24 hours, 3rd injection at 48 hours. Further information on the rehabilitation stages can be found in Section 4.3.3



**(Figure.4.4b)** Work flow diagram of the clinical study

### Statistical analyses:

Statistical analyses were performed using SPSS version 20.0 (IBM), p value <0.05 was considered to be significant. Non- parametric data analysis in the form of Mann-Whitely U test was used to compare the number of days lost between control and Actovegin treatment groups. This is due to the sub special group of subjects in this PhD and the large variation of number of days lost following hamstring injuries in literature. Furthermore, as discussed in Sections 2.4 and 4.3.5, the decision of return to play following muscle injuries are multifactorial, therefore normal distribution of data cannot be assumed and parametric analysis is not suitable to be used in this study.

## **4.5 Results**

All players involved in this study were given patient information sheets and gave their informed consent demonstratively recorded as a signed consent form (Appendix G). All injured players followed the same study protocol described in Section 4.4. All players in the Actovegin group experience minor muscle clamp immediately after the injection, which recovered within 5 minutes. All MRI scans were carried out in a local private hospital with a 1.5 Tesla MRI machine and was reported by the same Senior Musculoskeletal Consultant Radiologist on the same day as injury occurred.

### 4.5.1 Clinical Results

During the study period, 11 players sustained hamstring injuries that qualified for this study. All 11 players were examined by 3 clinicians on the same day as injury occurred, as discussed in Section 4.3.1.1. The results of injury grading were unanimous as demonstrated in Table 4.5.1.a. The mean age of injured players was 23, mean weight was 76 kg and mean height was 178 cm. 3 strikers, 3 midfielders, 2 wing forwards, 2 defenders and 1 wing backs sustained the injuries (Table 4.5.1.b). 3 injuries occurred during routine

training and 8 injuries during match day. All the grade 2 injuries occurred during match day in the second half of the game.

	<b>Clinical Diagnosis</b>			
	Clinician 1	Clinician 2	Clinician 3	<b>Overall</b>
Player 1	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 2	Grade 2	Grade 2	Grade 2	<b>Grade 2</b>
Player 3	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 4	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 5	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 6	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 7	Grade 2	Grade 2	Grade 2	<b>Grade 2</b>
Player 8	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 9	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 10	Grade 1	Grade 1	Grade 1	<b>Grade 1</b>
Player 11	Grade 2	Grade 2	Grade 2	<b>Grade 2</b>

**(Table 4.5.1.a)** Summary of 3 clinicians' assessments of injured players.

	Injury grade	Player's position	Event
Player 1	Grade 1	Midfielder	Training
Player 2	Grade 2	Defender	Match, 2 <sup>nd</sup> half
Player 3	Grade 1	Striker	Match, 1 <sup>st</sup> half
Player 4	Grade 1	Wing forward	Training
Player 5	Grade 1	Midfielder	Match, 1 <sup>st</sup> half
Player 6	Grade 1	Midfielder	Match, 2 <sup>nd</sup> half
Player 7	Grade 2	Striker	Match, 2 <sup>nd</sup> half
Player 8	Grade 1	Wing back	Match, 1 <sup>st</sup> half
Player 9	Grade 1	Defender	Match, 1 <sup>st</sup> half
Player 10	Grade 1	Striker	Training
Player 11	Grade 2	Wing forward	Match, 2 <sup>nd</sup> half

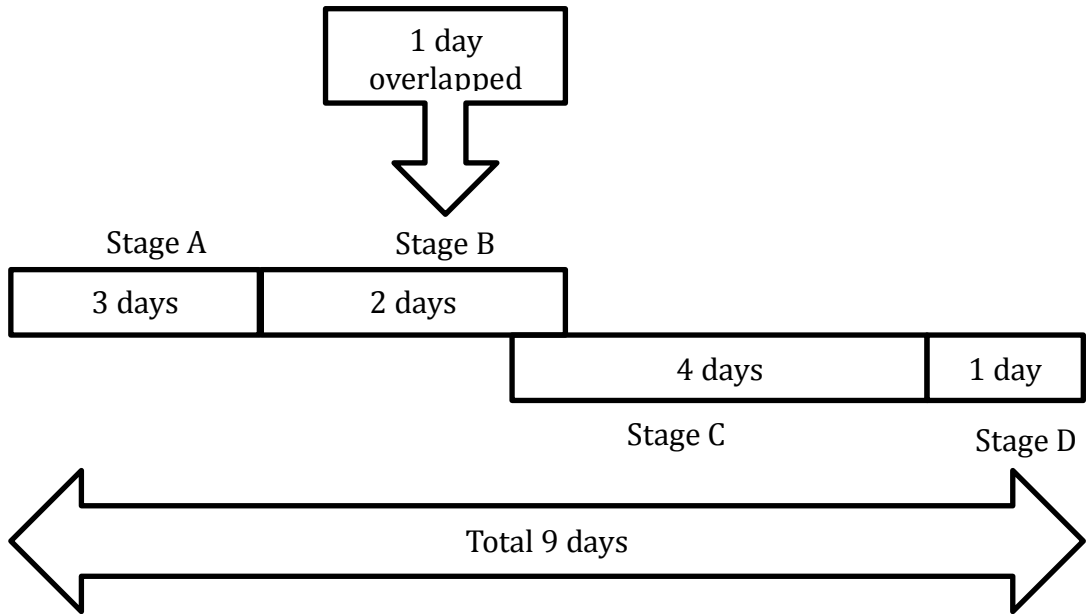
**(Table 4.5.1.b)** Summary of injured players' positions and event during which injuries occurred.

There were 6 right and 5 left sided injuries, 3 grade II injuries and 8 grade I injuries identified from the clinical examination. MRI scans on all players confirmed clinical diagnosis and defined the anatomical location of the injuries. 4 players had previous hamstring injuries but had been symptom free for at least 6 months. All players were able to progress through the rehabilitation program pain free. No recurrent muscle strains or injuries were recorded during rehabilitation. None of the players had any further injuries to the hamstrings during the remaining season. No adverse reactions were reported with Actovegin injections and all players in the Actovegin group received 3 doses of Actovegin as stated in the treatment protocol.

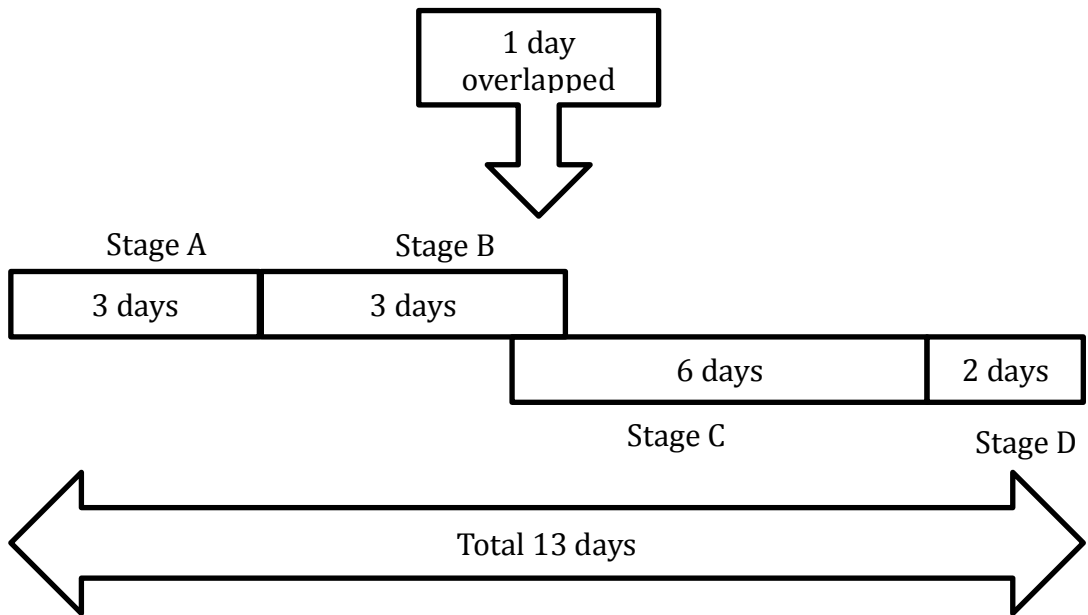
7 players had Actovegin treatment and 4 players opted-out of the injection therapy. In the Actovegin group, 3 players had grade II injuries and 4 players had grade I injuries. All 4 players in the control group had grade I injuries. (Table 4.5.1.c) The mean number of days lost in the control group was 20, with a range from 16 to 26 days. In the Actovegin group, the mean number of days lost with grade I injuries was 12, with a range from 9 to 15 days. Overall player's rehabilitation protocol summary can be found in Table 4.5.1.d. Player specific rehabilitation progress can be found in Figures 4.5.1.a to 4.5.1.k.

	Injury	treatment	Rehab Stage (days)				Overlapped day(s)	Total
			A	B	C	D		
Player 1	Grade 1	Actovegin	3	2	4	1	1	9
Player 2	Grade 2	Actovegin	3	3	6	2	1	13
Player 3	Grade 1	Actovegin	3	5	8	3	4	15
Player 4	Grade 1	Control	3	6	6	2	1	16
Player 5	Grade 1	Actovegin	3	4	3	1	1	10
Player 6	Grade 1	Actovegin	3	2	10	3	4	14
Player 7	Grade 2	Actovegin	3	5	14	2	3	21
Player 8	Grade 1	Control	3	7	12	4	0	26
Player 9	Grade 1	Control	3	6	10	4	2	21
Player 10	Grade 1	Control	3	5	8	1	0	17
Player 11	Grade 2	Actovegin	3	7	12	2	2	22

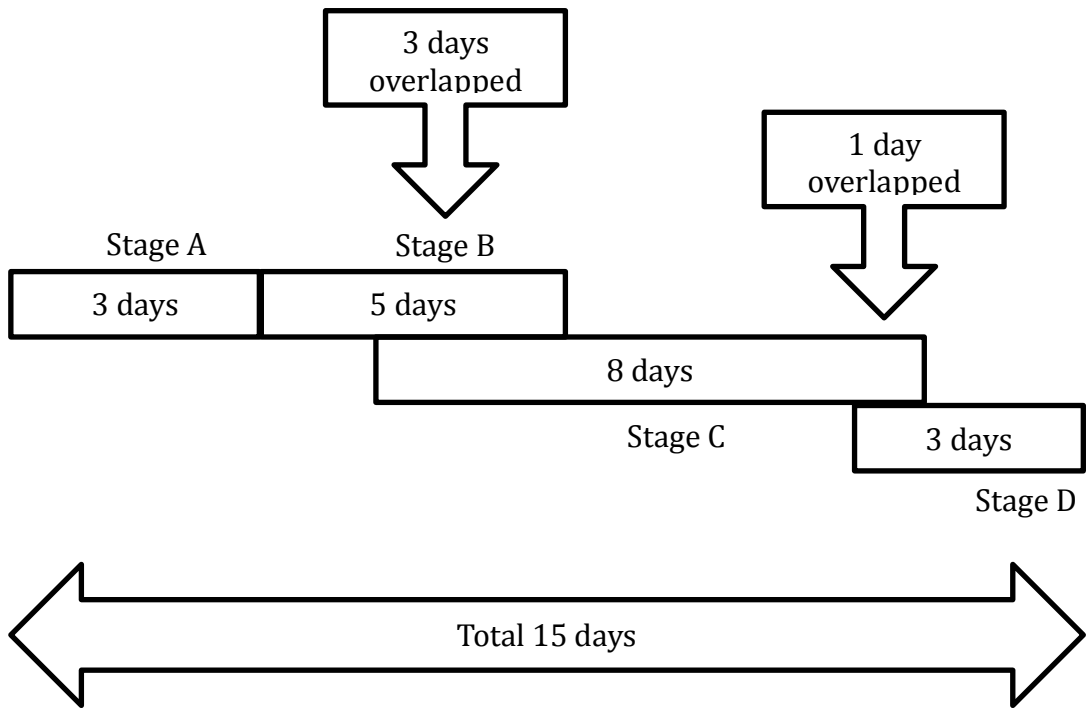
**(Table 4.5.1.d)** Summary of the rehabilitation stages for each player



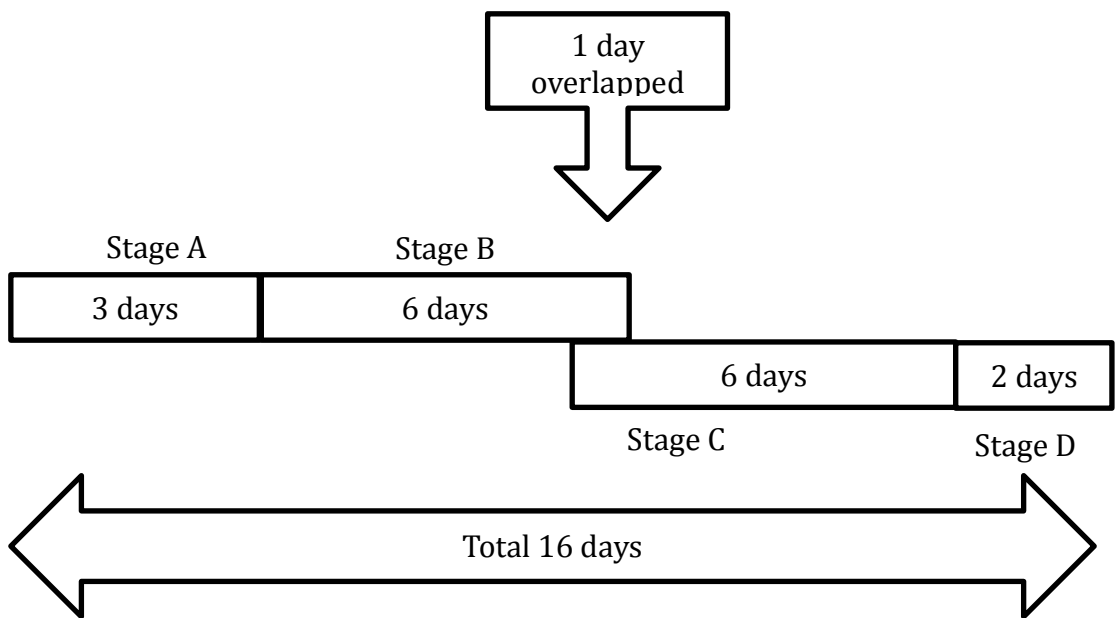
**(Figure 4.5.1.a)** Player 1 rehabilitation progress



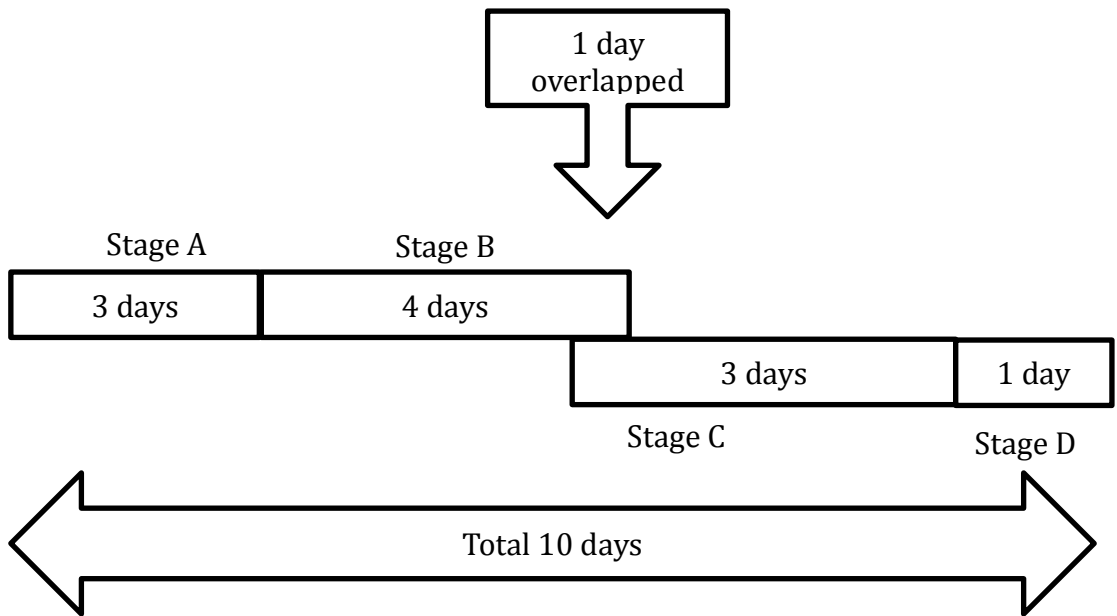
**(Figure 4.5.1.b)** Player 2 rehabilitation progress



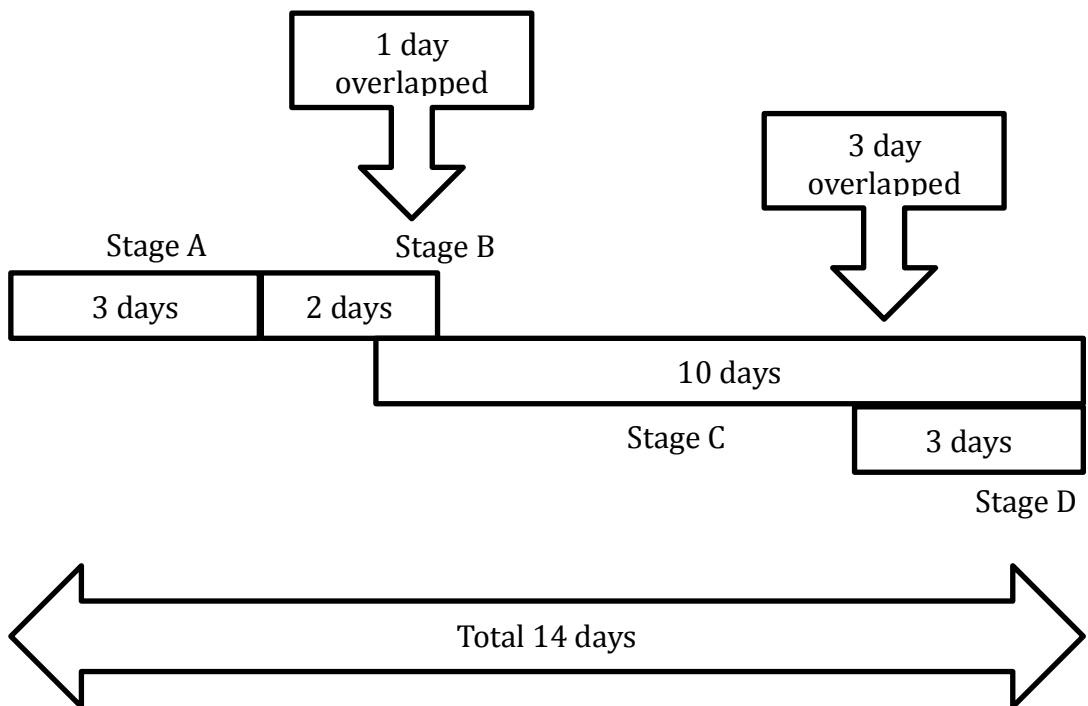
(Figure 4.5.1.c) Player 3 rehabilitation progress



(Figure 4.5.1.d) Player 4 rehabilitation progress

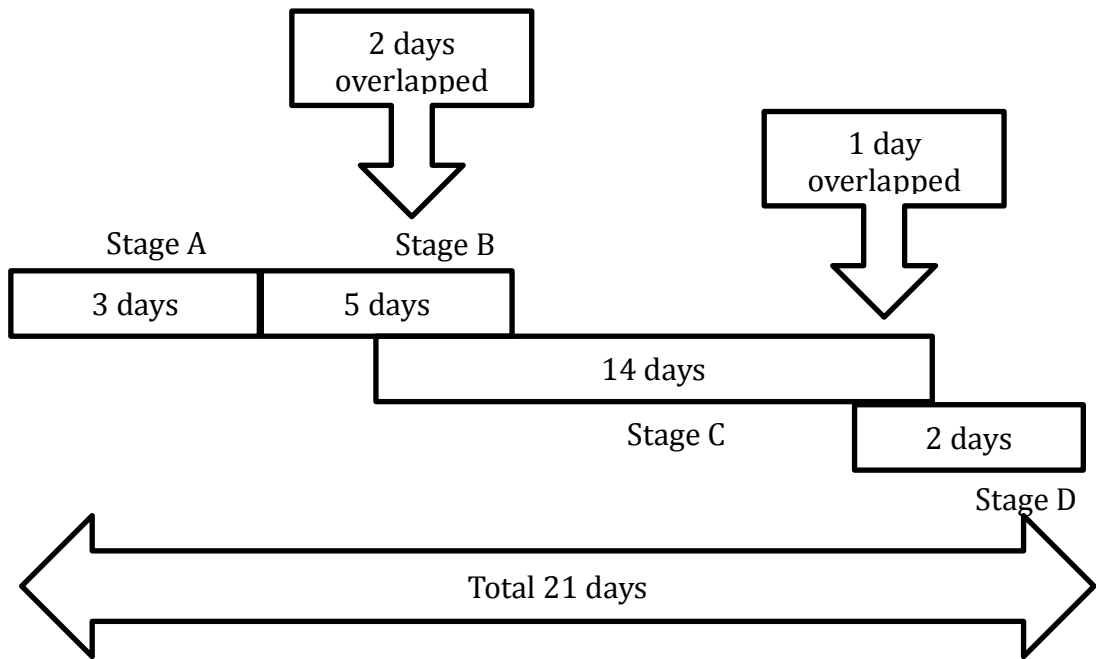


(Figure 4.5.1.e) Player 5 rehabilitation progress

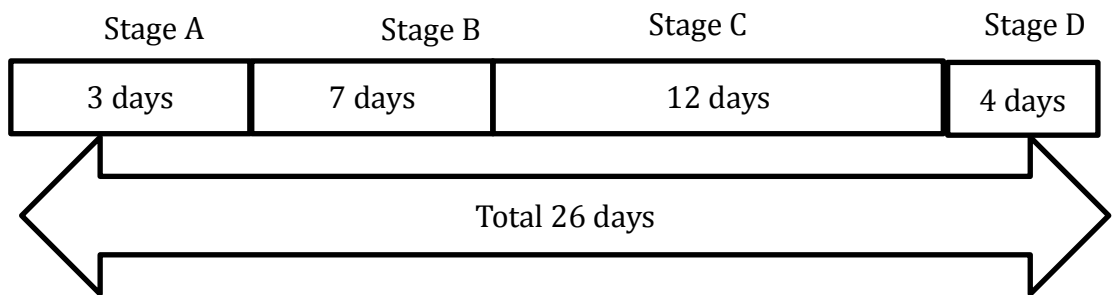


(Figure 4.5.1.f) Player 6 rehabilitation progress

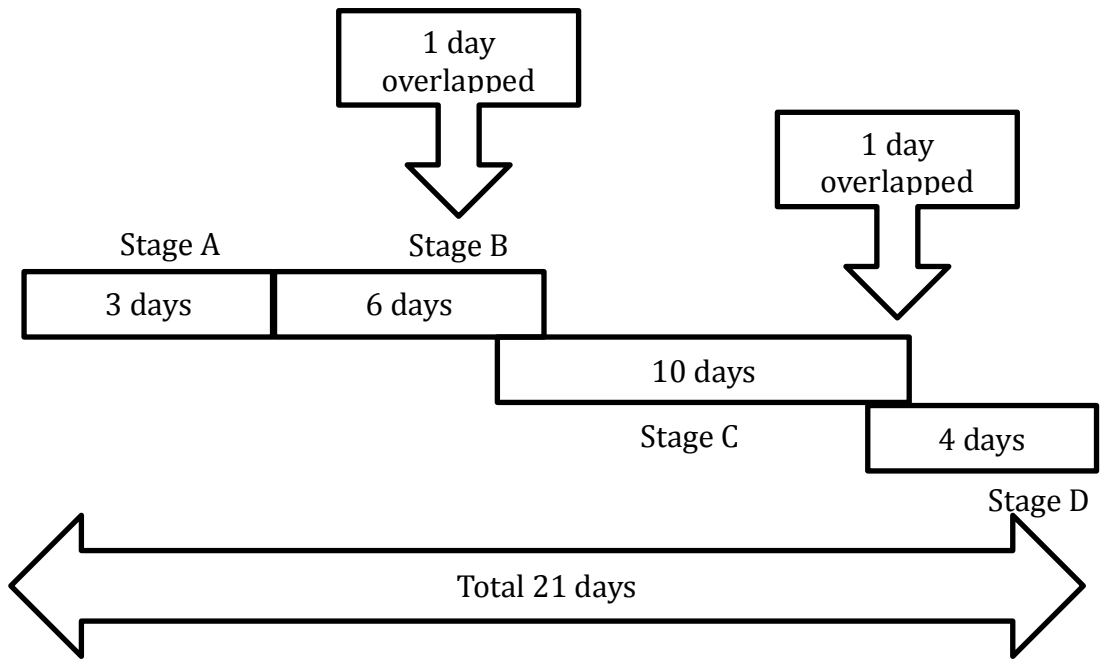




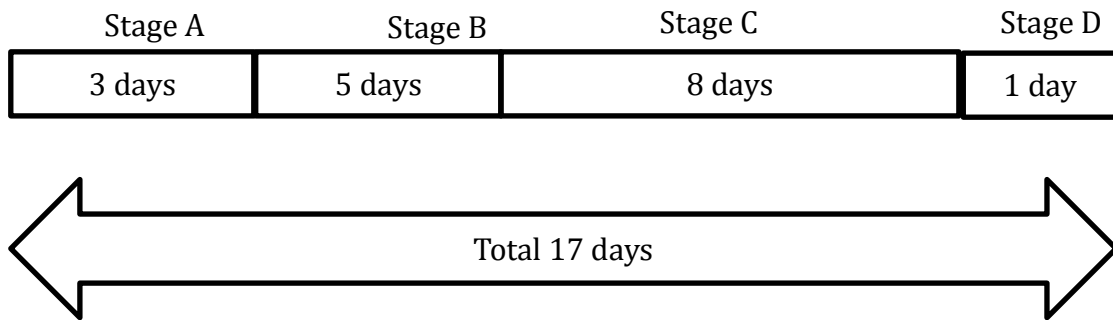
(Figure 4.5.1.g) Player 7 rehabilitation progress



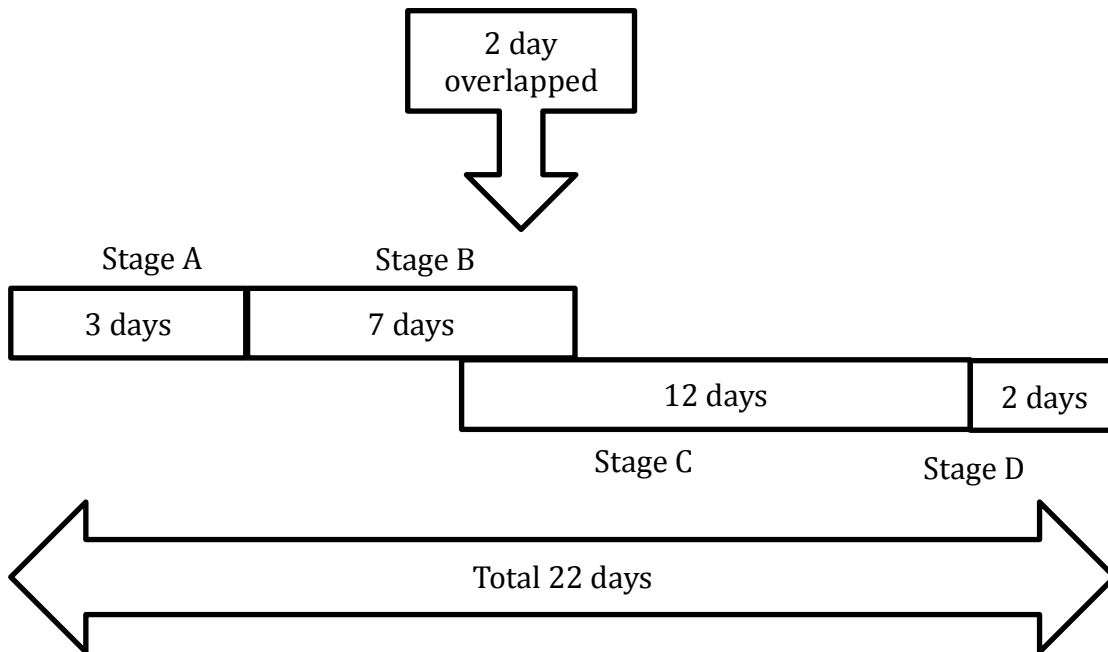
(Figure 4.5.1.h) Player 8 rehabilitation progress



**(Figure 4.5.1.i)** Player 9 rehabilitation progress



**(Figure 4.5.1.j)** Player 10 rehabilitation progress



**(Figure 4.5.1.k)** Player 11 rehabilitation progress

Mann-Whitney U non-parametric statistical analysis shows significant median difference between the Actovegin and the control group ( $p < 0.05$ ) in grade I hamstring injuries (Table 4.5.1.e). Players in the Actovegin treatment group were able to return to play 7 days earlier compared to physiotherapy alone (Table 4.5.1.f). For Grade II injuries, the median number of days lost in the Actovegin group was 21, with a range from 13 to 26 days. Statistical analysis cannot be performed, as there were no subjects with grade II injuries in the control group. Figure 4.5.1.L demonstrates the results for grade 1 injuries in a graphical format.

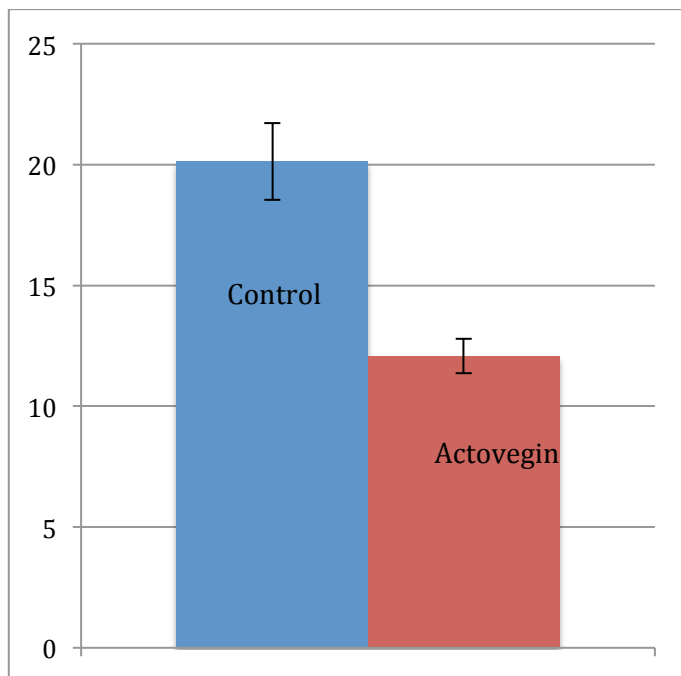
**SPSS 20 data output**

	days
Mann-Whitney U	0.00
Wilcoxon W	10
Z	-2.31
Asymp. Sig. (2-tailed)	0.021*
Exact Sig. [2*(1-tailed Sig.)]	0.029*

**(Table 4.5.1.e)** Mann-Whitney U-test confirmed that there was statistical difference in days lost between control and Actovegin groups for grade 1 hamstring injuries \*= Statistical significance

Age	Treatment	Injury	Days lost	Median
26	Actovegin	Grade II tear	22	21 S.D. 4.93 S.E.M. 2.85
24	Actovegin	Grade II tear	21	
18	Actovegin	Grade II tear	13	
22	Actovegin	Grade I tear	10	12* S.D. 2.94 S.E.M. 1.47
25	Actovegin	Grade I tear	9	
19	Actovegin	Grade I tear	14	
26	Actovegin	Grade I tear	15	
19	Control	Grade I tear	26	19* S.D. 4.54 S.E.M 2.27
28	Control	Grade I tear	16	
22	Control	Grade I tear	21	
24	Control	Grade I tear	17	

**(Table 4.5.1.f)** There was significant difference\* between the Actovegin and Control groups for individuals with grade 1 hamstring injuries. The median days lost for grade 1 injuries in the control group was 19 days compared to 12 in the Actovegin treatment group. \*= Statistical difference between groups



**(Figure 4.5.1.L)** Graph to show significant difference between number of days lost between Control and Actovegin groups for grade 1 hamstring injuries.

Due to the nature of the rehabilitation protocol with simultaneous progression of rehabilitation stages, it is not possible to completely isolate each stage and compare between players. Therefore, in-order to further analyse the data, it is assumed that once the next stage of the rehabilitation protocol is commenced the previous stage is terminated. This assumption enables the rehabilitation stage to be separated into 4 independent stages. Although this method of data manipulation may introduce bias into the following data analysis, this could help further distinguish the Actovegin and control group in the rehabilitation process.

It is important to note that in the data manipulation process, 3 days have been combined in the grade 1 control group, 10 days have been combined in the grade 1 Actovegin group and 6 days have been combined from the grade 2 Actovegin group. (Table 4.5.1.g)

	Injury	Treatment	Rehab stage (days)				Total
			A	B	C	D	
Player 1	Grade 1	Actovegin	3	1	4	1	9
Player 2	Grade 2	Actovegin	3	2	6	2	13
Player 3	Grade 1	Actovegin	3	2	7	3	15
Player 4	Grade 1	Control	3	5	6	2	16
Player 5	Grade 1	Actovegin	3	3	3	1	10
Player 6	Grade 1	Actovegin	3	1	7	3	14
Player 7	Grade 2	Actovegin	3	3	13	2	21
Player 8	Grade 1	Control	3	7	12	4	26
Player 9	Grade 1	Control	3	5	9	4	21
Player 10	Grade 1	Control	3	5	8	1	17
Player 11	Grade 2	Actovegin	3	5	12	2	22

(Table 4.5.1.g) Modified rehabilitation data, boxes in grey highlight the data that has been modified.

On further analysis of the modified data, as shown in Table 4.5.1.h, the median time of stage A remained the same in all groups, there was significant difference,  $p=0.029$ , in the Stage B rehabilitation protocol between control and Actovegin groups in grade I injuries. There were no significant difference in stage C and stage D of the rehabilitation programme between Actovegin and control groups for grade one-hamstring injuries. Table 4.5.6.1.i summarises the statistical analysis. Furthermore, injured players in the Actovegin group seem to spend less time in stages B and D with grade II injuries compared to the players in the control group with grade I injuries. (Figure 4.5.1.L)

Age	Treatment	Injury	A	B	C	D	Total Days lost
18	Actovegin	Grade II tear	3	2	6	2	13
24	Actovegin	Grade II tear	3	3	13	2	21
26	Actovegin	Grade II tear	3	5	12	2	22
		<b>Median</b>	<b>3</b>	<b>3</b>	<b>12</b>	<b>2</b>	
19	Actovegin	Grade I tear	3	1	7	3	14
22	Actovegin	Grade I tear	3	3	3	1	10
25	Actovegin	Grade I tear	3	1	4	1	9
26	Actovegin	Grade I tear	3	2	7	3	15
		<b>Median</b>	<b>3</b>	<b>1.5</b>	<b>5.5</b>	<b>2</b>	
19	Control	Grade I tear	3	7	12	4	26
22	Control	Grade I tear	3	5	9	4	21
24	Control	Grade I tear	3	5	8	1	17
28	Control	Grade I tear	3	5	6	2	16
		<b>Median</b>	<b>3</b>	<b>5</b>	<b>8.5</b>	<b>3</b>	

(Table 4.5.6.1.h) Breakdown of the median data for each stage of the rehabilitation programme with the modified data.

### Hypothesis Test Summary

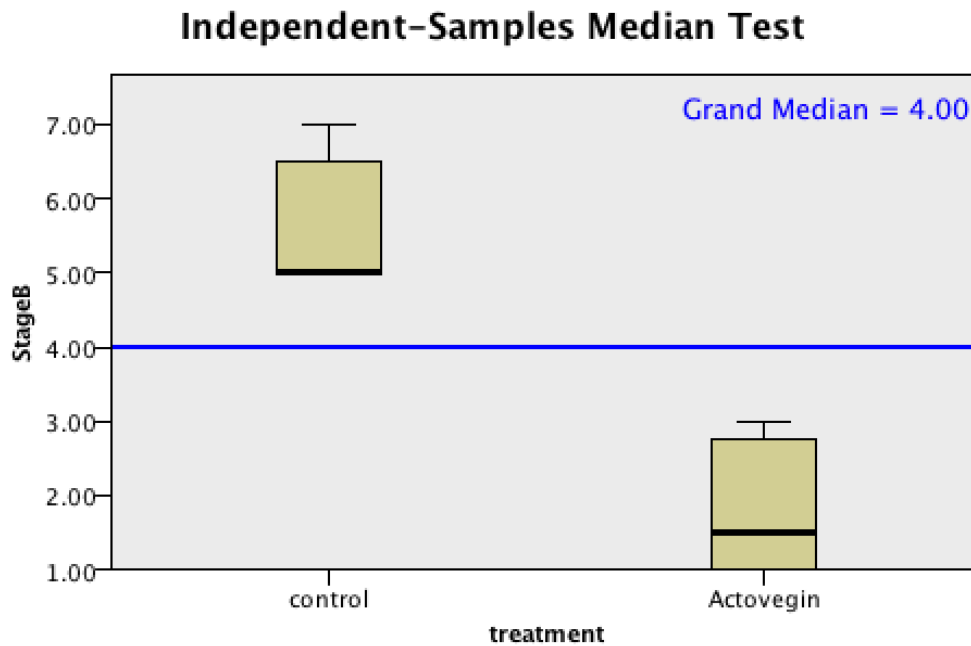
	Null Hypothesis	Test	Sig.	Decision
1	The medians of StageB are the same across categories of treatment.	Independent-Samples Median Test	.029 <sup>1,2</sup>	Reject the null hypothesis.
2	The distribution of StageB is the same across categories of treatment.	Independent-Samples Mann-Whitney U Test	.029 <sup>1</sup>	Reject the null hypothesis.
3	The distribution of StageB is the same across categories of treatment.	Independent-Samples Kolmogorov-Smirnov Test	.037	Reject the null hypothesis.
4	The medians of StageC are the same across categories of treatment.	Independent-Samples Median Test	.143 <sup>1,2</sup>	Retain the null hypothesis.
5	The distribution of StageC is the same across categories of treatment.	Independent-Samples Mann-Whitney U Test	.114 <sup>1</sup>	Retain the null hypothesis.
6	The distribution of StageC is the same across categories of treatment.	Independent-Samples Kolmogorov-Smirnov Test	.211	Retain the null hypothesis.
7	The medians of StageD are the same across categories of treatment.	Independent-Samples Median Test	1.000 <sup>1,2</sup>	Retain the null hypothesis.
8	The distribution of StageD is the same across categories of treatment.	Independent-Samples Mann-Whitney U Test	.486 <sup>1</sup>	Retain the null hypothesis.
9	The distribution of StageD is the same across categories of treatment.	Independent-Samples Kolmogorov-Smirnov Test	.699	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

<sup>1</sup>Exact significance is displayed for this test.

<sup>2</sup>Fisher Exact Sig.

(Table 4.5.6.1.i) Non-parametric analysis from SPSS with Mann-Whitney U test and Kolmogorov-Smirnov test for number of days spent in each stage of the rehabilitation programme with the modified data.



**(Figure 4.5.1.L)** Graph to show independent sample median test generated from SPSS for the difference between control and Actovegin group in the stage B rehabilitation protocol.

On further interpretation of the data, the control and treatment group of players who sustained grade I injuries were closely matched with age. Therefore further data analysis was performed for grade I injured players. There seems to be a trend to suggest that the total number of days lost is substantially reduced in a younger age group. There was a 12 day difference between the Actovegin treatment and control in the 19 year old injured players with very similar demographics. On the other hand, there was only 1 day of difference between the 26 and 28 years old players between the Actovegin and control group. Spearman's Rho correlation test performed in SPSS suggested that there was significant correlation  $-0.852$  ( $p=0.015$ ) between the age of the players and the number of days lost between control and treatment groups. This suggests that Actovegin treatment is associated with a reduced number of total days lost in a younger age group of professional football players with grade I muscle injuries. On further analysis of the individual stages of the rehabilitation process between players matched with age, there was no specific trend identified between the rehabilitation stages.



Age	Treatment	Injury	A	B	C	D	Total Days lost
19	Control	Grade I tear	3	7	12	4	26
19	Actovegin	Grade I tear	3	1	7	3	14
		Difference	0	6	5	1	12
22	Control	Grade I tear	3	5	9	4	21
22	Actovegin	Grade I tear	3	3	3	1	10
		Difference	0	2	6	3	11
24	Control	Grade I tear	3	5	8	1	17
25	Actovegin	Grade I tear	3	1	4	1	9
		Difference	0	4	4	0	8
28	Control	Grade I tear	3	5	6	2	16
26	Actovegin	Grade I tear	3	2	7	3	15
		Difference		3	-1	-1	1

(Table 4.5.6.1.j) Breakdown of the median data of each stage of the rehabilitation programme with the modified data and total number of days lost matched with the age of the players.

Sample size and power calculation:

In order to calculate the power of this pilot study and predict a sample size, the observed effect size of this study would need to be established by Cohen's d formula below:

$$d = \frac{\bar{X}_1 - \bar{X}_2}{s}$$

d = Cohen's d,  $X_1$  = mean of group 1,  $X_2$  = mean of group 2, S = pooled Standard deviation.

Pooled standard deviation can be calculated as:

$$s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2}}$$

$$S = \sqrt{\frac{(4-1)4.54^2 + (4-1)2.94^2}{8}}$$

$$S = 3.312$$

Therefore Cohen's d formula

$$d = \frac{19-12}{3.312}$$

$$d = 2.11$$

The Cohen's d value in this study is 2.11, which is larger than 1.3, which means that the effect size in this study is "Very Large". In order to determine the  $\beta$  value, to calculate the power of this study the formula below is used:

$$n = \frac{2\sigma^2(Z_\beta + Z_{\alpha/2})^2}{d^2}$$

n = total number, d = Cohen's ,  $\delta$  = standard deviation difference,  $Z_\beta = z$

Score for  $\beta$  value,  $Z_{\alpha/2} = z$  score for  $\alpha/2$  value = 1.96

Total number of players who sustained hamstring injuries = n = 8

Standard deviation difference between groups =  $\delta = 1.6$

$$8 = \frac{2(1.6)^2(Z_\beta + 1.96)^2}{2.11^2}$$

$$Z_\beta = 0.985$$

According to the Z table, 0.985 is equal to a  $\beta$  value of 0.168, the power of the study is equal to  $1 - \beta = 84\%$ .

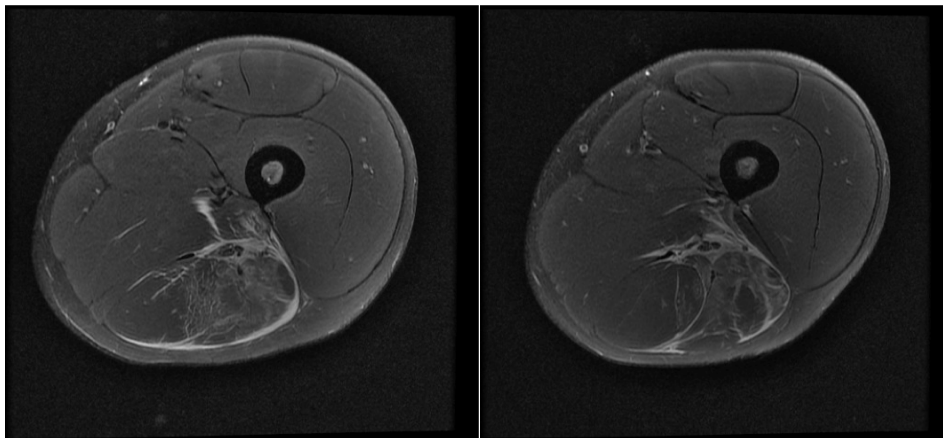
The effect size of this study is very large according to Cohen's d and the power is more than 80% with only 5% of alpha error level. Therefore, this pilot study is of a high standard and subsequent studies can be based from

this data. Furthermore, based on the data collected from this study, in order to produce a study with 95% power and 95% statistical significant, a sample size of 10 subjects (5 in each group) would be needed.

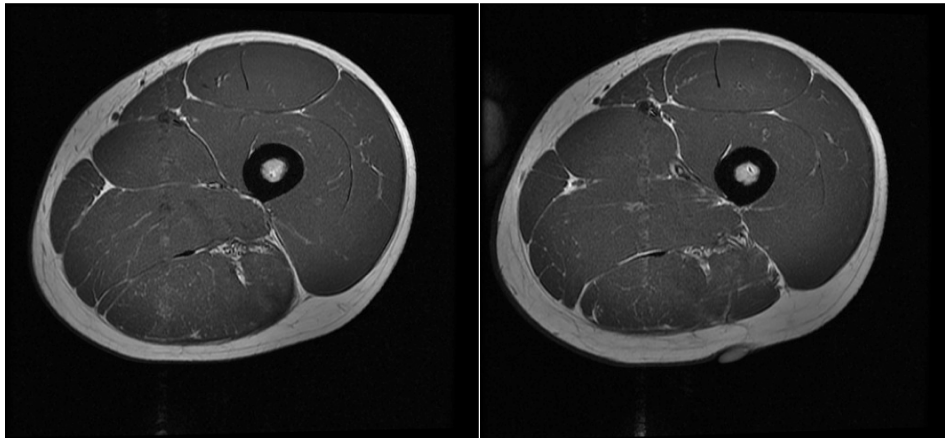
#### 4.5.2 MRI

According to the study protocol detailed in Section 4.4 and in appendix F, all injured players underwent MRI scanning to confirm clinical diagnosis. As discussed in Section 4.3.1.2, MRI is not essential for the diagnosis and grading of muscle injuries. It is use as a diagnosis adjunct to support clinical findings and also document the injury in graphical format.

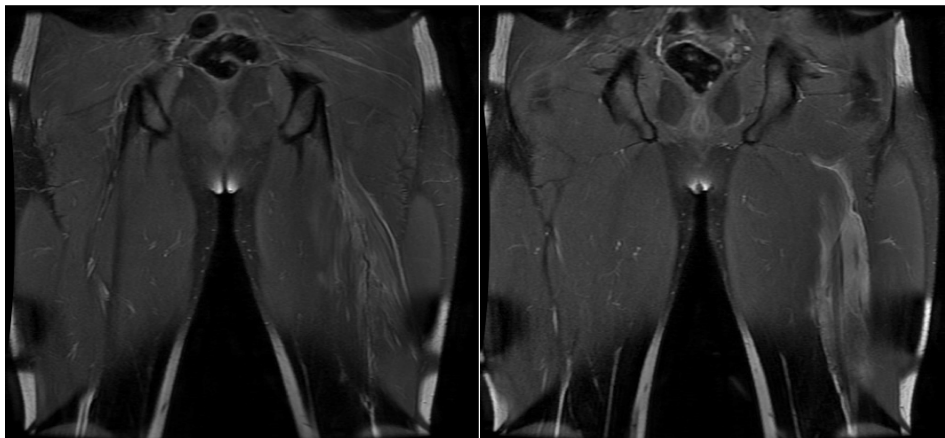
In this study, all injured players had an MRI scan within 24 hours of the injury, all scans were performed with a 1.5 tesla machine with Proton Density Fat Saturation (PD FS) and T1 spin echo (SE). Figure 4.5.2.a demonstrates an example of the MRI image of grade II hamstring injuries of one of the players in this study. The muscle oedema is demonstrated by the increased signal (white colour) in the muscle.



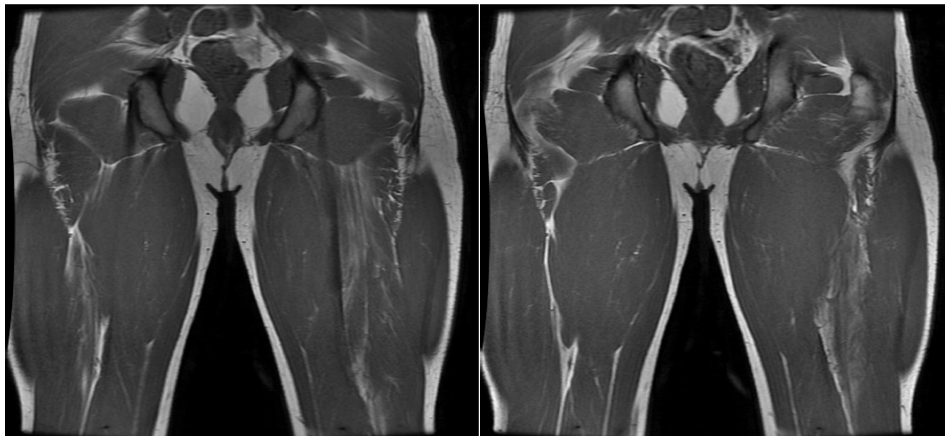
Axial PD FS



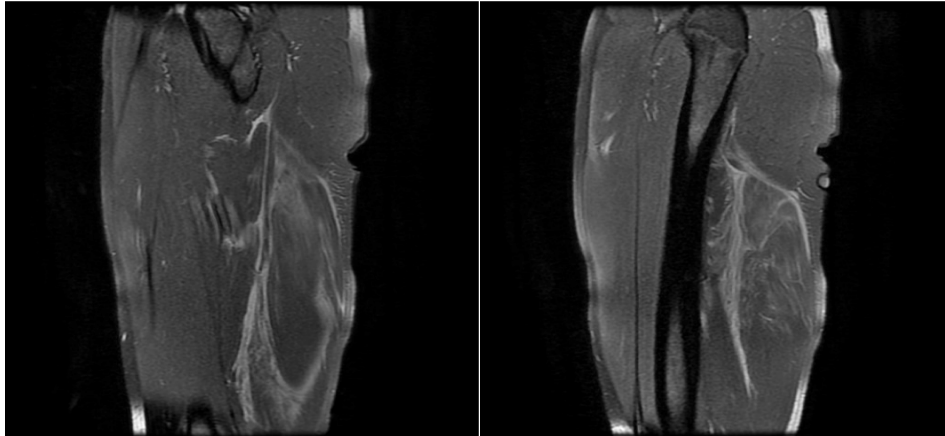
Axial T1 SE



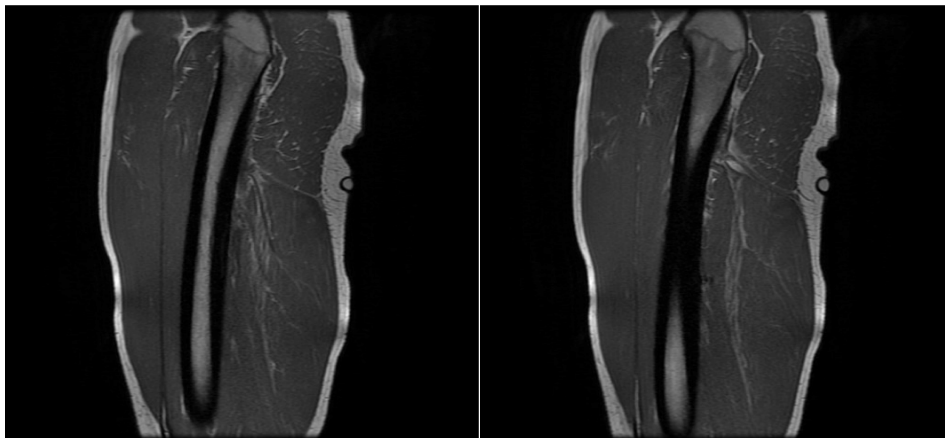
Coronal t1 SE



Coronal t1 SE



Sagittal PF FS



Sagittal t1 SE

**(Figure 4.5.2.a)** Selected images from the MRI images of grade II hamstring injuries of one of the players in this PhD.

Images were analysed in axial, coronal and sagittal planes by a certified Senior Consultant Radiologist. Out of the 11 players, 10 were reported to have sustained an injury to the biceps femoris (90%). 63% of the player's injuries arose from the musculotendinous junction. (Table 4.5.2.a) All MRI findings matched the clinical diagnosis made by the clinicians. (Table 4.5.2.b) Examples of MRI reports can be found in appendix H.

<b>MRI findings</b>	<b>Treatment</b>	<b>Side</b>
Grade II tear MTJ Biceps Femoris	Actovegin	Right
Grade II tear Biceps Femoris	Actovegin	Left
Grade II tear Biceps Femoris	Actovegin	Left
Grade I MTJ tear Biceps Femoris	Actovegin	Left
Grade I tear lateral Biceps Femoris	Actovegin	Right
Grade I tear distal Biceps Femoris	Actovegin	Right
Grade I tear MTJ Biceps Femoris	Actovegin	Right
Grade I MTJ tear Biceps Femoris	Control	Right
Grade I tear MTJ Semimembranosis	Control	Left
Grade I tear MTJ Biceps Femoris	Control	Left
Grade I MTJ tear Biceps Femoris	Control	Right

**(Table 4.5.2.a)** Report on location of hamstring injuries from MRI findings.  
MTJ - Musculotendinous junction

	Clinician 1	Clinician 2	Clinician 3	MRI
Player 1	Grade 1	Grade 1	Grade 1	Grade 1
Player 2	Grade 2	Grade 2	Grade 2	Grade 2
Player 3	Grade 1	Grade 1	Grade 1	Grade 1
Player 4	Grade 1	Grade 1	Grade 1	Grade 1
Player 5	Grade 1	Grade 1	Grade 1	Grade 1
Player 6	Grade 1	Grade 1	Grade 1	Grade 1
Player 7	Grade 2	Grade 2	Grade 2	Grade 2
Player 8	Grade 1	Grade 1	Grade 1	Grade 1
Player 9	Grade 1	Grade 1	Grade 1	Grade 1
Player 10	Grade 1	Grade 1	Grade 1	Grade 1
Player 11	Grade 2	Grade 2	Grade 2	Grade 2

**(Table 4.5.2.b)** Summary of 3 clinicians' assessments and MRI findings for injured players

## 4.6 Discussion

The clinical study in this PhD confirmed the hypothesis stated in Section 2.7, demonstrating that the Actovegin treatment regimen developed from the *in vitro* study (details in Section 4.3.2.1) could influence clinical outcome in treating acute muscle injuries. It is also the only study using Actovegin as a single agent in the treatment of muscle injuries.

The Actovegin treatment group returned to play in a median of 7 days earlier compared to physiotherapy alone in grade I hamstring injuries (further details in Section 4.5.1). For Grade II injuries, the median number of days lost in the Actovegin group was 2; unfortunately statistical analysis could not be performed, as there were no subjects with grade II injuries in the control group. However, it is only 2 days longer compared to grade I hamstring injuries treated in the control group. The results from the clinical study are comparable to literature. The physiotherapy protocol used in the clinical study was developed from the progressive agility and trunk stabilization exercises and icing (PATS) protocol described by Sherry and Best 2004. They reported that the average length for athletes to return to sport was 22.2 days (Sherry and Best, 2004), which is comparable to the median of 19 days in the control group (physiotherapy only) in the clinical study. For the Actovegin group, there has been no study in the literature reporting the use of the single Actovegin treatment for muscle injuries. The only Actovegin related study was reported by Pfister et al, its weaknesses and limitations have been discussed in Section 2.6.4; with their pharmacological cocktail of Actovegin and Traumeel mixture, the mean for return to sport was 22.2 days for grade 1 hamstring injuries (Pfister and Koller, 1990). Although it is not reasonable to perform direct comparison and statistical analysis between the clinical study in this PhD to the “active” treatment group described in Pfister et al, as there are many potential factors to influence recovery, the stand alone Actovegin treatment regimen described in this PhD seems to produce better results.

On further interpretation of the data, the control and treatment groups of players who sustained grade I injuries were closely matched with age. Spearman's Rho correlation test suggests that Actovegin treatment is associated with a reduced number of total days lost in a younger age group of professional football players with grade I muscle injuries. In this study the younger players seemed to take longer to return to play compared to the older players with grade 1 injuries, the effect of Actovegin treatment is more pronounced in terms of the reduction of number of days lost compared to the age matched control group. This suggests that the Actovegin treatment regimen has a larger effect in younger players. This could be due to the fact that younger players theoretically have a high metabolic rate compared to the older players; hence their cells would have a high-energy demand and are more dependent on ATP. Younger cells are also believed to have a better regeneration potential and therefore would require more ATP during the repair process. As discussed in 2.3.2, muscle strain injuries cause mechanical as well as biochemical damage to the cells, the speed and extent of muscle necrosis are dependent on the amount of ATP available and also the cellular metabolic rate. Hence cells that have a higher cellular metabolic rate would be more susceptible to ischaemic and metabolic insults following muscle strain injuries. Actovegin is thought to have a membrane stabilizing effect and improved energy balance in mitochondria and ATP production in ischaemic cells (further details in Section 2.5.4). Therefore, the younger cells with higher metabolic rate would have more benefit from Actovegin treatment as it could theoretically limit and contain the initial ischaemic and metabolic insult from the injury. Besides the effect of damage limitation, Actovegin could also be more beneficial to the younger cells in the biochemical process of muscle healing as discussed in Section 2.3.3. These facts could theoretically contribute to and explain the larger clinical effect with Actovegin treatment in younger patients.

All players in the study followed the same rehabilitation protocol and the results were further analysed according to the player's progression in each stage of the protocol. The Actovegin treatment group has a significantly shorter duration in stage B of the rehabilitation ( $p < 0.05$ ). This observation



further confirms the *in vitro* findings and hypothesis; Actovegin modulates the inflammatory process, which enhances the biochemical process of muscle healing. As the cells' recovery and repair becomes quicker, the effect of Actovegin treatment is seen in the early stages of the rehabilitation process.

The clinical findings supported the hypothesis developed from the results of the *in vitro* study. Actovegin can modulate the inflammatory process and influence the genotype expression of THP-1 and macrophages, therefore it can influence the healing process; further details can be found in Sections 3.4.2.3 and 3.4.3.3. The clinical study has demonstrated that the biochemical effects of Actovegin can be translated to influence clinical outcome in the treatment of acute muscle injuries.

# Chapter Five

## Discussion, Conclusion and Future Work

### 5.1 Discussion:

The aim of this PhD is to provide information on the role of Actovegin in muscle injuries and its efficacy as an injection therapy in the treatment of hamstring muscular injuries in professional footballers (Section 1.3). The *in vitro* study in this PhD thesis was the first study to investigate the role of Actovegin in the inflammatory process and demonstrated significant results to support the hypothesis. It confirmed that Actovegin could modulate the inflammatory process by influencing the CD68<sup>+</sup> and CD163<sup>+</sup> macrophages and CD163<sup>+</sup> THP-1 cells, which could influence the muscle healing process (Chapter 3). Based on the findings from the *in vitro* studies and data from previous literature, a stand alone single drug intramuscular Actovegin injection therapy regimen was developed to treat acute muscle injuries. The first clinical study using this stand-alone Actovegin treatment regimen was conducted in this PhD in professional footballer players and translated the *in vitro* findings to clinical practice, which confirmed the second hypothesis that Actovegin can influence clinical outcome in treating acute muscle injuries.

Overall, this PhD has suggested that Actovegin has an active role in the treatment of muscle strain injuries biochemically and clinically. Although the studies in this thesis were logical and performed to a high standard, there were some limitations and uncontrollable constraints. The *in vitro* cell studies were based on well-established isolated THP-1 and Macrophage cell-lines and the highest standard of qPCR analysis. It does not fully represent the biochemical environment in the muscle injury site in the human body. The inflammatory vs anti-inflammatory muscle healing model was based on published literature on cell line based studies. Although it is currently the most

widely acceptable theory, again it may not fully represent the biochemical environment in the muscle injury site in the human body. Furthermore, the qPCR data confirmed a change of mRNA, hence gene expression but it may or may not translate to final protein changes and effects in the complex muscle healing environment. Ideally, more complex cell types such as skeletal muscle cells should be used and mixed cells co-culture could further simulate the complex biochemical environment in the human body. Owing to the similarity of standard cell culture solution (FBS) and Actovegin, as discussed in Section 3.4, serum free cell culture models would be required for cellular investigation of the role Actovegin. Perhaps an animal model could be used as an study to simulate the complex biochemical environment of muscle healing. On the other hand, an animal model may not be reliable as Actovegin is a biological drug with bovine origin, the cross species effect may not translate to human muscle injuries. Therefore, a human clinical model is a more appropriate investigation for this drug. Owing to the logistical constrains and compliance with the professional football club and players, muscle biopsy following Actovegin treatment was not possible, otherwise it would have been the ideal opportunity to further understand the biochemical role of Actovegin in the treatment of muscle injuries.

Although the clinical results provided encouraging results, it is a non-blinded and non-randomised observational pilot study with subjective assessments for return to play. The potential psychological effects of injection therapy and placebo effect cannot be ruled out in this study; ideally a placebo injection should be used alongside Actovegin. As the treatment of muscle injuries is based on low-level evidence, there has not been any report or evidence on intramuscular isotonic saline injection therapy. Therefore, it is unethical to assume that intramuscular isotonic saline is a placebo and can be used as a control group. Alternatively, dry needling to the injury site could be used as a control to reduce the psychological effect of the needle entering the skin, but it will not mimic the injection effect of Actovegin. Furthermore, all players in the Actovegin group experienced minor muscle cramp immediately after injection, this effect may not be possible to mimic by any placebo substances. Psychological and placebo effects are important issues in all

medical therapies, researchers should always consider their potential impact and interpret the results with caution.

In the clinical study, the same diagnosis and grading of muscle injuries were recorded by three independent qualified practitioners, this provided a robust and reliable diagnostic method. As discussed in Section 4.3.1.2, MRI is not an essential tool for diagnosis or grading with the method used in this PhD described in Section 2.3.1. Therefore, the purpose of the MRI scan in this PhD was to document the injuries in a graphical format and was also used as a diagnosis adjunct to confirm the clinical findings. (For further details, please see Section 4.3.1.2) For future research, if any MRI grading methods were used for the diagnosis of muscle injuries, it would be advisable for the researcher to have at least two qualified radiologists to interpret the MRI findings. Furthermore, the patient should have at least 2 MRI scans by different machines to ensure reliability.

The number of subjects in the clinical study is relatively small, due to the competitive nature of the sport. It is not possible to standardise the rehabilitation regimen between clubs, therefore only a single football club was used, and hence the patient numbers in each group were small. On the other hand, these limitations could be seen as a strength for the study, as players in a professional football club are often closely matched with age and physical ability, they can be matched and produce a higher quality result. All players in the club are contracted to follow the same rehabilitation protocol and treated by the same team of physiotherapists, which significantly reduced the variability of the rehabilitation process. Therefore, a high quality clinical study can be performed with this subgroup of patients as demonstrated in the clinical study. The power calculation demonstrated in Section 4.5 suggested that the power of the clinical study is 84%, which is a high standard (>80%) for a clinical study. In order to improve the number of subjects available in the clinical study, amateur athletes or college athletes could be used. However, the compliance and variation in the rehabilitation process with these groups of athletes could affect the reliability of the study as demonstrated by Pfister et al during a similar studies (Pfister and Koller, 1990). Furthermore, the clinical

impact of a grade I muscle injury to an amateur athlete may not be as pronounced compared to a professional footballer. These footballers were in a different psycho-social and socio-economic group, therefore the clinical results may not translate.

Return to play and functional assessment following injury in the clinical study were based on clinical assessment by 3 clinicians independently. Although it is a powerful and reliable method of assessment, where all clinicians agreed with the outcome and the player could return to play, the study can be improved by using an objective assessment method to monitor the rehabilitation process. Isokinetic analysis has been tried, as described in Section 4.3.4. Due to the logistical constraints and the poor compliance for re-testing by the players, isokinetic testing is not a practical method for functional assessment in a professional football club. However, it may be a useful technique if the club had made this assessment compulsory for all the players and had a dedicated team of technicians for the isokinetic analysis. Traditional imaging techniques such as MRI may not be useful in monitoring the rehabilitation process, as currently there are no radiological benchmarks to monitor the muscle healing process. Therefore, clinical assessments remain as the gold standard of functional assessment in muscle injuries.

## 5.2 Conclusion:

The *in vitro* studies described in this PhD have provided essential evidence that Actovegin could affect the phenotype of macrophages and modulate the inflammatory process. These effects have translated to the clinical study and suggest that Actovegin intramuscular injection therapy could improve clinical outcome in the treatment of grade I hamstring injuries.

This thesis summarises the current evidence on Actovegin. Compared with conventional conservative RICE and NSAID therapy, Actovegin proposes an exciting and legal alternative for high performance athletes. From the studies, Actovegin injection therapy seems safe and well tolerated. More objective evidence is needed before further conclusions can be drawn for this new treatment of muscle injuries. Despite the positive experience with Actovegin in this PhD, Actovegin injection therapy should be reserved within elite professional athletes. Further evidence and clinical studies should be performed in different subgroups of populations to further investigate its efficacy. Although it may not be possible due to the limitations discussed in Section 5.1, a double-blinded multicentre randomised control trial could help to confirm its clinical efficacy in the general population.

Medicine is a form of science as well as an art. As modern sports physicians, we must apply the principles of Evidence Based Medicine when considering new therapies. Physicians should process this evidence carefully and pay attention to detail, especially when the published medical literature is limited. Further *in vitro* and clinical research must be encouraged to investigate the effects of Actovegin on muscle injuries before it can be labelled as effective treatment. Injection therapy could potentially be useful in the treatment of muscle injuries.

### 5.3 Future work:

The *In vitro* study in the PhD is the first study to investigate the role of Actovegin in the inflammatory process and demonstrated significant results. Future research could continue to explore this route by looking at different inflammatory cytokines with qPCR and micro array gene chip expression to further understand the mechanism of action. Protein expression studies such as western blotting should be used to confirm the genotype and phenotype expression changes demonstrated by qPCR. Furthermore, a time course experiment should be set up with the macrophages and THP-1 cell line to further analyse the effect of Actovegin in 48 and 72 hours of cultures. This can further identify the role of Actovegin in different stages of the inflammatory process. Finally, a human clinical muscle injection and biopsy model would be most beneficial to confirm all the *in vitro* findings.

Actovegin is a physiological mixture of bovine origin, further details can be found in Section 2.5. The HPLC experiment described in Section 3.3.2 has demonstrated peaks of contents in Actovegin. With the HPLC technique, each peak could be separated and be used to the same bioassays either as individual or as a mixture to identify the active ingredient(s). However, this approach may be difficult as there could be one or more active ingredient and the optimum concentration of these ingredient(s) may also have to be refined. Further studies can explore the compound with the peaks with bioassay and Nuclear magnetic resonance, in order to identify single or combined active ingredients within Actovegin.

In regards to the clinical aspect of the use of Actovegin in the treatment of muscle injuries, with the limitations of discussed in 5.1, future work should be focused on the duration and effective dose of Actovegin therapy. In order to minimise the placebo and psychological effect of injection therapy, a standard placebo substance needs to be established for injection therapy in muscular injuries. Further clinical studies should be done in a different subgroup of patients and ensure the clinical effect from this PhD is not group

specific and can be translated across different spectrums of patients. Finally, a double-blinded multicentre randomised control trial would be useful to confirm its efficacy.



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# **Appendix A**

**Documentation from the FA**

**Documentation from the global DRO**

Reference to page 46, 61

Dr Paul Lee  
281 Allensbank road  
Heath  
Cardiff  
CF14 3RA



Dear Dr Lee

Thank you for your recent correspondence regarding the status of Solcoseryl. Having consulted UK Sport on the status of this substance I can outline the following for you.

Solcoseryl is not currently a prohibited substance according to the World Anti-Doping Agency (WADA) List of Prohibited Substances and Methods 2009, however if the route of administration is via intravenous infusion, then a Therapeutic Use Exemption (TUE) approval would need to be secured in line with FA Doping Control Regulations.

Other routes of administration (oral, intramuscular injection, topical) are not prohibited and would not require a TUE. Please visit [www.thefa.com](http://www.thefa.com) for more information on checking the current status of medications and the TUE application process.

Please be aware however that The FA Doping Control department can not recommend or advocate the use of any specific treatment method or medication to treat a particular condition and the use of any prohibited substance or method remains the responsibility of the player at all times.

I hope this has satisfied your query; however should you require any further information please do not hesitate to contact me.

Yours sincerely

A handwritten signature in black ink, appearing to be 'P.P.', followed by the initials 'P.P.' in a simple font.

Mike Earl  
Doping Control Programme Manager  
The Football Association



**BIDDING NATION  
ENGLAND**

A handwritten signature in black ink, appearing to be 'P.P.', followed by the initials 'P.P.' in a simple font.



INGREDIENT STATUS

Ingredient: Actovegin

<b>Reference Number</b> 02100027943	<b>Nation of Purchase</b> United Kingdom
<b>Date and Time of Search</b> Saturday, May 15, 2010 9:02 PM (UTC)	<b>Sport</b> Football [FIFA]

Status

<b>Ingredient</b>	<b>Route of Administration</b>	<b>In-Competition</b>	<b>Out-of-Competition</b>
Actovegin	Injection - Intra-muscular	<input checked="" type="checkbox"/> <u>Not Prohibited</u>	<input checked="" type="checkbox"/> <u>Not Prohibited</u>
Actovegin	Injection - Intravenous	<input checked="" type="checkbox"/> <u>Not Prohibited</u>	<input checked="" type="checkbox"/> <u>Not Prohibited</u>
Actovegin	Injection - Local or Intra-Articular	<input checked="" type="checkbox"/> <u>Not Prohibited</u>	<input checked="" type="checkbox"/> <u>Not Prohibited</u>
Actovegin	Topical (dermatological)	<input checked="" type="checkbox"/> <u>Not Prohibited</u>	<input checked="" type="checkbox"/> <u>Not Prohibited</u>

WADA Classification(s): This ingredient is not currently included on the WADA Prohibited List.

Additional Information: If this substance is administered by intravenous *infusion*, as opposed to intravenous *injection*, it is Prohibited under Chemical and Physical Manipulation (M2).

An intravenous *injection* is the supply of a small volume of fluid or medication, in a rapid manner, by means of a simple syringe.

An intravenous *infusion* is the insertion of a specialized needle into a vein and the infusion of fluids at a predetermined rate from a reservoir usually above the level of the body.

See the FAQs for more details.



## Q&A on 2011 Prohibited List

[Share](#) 13

**What major changes does the 2011 List of Prohibited Substances and Methods include compared to the 2010 List?**

**Why was methylxanamine reclassified from a "non-specified" stimulant to a "specified" stimulant?**

**What is the status of caffeine?**

**What is the status of actovegin?**

Actovegin is not prohibited in sport under the WADA List of Prohibited Substances and Methods except if it is used by intravenous infusion.

Intravenous infusions are prohibited according to section M2 (Chemical and Physical Manipulation) of the Prohibited List. Intravenous injections with a simple syringe are permitted if the injected substance is not prohibited, the volume does not exceed 50 mL, and the intravenous injections are given at intervals equal or greater than six hours. Additional information can be found in the Medical Information on Intravenous Infusion, available [here](#).

- > World Anti-Doping Program
  - > Sport & Anti-Doping Organizations
  - > The Code
  - > International Standards
    - > Prohibited List
      - > 2011 Prohibited List (mobile)
      - > **Q&A on 2011 Prohibited List**
      - > Monitoring Program
    - > Testing
    - > Laboratories
    - > Therapeutic Use Exemptions
    - > Protection of Privacy and Personal Information
  - > Model Rules, Guidelines and Protocols
  - > Independent Observer Program
  - > Tools
  - > Governments
  - > Legal Library

# **Appendix B**

## **Actovegin drug EU licence**

Reference to page 60



**GMP-ZERTIFIKAT / CERTIFICATE OF GMP COMPLIANCE OF A MANUFACTURER**

Zertifikat Nr.: / Certificate No.: INS-480050-0011-002

**Teil 1 / Part 1**

Ausgestellt auf Basis einer Inspektion in Übereinstimmung mit /  
*Issued following an inspection in accordance with*

- Art. 111(5), Directive 2001/83/EC**
- Art. 80(5), Directive 2001/82/EC**
- Art. 15 of Directive 2001/20/EC**

Die zuständige Behörde **Österreichs** bestätigt wie folgt: /  
*The competent authority of **Austria** confirms the following:*

**Nycomed Austria GmbH**  
**St.-Peter-Straße 25**  
**A-4020 Linz**

wurde im Rahmen des nationalen Inspektionsprogramms inspiziert, in Verbindung mit der  
Geschäftszahl (Hersteller-Lizenznummer) /  
*has been inspected under the national inspection programme in connection with manufacturing  
authorisation no. **480050***

in Übereinstimmung mit / *in accordance with*

- Art. 40 of Directive 2001/83/EC
- Art. 44 of Directive 2001/82/EC
- Art. 13 of Directive 2001/20/EC

umgesetzt in folgende nationale Gesetzgebung /  
*transposed in the following national legislation:*

"Verordnung der Bundesministerin für Gesundheit und Frauen betreffend Betriebe, die Arzneimittel  
herstellen, kontrollieren oder in Verkehr bringen (Arzneimittelbetriebsordnung 2005 - AMBO 2005),  
BGBl. II Nr. 479/2004, zuletzt geändert durch das BGBl. II Nr. 156/2007".\*

und/ *and* \*

Ist ein Wirkstoffhersteller, inspiziert in Übereinstimmung mit /  
*Is an active substance manufacturer that has been inspected in accordance with*

- Art. 111(1) of Directive 2001/83/EC
- Art. 80(1) of Directive 2001/82/EC



**GMP-ZERTIFIKAT / CERTIFICATE OF GMP COMPLIANCE OF A MANUFACTURER**

Zertifikat Nr.: / Certificate No.: INS-480050-0011-002

umgesetzt in folgende nationale Gesetzgebung /  
*transposed in the following national legislation:*

"Verordnung der Bundesministerin für Gesundheit und Frauen betreffend Betriebe, die Arzneimittel herstellen, kontrollieren oder in Verkehr bringen (Arzneimittelbetriebsordnung 2005 - AMBO 2005), BGBl. II Nr. 479/2004, zuletzt geändert durch das BGBl. II Nr. 156/2007".\*

Aus der während der Inspektion des betreffenden Herstellers gewonnenen Kenntnis, zuletzt durchgeführt am /

*From the knowledge gained during inspection of this manufacturer, the latest of which was conducted on*  
**16<sup>th</sup> – 17<sup>th</sup> April 2008,**

kann angenommen werden, dass /  
*it is considered that it complies with*

den Richtlinien der Guten Herstellungspraxis entsprochen wird, festgehalten in /  
*The principles and guidelines of Good Manufacturing Practice laid down in*

Directive 2003/94/EC<sup>1</sup>/

Directive 91/412/EEC<sup>1</sup>/

der Richtlinie der GMP für Wirkstoffe / *The principles of GMP for active substances<sup>1</sup>.\**

Dieses Zertifikat spiegelt den Zustand der Betriebsstätte zum oben erwähnten Inspektionszeitpunkt wieder, falls mehr als drei Jahre verstrichen sind, sollte die ausstellende Behörde kontaktiert werden.  
*This certificate reflects the status of the manufacturing site at the time of the inspection noted above and should not be relied upon to reflect the compliance status if more than three years have elapsed since the date of that inspection, after which time the issuing authority should be consulted.*

Die Richtigkeit der Angaben kann bei der ausstellenden Behörde überprüft werden /  
*The authenticity of this certificate may be verified with the issuing authority.*

<sup>1</sup>These requirements fulfil the GMP recommendations of WHO.

(\*): Nichtzutreffendes streichen / delete that which does not apply



**GMP-ZERTIFIKAT / CERTIFICATE OF GMP COMPLIANCE OF A MANUFACTURER**

Zertifikat Nr.: / Certificate No.: INS-480050-0011-002

**GMP Zertifikat, Teil 2  
GMP-Certificate, Part 2**

**Teil 1 – HERSTELLUNGSTÄTIGKEITEN**

- Die erlaubten Herstellungstätigkeiten umfassen vollständige und teilweise Herstellung (einschließlich verschiedener Prozesse wie Umfüllen, Abpacken oder Kennzeichnen), Chargenfreigabe und -zertifizierung, Einfuhr, Lagerung und Vertrieb der genannten Darreichungsformen sofern nicht anders angegeben;  
- Die Qualitätskontrolle und/oder Freigabe und/oder Chargenzertifizierung ohne Herstellungsschritte sollten unter den entsprechenden Punkten spezifiziert werden;  
- Unter der relevanten Produktart und Darreichungsform sollte auch angegeben werden, wenn der Hersteller Produkte mit speziellen Anforderungen herstellt, z.B. radioaktive Arzneimittel oder Arzneimittel, die Penicilline, Sulfonamide, Zytostatika, Cephalosporine, Stoffe mit hormoneller Wirkung oder andere potenziell gefährliche Wirkstoffe enthalten (anwendbar für alle Bereiche des Teils 1 mit Ausnahme 1.5.2 und 1.6).

**Part 1 – MANUFACTURING OPERATIONS**

- authorised manufacturing operations include total and partial manufacturing (including various processes of dividing up, packaging or presentation), batch release and certification, importation, storage and distribution of specified dosage forms unless informed to the contrary;  
- quality control testing and/or release and batch certification activities without manufacturing operations should be specified under the relevant items;  
- if the company is engaged in manufacture of products with special requirements e.g. radiopharmaceuticals or products containing penicillin, sulphonamides, cytotoxics, cephalosporins, substances with hormonal activity, or other or potentially hazardous active ingredients this should be stated under the relevant product type and dosage form (applicable to all sections of Part 1 apart from sections 1.5.2 and 1.6)

Humanarzneimittel / *Human Medicinal Products*

Veterinärarzneimittel / *Veterinary Medicinal Products*

Prüfpräparate zur klinischen Prüfung / *Human Investigational Medicinal Products*

Phase I     Phase II     Phase III     Phase IV

<b>1.1</b>	<b>Sterile Produkte / Sterile products</b> (Liste der Arzneiformen / <i>list of dosage forms</i> )
1.1.1	Aseptisch hergestellt (Liste der Darreichungsformen) / <i>Aseptically prepared (List of dosage forms)</i>  1.1.1.4 Kleinvolumige flüssige Darreichungsformen / <i>Small volume liquids: Ampullen / Ampoules</i>
1.1.2	Im Endbehältnis sterilisiert (Liste der Darreichungsformen) / <i>Terminally sterilised (list of dosage forms)</i>  1.1.2.3 Kleinvolumige flüssige Darreichungsformen / <i>Small volume liquids: Ampullen, Fläschchen / Ampoules, Vials,</i> 1.1.2.5 Andere endsterilisierte Produkte / <i>Other terminally sterilised prepared products: Augentropfen / Eye Drops</i>
<b>1.2</b>	<b>Nichtsterile Produkte / Non-Sterile products</b>
1.2.1	Nichtsterile Produkte (Liste der Darreichungsformen) / <i>Non-sterile products (list of dosage forms)</i>  1.2.1.6 Flüssige Darreichungsformen zur inneren Anwendung / <i>Liquids for internal use: Lösungen / Solutions</i> 1.2.1.13 Tabletten / <i>Tablets</i>



**GMP-ZERTIFIKAT / CERTIFICATE OF GMP COMPLIANCE OF A MANUFACTURER**

Zertifikat Nr.: / Certificate No.: INS-480050-0011-002

	1.2.2 nur Chargenfreigabe / <i>Batch certification only:</i> Lösungen, Tabletten / <i>solutions, tablets</i>
<b>1.3</b>	<b>Biologische Arzneimittel / <i>Biological medicinal products</i></b>
	1.3.1 Biologische Arzneimittel / <i>Biological medicinal products</i>  1.3.1.1 Blutzubereitungen / <i>Blood products:</i> Resorbierbare Wundauflage (Kollagen, humanes Fibrinogen, Thrombin, Aprotinin) / <i>absorbable wound dressing (collagen, fibrinogen human, thrombin, aprotinin)</i>  1.3.1.6 Produkte menschlicher oder tierischer Herkunft / <i>Human or animal extracted products:</i> Entproteinisiertes Kalbsbluthämoderivat / <i>deproteinized hemoderivative of calf blood</i>
<b>1.4</b>	<b>Andere Produkte oder Herstellungstätigkeiten</b> (jede andere relevante Herstellungsaktivität/ Produktart, die oben nicht erwähnt ist, z.B. Sterilisation von Wirkstoffen, Herstellung von biologischen Ausgangsstoffen (sofern durch nationale Vorschriften vorgesehen), pflanzliche oder homöopathische Produkte, Bulk oder vollständige Herstellung usw.) <b>Other products or manufacturing activity</b> ( <i>any other relevant manufacturing activity/product type that is not covered above e.g.: sterilisation of active substances, manufacture of biological active starting materials (when required by national legislation), herbal or homeopathic products, bulk or total manufacturing, etc.</i> )
	1.4.1 Herstellung von: / <i>Manufacture of:</i>  1.4.1.4 Anderen / <i>Others:</i> <ul style="list-style-type: none"> <li>• Wirkstoff (Bulk, Endprodukt) / <i>API, Drug Substance (Bulk):</i> Aprindine hydrochloride, Azintamide, Barnifylline hydrochloride, Celiprolol hydrochloride, Distigmine bromide, Etamivan, Hexobendine dihydrochloride, Hexoprenaline sulfate, Lornoxicam, Midodrine hydrochloride, Morphine-6-glucuronide, Podophyllotoxin, Sulfametrole, Suxamethonium chloride, Thonzonium bromide, Warfarin sodium</li> <li>• Hochtoxische Stoffe / <i>specifically toxic substances</i></li> <li>• Wirkstoff (Ausgangsmaterial, Zwischenprodukt) / <i>API starting material, (raw material, intermediate)</i></li> <li>• Arzneimittel für Klinische Versuche / <i>Drugs for clinical trials:</i> Tabletten, Ampullen, Schwämme / <i>tablets, ampoules, sponge</i></li> <li>• Salbengrundlage, Lebensmittelzusätze / <i>ointment base, food supplements</i></li> </ul> 1.4.3 Andere / <i>Others:</i> Verblindung / <i>Blinding:</i> Tabletten, Ampullen, Schwämme / <i>tablets, ampoules, sponge</i>
<b>1.6</b>	<b>Qualitätskontrolle / <i>Quality control testing</i></b>
	1.6.1 Mikrobiologisch: Sterilität / <i>Microbiological: sterility</i> 1.6.2 Mikrobiologisch: Prüfung nicht steriler Produkte / <i>Microbiological: non-sterility</i>

CER99X 22367DM / 4.0



**GMP-ZERTIFIKAT / CERTIFICATE OF GMP COMPLIANCE OF A MANUFACTURER**

Zertifikat Nr.: / Certificate No.: INS-480050-0011-002

Wirkstoffherstellung: siehe 1.4.1.4. / *Manufacture of active substance: referring to item 1.4.1.4.*

Mögliche Einschränkungen oder Erklärungen bezüglich des vorliegenden Zertifikats /  
*Any restrictions or clarifying remarks related to the scope of this certificate:*

Keine / *None*

Für das Bundesamt für Sicherheit im Gesundheitswesen /  
*For the Federal Office for Safety in Health Care*



Leg. Verm. Nr. 414

Die Unterschrift von

*DI. R. Schwarz*

wird hiermit beglaubigt,  
Wien, am **18. Feb. 2009**



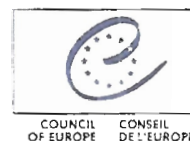
**Gerlinde Fromm**

**Gebühr entrichtet**



European Directorate for the  
Quality of Medicines & HealthCare

Certification of Substances Division



**Certificate of suitability**  
**No. R0-CEP 2004-235-Rev 02**

1 *Name of the substance:*  
2 **DEPROTEINISED HEMODERIVATIVE OF CALF BLOOD**  
3 Actovegin concentrate

4 *Name of holder:*  
5 **NYCOMED AUSTRIA GMBH**  
6 St Peter-Strasse 25  
7 Austria-4020 Linz

8 *Site(s) of production:*  
9 **NYCOMED AUSTRIA GMBH**  
10 St Peter-Strasse 25  
11 Austria-4020 Linz

12 **THIS CERTIFICATE SUPERSEDES THE PREVIOUS CERTIFICATE**  
13 **R0-CEP 2004-235-REV 01**

14 After examination of the information provided on the origin of raw material(s) and type of  
15 tissue(s) used and on the manufacturing process for this substance on the site(s) of  
16 production mentioned above, we certify that the substance **DEPROTEINISED**  
17 **HEMODERIVATIVE OF CALF BLOOD** meets the criteria described in the current  
18 version of the monograph Products with risk of transmitting agents of animal spongiform  
19 encephalopathies no. 1483 of the European Pharmacopoeia, current edition including  
20 supplements.


21 – countries of origin of source materials: Germany, France, Austria, Italy,  
22 Poland, Belgium, Spain,  
23 Luxembourg, The Netherlands,  
24 Lithuania, Latvia, Denmark, Ireland  
25 – nature of animal tissues used in manufacture: Bovine calf blood

26 The submitted dossier must be updated after any significant change that may alter the  
27 quality, safety or efficacy of the substance, or that may alter the risk of transmitting  
28 animal spongiform encephalopathy agents.

29 Manufacture of the substance shall take place in accordance with a suitable quality  
30 assurance system such as GMP, and in accordance with the dossier submitted.

Address: 7, allée Kastner, CS 30026 - F 67081 Strasbourg (France)  
Telephone: 33 (0) 3 88 41 30 30 Fax: 33 (0) 3 88 41 27 71 e-mail: cep@edqm.eu  
Internet : <http://www.edqm.eu>

- 31 Failure to comply with these provisions will render this certificate void.
- 32 The certificate is valid provided there has been no deterioration in the TSE status of the  
33 country(ies) of origin of the source material.
- 34 This certificate is granted within the framework of the procedure established by the  
35 European Pharmacopoeia Commission [Resolution AP-CSP (93) 5 as amended] for a  
36 period of five years starting from **16 December 2004**. Moreover, it is granted according  
37 to the provisions of Directive 2001/83/EC and Directive 2001/82/EC and any  
38 subsequent amendment, and the related guidelines.
- 39 This certificate has:  
40 lines.

  
On behalf of the  
Director of EDQM & HealthCare



Strasbourg, 28 September 2007

DECLARATION OF ACCESS (to be filled in by the certificate holder under their own responsibility)

<p style="text-align: center;"><b>Nycomed Austria GmbH</b>, as holder of the certificate of suitability</p> <p style="text-align: center;"><b>R0-CEP 2004-235-Rev 02 for DEPROTEINISED HEMODERIVATIVE OF CALF BLOOD</b></p> <p>hereby authorises ..... (name of the pharmaceutical company)</p> <p>to use the above-mentioned certificate of suitability in support of their application(s) for the following Marketing Authorisation(s): (name of product(s) and marketing number(s), if known)</p> <p>The holder also certifies that no significant changes to the operations as described in the CEP dossier have been made since the granting of this version of the certificate</p> <p>Date and Signature (of the CEP holder):</p>
---

Address: 7, allée Kastner, CS 30026 - F - 67081 Strasbourg (France)  
Telephone: 33 (0) 3 88 41 30 30 - Fax: 33 (0) 3 88 41 27 71 - e-mail: cep@edqm.eu  
Internet : <http://www.edqm.eu>





Nycomed Austria GmbH  
St.-Peter-Strasse 25  
A-4020 Linz, Austria  
Postal address:  
P.O. Box 122  
A-4021 Linz, Austria  
T +43 (732) 6919-0  
F +43 (732) 65 55 68

## To whom it may concern

July 20<sup>th</sup>, 2006 PQS/Emau  
Eva Maurhart, DI  
Tel: (+43-70) 6919-4147  
Fax: (+43-70) 6919- 4109  
Email: emau@nycomed.com

## Compliance with the TSE Guideline

To the best of our current knowledge, it is hereby confirmed that all exipients used for the manufacturing of the medical product

### ACTOVEGIN SOLUTION FOR INJECTION 200MG PER 5ML

manufactured at NYCOMED Austria GmbH, are in compliance with the *Note for Guidance on minimising the risk of transmitting spongiform encephalopathy agents via human and veterinary medicinal products* (EMA 410/01 Rev. 2), referred to in the Directive 2001/83/EC.

We confirm that the used exipients (water for injection as well as hydrochloric acid) are of non-animal origin.

### Nycomed Austria GmbH

i.A. Eva Maurhart  
DI Eva Maurhart

Quality Assurance Biological Products

i.A.   
Mag. Michael Huber

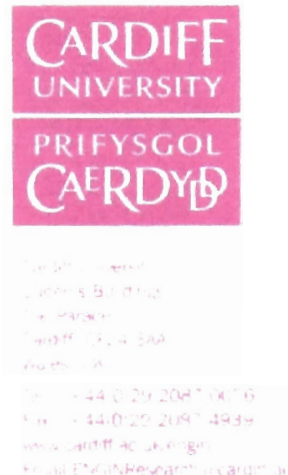
Quality Assurance Biological Products

# **Appendix C**

## **Ethical approval**

**Reference to page 112, 146**

Cardiff School of Engineering  
Director of School Professor H.R. Thomas BSc, MSc, DIC, PhD, DSc, FRSE, FICE, FRCR, FRS  
Research Office:  
Deputy Director of School Research Professor P.J. Tasker BSc, PhD



To whom it may concern,

**RE: ETHICAL APPROVAL**

**Title of Project:** A Pilot to Study an Intramuscular Injection Therapy of Traumeel and Actovegin in Elite Football Players with Muscular Injury

**Researcher:** Paul Lee

**Supervisor:** Prof. Len Nokes

I confirm that the above project has been subject to the School's ethical approval process.

On behalf of the Cardiff School of Engineering Research Committee, I approve the attached application.

Professor PJ Tasker, Chair, Research Committee  
Deputy Director of School, Research

2<sup>nd</sup> July 2009

# **Appendix D**

## **Kincom Protocol**

**Reference to page 132**

## KinCom Protocol

5 players per session

Wearing shorts for the hamstring testing

Barefooted on ankle testing

1. Age
2. Weight
3. Height
4. Position of play
5. Previous injuries

Warm up – 5 minutes on treadmill at their own speed

HAMSTRING and Quadriceps

**RIGHT** side first

Players to KinCom, Strapped in, sitting position

Knee to pivot point

Strapped load cell just above medial malleolus

Set up machine

- Overlay
- Knee
- Flex (concentric / eccentric)
- Anatomical 0 = full extension knee
- Direction of movement = down
- Stop angle = Flex 90
- Start angle = Ext 0

Angle velocity 60 /s, Flex 50N, Ext 50N, min 20N, Max 2000N

Gravity correction off

Instructions:

“This test is not like a weight machine, the machine will have a fixed speed, it will not move faster if you pull harder. It will record your power.”

“Pull the machine by bending your knee” (Flex)

“Pull the machine by bending your knee, and resist it as your knee extends”

1 x learning

2 x sub max testing

Rest for 30 seconds

Best of 3 for measurement, Save data

Reverse the settings for Quadriceps (2 sub max, then best of 3)

**LEFT** side test

2 x sub max testing

Rest for 30 seconds

Best of 3 for measurement, Save data

Reverse the setting for Quadriceps(2 sub max, then best of 3)

2 x sub max testing

Rest for 30 seconds

Best of 3 for measurement, Save data

# **Appendix E**

## **Patient information sheet for clinical study**

**Reference to page 146**

## Study information sheet

### **A pilot study of intramuscular injection therapy with Actovegin on elite football players with muscular injuries.**

*We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.*

*All players in Cardiff City Football Club are being invited to take part.*

*It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive from the medical team.*

#### **Background**

Muscle injuries are one of the most common sport related injuries. Currently, the most common treatment option is rest, ice, compression and elevation.

In 2008, an article in the British Journal of Sports Medicine summarised that currently almost all our knowledge in the treatment of muscular injuries is very poor. It highlighted the importance of injection therapies and called for further studies into Traumeel and Actovegin.

#### **Study Drug:**

Actovegin: It is believed to improve the utilization of oxygen and promote uptake of nutrients into the cells.

We have written confirmation from the WADA, FA and UK sport that Actovegin are not prohibited via intra-muscular injection.

#### **What the study involves**

As we don't know what treatment is the best for muscular injuries. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same to start with, each participant is put into a group by chance (randomly).

Following clinical examination by the club doctor and MRI confirmation of a muscle tear that does not require surgical repair, you will be randomised into two groups. You will either receive a course of 3 Actovegin injections, if you would like to opt out for the Actovegin injection, you will be invited to join the control group. You will undergo standard physiotherapy provided by the club.

**Safety**

Actovegin have been used for over 60 years in Europe. There are no known reports of allergy associated with Traumeel available in the medical literature.

GMC registered doctors who are trained in life support and are able to deal with anaphylactic reactions will perform the injection therapies. Oxygen and adrenaline will be available at all times.

**After the study**

All information collected will be anonymous and treated confidentially. All participants' personal data and results will be stored in a password protected database. All data will be anonymised before publishing.

**Taking part**

Please note that taking part in the study is entirely voluntary. You are free to withdraw at any time. Choosing not to take part in the study will not in any way affect your medical management. No money is offered for taking part in the study.

This research is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Cardiff University's Research Ethics Committee.

If you have any more question please feel free to ask any of the research doctors. We hope that the findings of this study will be of benefit to muscular injuries in the future. Thank you for considering taking part.

*If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number). If you remain unhappy and wish to complain formally, you can do this through the Cardiff City Football club or Cardiff University Complaints Procedure (or Private Institution). Details can be obtained from the Club or University.*

**Investigators**

Chief investigator - Prof. Len Nokes

Principal investigator - Dr. Paul Lee



# **Appendix F**

## **Clinical Study protocol**

**Reference to page 146,164**

## Cardiff University Protocol

**A pilot study into intramuscular injection therapy with Actovegin® on elite football players with muscular injuries.**

**Prospective Observational Study**

**Short title**

**ActoFC study**

<b>Protocol version number and date:</b>	3.0.0	
<b>EudraCT number:</b>	2008-014603-31	
<b>Sponsor:</b>	Cardiff University	
<b>Protocol authorised by:</b>	Professor Len Nokes, Dr. Paul Lee	
<i>Name and Role</i>	<i>Date</i>	<i>Signature</i>

Professor Len Nokes, Dr. Paul Lee		
<i>Name and Role</i>	<i>Date</i>	<i>Signature</i>

**Study Management Group**

<u>Chief Investigator:</u>	<b>Professor Len Nokes</b> , <i>BEng, MSc, PhD, MB,BCh, MD, FIMechE, Ceng, DipSEM, FFSEM</i>  Director of Medical Engineering Medical Engineering Research Unit, Cardiff University Tel: 029 2087 5907 Fax: 029 2087 5907 Email: Nokes@cardiff.ac.uk
<u>Principal Investigator:</u>	<b>Dr. Paul Lee</b> , <i>MB BCh, PgDip(SEM), MRCS, MFSEM</i>  PhD student Medical Engineering Research Unit, Cardiff University Tel: 077 6461 4688 Email: paul@medtaste.co.uk
<u>Principal site:</u>	Cardiff City Football Club (medical centre)  Responsible physician: Chief and Principal Investigator
<u>Collaborators:</u>	
Cardiff City football club	<b>Mr. Sean Connely</b>  Chief Physiotherapist Cardiff City football club
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**Sponsor**

Cardiff University is the research Sponsor for the study. For further information regarding Sponsorship related information please contact the Research and Commercial Division at:

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**Funder**

Cardiff City Football Club

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**List of Abbreviations**

BNF	British Nation Formula
BSE	Bovine Spongiform Encephalopathy
CI	Chief investigator
FA	Football association
IA	Intra arterial injection
IM	Intra muscular Injection
IV	Intra venous injection
IPO	Inositol phosphate oligosaccharides
PI	Principal investigator
NSAIDS	Non-steroidal anti inflammatory drug
TSE	Transmissible spongiform encephalopathies
WADA	World Anti-Doping Agency

### **Brief Summary**

Muscle injuries are one of the most common sport related injuries. In 2008, an article in the British Journal of Sports Medicine entitled "The early management of muscle strains in the elite athlete: Best practice in the world with a limited evidence" summarised that currently almost all our so-called knowledge has a basis of level 4 or level 5. The panel continued to highlight the importance of injection therapies and called for further studies into Actovegin®.

Actovegin® is manufactured by NYCOMED Austria. It is believed to improve the utilization of oxygen and to promote the uptake of nutrient media into the cell, hence promote wound healing. It is used as an intravenous infusion to treat acute stroke, postpartum haemorrhage and as a topical form to treat skin ulcers over 40 years in Europe. Its safety and efficiency has been proven by many published studies. We have confirmed with UKsport, World Anti Doping Agency and The Football Association (FA) that intramuscular injection of both of our study drugs are not prohibited for either in or out of competition use in the UK.

The aim of our study is to provide high quality and objective evidence for this combination of injection therapy in muscular injuries. Our hypothesis is that this combination of injection therapy can reduce the number of days required for players return to play after muscular injuries and also reduce the subjective perception of pain.

### **Lay summary**

Muscle injuries are the most common sport related injuries. Currently almost all the treatment methods were based on very poor scientific evidence. Injection therapy for treating muscle injuries was considered by the panel of experts in the Royal College of Surgeon Faculty of Sport and Exercise Medicine to be the current best practice. Actovegin® - a drug that has been used by clinicians across Europe, China and Russia for over 60 years, was recommend by this panel for injection therapy.

Nevertheless very little is known regarding the effects of Actovegin® on muscle injuries. Therefore, this study will compare the clinical outcome of two groups of elite football players who sustain muscle injury and are treated with Actovegin® or Placebo. Scientific laboratory based experiment with also be performed to supplement clinical findings.

### **Background**

Muscle injuries are one of the most common sport related injuries, their incidence varying from 30-55%(Noonan and Garrett, 1999, Verrall et al., 2001, Jarvinen et al., 2000). According to the World Health Organization musculoskeletal injuries are the most common cause of long term pain and physical disability, which affects hundreds of millions of people in the world(Woolf and Pfleger, 2003). In an audit published by The Football Association (FA) in 2004 it suggested that 12% of all injuries were hamstring injuries, it is 2.5 times more common than quadriceps injuries(Woods et al., 2004). In the 2 seasons from 1997 to 1999, 749 hamstring injuries were reported across the 91 British football clubs(Woods et al., 2004). It was accounted for loss of 90 training days and 15 matches per club per season(Woods et al., 2004).

Muscle injury is often assessed and diagnosed by clinical examination. Ultrasonography and Magnetic Resonance Imaging can be useful in confirming the diagnosis and aid the clinician in making treatment decisions. Muscle strains can be classified into three categories according to severity: Grade 1 (mild) a tear of few muscle fibres, minor swelling and discomfort with no or minimal loss of strength and restriction of movement. Grade 2 (moderate) a greater damage of muscle with clear loss of strength. Grade 3 (severe) a tear extending across the whole muscle belly, result in total loss of function.

#### Current management:

Rest, immobilization(Jarvinen et al., 2000), physical therapy(Cibulka et al., 1986) and non-steroidal anti-inflammatory drugs (NSAIDS)(Reynolds et al., 1995) have been the mainstay of therapy for grade 1 and 2 muscle injuries (Kasemkijwattana et al., 2000). For Grade 3 muscle injuries, surgical repair was essential (Jarvinen et al., 2000, Taimela et al., 1997).

Immobilization can lead to improved granulation of the injured muscle and promote healing, but it will cause significant atrophy of healthy myofibers and joint stiffness(Jarvinen et al., 2000). Although some studies have shown that the administration of NSAIDS promotes muscle healing by reducing degeneration and inflammation (Abramson and Weissmann, 1989, Cheung and Tidball, 2003) other research has demonstrated that NSAIDS are detrimental to the entire healing process (Obremsky et al., 1994, Mishra et al., 1995, Almekinders and Gilbert, 1986, Shen et al., 2005). Recently new treatment options such as growth factors injection therapy have shown good therapeutic results. However, its performance enhancing properties, growth factors, autologous blood and autologous conditioned serum was prohibited by World Anti-Doping Agency (WADA) under section M1 and S2 from the WADA prohibited list(WADA, accessed 03/08/09).

#### Proposed treatment:

The damage caused by the strain of the skeletal muscle is classified as shearing injury(Jarvinen et al., 2000). The mechanical force tears the entire myofiber, damages its plasma membrane and leaving the sarcoplasm open at the end of the stumps and initiated necrosis. It will extend along the whole



length of the ruptured myofiber which causes inflammation and further cell damage(Jarvinen et al., 2000). Actovegin® believed to improve the utilization of oxygen and to promote the uptake of nutrient media into the cell (Hoyer and Betz, 1989, Kanowski et al., 1995, Schoenwald et al., 1991), therefore was thought to improve wound healing and prevent further necrosis. Clinically, it is used as an intravenous infusion to treat acute stroke(Derev'yannykh et al., 2008, Boyarinov et al., 1998), postpartum haemorrhage(Appiah, 2002) and as a topical form to treat skin ulcers(Biland et al., 1985). Recently, it has also been used as intra-arterial infusion to treat long bone fractures(Khomutov et al., 1999) and radiation damage(Beetz et al., 1996).

Actovegin® is a deproteinised haemodialysate of ultra filtered calf serum of animals under 8 months of age, produced by Nycomed Austria GmbH. It is licensed for the treatment of disturbances in the cerebral circulation, peripheral blood disturbances (arterial angiopathy, ulcers), skin grafting and burns, wound-healing, and mucous membrane lesions. It is not in the British National Formulary (BNF), but has been used by clinicians across Europe, China and Russia for over 60 years. It contains physiological electrolytes, essential trace elements, amino acids, oligopeptides, inositolphospho-oligosaccharides (IPOs), carbohydrate and nucleosides with a molecular weight of no more than 6000 Daltons. Although, there have not been any randomised control trials in relation to its efficacy in the treatment of muscle injuries, it has been used by many high profile sports doctors across the UK and Europe in treating muscle tears. In 2006, an article from the Times newspaper reported the comment from Dr. Hans-Wilhelm Müller-Wohlfahrt, team doctor for the German national football team and Bayern Munich Football Club regarding the use of Actovegin®. In this report Dr. Müller-Wohlfahrt discussed his experience with Actovegin® and the success of its treatment with high profile sports people such as Maurice Green, Asafa Powell, Diego Maradona, Darren Gough and Paula Radcliffe(Crompton, 2006). "I am an empirical doctor and, over 30 years, I have treated so many that nobody can tell me it doesn't work. Nobody I have seen has had an adverse effect, or an allergic or other reaction."

### **Doping Control**

Intramuscular Actovegin® is not prohibited for either in or out of competition use according to UK sport and WADA(WADA, accessed 03/08/09). We have also received written confirmation from the FA which states that Intramuscular injection of Actovegin® are not prohibited for either in or out of competition use. According to the latest information by UK Sport, Actovegin® is listed as a legitimate drug that can be used both in, as well as out-of-competition.

**Safety**

There are many clinical studies which have demonstrated its safety and efficacy (Derev'yannykh et al., 2008, Boyarinov et al., 1998, Appiah, 2002, Biland et al., 1985, Khomutov et al., 1999, Beetz et al., 1996). A recent randomized double-blind multicentre study with 562 patients further reinforced the safety profile with Actovegin®(Ziegler et al., 2009).

The manufacturer Nycomed Austria GmbH confirms that all measures are in place to guarantee the Transmissible Spongiform Encephalopathies (TSE) safety of Actovegin®(Nycomed). According to the latest guidelines from the European Medicines Agency (EMEA/410/01 final issued in February 2001) and the Final Opinion of the Scientific Steering Committee on the geographical Bovine Spongiform Encephalopathy (BSE) risk (issued in July 2002) the safety of a medicinal product is determined by 4 important factors:

1. Animals as source of material: the most satisfactory source of materials is from countries which are free of BSE and have appropriate surveillance systems. Materials may be used from countries with a low BSE incidence.
2. Parts of animal bodies and body fluids used as starting materials: tissues and body fluids are categorised in four categories (from category I = high infectivity like brain to category IV= safest category, no detectable infectivity like blood and milk).
3. Age of animals: the sourcing from young animals is seen as very important safety factor.
4. A production process should be designed which is thought to remove or inactivate TSE agents.

Austria is officially categorised as a BSE and Scrapie free country by the World Organization for Animal Health and the Scientific Steering Committee of the European Union. The calf blood used as raw material for Actovegin® derives from calves born, raised and slaughtered in Austria. Blood is in the safest tissue category (IV). The calves were never fed animal carcass fodder and are declared fit for human consumption, which has been proven by veterinary certificates(Nycomed). Moreover the traceability of every Actovegin® batch back to the individual calves as blood donors is ensured. The mother cows of the calves can also be traced. The manufacturing process of Actovegin® is BSE validated, thus proven to be capable of removing hypothetically present TSE agents(Nycomed).

### **Trial Objectives and Purpose**

In professional elite-level athletes, Orchard et al (2008) summarised that currently almost all our so-called knowledge in the treatment of muscle injuries was based on very poor scientific evidence (Orchard et al., 2008). Dr. Mueller-Wohlfaht's Actovegin® injection treatment regime for treating muscle injuries was considered by the panel of experts to be the current best practice (Orchard et al., 2008). In the season of 2008-2009, we employed Dr. Mueller-Wohlfaht's treatment regime to treat grade 1 and 2 hamstring injuries in professional football players in Cardiff. All 8 players reported they "felt better" after the initial injection in terms of pain and mobility. According to the team physiotherapist, the injured players reached rehabilitation key stages earlier than expected and, in some cases, players returned to play 10 days earlier than expected with the same type of muscle injuries.

In terms of the professional elite athlete, shortened recovery time could mean continuing training, increased game play and benefit to the team and club. As our preliminary experience suggests that Actovegin® is safe in patients and may even be associated with improved outcomes. This evidence requires validation through a prospective randomised trial. Therefore, a PhD study under the supervision of Professor Len Nokes, titled "A pilot study into intramuscular injection therapy of Actovegin® in elite football players with muscular injury" has been approved by Cardiff University and supported by Cardiff City Football Club.

### **Aims**

Evidence obtained so far with regard to the efficacy of Actovegin® in muscle injuries remains largely anecdotal. Despite the lack of scientific understanding of the biological action of Actovegin®, it is predicted that the use of Actovegin® in athletes will become more widespread in the future. Pharmacodynamic studies suggested that Actovegin® can increase the respiration capacity of cells, improve oxygen and glucose utilisation, optimisation of mitochondria and exhibit protective effect on hypoxic cells (Obermaier-Kusser et al., 1989, Kuninaka et al., 1991).

The aims of this proposed is to extend the preliminary clinical study on the efficacy of Actovegin® treatment in muscle injuries

**Trial Design****Hypothesis for Clinical Trial**

Intra muscular Actovegin® injections can reduce number of days for the player to return to training with the first team following from grade I or II muscle injuries.

**Primary outcome**

Number of days from injury for the player to return to training with the first team

**Secondary outcome:**

Player's perception of pain and effectiveness of treatment

Player's isokinetic and isometric data

**Selection and withdrawal of Subjects****Inclusion Criteria:**

Informed consent  
> 18 years  
Clinical and MRI diagnosis of muscle injuries  
Grade 1 or 2 muscle tear  
No previous allergic reaction to Actovegin®

**Exclusion Criteria:**

Grade 3 or more muscle injuries  
Fractures  
Other soft tissue injuries besides muscle tears

**Withdrawal Criteria:**

Acute or delayed hypersensitivity reaction  
Infection  
Request of the player  
Discretion of the investigator or club management

**Study design:**

A team of elite football players (n=21)

Pre-season physical and isokinetic testing

The study will be explained to all players by the CI and PI.

All players will receive study information sheets

Informed consent will be taken with the consent form

Follow this group of player for the season as team physician

Clinical examination and MRI to confirm diagnosis of grade 1 and 2 muscle tear.

Players will be divided into 2 groups:

- 1) Actovegin® 3ml
- 2) Physio only

Injection sites will be cleaned with alcohol wipes and 1 orange needles will be used for the injections. 2ml injected directly to the injury site,

- 1<sup>st</sup> injection takes place at diagnosis
- 2<sup>nd</sup> injection 1 day after 1<sup>st</sup> injection
- 3<sup>rd</sup> injection 2 days after 1<sup>st</sup> injection

Both groups (control and treatment) will undergo same physiotherapy protocol provided by the club.

The club will monitor players' recovery progress; complete recovery will be deemed as the player's return to training with the first team.

Day 0	Day 1	Day 2		Primary outcome	
1 <sup>st</sup> Injection	2 <sup>nd</sup> Injection	3 <sup>rd</sup> Injection		Isokinetic testing	Return to play
Injury confirmed by MRI				Deemed fit to return	

1 <sup>st</sup> Injection	2 <sup>nd</sup> Injection	3 <sup>rd</sup> Injection	Isokinetic testing	Return to play
---------------------------	---------------------------	---------------------------	--------------------	----------------

**Adverse events**

Any adverse event during the study, whether or not related to the test medication will be assessed by the principal investigator and documented with the adverse events report form. (Appendix C). They will be reviewed within 24hours by the Chief and Principal investigators.

GMC registered doctors who are trained in life support and are able to deal with anaphylactic reactions will perform the injection therapies. Oxygen and adrenaline will be available at all times.

**Serious adverse events**

1. Death
2. Anaphylactic reactions

Although it is very unlikely that serious adverse events will occur, these will be recorded on the adverse event forms (Appendix C) and will be reviewed within 24hours by the Chief and Principal investigators. Notification will also be given to the MHRA, Cardiff University R&D office and NRES.

**Statistics**

As this is a pilot study, approximately 18 players will be enrolled to the study over the study period. The study will have 80% power at a 5% significance level. Through the power calculation, this study can detect a standard difference of 1.5 standard deviations.

**Data collection:**

All data will be entered using a password protected excel spreadsheet (Data A). Personal information will be stored as a separately protected excel spreadsheet (Data B). A unique identification number will be used to link between the databases. Only the CI and PI will have the password to access these spreadsheets.

All participants' personal data and results will be coded and anonymised, and stored in a password protected database. Any announcement or publication of results and publicity concerning the study will require approval of all investigators. Such publications will be in the names of all the investigators. All publications, posters, oral presentations at scientific meetings, seminars, and any other forum will only contain the group mean data, no identifiable data will be included.

**Quality assurance:**

PI or CI will perform data entry. A hard copy will be printed (Data A only), countersigned and dated by PI and CI each time data is entered into the database. This paper copy will be filed into a locked cabinet and reviewed by the monitoring committee at monthly meetings.



**Regulatory Approval**

Ethics/regulatory approval

Ethic approval was granted via Cardiff University, Cardiff School of Engineering Research Committee. 2<sup>nd</sup> July 2009.

NHS ethic approval is currently in process

MHRA regulatory approval is in progress

ISRCTN registration number:

EudraCT number: 2009-014603-31

**Indemnity**

Cardiff University holds 'Clinical Trial' insurance which applies to this trial. Note that insurance cover is not automatic and will need to be agreed on a trial-by-trial basis. The Clinical Trial insurance provides negligent harm cover for the design and management of the trial. Cover for clinical negligence will be provided by PI and CI personal medical insurance indemnity.

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# **Appendix G**

## **Consent forms for the clinical study**

**Reference to page 148**

### CONSENT FORM

**A pilot study of intramuscular injection therapy with Actovegin on elite football players with muscular injuries.**

PLEASE INITIAL NEXT TO EACH NUMBER

1. I confirm that I have read and understand the information sheet dated..... for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Name of Person taking consent	Date	Signature

**1 copy for participant; 1 copy for researcher file**

_____	_____	_____
Name of Person	Date	Signature

# **Appendix H**

## **MRI reports**

**Reference to page 167**

[REDACTED]

## IMAGING REPORT

KL/MB



31737 Hospital  
[REDACTED]  
[REDACTED]  
[REDACTED]  
[REDACTED]  
[REDACTED]  
[REDACTED]  
[REDACTED]

Dear Dr N [REDACTED]

Re: M [REDACTED]  
[REDACTED]

Thank you for referring this patient

### [REDACTED] MRI RIGHT HAMSTRING

Examination No: 15 [REDACTED]

A skin marker was placed at the site of symptoms for the patient and lies over the proximal hamstring. The scan confirms a Grade 1 musculotendinous tear of the long head of biceps near its origin on the conjoint tendon. This is at the site of the patients symptoms. In addition, there is an older tear of the vastus lateralis muscle at its origin. This a small Grade 2 tear.

Yours sincerely

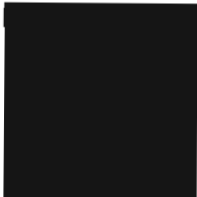
**DR K L [REDACTED]**  
Consultant Radiologist

Disc with patient and report to Dr L [REDACTED]



**IMAGING REPORT**

KL/KP



Spire  
Cardiff Hospital

Crosscaddan Road  
Pontwyn  
Cardiff  
CF23 3XJ

Tel: 029 2073 5515  
Fax: 029 2073 5821

[www.spirehealthcare.com/cardiff](http://www.spirehealthcare.com/cardiff)

Dear Dr N [REDACTED]

Re: M [REDACTED]  
[REDACTED]

Thank you for referring this patient.

**[REDACTED] MRI LEFT THIGH**

Examination No: 20 [REDACTED]

A skin marker was placed at the site of the patient's symptoms and this was very close to the injection site. The scan confirms what is predominantly a Grade I tear of rectus and I find it difficult to completely exclude a small Grade II tear.

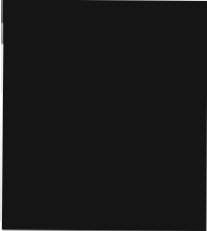
Yours sincerely

**DR K L [REDACTED]**  
Consultant Radiologist

CD with patient, report to L [REDACTED]

## IMAGING REPORT

KL/KS



Cardiac MRI - 05/01/18

Clonkeadain Road  
Trenton  
Pa 17139  
USA

Tel: 610 500 5515  
Fax: 610 500 5521

[www.heartscan.com/cardiac](http://www.heartscan.com/cardiac)

Dear Dr N [REDACTED]

Re: K [REDACTED]

Thank you for referring this patient.

MRI SCAN RIGHT THIGH: [REDACTED]

Examination No: 19 [REDACTED]

A skin marker was placed over the area of concern to the patient. This lies at the distal edge of a grade I musculotendinous tear of the longhead of biceps femoris about 5cm distal to the ischial tuberosity. The examination is otherwise unremarkable.

Yours sincerely

Dr K L [REDACTED]  
Consultant Radiologist

Disc with patient ~ report to Dr N [REDACTED]

**IMAGING REPORT**

KL/CM



**Spire**  
Cardiff Hospital

Croescadarn Road  
Pentwyn  
Cardiff  
CF23 8XL

Tel 029 2073 5515  
Fax 029 2073 5821

[www.spirehealthcare.com/cardiff](http://www.spirehealthcare.com/cardiff)

Dear Dr N [REDACTED]

Re: Mr P [REDACTED]  
Cardiff City Fc Ninian Park Road Cardiff O0 000

Thank you for referring this patient

**MRI LEFT THIGH** [REDACTED]

Examination No 19 [REDACTED]

The scan confirms a grade 2 musculotendinous tear of the long head of biceps femoris about 9cms below the ischial tuberosity. The skin marker that he has put on is quite accurate in demonstrating the level of the injury.

Yours sincerely

DR K L [REDACTED]  
Consultant Radiologist

Disc with patient. report to Dr L [REDACTED]

# Published paper

Our Experience on Actovegin, is it Cutting Edge?



Actovegin – Cutting edge Sports Medicine or “Voodoo” Remedy?



# Conference presentation

UK SEM conference 2010



# Conference presentation

# Our Experience on Actovegin, is it Cutting Edge?

## Authors

P. Lee<sup>1</sup>, A. Rattenberry<sup>1</sup>, S. Connelly<sup>1</sup>, L. Nokes<sup>1</sup>

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## Key words

- Actovegin
- muscle injuries
- doping

## Abstract

Muscle injuries are one of the most common sport related injuries, their incidence varying from 30–55% in all sports injuries. They account for the loss of 90 training days and 15 matches per club per season in elite football (soccer). In recent years, the use of Actovegin<sup>®</sup> in sports medicine has caused a lot of controversy in many sports disciplines. Although it is unlikely for this deproteinised substance to have oxygen-enhancing capacity, there is an anecdotal belief that Actovegin<sup>®</sup> can increase an athlete's performance. Actovegin<sup>®</sup> is produced by Nycomed

Austria GmbH and has been used by doctors across Europe, China and Russia for over 60 years. Nevertheless, very little is known regarding the effects of Actovegin on muscle injuries. This article reviews the current evidence on Actovegin<sup>®</sup>, its legal status with sports governing bodies and its potential role in sport injuries. We will also report our experience with this drug in treating muscle injuries. In this pilot study, players in the Actovegin treatment group were able to return to play 8 days earlier (95% CI -1.249 to -14.7510) compared to physiotherapy alone ( $p=0.033$ ). No adverse reactions were recorded in any of the participants.

## Clinical Relevance

There are numerous anecdotal beliefs and there is much media attention regarding Actovegin injection therapy, but the published scientific evidence and clinical trials are largely ignored. This short review will "reintroduce" the scientific evidence around this contentious topic and will aid sports clinicians to examine evidence with an open mind. From our experience with this pilot study, Actovegin injection therapy seems safe and well tolerated. Further objective evidence is needed before any meaningful conclusions can be drawn for this new treatment of muscle injuries.

accounted for the loss of 90 training days and 15 matches per club per season [29]. A recent prospective cohort study published with the Union of European Football Associations (UEFA) confirmed that hamstring injuries were the single most common injuries in professional football players, representing 17% of overall injuries [11]. Similar observations regarding hamstring injuries were reported by Bennell et al. [5] in Australian Rules footballers, with hamstring injuries accounting for 86.4 injuries per 10000 playing hours.

Hamstring injuries are often diagnosed and managed by the team clinician. Ultrasonography and Magnetic Resonance Imaging (MRI) can be useful in confirming the diagnosis and aiding the clinician to make decisions with regards to treatment. Rest, immobilization [13], physical therapy and sometimes non-steroidal anti-inflammatory drugs (NSAIDs) [23] have been the mainstay of therapy for grade 1 and 2 muscle injuries [14]. Immobilization can lead to improved granulation of the injured muscle and promote healing, but it will cause significant atrophy of healthy myofibers and joint stiffness [13]. Although some studies have shown that the administration of NSAIDs promotes muscle healing by reducing degenera-

## Introduction

Muscle injuries are one of the most common sports related injuries, their incidence varying from 30–55% [13,27]. An audit published by The Football Association (FA) in 2004 suggested that 12% of all injuries were hamstring injuries. They are 2.5 times more common than quadriceps injuries [3,29]. In the 2 seasons from 1997 to 1999, 749 hamstring injuries were reported across the 91 British football clubs [29]. They

ian Rules footballers, with hamstring injuries accounting for 86.4 injuries per 10000 playing hours.

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November 16, 2010

## Bibliography

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open mind. From our experience with this pilot study, Actovegin injection therapy seems safe and well tolerated. Further objective evidence is needed

tion and inflammation [1], other research has demonstrated that NSAIDs are detrimental to the entire healing process [19,25]. Recently, new treatment options such as growth factor injection therapy have shown good therapeutic results. However, due to their performance enhancing and anabolic properties, growth factors are prohibited by the World Anti-Doping Agency (WADA) under section M1 and S2 of the WADA prohibited list [28]. In 2008, a best practice statement summarised that currently almost all our knowledge in treating muscle injuries has a basis of level 4 or level 5 [20]. This panel of experts also concluded that Actovegin injection therapy could be important in the treatment of muscle injuries [20]. Although its intramuscular use is not prohibited by the regulatory authority, World Anti-Doping Agency (WADA) [28], athletes and coaches have reservations about its use. In this paper, we have reviewed the evidence for Actovegin and report our experience with this drug in treating muscle injuries in our pilot study.

## Background

Actovegin® is a deproteinised haemodialysate produced by Nycomed Austria GmbH. Clinically, it is used as an intravenous infusion to treat acute stroke [7,10], postpartum haemorrhage [2] and as a topical form to treat skin ulcers [6]. Recently, it has also been reported to be used as an intra-arterial infusion to treat long bone fractures [15] and radiation damage [4]. Clinicians across Europe, China and Russia have used Actovegin for over 60 years [4,18,22,30]. It contains physiological components, electrolytes and essential trace elements, 30% of organic components are amino acids, nucleosides, intermediary products of carbohydrates and fat metabolites [18]. It does not contain growth factors or hormone like substances, as it is ultra filtered to 6000 Daltons. It can be administered as tablets, topical formulations, injections or infusions via intramuscular, intravenous or intra-arterial routes [18].

Many studies have tried to identify the active ingredients in this mixture, but have been unsuccessful. Schoenwald et al. [24] suggested that the active fractions in Actovegin were strongly negatively charged and were thought to be phosphorylated and/or sulfated oligosaccharides of approximately 3000 Daltons in molecular weight and different to the IPO fraction reported by other studies. This fraction is also resistant to proteinase K digestion, which suggests that it is unlikely to contain peptides [24]. Actovegin also showed a synergistic effect on cell proliferation with growth factors such as Epidermal Growth Factor (EGF), Basic Fibroblast Growth Factor (bFGF), and Endothelial Cell Growth Factor (ECGF), causing an increase in cell numbers, increase activity of acid phosphatase and an improved level of thymidine incorporation compared to Actovegin alone [24]. Therefore, the active compounds in Actovegin are unlikely to be these growth factors or their derived fragments [24]. The trivial nutritive effects of Actovegin were excluded as a mixture of the same level of amino acids and substrates did not stimulate proliferation or have insulin like activity in vivo [24].

## Clinical Evidence

Although the active ingredients within Actovegin are yet to be identified, there are many clinical studies to confirm its safety and effectiveness. There has been one case report of a possible

anaphylactic reaction related to the use of intravenous Actovegin injection by an amateur sportsman. In this report, the so-called "anaphylactic reaction" was not confirmed with any biochemistry and the patient improved with antibiotics. The author later stated in the communication letter that this patient had taken Actovegin once before with no adverse reaction. Therefore, the most likely cause for this acute shock was due to bacterial contamination during injection not anaphylactic reaction to the drug [16]. A double-blind placebo controlled single centre study with recreational athletes demonstrated that ultrasound guided para-tendon injection of Actovegin was effective in the treatment of Achilles tendinitis [22]. The tendon cross section measurement was reduced significantly ( $p < 0.0001$ ), patients' physical activity and perception to pain was also improved ( $p < 0.002$ ) in the treatment group [22]. The overall patient satisfactory score was also significantly better in the Actovegin group ( $p < 0.0001$ ) and no adverse event was reported in this study [22]. Although it is a relatively small-scale study with limited power, it is a well-conducted study that was featured in a Cochrane review. Ziegler et al. [31] reported a double-blind multicentre randomized control study with 567 patients treated with daily high dose intravenous Actovegin infusion for symptomatic diabetic polyneuropathy. No anaphylactic reactions were reported in this study after 5620 infusions of the maximum dose of Actovegin. The adverse effect profile was no different compared to placebo [31].

## Legal Status

Besides its clinical properties, there are anecdotal beliefs amongst athletes that Actovegin possesses an oxygen carrying capacity and has the potential to enhance oxygen uptake, which leads to better performance. Although these claims are not based on any objective scientific evidence, the IOC announced in December 2000 that Actovegin was banned under the classification of blood-doping agents. 2 months later, however, the IOC lifted the ban as there was no evidence that Actovegin actually enhances performance [26]. In a recent study with 567 diabetic patients, no improvement of muscle strength or condition was found after treatment with the maximum recommended dose of Actovegin for 160 days [31]. Currently, intramuscular use of Actovegin is not prohibited in or out of competition according to the latest search of the Global Drug Reference Online (Global DRO), which is approved by UK Anti-Doping (UKAD), the Canadian Centre for Ethics in Sport (CCES), the United States Anti-Doping Agency (USADA) and WADA [9].

## Evidence on Actovegin in Muscle Injuries

The treatment of muscle tears with intramuscular Actovegin was first published by Pfister and Koller in 1990 [21]. Their partially blinded case control study with 103 patients, showed a reduction in recovery time with the treatment group of 5.5 weeks compared with 8.3 weeks for the control group [21]. However, in this study, the diagnosis of specific muscle injuries was only based on clinical finding and was not graded according to MRI. Patients were recruited from various sports and levels and the treatment regimen and rehabilitation protocol were not standardised. Actovegin was mixed with local anaesthetics before injection, therefore its pharmacodynamics and pharma-



cokinetics were altered. The final outcomes were based on patients and various clinicians' subjective observations and there was no pre injury data to compare outcomes. Despite these limitations, it is the first published study regarding the use of Actovegin as an intra-muscular injection and no adverse events were reported in this paper. Wright-Carpenter et al. [30] compared Autologous Conditioned Serum (ACS) to Actovegin in a small non-randomised study. The Actovegin group was created by the retrospective analysis of the Pfister and Koller study, therefore should not be seen as new evidence. Ziegler et al. [31] reported a double-blind multicentre randomized control study with 567 patients treated with 20 daily infusions followed by 140 days of oral Actovegin. Although muscle assessment was not the primary objective in this study, it suggests that Actovegin does not have anabolic or ergogenic activity in terms of muscle development. It does not improve muscle strength ( $p=0.731$ ) or muscle reflex ( $p=0.571$ ) [31]. Furthermore, there was no significant difference in the adverse event rate compared to placebo [31].

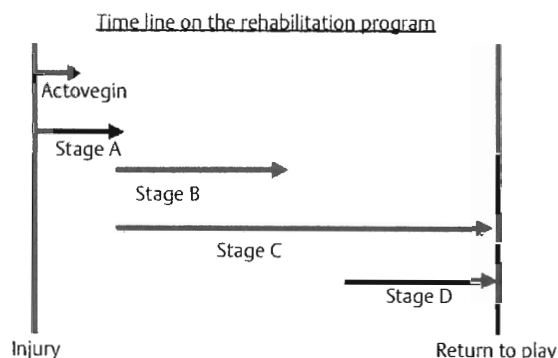
In 2006, an article from the Times newspaper reported a comment from Dr. Hans-Wilhelm Müller-Wohlfahrt, team doctor for the German national football team and Bayern Munich Football Club regarding the use of Actovegin®. In this report Dr. Müller-Wohlfahrt discussed his experience with Actovegin® and the success of its treatment with high profile sports people such as Maurice Green, Asafa Powell, Diego Maradona, Darren Gough and Paula Radcliffe [8] "I am an empirical doctor and, over 30 years, I have treated so many that nobody can tell me it doesn't work. Nobody I have seen has had an adverse effect, or an allergic or other reaction." [8]. Although it is not a published clinical study, his 30 years specialist experience with muscle injuries in elite athlete should not be overlooked [20]. There is no study that reports the use of intramuscular injection of Actovegin as a single therapy to treat muscle injuries. Therefore, we will report our experience with this drug in this pilot study.

## Method

Prospective data was collected during the 2008–2009 season from a professional football team competing in the UK Championship League for players who sustained grade 1 or 2 hamstring injuries according to the grading system described by Jarvinen et al. [13].

After initial assessment and diagnosis by the team physician or physiotherapist, all injured players underwent MRI to confirm the diagnosis. Actovegin injection therapy was initiated immediately once muscle fiber tears were defined as moderate strains, characterized by a stretch injury with detection of bleeding on MRI scanning (grade I or II tears) [13]. Players with normal MRI findings or grade III tears were excluded in this report. Players who refused Actovegin treatment were allocated to the control group. All players in the Actovegin treatment group followed the same injection protocol (◊ Fig. 1) of 3 intramuscular injection therapies and the same hamstring specific rehabilitation protocol. There was no blinding or randomization in this study. 2 ml of Actovegin were injected into the injury site under direct palpation. The injection process was repeated after 24h (◊ Fig. 1). Players who opted-out of the injection therapy followed the same rehabilitation protocol. All players were discouraged from taking any anti-inflammatory (NSAIDs) or oral supplements during the rehab period. Players were able to return to training

Day 0	Day 1	Day 2	Rehab	Deemed fit to return by the team physio	Return to play
1 <sup>st</sup> Injections	2 <sup>nd</sup> Injections	3 <sup>rd</sup> Injections			
Injury confirmed by MRI					
Stage A			B,C,D		



**Fig. 1** Injection and rehabilitation regimen.

Rehabilitation regimen:

Assessment for lumbar/sacral and pelvic dysfunction carried out on daily basis during rehabilitation. Any dysfunction is treated accordingly.

Stage A: RICE, gentle movement (3 days)

Low- to moderate-intensity sidestepping, grapevine stepping, single-leg stand progressing from eyes open to eyes closed, prone abdominal body bridge

Stage B: Mobilize hamstring to full range, core stability exercise (2–7 days)

Moderate- to high-intensity sidestepping, grapevine stepping, single-leg stand windmill touches, push-up stabilization with trunk rotation, fast feet in place, proprioceptive neuromuscular facilitation trunk pull-downs with Thera-Band, symptom-free practice without high-speed maneuvers

Stage C: Strengthening exercise, core stability exercise, proprioceptive, CV (3–14 days)

High-intensity sidestepping, grapevine stepping, single-leg stand windmill touches, swiss ball stabilization with trunk rotation, proprioceptive neuromuscular facilitation trunk pull-downs with Thera-Band, running with ball

Stage D: Sudden movement, stop start, cutting (1–4 days)

Swiss ball stabilization with trunk rotation, nordic hamstring curl, cutting exercise with ball, sudden stop and start running with ball.

with the first team once they had completed the rehabilitation program and passed the assessment by the team physiotherapist as per stage D in the rehabilitation program (◊ Fig. 1).

Statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago). Actovegin treatment group was compared to Control group using Student's 2 two-tail unpaired t-test,  $p$ -value  $<0.05$  was considered to be significant. Our study has been performed in accordance with the ethical standards of the IJSM [12]. It has been ethically reviewed and approved by the Cardiff University.

## Results

There were 11 MRI confirmed hamstring injuries in the club during the 2008 and 2009 seasons. The mean age of injured players was 23 and all injuries occurred at the musculotendinous junction of biceps femoris. There were 3 grade II injuries and 8 grade I injuries. 4 players had previous hamstring injuries but had been symptom free for at least 6 months. All players were able to progress through the rehabilitation program

**Table 1** Results for Actovegin and control group.

Season	Age	Treatment	MRI findings	Day loss	Means
2008	26	Actovegin	grade II tear	22	18.7
2009	24	Actovegin	grade II tear	21	S.D. 4.93
2009	18	Actovegin	grade II tear	13	S.E.M. 2.85
2008	22	Actovegin	grade I tear	10	12*
2009	25	Actovegin	grade I tear	9	S.D. 2.94
2009	19	Actovegin	grade I tear	14	S.E.M. 1.47
2009	26	Actovegin	grade I tear	15	
2008	19	control	grade I tear	26	20*
2008	28	control	grade I tear	16	S.D. 4.54
2008	22	control	grade I tear	21	S.E.M 2.27
2008	24	control	grade I tear	17	

\*  $p < 0.05$  between groups, S.D. = Standard Deviation, S.E.M. = Standard Error Mean

pain free. No recurrent muscle strains or injuries were recorded during rehabilitation. No adverse reactions were reported with Actovegin injections and all players in the Actovegin group received 3 doses of Actovegin as stated in the treatment protocol.

7 players had Actovegin treatment and 4 players opted-out of the injection therapy. In the Actovegin group 3 players had grade II injuries and 4 players had grade I injuries. All 4 players in the control group had grade I injuries. The mean number of days lost in the control group was 20 with a range from 16 to 26 days; in the Actovegin group the mean number of days lost with grade I injuries was 12 with a range from 9 to 15 days (Table 1). There was a significant statistical difference when the Actovegin group was compared to the control group ( $p = 0.033$ ). In grade I hamstring injuries, players in the Actovegin treatment group were able to return to play 8 days earlier (95% CI -1.249 to -14.7510) compared to physiotherapy alone. For Grade II injuries, the mean number of days lost in the Actovegin group was 18.67 with a range from 13 to 26 days. Statistical analysis cannot be performed as there were no subjects with grade II injuries in the control group.

## Discussion

It is evident that hamstring injuries are very common in sports with high demands on speed and power such as football (soccer). The incidence during our study was comparable to recent literature [11,29]. The Actovegin injection therapy regimen described in this paper for grade I hamstring injuries appears to significantly reduce the number of days for return to play, with a mean of 8 days reduction compared with rehabilitation therapy alone ( $p < 0.05$ ). It is difficult to perform full economic costing for elite football players, as it is impossible to attach a value for a player to be able to play the next game.

In vivo and in vitro studies suggest that Actovegin contains some active components, although they are yet to be identified. It has been used by clinicians across Europe, China and Russia for over 60 years to treat stroke and diabetic neuropathies [6,15,18,22,30]. There is limited evidence on its role in the treatment of muscle injuries and no evidence regarding any performance enhancing properties. There has only been one published clinical study to investigate its role in muscle injuries when mixed with local anaesthetics [21]. Although an unpublished case series with Dr. Hans-Wilhelm Müller-Wohlfahrt's injection regimen seems to have good results, it has also been mixed with Traumeel and local anaesthetics [8]. There have been no clinical

studies investigating the effect of stand-alone Actovegin therapy in muscle injuries. From our review, Actovegin is a drug that has 60 years of track record in clinical use and it has a well-established safety profile. The only anaphylactic case report published could be discounted, as the cause is most likely to be bacterial contamination of injection site. There is no evidence of an ergogenic effect with Actovegin. Many official governing bodies including WADA, UKAD, CCES and USADA do not prohibit its use intramuscularly. On the other hand, it is not on the British National Formulary (BNF) and Medicines and Healthcare products Regulatory Agency (MHRA) in the UK and Food and Drug Administration (FDA) in the USA have not approved its use.

In professional elite-level athletes, Orchard et al. [20] summarised that currently almost all our so-called knowledge in the treatment of muscle injuries was based on very poor scientific evidence. The career lifespan for the professional elite athlete is often short lived, shortened recovery time could mean continuing with training, increased game play and benefit to the team and club.

Due to the unique relationship between sports physicians and athletes, they are often under pressure to seek the latest "active" or "cutting edge" treatments [17]. Athletes are often not interested in being part of a Clinical Trial. Therefore, it is not always possible to get a large number of participants who are professional athletes. There is also much publicity about the use of this drug based on anecdotal assumptions on its questionable potential ergogenic properties [17]. Actovegin is not licensed to treat muscle or soft tissue injuries and its evidence is limited. Nevertheless, there is published evidence demonstrating its efficacy and safety [2,6,7,15,22,31].

## Limitations

This is a non-blinded and non-randomised observational pilot study with subjective assessments for return to play. The potential psychological effect of injection therapy and placebo effect cannot be ruled out in this study; ideally a placebo injection should be used alongside Actovegin. Due to the competitions of the sport, it is not possible to standardise the rehabilitation regimen between clubs, therefore only a single football club was used, hence the patient numbers in each group were small. The power of this study is 84%.

In our study, all the injections were performed by one of the authors (LN). Injection sites were marked with permanent marker on the first injection, under direct palpation of the maximum tenderness point of the muscle. Ultrasound guidance was not used as our clinical marking corresponded to the location of injury on the MRI.

## Conclusion

This article summarises the current evidence on Actovegin, there is no evidence that Actovegin can enhance athletes' performance. Although our report is based on a non-randomised observational pilot study with subjective assessments for return to play, the impact of Actovegin injection therapy must not be overlooked. Compared with conventional conservative RICE and NSAID therapy, Actovegin proposes an exciting and legal alternative for high performance athletes. From our experience, Actovegin injection therapy seems safe and well tolerated. More



objective evidence is needed before any meaningful conclusions can be drawn for this new treatment of muscle injuries. Despite our positive experience with Actovegin, we do not advocate its use until further high quality evidence can be obtained. Medicine is a form of science as well as an art. Physicians should process the evidence (in whichever form that it may come) with an open mind. Premature conclusions should not be drawn based on limited evidence. Further research must be encouraged to investigate the effects of Actovegin on muscle injuries. Injection therapy could potentially revolutionise the treatment of muscle injuries, it should not be regarded as "Snake oil".

### Acknowledgements

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# Actovegin<sup>®</sup> — Cutting-edge Sports Medicine or “Voodoo” Remedy?

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## Abstract

Actovegin<sup>®</sup> is a deproteinized serum extract of bovine origin, and in recent years it has been used widely in treating sport injuries with many anecdotal reports of success. However, the use of Actovegin<sup>®</sup> in sport medicine has caused a substantial amount of controversy, especially concerning its supposed oxygen-enhancing capacity and an anecdotal belief that its use can increase an athlete's performance. In 2009, a sports physician was arrested with this “performance-enhancing drug,” while an editorial in a sports medicine journal strongly questioned the evidence base for using this drug for acute muscle injury. There is also a report that suggested that Actovegin<sup>®</sup> might have induced anaphylactic shock in a cyclist. In this review, we have systematically examined the current evidence on Actovegin<sup>®</sup>. Its mechanism of action, clinical evidence, legal status with sports governing bodies, and its potential role in sport injuries will be discussed.

## Introduction

Actovegin<sup>®</sup> has received a great deal of media attention in the recent years, especially surrounding its use in sports medicine. In 2009, a sports physician was arrested with this performance-enhancing drug, whereas an editorial in a sports medicine journal strongly questioned the evidence base for using this drug for acute muscle injury (12). There is also a report that suggested that Actovegin<sup>®</sup> might have induced anaphylactic shock in a cyclist (20). However, there also are good safety results from a large, multicenter, randomized, control trial (36). Nevertheless, Actovegin<sup>®</sup> has received much publicity, and there also are many anecdotal beliefs surrounding this drug (12). Currently, Actovegin<sup>®</sup> is not on the World Anti-Doping Agency's (WADA) prohibited substance list (34). However, many athletes, coaches,

and doctors still have reservations about its use in sports medicine. In this article we review the evidence of the efficacy of Actovegin<sup>®</sup> as a drug and discuss its potential role in sports medicine.

## Methods

A comprehensive literature search was performed on MEDLINE (1950 to 2010), PubMed, Embase (1980 to 2010), the Allied and Complementary Medicine Database (1985 to 2010), ScienceDirect, Scopus, BIOSIS, the Cochrane Library, and Google. The title, abstract, and key words were searched using the terms “Actovegin<sup>®</sup>,” “injection therapy,” and “muscle injuries.” No language restrictions were imposed; original articles were obtained if possible and translated to English. Only relevant articles were included in this review after discussion between the authors.

## Background

Actovegin<sup>®</sup> is a deproteinized hemodialysate of ultrafiltered calf serum from animals under 8 months of age, produced by Nycomed GmbH, Linz, Austria. Austria is officially categorized as a bovine spongiform encephalopathy-, transmissible spongiform encephalopathy-, and scrapie-free country by the World Organization for Animal Health and the Scientific Steering Committee of the European Union. The manufacturing process of Actovegin<sup>®</sup> is bovine spongiform encephalopathy validated, thus proven to be capable of removing hypothetically present transmissible spongiform encephalopathy agents (23). It is ultrafiltered to 6,000 Da; therefore, it does not contain protein, growth factors, or hormonelike substances. Actovegin<sup>®</sup> contains physiological components, electrolytes, and essential trace elements. Amino acids, nucleosides, and intermediary products of carbohydrate and fat metabolites constitute approximately 30% of organic components in Actovegin<sup>®</sup> (23). The active ingredients in this mixture have yet to be identified. Actovegin<sup>®</sup> can be administered as tablets, topical formulations, injections,

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or infusion via intramuscular, intravenous, or intra-arterial routes (23).

Actovegin® is a licensed drug in Europe, China, and Russia and has been used by clinicians for more than 60 yr (3,23,28,35). Clinically, it has been used as an intravenous infusion to treat acute stroke (5,11) and as a topical form to treat skin and mouth ulcers (4). It also has been reported to be used as an intra-arterial infusion to treat long-bone fractures (17) and postpartum hemorrhage (2). Solcoseryl (Valeant Pharmaceuticals International, Moscow) is another widely used drug in the same calf serum hemodialysate group, but there has been no published report comparing the ingredients or biological actions between Actovegin® and Solcoseryl. In this review, we will only focus on the evidence around Actovegin®.

### Mechanisms

During the past 60 yr, researchers have endeavored to identify the active ingredients in Actovegin® but have been unsuccessful. Studies *in vitro* have suggested that it promotes oxidative metabolism and shifts the redox balance of cells into the direction of oxidized substrates. Actovegin®, therefore, initially was thought to have protective effects against hypoxic cell injury (30). Hoyer and Betz (14) demonstrated that Actovegin® does not directly influence cells during the ischemic period because the intracellular levels of glucose and lactate are similar to untreated animal cells during this period. Therefore, its mechanism of action is thought to improve the efficacy of a cell's energy balance in the postischemic metabolic events and interrupt the process of cell damage to avoid further cell death (14).

Inositol phosphate oligosaccharides (IPO) are one of the putative ingredients in Actovegin® (24). The IPO have been shown to have a partial insulin-like effect on the glucose transport activity of adipocytes but do not induce carrier translocation or stimulate insulin receptor kinase *in vitro* or *in vivo* (24). It has been reported that IPO activate glucose transporters, hence promoting glucose uptake by cells (10). IPO can contribute up to 50% of the maximum insulin effect on glucose transport and also can stimulate the activity of certain enzymes including pyruvate dehydrogenase, the key enzyme of the citric acid cycle (24). It has a synergistic effect with insulin and promotes glucose activity when insulin levels are suboptimal but does not alter the peak effect (24). A strongly negatively charged sulfated oligosaccharide of approximately 3,000-Da molecular weight also has been isolated from Actovegin®, which is different from the IPO fraction reported by the other studies (30). This fraction has a similar effect to IPO but with less effectiveness.

Actovegin® has a synergistic effect on cell proliferation *in vivo* with epidermal growth factor, basic fibroblast growth factor, and endothelial cell growth factor, causing an increase in cell numbers, an increased activity of acid phosphatase, and an improved level of thymidine incorporation compared with controls (30). These effects are resistant to proteinase K digestion; therefore, the active compounds of Actovegin® are unlikely to be growth factors or their derived fragments (30). The trivial nutritive effects of Actovegin® were excluded because a mixture of the same

level of amino acids and substrates did not stimulate proliferation or have insulin-like activity *in vivo* (30).

Actovegin® is likely to have membrane-stabilizing effects in ischemic cells. This may be due to the presence of negatively charged oligosaccharides, shifting the cells to the direction of oxidized substrates. At the immediate postischemic period, these factors may help cellular recovery. Its insulin and growth factor synergistic properties also could be beneficial and make the initial recovery period more efficient. Therefore, Actovegin® is assumed to be useful in circulatory disturbances and postischemic events.

A recent study *in vivo* indicated that Actovegin® could regulate the expression of cell surface receptors on macrophages, which indicates their phenotypic subgroups in the inflammatory sequence leading to muscle repair (18). Furthermore, Actovegin® can upregulate some of the inflammatory mediators that may have an important role in regulating the inflammatory process in muscle healing (18).

### Clinical Reports Not Related to Muscle Injuries

Although the active ingredients in Actovegin® are yet to be identified, many clinical studies have indicated its safety and effectiveness. There has been one case report of a possible anaphylactic reaction related to the use of intravenous Actovegin® injections by an amateur cyclist. In this report, the diagnosis of "anaphylactic reaction" was not confirmed with any biochemical testing, and the patient improved with broad-spectrum antibiotics. The author later stated in the communication letter that this patient had used intravenous Actovegin® once before with no adverse reaction; thus, an anaphylactic response is unlikely. However, it is possible that the first use of Actovegin® has primed the immune cells to react severely after the second or subsequent administrations. Because the patient improved on broad-spectrum antibiotics, the most likely cause for this acute shock was due to bacterial contamination during injection, not anaphylactic reaction to the drug (20).

Pfrringer *et al.* (28), in a double-blind, placebo-controlled, single-center study with 60 recreational athletes, demonstrated that ultrasound-guided paratenon injection of Actovegin® was effective in the treatment of Achilles tendinitis. The tendon cross-sectional measurement was reduced significantly ( $P < 0.0001$ ); patients' physical activity and perception to pain also were improved ( $P < 0.002$ ) in the treatment group (28). The overall clinical outcome, which is measured by patients' satisfactory score, was significantly better in the Actovegin® group ( $P < 0.0001$ ), and no adverse event was reported in this study (28). Although it is a relatively small-scale study with limited power, it is a well-conducted study that was featured in a Cochrane Review. Ziegler *et al.* (36) reported a double-blind, multicenter, randomized, control study with 567 patients with type 2 diabetes, in which 281 patients were treated with 20 daily high-dose intravenous Actovegin® infusions followed by 1,800 mg of Actovegin® daily for 120 d in the treatment of symptomatic diabetic polyneuropathy compared with placebo. Total symptom score of the lower limbs, vibration perception threshold, Neuropathy Impairment Score of the Lower Limbs, and quality of life (SF-36) were used as patient-reported outcome measures, which all showed a statistically significant difference compared with placebo

(30). This fraction has a similar effect to IPO but with less effectiveness.

Actovegin® has a synergistic effect on cell proliferation

well-conducted study that was featured in a Cochrane Review. Ziegler *et al.* (36) reported a double-blind, multicenter randomized control study with 567 patients with

and baseline scores ( $P < 0.05$ ). Furthermore, no anaphylactic reactions were reported with this study after 5,620 infusions, and the adverse effect profile was no different compared with placebo (36).

#### Legal Status

Besides its clinical properties, there are anecdotal beliefs among athletes that Actovegin® possesses an oxygen-carrying capacity and has the potential to enhance oxygen uptake, which leads to better performance. Although these claims are not based on any objective scientific evidence or published clinical reports, the International Olympic Committee announced in December 2000 that Actovegin® was banned under the classification of blood-doping agents. Two months later, however, the International Olympic Committee lifted the ban because there was no evidence that Actovegin® actually enhances performance (32). So far, there have not been any sports-related studies or performance testing with this drug on healthy individuals. Currently, intramuscular use of Actovegin® is not prohibited in or out of competition for any given sport according to the latest search (2010) in the Global Drug Reference Online, which is approved by U.K. Anti-Doping, the Canadian Centre for Ethics in Sport, the U.S. Anti-Doping Agency, and WADA (13). According to the latest 2011 WADA prohibited list, Actovegin® is not prohibited in any sports. However, WADA has issued a specific guidance on Actovegin® on its Web site. According to section M2 of the WADA code, the volume of intravenous injection of any nonprohibited substance must not exceed 50 mL with a simple syringe, and further serial injections must be at least 6 h apart (34). Therefore, it should be stated clearly here that Actovegin® cannot be administered by intravenous infusion or single intravenous injection with a volume exceeding 50 mL (34).

#### Muscle Injuries

Muscle injuries are one of the most common sports-related injuries; their incidence varies from 30% to 55% (15,22,33). Rest, immobilization (15), physical therapy (7), and nonsteroidal antiinflammatory drugs (NSAID) (29) have been the mainstay of therapy for muscle injuries (16). Immobilization may lead to improved granulation of the injured muscle and promote healing, but it will cause significant atrophy of healthy myofibers and joint stiffness (15). Although some studies have shown that the administration of NSAID promotes muscle healing by reducing degeneration and inflammation, other studies have demonstrated that NSAID are detrimental to the entire healing process (1,21,25,31). Recently, new treatment options such as growth factor injection therapy have shown good therapeutic results. However, because of their performance-enhancing properties, growth factor hormones are prohibited by WADA under sections M1 and S2 from the WADA prohibited list (34). In 2008, Orchard *et al.* (26), in a best practice statement, suggested that the evidence on current treatments in muscle injuries is low; further research in new treatment such as Dr. Müller-Wohlhahrt's Actovegin® and Traumeel (Heel, Inc., New Mexico) injection therapy should be explored.

A recent comprehensive review of the cellular and molecular mechanisms of muscle regeneration suggested that

muscle repair after muscle strains is initiated by necrosis. Posttraumatic necrosis of the myofibers stimulates an inflammatory response mediated by macrophages (8). This, followed by differentiation of satellite cells then maturation, leads to new myofiber formation (8). This process is different from the sequence of events observed during embryonic myogenesis.

Macrophages traditionally have been viewed as scavengers only involved in the removal of necrotic debris; however, recent studies have suggested that they also may have an active role in promoting muscle regeneration (8,18). Muscle fiber necrosis leads to dissolution of the plasma membrane causing rapid disintegration of myofibrils (8). This activates the complement cascade causing chemotaxis of leukocytes and monocytes from blood. Recent studies suggest that there are two distinct subpopulations of macrophages sequentially involved in this process (8). The early invading "phagocytic" macrophages (CD68<sup>+</sup>), characterized by the expression of the CD68 cell surface marker and lacking the CD163 marker, reach the highest concentration in damaged muscle at about 24 h after the onset of injury and then rapidly decline. These CD68<sup>+</sup> macrophages are proinflammatory phagocytes that secrete inflammatory cytokines such as the tumor necrosis factor and interleukin-1 and are responsible for the removal of necrotic debris. After 48 h, a second subpopulation of "nonphagocytic" macrophages (CD163<sup>+</sup>), characterized by the expression of the CD163 surface marker and lacking the CD68 marker, was then found and reaches a peak at day 4 after the initial injury. This CD163<sup>+</sup> subgroup displays an antiinflammatory property and secretes cytokines, such as interleukin-10, which contributes to the termination of inflammation (6,8).

Therefore, macrophages have a central regulatory role in the muscle response to injury (6,8). A recent report suggested that Actovegin® may cause up-regulation of the CD68<sup>+</sup> subgroup of macrophages and the precursor of the CD163<sup>+</sup> subgroup *in vitro* (6,8,18). However, it is only a preliminary laboratory-based gene expression report; it may or may not translate to clinical practice. Therefore, premature conclusions must not be drawn until more research is published in this area.

#### Evidence on Actovegin® in Muscle Injuries

The treatment of muscle tears with intramuscular Actovegin® was first published by Pfister and Koller (27) in 1990. Their partially blinded case control study with 103 patients showed a reduction of recovery time with the treatment group of 5.5 wk compared with 8.3 wk for the control group (27). However, in this study, patients were recruited from various sports and levels, and the treatment regimen and the rehabilitation protocol were not standardized. The diagnosis of muscle injuries was based only on clinical findings and was not graded according to magnetic resonance imaging. Actovegin® was mixed with local anesthetics before injection; therefore, its pharmacodynamics and pharmacokinetics were altered. The treatment regimen in this study was not standardized, the number of injections ranged from three to eight, and the final outcomes were based on patients' and various clinicians' subjective observations, and there were no preinjury data to compare outcomes. Despite



the limitations of the study, it is the first published study regarding Actovegin's use as an intramuscular injection in the treatment of muscle injuries, and no adverse events were reported in this article. Since Pfister and Koller (27), there had not been any published report in the medical literature regarding the use of Actovegin® in muscle injuries until Wright-Carpenter *et al.* (35) in 2004. In this small non-randomized study, autologous conditioned serum was compared with Actovegin®. The Actovegin® group in this study was created by the retrospective analysis of the study of Pfister and Koller; therefore, it should not be seen as new evidence.

Ziegler *et al.* (36) reported a double-blind, multicenter, randomized, control study with 567 diabetic patients treated with Actovegin® for diabetic neuropathies, which was discussed above. Although muscle assessments were not the primary objective for this study, it was measured as a secondary objective to monitor the progression of treatment. There was no significant difference in the adverse event rate compared with placebo; Actovegin® did not improve muscle strength ( $P = 0.731$ ) or muscle reflex ( $P = 0.571$ ) (36). Therefore, it is reasonable to conclude that Actovegin® is a safe drug and does not have anabolic or ergogenic effects on muscles.

Lee *et al.* (19) reported the first single Actovegin® intramuscular injection therapy on high-level professional footballers. The therapy regimen described by Lee *et al.* for magnetic resonance imaging-proven grade I hamstring injuries seems to significantly reduce the number of days for return to play, with a mean of 8 d of reduction compared with rehabilitation therapy alone ( $P < 0.05$ ). Although this is an observational study with only 11 subjects and without a placebo injection group, potential psychological effects of injection cannot be ruled out. Nevertheless, this study provided important pilot data regarding the use of single Actovegin® intramuscular injection therapy for muscle injuries.

#### Discussion

*In vivo* and *in vitro* studies suggest that Actovegin® contains some active components, although they are yet to be identified. It is a licensed drug that has been used by clinicians across Europe, China, and Russia to treat stroke and diabetic neuropathies (4,17,23,28,35). There is limited evidence on its role regarding the treatment of muscle injuries and no evidence regarding any performance-enhancing properties (27). Recently, there have been *in vitro* research data suggesting that Actovegin® may have a role in the modulation of the inflammatory process in muscle repair after traumatic injuries (6,8,18).

An unpublished case series with high-profile sports people such as Maurice Green, Asafa Powell, Diego Maradona, Darren Gough, and Paula Radcliffe suggested that Dr. Hans-Wilhelm Müller-Wohlfahrt's injection regimen with a mixture of Actovegin®, Traumeel, and local anesthetics seems to have good anecdotal results (9,26). There has been only one small clinical study investigating the effect of stand-alone Actovegin® therapy in muscle injuries. Our review describes the clinical use and evaluation of Actovegin® for more than 60 yr with an apparently good safety profile. The only anaphylactic case report published could be discounted

because the cause is most likely to be bacterial contamination of the injection site. Actovegin® does not improve muscle strength or muscle reflex, and there is no evidence of an ergogenic effect reported.

The mechanism of action with Actovegin® can potentially protect ischemic cells, modulate the inflammatory process, and help the initial phase of recovery from acute muscle injuries become more efficient. Skeletal muscle is a very vascular structure and is highly energy dependent. Once injured, blood flow often is disturbed, which leads to cell ischemia and energy imbalance. Therefore, Actovegin® injection therapy to the injury site might aid recovery and limit further ischemic effects and control cellular damage from the initial injuries.

Official governing bodies including WADA, U.K. Anti-Doping, the Canadian Centre for Ethics in Sport, and the U.S. Anti-Doping Agency do not prohibit its use intramuscularly. On the other hand, it is not on the British National Formulary, and the Medicines and Healthcare products Regulatory Agency in the United Kingdom and the Food and Drug Administration in the United States have not approved its use. Although it is not uncommon in modern medical therapy, the intramuscular use of Actovegin® to treat muscle injuries is off-license.

In professional elite-level athletes, Orchard *et al.* (26) summarized that currently, almost all our so-called knowledge in the treatment of muscle injuries was based on very poor scientific evidence. The career lifespan for the professional elite athlete often is short-lived; shortened recovery time could mean continuing with training, increased game play, and benefit to the team and club.

Because of the unique relationship between sports physicians and athletes, they often are under pressure to seek the latest "active" or "cutting-edge" treatments (12). Athletes often are not interested in being part of a clinical trial. Therefore, it is not always possible to get a large number of participants who are professional athletes. There is also much publicity about the use of this drug on the basis of anecdotal assumptions on its questionable theoretical ergogenic properties and placebo effects (12). To obtain the highest level of evidence and rule out placebo effects with intramuscular Actovegin® therapy, a control must be established first. Because the traditional treatment of muscle injuries is based on low-level evidence, there has not been any report or evidence on intramuscular isotonic saline injection therapy. Therefore, it is unethical to assume that intramuscular isotonic saline is a placebo and can be used as control group. Nevertheless, Actovegin® is not licensed to treat muscle or soft tissue injuries, and its evidence is limited. There is published evidence demonstrating its efficacy and safety for a variety of conditions not related to muscle injuries (2,4,5,17,28,36).

As modern sports physicians, we must apply the principles of evidence-based medicine when considering new therapies. Physicians should process these evidences carefully and pay attention to detail, especially when the published medical literatures are limited. This article summarizes the current evidence on Actovegin®. Through our research, there is no evidence that Actovegin® can enhance an athlete's performance. Further research must be encouraged to investigate the effects of Actovegin® on muscle injuries before

after traumatic injuries (6,8,18).

An unpublished case series with high-profile sports people such as Maurice Green, Asafa Powell, Diego Maradona

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it can be labeled as an effective treatment. Injection therapy could potentially be useful in the treatment of muscle injuries; therefore, it should not be regarded as a "witchcraft" remedy. Currently, Actovegin® falls somewhere in between cutting-edge and "voodoo."

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