

**Effect of Extracorporeal Shock Wave Therapy on Equine Umbilical Cord Blood  
Mesenchymal Stromal Cells *in vitro***

by  
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## ABSTRACT

### EFFECT OF EXTRACORPOREAL SHOCK WAVE THERAPY ON EQUINE UMBILICAL CORD BLOOD MESENCHYMAL STROMAL CELLS *IN VITRO*

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Extracorporeal shock wave therapy (ESWT) has been shown to induce different biological effects on a variety of cells, including regulation and stimulation of their function and metabolism. ESWT can promote different biological responses such as proliferation, migration, and regenerations of cells. Recent studies have shown that mesenchymal stromal cells (MSCs) secrete factors that enhance regeneration of tissues, stimulate proliferation and differentiation of cells and decrease inflammatory and immune-reactions. Clinically, the combination of these two therapies has been used as treatment for tendon and ligament lesions in horses; however, there is no scientific evidence supporting this combination of therapies *in vivo*. Therefore, the objectives of the study were to evaluate if ESWT affects equine umbilical cord blood mesenchymal stromal cells (CB-MSCs) proliferative, metabolic, migrative, differentiation, and immunomodulatory properties *in vitro*. Equine CB-MSCs were treated using an electrohydraulic shock wave generator attached to a water bath. All experiments were performed as triplicates. Proliferation, viability, migration and immunomodulatory properties of the cells were evaluated. Equine CB-MSCs were induced to evaluate their trilineage differentiation potential. ESWT treated cells had increased metabolic activity, showed positive adipogenic, osteogenic, and chondrogenic differentiation, and showed

higher potential for differentiation towards the adipogenic and osteogenic cell fates. ESWT treated cells showed similar immunomodulatory properties to non-ESWT treated cells. Equine CB-MSCs are sensitive to ESWT treatment and showed increased metabolic, adipogenic and osteogenic activity, but unaltered immunosuppressive properties. *In vivo* studies are warranted to determine if synergistic effects can be obtained in the treatment musculoskeletal injuries if ESWT and equine CB-MSC therapies are combined.

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## **DECLARATION OF WORK PERFORMED**

I declare that, with the exception of the items below, all work in this thesis was performed by me, Ramés Salcedo.

The procedures associated with the mixed lymphocyte reaction were performed by Olivia Lee, she also wrote the material and methods for that section.

The statistical analysis was performed with the guidance of William Sears, Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario.

## TABLE OF CONTENTS

	Page
Abstract	ii
Acknowledgements	iv
Declaration of Work Performed	v
Table of Contents	vi
List of Tables	ix
List of Figures	x
List of Abbreviations	xi

### CHAPTER I: Literature Review

1.1	Extracorporeal Shock Wave Therapy (ESWT)	1
1.1.1	History of ESWT	1
1.1.2	Principles of ESWT	2
1.1.3	Mechanotransduction	4
1.1.4	Molecular effects	5
i)	Mitogen-activated protein kinase (MAPK)	5
ii)	Growth factors	6
1.1.5	Other mechanisms of action	7
1.2	Mesenchymal stromal cells (MSCs)	8
1.2.1	Definitions and Nomenclature	8
1.2.2	Sources	9
1.2.3	Mechanism of action	10
1.2.4	Equine Cell-Based Therapies	11
i)	Equine Cell-Based Therapies for Soft Tissue Injuries	12
ii)	Equine Cell Based Therapies for Joint Disease	13
1.2.5	ESWT as augmentative treatment on MSCs	15
1.3	References	15

## **CHAPTER II: Introduction to Shock Wave Application to Cell Culture**

2.1	<i>In vitro</i> Shock Wave Treatment	25
2.2	Pilot Study	26
2.3	References	27

## **CHAPTER III: Effect of Extracorporeal Shock Wave Therapy on Equine Umbilical Cord Blood Mesenchymal Stromal Cells *in vitro***

3.1	Abstract	29
3.2	Background	30
3.3	Material and Methods	32
	3.3.1 Cell culture	32
	3.3.2 <i>In vitro</i> extracorporeal shock wave treatment	32
	3.3.3 Proliferation	33
	3.3.4 Metabolic activity	34
	3.3.5 Migration (Scratch assay)	34
	3.3.6 Trilineage differentiation	34
	3.3.7 Mononuclear cell suppression assay	38
3.4	Statistical analysis	39
3.5	Results	40
	3.5.1 Proliferation	40
	3.5.2 Metabolic activity	41
	3.5.3 Migration	41
	3.5.4 Trilineage differentiation	41
	3.5.5 Mononuclear cell suppression assay	42
3.6	Discussion	42
3.7	Conclusion	47
3.8	References	48
3.9	Figures	59

## **CHAPTER IV: Summary and Conclusions**

4.1	References	61
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## **CHAPTER V: Appendices**

5.1	Figures	64
5.2	Tables	66

## LIST OF TABLES

		Page
Table 1	Extracorporeal shock wave generators used in equine veterinary medicine	66

## LIST OF FIGURES

		Page
Figure 1	Physical characteristics of extracorporeal shock waves	64
Figure 2	Wave propagation of fESWT and rESWT	64
Figure 3	Water bath for ESWT application	65
Figure 4	Pilot study experimental design	65
Figure 5	Experimental design	54
Figure 6	Proliferation - Population doubling times	55
Figure 7	Metabolic activity	55
Figure 8	Representative scratch assay	56
Figure 9	Trilineage differentiation	57
Figure 10	Mononuclear cell suppression assay	58

## LIST OF ABBREVIATIONS

ASCs	Adipose derived stem cells
ATP	Adenosine triphosphate
CB-MSCs	Umbilical cord blood mesenchymal stromal cells
CGRP	Calcitonin gene-related peptide
BrdU	Bromodeoxyuridine
DMEM	Dulbecco's modified eagle's medium
EFD	Energy flux density
ERK	Extracellular signal-regulated kinase
ESWT	Extracorporeal shock wave therapy
ErK1/2	Extracellular signal-regulated kinase
fESWT	Focal extracorporeal shock wave therapy
hBMSCs	Human bone marrow stromal cells
IGF	Insulin like growth factor
LSA	Lymphocyte suppression assay
MAPK	Mitogen-activated protein kinase
MHz	Megahertz
MNC	Mononuclear cell fraction
MPa	Megapascals
MSCs	Mesenchymal stromal cells
OA	Osteoarthritis
PBS	Phosphate buffered saline

rESWT	Radial wave generator
SW	Shock wave
SDFT	Superficial digital flexor tendon
VEGT	Vascular endothelial growth factor

## CHAPTER I

### Literature Review

#### 1.1 Extracorporeal Shock Wave Therapy (ESWT)

##### 1.1.1 History of ESWT

The first time the influence of shock waves (SW) on tissues was observed was during the Second World War. Explosion of bombs in the water damaged the lungs of the shipwreck sailors [1]. Likewise, it is by accident that in 1966, the effect of SW was discovered while conducting experiments with high velocity projectiles. An employee touched a plate while it was being impacted by a projectile and the shock waves were transmitted to the hand and then the body [1,2]. Since then, systematic investigations for a possible use for SW in medicine was performed. In 1971, the breakdown with shock waves of uroliths without direct contact with the stone was reported, uroliths were implanted in the renal pelvis of dogs and then the uroliths were disintegrated [2]. In the 1980's extracorporeal shock wave became established as the modality of choice to perform lithotripsy, "*act of breaking stones*" [3]. The first clinical treatment of a human patient suffering of renal calculi was performed in 1980; and for gallbladder stones was performed in 1985 in Germany [1].

After SW lithotripsy became popular and more commonly performed, it was noted that it was associated with an increased in bone mineral density of the pelvis. This increased in osteogenic activity was subsequently investigated by Haupt (1992), who showed a significantly better

radiologic healing and stronger mechanical stability in fractured rat femurs treated with SW [4].

The first clinical investigations and treatment for musculoskeletal conditions in humans were performed with lithotripters, which had been designed for SW therapy in urology [1]. The need to treat different anatomical areas made necessary the development of new equipment, which is how, in 1993, the first orthopedic shockwave device, OssaTron (HMT AG, Lengwil, Switzerland) was developed [1,5]. In the same year the first reports of extracorporeal shock wave therapy (ESWT) for treatment of chronic calcifying tendonitis of the rotator cuff were published demonstrating encouraging results [6]. Other conditions that were successfully treated with ESWT were non union and delayed union in long bone fractures, avascular necrosis of the femoral head, stress fractures, patellar tendinopathy, Achilles tendinopathy, and plantar fasciitis [7].

In Germany, an estimated of 60,000 – 100,000 patients are treated annually with ESWT for orthopedic conditions, exceeding the number of patients treated for lithotripsy with the same modality [8]. In the year 2000 the Food and Drug Administration of the United States gave the first approval of ESWT for the treatment of plantar fasciitis, followed in 2002 by approval for lateral epicondylitis [9]. As a result of the positive outcomes in humans, ESWT was used as a treatment in horses for different orthopedic conditions, such as proximal suspensory desmitis and osteoarthritis of the distal tarsal joint [10,11].

### **1.1.2 Principles of ESWT**

The SW is a transient pressure disturbance that propagates in three-dimensional space with a sudden rise from ambient pressure to its maximum pressure at the wave front. The basic physical

properties of a SW can cause expansion and contraction within a medium, effecting a change in the material density. The SW is characterized by high peak pressures up to 100 MPa (500 bar), a fast initial rise in pressure during a period of less than 10 ns, a low tensile amplitude of 10 MPa, a short life cycle of approximately 10  $\mu$ s, and a broad frequency spectrum, in the range of 16 Hz to 20 MHz. The measured shock wave rise time is in the 30 ns (Figure 1). Focal extracorporeal shock wave (fESWT) therapy uses pressure waves generated outside of the body that can be focused to a specific tissue/site inside the body. The fESWT should not be confused with ballistic or radial wave generators (rESWT), from which the acoustic pressures generated are not focused to a specific point within the tissues [12,13].

There are three types of fESWT generators used in medicine: electrohydraulic, electromagnetic and piezoelectric. Most of the generators used to treat musculoskeletal conditions in horses are electrohydraulic (Table 1). The electrohydraulic generator uses the tips of two electrodes as a point source. This electrode is placed in the first focal point of a semi ellipsoid and high voltage is switched to the tips of the electrode. Between these tips, an electrical spark is generated and a shock wave is released right by the vaporization of water between the tips. The spherical shock waves are reflected by a metal ellipsoid and focused into the second focal point for which the therapy is adjusted to the therapeutically volume inside the patient's body [12,13].

The physical parameters used to measure shock waves are: pressure distribution which is the pressure measured in megapascals generated by a SW as a function of time and space. Energy density or energy flux density (EFD) is a measure of the energy per square area that is being released by the sonic pulse at a specific point (e.g. 0.15 mJ/mm<sup>2</sup>). Total energy or total pulse

energy is the sum of all energy densities across the beam. It describes the total acoustical energy per released SW at the second focal point [12,13].

Devices that deliver ballistic or radial waves consist of low cost generators and have become widely available; however, these are different from the previously described generators (fESWT). The generated waves spread in a radial manner resulting in energy loss proportional to  $1/\text{radius}$ . As the distance between the source and target is doubled, only one fourth of the energy will hit the target. The maximum energy is highest at the source or, when coupled to tissue, on the skin surface and dissipates as distance increases. To produce this type of waves, a projectile is accelerated to high speed by compressed air. When the projectile hits the probe installed in the hand piece, the impact energy is partially transformed into shock wave energy, then transmitted through the probe and coupled at the probe tip (Figure 2) [12,13].

### **1.1.3 Mechanotransduction**

Mechanotransduction is the mechanism by which cells convert mechanical stimulation into biochemical responses and could be referred to as a “biological pathway”. This mechanical intervention can influence cellular functions such as migration, proliferation and differentiation [14,15].

The mechanism of action of ESWT is based on mechanotransduction and has been partially investigated and described [16]. Different molecules, cellular components and extracellular structures are involved in the mechanotransduction pathways like ion channels, cadherins, gap

junctions, integrins and microfilaments [17].

There are some published data of the mechanism by which ESWT creates a response from different cells through the previously mentioned mechanotransduction pathways. For example, after treating human bone marrow cells with ESWT, a rapid cell membrane hyperpolarization was observed, which can last up to 5 minutes, after which the cell membrane returns to its normal state; this effect was thought to be mediated via calcium activated channels [17]. Furthermore, a transient and reversible cytoskeletal damage was observed after ESWT was applied to human renal carcinoma cells. Only actin and tubulin were affected, vimentin was not and the cells reorganize their cytoskeletal structure 3 hours after treatment [18]. Lastly, when studying the activation of angiogenic signaling pathways, the levels of beta 1-integrin are significantly increased after ESWT was applied to human umbilical vein endothelial cells [19].

#### **1.1.4 Molecular effects**

##### **i) Mitogen-activated protein kinase (MAPK)**

The mitogen-activated protein kinase (MAPK) signaling pathway is a phosphorylation cascade that transmits signals from different cell surface receptors to cytosolic and nuclear targets in different cells [20]. This cascade is involved in a variety of biological effects, such as proliferation, differentiation, cell attachment, protein synthesis and apoptosis [21,22]. ESWT when applied to bone tissue was found to significantly increase extracellular signal-regulated kinase (ERK) and p38 kinase activities; these signals leads to an increase in cell proliferation, and

stimulation of bone morphogenesis [23]. Chen *et al.* (2004) suggested that the mitogen-activated protein kinases (MAPK) have the ability to regulate mechanotransduction of ESWT by triggering mitogenic and osteogenic responses, which was shown in a segmental bone defect model in rats [23]. Another study demonstrated that ESWT increase p38 kinase and focal adhesion kinase (FAK) kinase activities within T cells and these effects promoted the proliferation of the Jurkat T cells and production and secretion of interleukin-2 (IL-2) [24].

## ii) Growth factors

Vascular endothelial growth factor (VEGF) is considered a potent angiogenic factor that is up-regulated in certain diseases such as cancer. Besides endothelial cells, its receptors are expressed on numerous types of cells. VEGF plays a role in different normal physiological functions such as bone formation, hematopoiesis, and wound healing [25,26]. After ESWT was applied to rat adipose tissue-derived stem cells, it was observed that VEGF and nerve growth factor (NGF) were increased significantly compared to untreated cells. This increase in expression was maintained through several passages, which was translated in an accelerated neovascularization and innervation rate *in vivo* [27]. In another study the effect of ESWT on bone regeneration was evaluated: up-regulation of the expressions of bone morphogenic protein (BMP2) and VEGF was augmented compared to the control. These results indicate an increase in angiogenesis and bone regeneration. BMP2 and VEGF are thought to act as modulators that promote migration, proliferation, and maturation of bone [28].

Transforming growth factor-beta (TGF- $\beta$ ), is a superfamily of cytokines that produce a

wide range of effects such as epithelial cell growth, differentiation, motility, organization, apoptosis, and tumorigenesis. These proteins are produced by a subset of T-cells but can be expressed by all cells. Some major sources of TGF- $\beta$  include platelets, macrophages, neutrophils, bone and soft tissues [29]. Conflicting information has been published regarding the effect of ESWT on the regulation of TGF- $\beta$ . No changes in the expression levels were observed in human osteoblasts after treatment with ESWT when compared with the control cells [30]. This was in contradiction of the results of Wang *et al.* (2009) who showed a significantly higher serum levels of TGF- $\beta$ 1, VEGF and BMP-2 after treatment of long bone non union fractures with ESWT [31]. Additionally, Chen *et al.* (2004) showed an increase expression of TGF- $\beta$ 1 and insulin like-growth factor (IGF-1) when tendons were treated with ESWT after experimental tendinitis was performed to evaluate tendon healing [32].

#### **1.1.4 Other mechanisms of action**

The analgesic effect of ESWT is well known, it has been previously reported in humans, sheep and horses. Initially it was shown in humans after treating heel spurs, that pain decrease for 3 to 4 days after treatment [13,33,34]. The exact mechanism is not known and there are several hypotheses been investigated.

Ochiai *et al.* (2007) evaluated the effect of SW in a model of osteoarthritis (OA) in rats; they found that the neuropeptide calcitonin gene-related peptide (CGRP) that is expressed by nociceptors and is thought to play a role in the sensation of joint pain was reduced in neurons in the dorsal root ganglion of rats exposed to ESWT. The authors suggest that this reduction in

expression was responsible for the reduction in pain that was observed for up to 2 weeks after treatment *in vivo* [33]. The same group evaluated the presence of activating transcription factor 3 (ATF3) and growth-associated phosphoprotein (GAP-43) *ex vivo*, these two markers are specifically expressed after nerve injury and axonal regeneration respectively, after treating rats with ESWT, there was a significant increase in this two markers, which means that ESWT induce injury to the nerves around the treated area [34].

A study in sheep evaluated the neuropeptides substance P and calcitonin gene-related peptide (CGRP) in skin, nerves and periosteum after treatment using rESWT and fESWT. There were no significant differences in the concentrations of neuropeptides; however, histologically there was a significant increase in perineural inflammation after treatment, suggesting that inflammation is one of the factors that contribute to analgesia [35].

Considerable research is still needed to further elucidate the mechanism of action of ESWT and despite this, the use of ESWT for the treatment of musculoskeletal conditions in humans and animals has been advocated by the literature with increase in clinical success.

## **1.2 Mesenchymal stromal cells (MSCs)**

### **1.2.1 Nomenclature and Definition**

In 2005, the International Society for Cellular Therapy (ISCT) tried to standardized the terminology to the plastic-adherent cells isolated from bone marrow, adipose tissue or other tissues

with multipotent differentiation capacities. It was proposed that these plastic adherent cells described as mesenchymal stem cells be termed multipotent mesenchymal stromal cells [36]. The following year the ISCT described the minimal necessary criteria to define human mesenchymal stromal cells (MSCs): human MSCs must be plastic-adherent in standard culture conditions, express the surface markers CD105, CD73 and CD90, be absent of expression of surface markers CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, and finally, be capable of trilineage differentiation (osteoblast, adipocytes and chondroblast) *in vitro* [37].

In horses MSCs remain uncharacterized, and the expression of markers varies between reports. They may express CD 29, CD 44, or CD 90 and lack or have low expression of CD4, CD8, CD11a/18, CD 45, CD73, CD79a, CD105, or MHCII [38,39].

### **1.2.2 Sources**

MSCs were originally described to originate from the bone marrow. Nowadays they have been isolated from many other tissue sources, including adipose tissue, umbilical cord, skeletal muscle, dermis, lung, dental pulp, and peripheral blood [40]. Additionally, fetal MSCs have been derived from amnion, umbilical cord blood and umbilical cord tissue, as well as been isolated from several other tissues [41,42]. The most studied and used source of equine MSCs in clinical cases are from bone marrow and adipose tissue. Bone marrow is obtained from the sternum or tuber coxae of the standing horse and fat to derive adipose MSCs is obtained from the adipose tissue located to the side of the tail head [42]. Equine cord blood derived MSCs (CB-MSCs) can be isolated from fresh umbilical cord blood [43], and it has been suggested that these cells might have

superior immune tolerance, proliferative potential, and differentiation potency than MSCs derived from other tissues. Furthermore, the ability to collect these cells non-invasively makes them attractive for clinical use [44,45].

Allogeneic cells have been shown in different clinical trials to be safe. There are several advantages of using these cells, they can be cultured, expanded and stored long term before clinical use [46,47]. On average 15-30 million cells are injected to treat musculoskeletal injuries [48,49]. On the other hand, studies have shown age-related changes on BM-MSCs (e.g. loss of proliferation, differentiation potential, increase in senescent cell numbers) that can be deleterious to the quality of the cells. Additionally, a regression in osteogenic potential has been observed with donor age regardless of species and a longer time is needed after collection for expansion in culture in order to reach enough concentrations for therapeutic use [47].

### **1.2.3 Mechanism of action**

The effects of MSCs have initially been explained by a functional integration of the MSCs into the tissue after transplantation. It was believed that after administration, the MSCs would engraft and differentiate resulting in regeneration of the injured tissue. However, follow up studies observed that the MSCs did not engraft in sufficient numbers to provide tissue healing [50]. It is now accepted that this mechanism is not fully responsible for all the effects noticed after MSCs implantation.

We now know that MSCs exert their effect on the tissues by several other mechanisms, which include paracrine activity of growth factors, cytokines, and hormones cell–cell interactions, and the release of extracellular vesicles containing reparative peptides and microRNAs. These secreted factors can accelerate progenitor cells self-renewal, stimulate angiogenesis, minimize apoptosis and inflammation, and modulate immune responses [50,51]. As a result of their regenerative, anti-inflammatory, immunomodulatory and trophic functions, MSCs are an interesting option for equine cell-based therapies.

#### **1.2.4 Equine Cell-Based Therapies**

Cell based therapy involves the administration of different types of cells, with the idea that they can facilitate the replacement or repair of damaged tissue or cells [52]. Equine cell based therapies utilize autologous conditioned serum (ACS), platelet rich plasma (PRP), and expanded or non expanded MSCs [53].

These cells can be administered by different ways: intravenous, intra-arterial or directly transplanted into the affected region [52]; the source can be the own patient (autogenous) or a donor from the same species (allogeneic) [46]. Between the different cell sources used for soft tissue injuries in horses, bone marrow and adipose tissue are the most common used [42], and there are only two reports using equine CB-MSCs for clinical cases with musculoskeletal injuries [48,54].

## i) Equine Cell-Based Therapies for Soft Tissue Injuries

From soft tissue injuries, the most common is the tendinitis of the superficial digital flexor tendon (SDFT), which is frequently preceded by degenerative changes in the extracellular matrix and the healing response is usually slow and inadequate with a high risk of re-injury [55]. In an experimental model of collagenase-induced tendinitis in the SDFT in horses, bone marrow derived mesenchymal stromal cells (BM-MSCs) were used to evaluate tendon healing in vivo. Treated tendons showed a significant improved histological scores compared to their controls. No difference was observed between the tendons treated with MSCs and insulin-like growth factor (IGF) [56]. Another horse study surgically induced a core lesion in the SDFT and evaluated the effect of a single intra-lesional treatment with adipose-derived mesenchymal stromal cells (ASC). The treatment was not superior to the administration of autologous inactivated serum when evaluating histological and biochemical characteristics. Only a positive influence on the collagen crosslinking of the scar tissue was found that can be translated in tensile stress resistance [57].

Goodwin *et al.* (2012) reported about the outcome of naturally occurring SDFT injuries in Thoroughbred racehorses: from the 113 horses with available follow up the were treated with MSCs in bone marrow supernatant, the re-injury rate was 27.4%, which was significantly less when compared to other treatments modalities (re-injury rate of 44 to 56%) [58,59,60]. Another clinical study followed up horses treatment with different soft tissue injuries (SDFT tendinitis, suspensory ligament desmitis) with allogeneic CB-MSCs. It showed that 77% of the treated horses

returned to work at the same or higher level of exercise. One of the limitations of this study was the lack of a control group [48]. The same limitation was observed in a clinical report, where 6 racehorses with SDFT tendonitis were treated with CB-MSCs intra-lesional injections. Four out of six horses were included in the success group (7 – 10 starts after injury) [54].

The evidence in experimental studies and clinical cases support the use of MSCs in the treatment of soft tissue injuries in the horse. The healed tendons and ligaments appear to be stronger and the re-injury rates are lower compared to historical controls.

#### ii) Equine Cell-Based Therapies for Joint Disease

The use of cell-based therapies for the treatment of joint disease in the horse has demonstrated beneficial responses after their intra-articular administration [61]. The administration of intra-articular allogeneic CB-MSCs in experimentally induced synovitis after the administration of lipopolysaccharide (LPS), showed a decrease in the total nucleated, neutrophil and mononuclear cell numbers, suggesting that the intra-articular administration of MSCs may have a therapeutic use for treating joint inflammation [62]. In a study using the carpal osteochondral fragment model to create osteoarthritis, found that administration of BM-MSCs to the injured joint, decreased synovial levels of prostaglandin (PGE<sub>2</sub>), [63] supporting the anti-inflammatory effect previously reported. Bertoni *et al.* (2019) compared the intra-articular administration of BM-MSCs and CB-MSCs to healthy fetlocks, finding no significant differences in synovial fluid parameters (protein, cell count and PGE<sub>2</sub>), but there was a significant decrease of C-terminal telopeptide of type II collagen which is a marker of inflammation and cartilage

degeneration on the MSC groups compared to the control. Furthermore, BM-MSCs induced significantly more synovial effusion than CB-MSCs and the administration of 10 million cell per joint triggered more inflammatory signs than 20 million [64].

A prospective case series found that MSCs from BM-MSCs appeared to be safe when administered postoperatively for meniscal, cartilage and/or ligamentous lesions in the stifle. Furthermore, horses with meniscal lesions had a rate of 75% of return to work, which was higher than the previously reported rates of 60-63%. They also reported that 3 horses sustained joint flares following the BM-MSCs administration, of which none had long term effects [49]. A recent abstract reported the adverse reactions after administration of allogeneic BM-MSCs into joints and soft tissues (tendon and ligaments) in 164 clinical cases; only 3 synovial structures and 7 soft tissue administrations developed an adverse reaction. The authors concluded that allogeneic administration was a safe procedure for horses with varied musculoskeletal injuries [65]. Broeckx *et al.* (2019) recently reported their results of a randomized, multicenter, double-blinded and placebo-controlled study. Allogeneic chondrogenic induced mesenchymal stem cells were injected intra-articularly in horses with early signs of degenerative joint disease in the metacarpophalangeal/metatarsophalangeal joints. No adverse events were observed and there was a significant clinical improvement in lameness scores, response to flexion and joint effusion when compared to the control group [66].

The results of the experimental studies and the clinical studies support the use of MSCs in the treatment of joint disease in the horse. The administration of the MSCs is safe, decreases inflammation and there is a clinically-relevant improvement.

### 1.2.5 ESWT as augmentative treatment on MSCs

A variety of cells in culture, including MSCs, have shown to be sensitive to ESWT. Human BM-MSCs showed increased proliferation, migration and the rate of apoptosis activation was reduced after treatment with ESWT, and all treated cells maintained their differentiation potentials [67]. Rat ASC responded to ESWT with an elevation of mesenchymal markers and higher capacity to differentiate towards adipogenic and osteogenic lineages with minimal changes in proliferation [68]. When equine adipose tissue-derived mesenchymal stem cells were treated with ESWT, they showed an increased proliferation, but no effects were observed in their differentiation potential [69]. The increased proliferation after shockwave treatment may be mediated through purinergic receptors via downstream ErK1/2 signals following activation by extracellular adenosine triphosphate (ATP) [70]. Further studies are needed to further understand the augmentative effect that ESWT may have on MSCs.

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## CHAPTER II

### Shock Wave Application to Cell Culture (Pilot Study)

#### 2.1 Introduction to *in vitro* Shock Wave Treatment to MSCs

Extracorporeal shock wave therapy (ESWT) is well known for its positive effects in musculoskeletal conditions in humans and horses. Nowadays the number of *in vitro* experiments in cell cultures is increasing continuously. Research has shown some of the effects that ESWT has on cells. Depending on the type and energy levels the effects can go from positive regeneration, increased proliferation and differentiation, to negative effects such as apoptosis [1,2]. Different methods of applying shock waves with a wide range of energy protocols have been described in the literature, making comparison of results challenging. In this study the effects of *in vitro* application of ESWT to equine umbilical cord blood mesenchymal stromal cells (CB-MSCs) were evaluated using a modification of the model for in-vitro shock wave treatment (IVSWT) water bath [1]. This study aims to describe the response of a specific cell type (CB-MSCs) to ESWT and give us a better understanding of the effect of shockwave on this type of cells.

Information regarding energy flux densities used *in vitro* available in the literature varies between 0.08 to 0.16 mJ/mm<sup>2</sup> and number of pulses between 25 to 1000, but the optimal protocols are unknown. From the different methods of shock wave application to cell cultures that are described in the literature, three experimental set ups were investigated [1,2,3].

In an attempt to mimic *in vivo* conditions Suhr *et al.* (2013) used freshly prepared pork skin as an absorbing support surface, and to optimize coupling to the probe ultrasound gel was placed on top of the skin. They reported an increased proliferation and migration of the treated cells [2]. A different setting was reported by Nurzynka *et al.* (2008) where the probe was kept in contact with the flask containing the cells by means of a water-filled cushion covered with ultrasound gel; the results showed an increased proliferation and differentiation. The authors recognized as one of their limitations the presence of a liquid air interface that might interfere with the primary wave [3]. In 2014, Holfeld raised some concerns about the retroreflection of the waves after passing the cell culture; it was believed that the waves could reflect on the surface were the flask was resting after passing through the cells and therefore damage the cells. In order to counteract that effect, they created a water bath (IVSWT) in which the flask containing the cells were positioned while being treated. The IVSWT Water Bath used a thin membrane to couple the probe and has an absorber placed at the far end of the bath to destruct the coming waves and eliminate the reflection [1] (Figure 3).

## **2.2 Pilot Study**

In the first part of the project, a pilot study was performed to compare different shock wave application set ups and energy protocols. The equine CB-MSCs were thawed and seeded in T-25 cell flasks at a cell density of 5000 cells/cm<sup>2</sup> and treatment was performed at a confluence of 90%. The first setting consisted of a water-filled cushion in direct contact with the flask and the probe. The second setting was the flask in direct contact with the probe and the last setting was the IVSWT water bath previously described by Holfeld *et al* [1]. Four different energy protocols ( 1)0.01

mJ/mm<sup>2</sup> 3 Hz 300 impulses, 2)0.01 mJ/mm<sup>2</sup> 6 Hz 300 impulses, 3)0.15 mJ/mm<sup>2</sup> 3 Hz 300 impulses, 4)0.15 mJ/mm<sup>2</sup> 6 Hz 300 impulses) were evaluated for each setting and the outcomes evaluated were: metabolic activity and population doubling time (Figure 4).

The different set ups tested (described above) provided different results in their metabolic activity, which are summarized in figure 5. Based on the best outcomes on metabolic activity as observed in the Alamar blue assay, it was decided to use the IVSWT water bath with an energy flux density of 0.1 mJ/mm<sup>2</sup>, frequency of 6 Hz and 300 impulses.

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## CHAPTER III

### **Effect of Extracorporeal Shock Wave Therapy on Equine Umbilical Cord Blood Mesenchymal Stromal Cells *in vitro***

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### 3.1 Abstract

Extracorporeal shock wave therapy (ESWT) has been shown to induce different biological effects on a variety of cells, including regulation and stimulation of their function and metabolism. ESWT can promote different biological responses such as proliferation, migration, and regenerations of cells. Recent studies have shown that mesenchymal stromal cells (MSCs) secrete factors that enhance regeneration of tissues, stimulate proliferation and differentiation of cells and decrease inflammatory and immune-reactions. Clinically, the combination of these two therapies has been used as treatment for tendon and ligament lesions in horses; however, there is no scientific evidence supporting this combination of therapies *in vivo*. Therefore, the objectives of the study were to evaluate the effects of ESWT on equine umbilical cord blood mesenchymal stromal cells (CB-MSCs) proliferative, metabolic, migrative, differentiation, and immunomodulatory properties *in vitro*. Equine CB-MSCs were treated using an electrohydraulic shock wave generator attached to a water bath. All experiments were performed as triplicates. Proliferation, viability, migration and immunomodulatory properties of the cells were evaluated. Equine CB-MSCs were induced to evaluate their trilineage differentiation potential. ESWT treated cells had increased metabolic activity, showed positive adipogenic, osteogenic, and chondrogenic differentiation, and showed higher potential for differentiation towards the adipogenic and osteogenic cell fates. ESWT treated cells showed similar immunomodulatory properties to none-ESWT treated cells. Equine CB-MSCs are sensitive (not sure what this means – are sensitive) to ESWT treatment and showed increased metabolic, adipogenic and osteogenic activity, but unaltered immunosuppressive properties. *In vivo* studies are warranted to determine if synergistic effects can be obtained in the treatment musculoskeletal injuries if ESWT and equine CB-MSC therapies are combined.

## 3.2 Background

Extracorporeal shock wave therapy (ESWT) is one of the leading treatments of specific orthopedic diseases in humans such as plantar fasciitis and lateral epicondylitis and more recently it has been used to treat Achilles and patellar tendinopathies, [1,2]. ESWT is also a common treatment for tendon and ligament injuries in the horse particularly suspensory ligament desmitis [3]. At present it is also used in horses for osteoarthritis when other treatments are ineffective [4]. ESWT uses acoustic waves generated mechanically outside of the body that can be focused to a specific point within the body. Shock waves used for medical purpose are generated in a fluid medium, by one of three different generators: electrohydraulic, piezoelectric or electromagnetic. The shape of the acoustic wave is characterized by an initial positive rapid phase, of high amplitude, followed, by a sudden phase of mild negative pressure, and then returns to the ambient values. Their peak pressure is high - up to 100 mpa (500 bar) with a rapid rise (<10 ns) in pressure, of short duration (<10 ms) and a broad range of frequency [5,6].

A variety of cells in culture, including MSCs, have shown to be responsive to ESWT. Human bone marrow stromal cells (hBMSCs) showed increased proliferation, migration and the rate of apoptosis activation was reduced after treatment with focused ESWT, and all treated cells maintained their differentiation potentials [7]. Rat adipose derived stromal cells (ASCs) responded to ESWT with an elevation of mesenchymal markers and higher capacity to differentiate towards adipogenic and osteogenic lineages with minimal changes in proliferation [8]. When equine adipose tissue-derived mesenchymal stromal cells were treated with ESWT, they showed an increased proliferation, but no effects were observed in their differentiation potential [9]. The

increased proliferation after shockwave treatment may be mediated through purinergic receptors via downstream ErK1/2 signals following activation by extracellular ATP [10].

MSCs are multipotent cells capable of differentiation into osteogenic, adipogenic and chondrogenic lineages [11]. Bone marrow aspirates and adipose tissue are the most studied and used sources of MSCs for clinical cases in the horse [12]. It has been suggested that MSCs from umbilical cord blood (CB-MSCs) might have superior immune tolerance, proliferative potential, and differentiation potency [13]. MSCs may function in two different ways, first in a progenitor function by direct tissue integration and second in a non-progenitor function through secretory products that have trophic and immunosuppressive effects [14]. Recent studies have shown that MSCs secrete factors that enhance regeneration of injured tissue, stimulate proliferation and differentiation of endogenous cells and decrease inflammatory and immune reactions [14–16].

Clinically, the combination of ESWT and MSC therapies have been used as treatment for tendon and ligament lesions in horses without scientific evidence supporting this approach. We hypothesized that ESWT will affect the progenitor functions and non-progenitor functions of equine CB-MSCs. The aim of this study was to evaluate if ESWT affects equine CB-MSCs proliferative, metabolic, migrative, differentiation, and immunomodulatory properties *in vitro*.

### **3.3 Material and Methods**

#### **3.3.1 Cell culture**

The equine CB-MSC were established as previously described by Koch *et al.* [17]. Briefly, the mononuclear cell fraction (MNCF) was cultured and non-adherent cells were removed through successive complete medium changes. When cell numbers allowed, the cells were passaged and expanded until they were cryopreserved for later use.

The three cryovials of equine CB-MSC used in these studies had been stored in liquid nitrogen from 1 to 9 months prior to the study and all contained cells at passage 3. The cells were thawed, seeded at cell density of 5,000 cells/cm<sup>2</sup> culture surface area and expanded until ~80% confluence to obtain sufficient cell numbers for all the experiments and assays of these studies.

#### **3.3.2 In vitro extracorporeal shock wave treatment**

The *in vitro* shock wave treatment was performed as described by Holfeld *et al.* using an electrohydraulic shock wave generator (VersaTron, Pulse Veterinary Technologies, Alpharetta GA, USA) [18]. Equine CB-MSCs were treated at a confluence of 90% in T-25 cell culture flasks. The flasks were placed in front of the applicator (R80 Trode, focal depth 50 mm – 110mm) at a distance of 5 cm from the probe to the cell layer inside the water container in direct contact with pre-warmed (37°C) water. The focused shock waves were applied with the following parameters: an energy flux density of 0.1 mJ/mm<sup>2</sup>, frequency of 3Hz and 300 impulses (Figure 3).

The control group was maintained in the same culture conditions, consisting in equilibrated expansion media or differentiation media without shock wave exposure. All cell culture experiments were performed as triplicates from 3 different donors. The process is shown in flow chart (Figure 6).

### 3.3.3 Proliferation

Cells were detached using trypsin 0.025% (Lonza, Walkersville, MD, USA) and re-seeded in 6 well culture flasks. To document cell morphology, digital images were obtained prior to detachment at passage 5, 6 and 7 using phase-contrast microscopy and Q-Capture software (Q-Imaging, Surrey, BC, Canada).

Doubling times for passage 5, 6 and 7 were calculated according to Tessier *et al.*:

$$1. \text{Cell-doubling number (CD)} = \ln(N_h/N_s) / \ln 2$$

$\ln$  = natural Logarithm

$N_h$  = harvest cell number

$N_s$  = seed cell number

$$2. \text{Cell doubling time (DT)} = CT/CD$$

$CT$  = cell culture time [19].

### **3.3.4 Metabolic activity**

Equine CB-MSC, were seeded in 96-well plates at 5,000 cells/cm<sup>2</sup> in 300 µl of culture media. The following day the culture media was changed. On day 4, the culture media was replaced with 300 µl of 10% resazurin (Sigma Aldrich, Oakville, ON, Canada) in phosphate buffered saline (PBS), incubated at 37°C for 4 h protected from light and fluorescence was read by a plate reader (Spectramax i3, Molecular Devices, Sunnyvale, CA, USA) at 585 nm using an excitation wavelength of 555 nm.

### **3.3.5 Migration (Scratch assay)**

For the scratch migration assay, equine CB-MSC were seeded at 4 x 10<sup>4</sup> cells in 70 µl into each dish (Culture-Insert 2 well in µ-Dish 35mm, high Ibidi cat# 80206) and incubated, cells were grown to confluence. The Culture-Insert 2 well was removed creating a physical gap (scratch) within the cell monolayer. The cell monolayer was washed with PBS to remove debris and 2 ml of culture medium were added. Images were taken at 0, 12, 24, 36, and 48-hour time points or until complete closure of the scratch wound was observed using a motorized inverted microscope (IX81, Olympus America Inc., Melville, NY, USA). The dishes were incubated between each time point. After all images were acquired, measurements of the surface area of each scratch at each time points were performed with Photoshop (Adobe Systems Incorporated, NY, USA).

### **3.3.6 Trilineage differentiation**

For adipogenesis, equine CB-MSC were seeded at 2.1 x 10<sup>4</sup> cells/ml in 6 well plates, media was changed every 2-3 days. After the cells reached 100% confluence the cells were exposed to

adipogenic induction media (BulletKit, Adipogenesis, Lonza). The induction medium consisted of 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), 10 µg/ml recombinant human (rh) insulin, 0.2 mM indomethacin and 10% fetal calf serum (FCS) in DMEM. Media was changed every 2-3 days as previously described, Koch *et al.* [17]. Adipogenic potential was assessed after 14 days of exposure to induction media by Oil Red O staining to observe lipid droplets and AdipoRed™ assay (Lonza).

For Oil Red O staining, all the media was aspirated off the wells, washed once with 2 ml of PBS, then replaced with 2 ml of 10% formalin (Fischer Scientific, Whitby, ON, Canada) for 30 min at room temperature to fixate the cells, the formalin was replaced with 2 ml of sterile water (Milli-Q water, Millipore, Mississauga, ON, Canada) for 2 minutes and then replaced with 60% isopropanol (Fischer Scientific) for 5 minutes, the isopropanol was replaced with 2 ml of Oil Red O (Sigma-Aldrich,) working solution [3 parts (300 mg Oil Red O in 100 ml of 99% isopropanol) mixed with 2 parts sterile water], after 5 minutes the Oil red O solution was washed off indirectly with tap water. Two ml of Harris hematoxylin (Sigma Aldrich) were added to the wells for 1 minute before being aspirated and the wells washed with warm tap water.

The AdipoRed Assay (Lonza) was performed according to the manufacturer's protocol for 96 well plates. Briefly, equine CB-MSC were induced for 14 days. The supernatant was removed and rinsed with 200 µl PBS. Then each well was filled with 200 µl of PBS, 5 µl of reagent were injected followed by shaking for 1 second. The fluorescence was measured with excitation at 485 nm and emission at 520 nm as previously described by Koch *et al.* [17].

Chondrogenesis differentiation was performed in pellet culture as previously described by Co *et al.* [20]. Approximately  $2.5 \times 10^5$  CB-MSCs were suspended in 200  $\mu$ l of chondrogenic medium, containing High Glucose Dulbecco's modified Eagle medium (HG-DMEM) (Sigma Aldrich; Oakville, ON), 200 mM Glutamax (Invitrogen; Burlington, ON, Canada), 100 mM sodium pyruvate (Invitrogen), 1X Antibiotic Antimycotic (ABAM) solution (Invitrogen), 0.1 mM dexamethasone (Sigma Aldrich), 100 mg/ml ascorbic acid-2 phosphate (Sigma Aldrich), 40 mg/ml proline (Sigma Aldrich), 1 ITS (BD Biosciences; Mississauga, ON, Canada), and 10 ng/ml TGF- $\beta$ 3 (R&D Systems; Minneapolis, MN, USA), the cells were plated in V-bottom polypropylene 96 well plates (Phenix #MPG-651201) and centrifuged at 200x g for 10 min and incubated for 3 weeks. Media was changed every 2-3 days. Chondrogenic potential was assessed by toluidine blue to visualize glycosaminoglycan (GAG) and biochemistry.

For toluidine blue stain, the pellets were fixed in 10% formalin, dehydrated in isopropanol and embedded in paraffin and sectioned (5  $\mu$ M). Slides were deparaffinized and rehydrated prior to staining. The stock solution of Toluidine blue contained 1 g powder (Sigma Aldrich) in 100 ml 70% EtOH diluted to 10 % with 1% NaCl. Sections were stained in Toluidine blue working solution for 2 min and washed in three changes of distilled water.

For biochemistry evaluation, the pellets were digested in 40 mg/ml papain (Invitrogen; Burlington, ON, Canada), 20 mM ammonium acetate, 1 mM EDTA, and 1 mM dithiothreitol (DTT) (Sigma Aldrich) for 48 h at 65°C vortexing at 24 h. Stored at -40°C until further analysis. The proteoglycan content of the digest was estimated by quantifying the amount of sulfated glycosaminoglycans (GAG), using the dimethylmethylene blue dye and spectrophotometry with a

wavelength of 525 nm. The standard curve for the proteoglycan content assay was generated using chondroitin sulfate (Sigma Aldrich). DNA content was assessed using a commercial kit: DNA quantitation kit e Fluorescence assay (Sigma Aldrich) in a 96-well plate. The assay was carried out according to manufacturer's instructions.

For osteogenesis, equine CB-MSC were seeded at a density of 3,000 cells/cm<sup>2</sup> in 6 well plates, media was changed every 2-3 days. After the cells reached 100% confluency the cells were exposed to osteogenic induction media (BulletKit, osteogenic, Lonza). The induction medium consisted of DMEM-LG, 10% FBS, 1% L-glutamine, 1% antibiotic-antimycotic (ABAM), 100 nM dexamethasone, 10 mM  $\beta$ -glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. Media was changed every 3-4 days. Osteogenic potential was assessed by Alizarin Red S staining for calcium deposition and semi-quantitative alkaline phosphatase enzyme activity assay.

For Alizarin Red S staining, the pH of the Alizarin solution was adjusted to 4.1- 4.3 with ammonium hydroxide. The media was aspirated from each well and the cells were fixed in ice-cold 70% ethanol for 5 min at room temperature. Alcohol was aspirated and the wells were rinsed twice (5 min) with water, the water was aspirated, and 1 ml of 2% Alizarin Red S solution was added, it was incubated for 3 minutes at room temperature and removed, wells were washed 5 times with 2 ml of water.

For Alkaline phosphatase enzyme activity assay the culture wells were rinsed twice with PBS, 0.5 ml of PBS was added to the well and the cells were scraped off and transferred to a 1.5 ml Eppendorf tube. The wells were rinsed with 0.5 ml PBS, which was transferred to the Eppendorf

tube as well. The sample was centrifuged at 5000  $\times$  g for 8 seconds. All the PBS was aspirated, and the cell pellet was resuspended in 0.1 ml lysis buffer by pipetting the cells up and down several times. The lysis buffer consisted of (500 ml lysis buffer: 250 mg sodium deoxycholate, 5 mg phenylmethyl sulfonyl fluoride (PMSF), 5 mg aprotinin, 500 ml nonidet P-40, 500 ml 10% (wt/vol) SDS). The Eppendorf tubes were left on ice for 5 minutes and vortexed for 30 seconds, then they were microcentrifuged at maximum speed for 10 minutes at 6°C. Fifty microliters of the supernatant were added in triplicate from each osteo-induced and control sample to a well in a 96 well plate, then the kit, p-Nitrophenyl Phosphate Liquid Substrate System (Sigma-Aldrich), was used and 50  $\mu$ l of p-nitrophenyl phosphate (pNPP) were added to each well. The absorbance was read at 405 nm on a microplate reader as soon as possible after adding the pNPP (time 0) and subsequent every 5 minutes for 20 minutes. The absorbance was read against 100  $\mu$ l of undiluted pNPP. Covering it with aluminium foil screened the microplate from light between readings.

### **3.3.7 Mononuclear cell suppression assay**

A pooled peripheral blood mononuclear cell (MNC) donor MSA was used to evaluate equine CB-MSCs for their ability to suppress MNC proliferation *in vitro*. Briefly, MNCs from five unrelated donors were mixed in equal ratios and stimulated with the mitogen concanavalin A (Sigma Aldrich). Negative control pooled MNCs were not stimulated with mitogen. Treated and non-treated CB-MSCs were irradiated (20 Gy) and mixed with stimulated pooled MNCs at a ratio of 1:10 (CB-MSCs:MNCs) in MNC media (RPMI 1640 with 10% horse serum, 1% L-glutamine and 1% pen/strep) and then seeded in 48 well plates. Reactions were incubated for four days at 38°C in 5% CO<sub>2</sub>. After five days of co-culturing, cells were stained with

Bromodeoxyuridine (BrdU) and assessed with a BrdU ELISA kit (Roche, Mississauga, ON, Canada) following the manufacturer's protocol.

### **3.4 Statistical analysis**

For statistical analysis, the effect of ESWT delivered on equine CB-MSCs was evaluated. A statistical program (SAS 9.2, Proc MIXED, SAS Institute Inc., Cary, North Carolina, USA) was used to fit a general linear mixed model. For proliferation the design was a two-factor factorial in a randomized complete block design with fixed effect factors treatment and passage. Interactions were also included in the model. For metabolic activity the data was divided by a million for presentation. The design was also a two-factor factorial in a randomized complete block design with fixed effect factor cell culture and treatment. For migration the design was a two-factor factorial in a randomized complete block design with fixed effect factors treatment and time. To accommodate time being a repeated measure, the following correlation structures (offered by SAS) were attempted: ar(1), arh(1), toep, toep(2)-toep(5), toeph toeph(2)-toeph(5) un un(2)-un(5), and one was chosen based on Akaike Information Criterion (AIC). For adipogenesis the design was a two-factor factorial in a randomized complete block design by subsampling with fixed effect factors cell culture and treatment. And for osteogenesis to accommodate time being a repeated measure AIC was chosen. Repeated measures were nested in cell culture by treatment. Again, these error structures were attempted: ar(1) arh(1) toep toep(2)-toep(4) toeph toeph(2)-toeph(4) un un(2)-un(4). For the mononuclear cell suppression assay, a one-way ANOVA was performed with Tukey's multiple comparisons test. Differences were considered significant at  $p \leq 0.05$ .

To assess the ANOVA assumptions, comprehensive residual analyses were performed. The

assumption of normality was formally tested by use of Shapiro-Wilk, Kolmogorov-Smirnov, Cramér-von Mises, and Anderson-Darling tests. Residuals were plotted against the predicted values and explanatory variables that were used in the models to look for outliers, bimodal distributions, the need for data transformations, or other issues that should be addressed.

## **3.5 Results**

### **3.5.1 Proliferation**

Treated and untreated equine CB-MSC showed an elongated, spindle-shape fibroblast-like morphology. There were no obvious morphological differences between the treated and the untreated equine CB-MSCs.

One outlier was identified, but was retained in the analysis. The data was transformed using log based 2 and the assumption of the ANOVA analysis was adequately met and unequal variances in passages were accommodated. Population-doubling time was measured from passage 5 to 7. Mean (95% confidence interval (CI)) doubling time in days was as follows: passage 5, 1.67 days (0.83 – 3.14 days) for treated equine CB-MSC and 1.39 days (0.80 – 3.03 days) for untreated CB-MSC, for passage 6, 1.43 days (0.68 – 2.41 days) for treated equine CB-MSC and 1.46 days (0.67 – 2.39 days) for untreated equine CB-MSC and for passage 7, 2.42 days (1.11 – 4.30 days) for treated equine CB-MSC and 2.39 days (1.11 – 4.35 days) for untreated equine CB-MSC. There were no differences ( $p > 0.05$ ) between treated and untreated equine CB-MSC (Figure 7).

### **3.5.2 Metabolic activity**

The data was divided by a million to allow for statistical analysis. Treated equine CB-MSC had higher metabolic activity than untreated equine CB-MSC as evaluated by the Alamar blue assay ( $p = 0.0002$ ). Mean relative fluorescent units (RFU) was 102.29 (84.95 – 119.62 RFU) for treated equine CB-MSC and 65.10 RFU (47.76 – 82.43 RFU) for untreated equine CB-MSC (Figure 8).

### **3.5.3 Migration (Scratch assay)**

Data was mildly non-normal, unequal variances in time was accommodated, and the assumptions were not well met. For the scratch assay, the results obtained at the 60-h time point were removed from the analysis as most of the scratches were already closed. For all the experiments, the time to closure among groups was similar. There was no difference for each area measured between the treated and the untreated equine CB-MSC (Figure 9).

### **3.5.4 Trilineage differentiation**

Positive trilineage potency was observed for the treated and the untreated equine CB-MSC, no subjective differences were observed for the differentiation capacities when evaluated by Oil Red O, Alizarin Red S and Toluidine blue staining, respectively. Significantly increased adipogenic differentiation occurred in the treated equine CB-MSC compared to the untreated cells based on a higher intracellular lipid formation ( $p = 0.0002$ ). Significant osteogenic differentiation

occurred in the treated equine CB-MSC compared to the untreated cells as demonstrated by a higher alkaline phosphatase activity in the initial minutes ( $p < 0.0001$ ) (Figure 10). There were no differences for chondrogenesis as evaluated on biochemistry and wet mass.

### **3.5.5 Mononuclear cell suppression assay**

To investigate the immunomodulatory potency of equine CB-MSC treated with ESWT, we used a pooled mononuclear cell suppression assay (pMSA) method to compare MNC suppression of equine CB-MSC with and without ESWT. Treated and non-treated equine CB-MSC were co-culture with pooled MNCs for 96 hours and MNC proliferation was assessed with Brdu ELISA. Both treated and non-treated equine CB-MSC were able to suppress MNC relative to the positive control. The ESWT treated and non-treated MSCs suppressed MNCs to similar extents (Figure 11).

## **3.6 Discussion**

In the present study we demonstrated that equine CB-MSCs responds to ESWT stimulation. The proliferation of these cells was not adversely influenced by shockwave treatment but the metabolic activity of the treated cells was increased. Others have reported similar findings where proliferation of human adipose-derived stem cells (hASCs) and rat adipose-derived stem cells (rASCs) were not different between the untreated control group and the ESWT group [8]. In contrast to those results, Suhr *et al.* observed that the application of ESWT to human bone marrow stromal cells (hBMSC) stimulated proliferation [7]. Weihs *et al.* showed an increase in both

metabolic activity and in proliferation in a dose and time dependent effect [10]. Varying treatment protocols, ESWT generators and application, species and stem cell sources could all account for the observed differences in proliferation and metabolic activity. MSCs isolated from different tissues possess a different ability to differentiate, different surface marker expression and proliferative capabilities [21,22]. Also MSCs responds differently to the same stimulus, as observed when human adult and neonatal cells from the same individual were induced for neuronal transdifferentiation potential and showed different neuronal features [21]. Similarly, cells from the umbilical cord blood have shown increased proliferation and potency compared to bone marrow or adipose tissue [22]. As a result of this different response, a protocol utilizing the most appropriate cell source and cell type is essential to obtain the best response to treatment with ESWT.

In the current study, shock waves were applied via a water bath. The water bath was specifically designed to allow propagation of the shock waves after passing the cell culture, as it has been suggested that waves that are not propagated will get reflected at the culture medium and will disrupt the upcoming waves as well as the cell monolayer [18]. Conflicting information about *in vitro* application of shock waves is published. Some authors recommend application of the shock waves to flasks with an MSC cell monolayer in a water bath as we reported here; while others suggest applying shockwaves directly to tubes or flasks containing a MSC cell suspension through an interface (water-cushion, pig skin) [7,23]. The advantages of using the water bath in this study standardizes conditions, like temperature and distance, as well as being a model that simulates the *in vivo* conditions present during treatment with ESWT. One of the disadvantages of the method we used in our study, is that some detachment of the cells in the monolayer can occur, and

furthermore, the cells located at the periphery might receive lower doses of ESWT [24].

The beneficial effects of ESWT on wound healing have been shown on different species (25). Previous studies reported an increased expression in shockwave treated tissues of various growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF-B1) and insulin like growth factor 1(IGF-1). The increase in growth factor levels is thought to stimulate healing by increasing neovascularization, fibroblastic activity and proliferation [25,26]. Other studies in horses have shown no difference in overall healing time but significant improved quality of healing by a decrease in granulation tissue [27]. Studies *in vitro* have shown an increase in migration of hASC, as shown by the scratch assay, where treated cells filled the scratch faster than the control [24]. We could not reproduce this finding in our study as we did not see a difference in migration across the scratch after shockwave treatment. This reported difference in biological behavior might be attributed to the distinct cell types or treatment protocols, as previously mentioned.

We have shown that equine CB-MSCs maintain their multilineage differentiation potential after treatment with ESWT. Treated cells do not only maintain but also increase their potency towards adipogenic and osteogenic lineage. There is growing evidence suggesting that ESWT has an effect on MSCs differentiation [7,9,10]. Until now, the only experiment assessing the response of equine MSCs to ESWT reported that shock waves stimulate a higher potential for differentiation towards adipogenic, osteogenic and chondrogenic lineages of equine ASCs [9]. In contrast, Suhr *et al.* found that the adipogenic differentiation was unaffected but the osteogenic and chondrogenic differentiation potentials were reduced in hBMSC [7]. Similar to our results, Leone *et al.* found an

improved differentiation towards adipogenic and osteogenic lineages in tendon-derived stem/progenitor cells (hTSPCs) [28].

In the search of MSCs with an increased rate of survival after transplantation, Heneidi *et al.* isolated and characterized a new population of adipose stem cells (ASCs) named: multilineage differentiating stress-enduring cells. The cells were isolated using severe stress conditions [29]. In our study after exposing the equine CB-MSCs to ESWT, an increase in adipogenic potency was observed in addition to an increase in metabolic activity. It is possible that ESWT can precondition the cells, allowing them a superior performance when transplanted. Furthermore, the combination of ESWT with ASCs has been shown beneficial, as observed in experimental studies of brain damage in rats where this combination of treatments reduces the effect of local and systemic inflammatory immune reactions, oxidative stress and apoptosis and also reduce damage to the mitochondria in the brain [30].

Several *in vitro* and *in vivo* studies have demonstrated the enhancement of osteogenic differentiations of different type of cells after ESWT by measuring BMP-2 expression, alkaline phosphatase (ALP) activity, and calcium deposits [31,32]. This is in agreement to our findings, as we observed an increase in ALP activity, which is a recognized biochemical marker of osteoblast activity [33]. Furthermore, it is believed that TGF $\beta$ 1 is responsible for the increased activity of osteoblasts [34].

Effects on differentiation and proliferation on different type of cells after ESWT can be explained by mechanotransduction, which is how cells respond to mechanical stimulation and convert these stimulations into biochemical signals that can alter growth factor expression and

cellular adaptation [35]. The complete mechanism of action still remains unknown but there is evidence indicating that ESWT can stimulate the mitogen-activated protein kinase (MAPK) cascade [36,37]. As reported by Weihs et al., shock wave treatment initiates the release of ATP activating purinergic receptors that enhance proliferation by increasing ERK<sup>1/2</sup> signaling [10].

The contribution of the immunomodulatory ability of MSCs to tissue regeneration and healing have been long discussed. Using a mononuclear cell suppression assay (MSA), we observed that ESWT did not affect the mononuclear cell (MNC) suppressive potency *in vitro*. Our study is the first to examine the effect that equine CB-MSCs treated with ESWT has on MNC suppression. A previous study compared the surface markers and cytokine secretions of macrophages, and have demonstrated that the expression of classical pro-inflammatory M1 marker CD80 and COX2 decreased after ESWT, shifting the monocytes into the anti-inflammatory M2 phenotype. ESWT had also reduced the secretion of pro-inflammatory cytokines by M1 macrophages [39]. ESWT may influence MNC subpopulations when applied directly, but whether ESWT can effect MNCs indirectly through modifying MSCs still requires more investigation. It is possible that ESWT can influence MSC surface marker expression and cytokine levels, which would subsequently affect MNCs. However, this effect cannot be observed on the level of MSC induced MNC suppression. In addition, it is also possible that varying the energy level of the ESWT could change the outcome we have observed.

In previous experimental studies in horses *in vivo*, desmitis of the suspensory ligament or tendinitis of the superficial digital flexor tendon was induced with collagenase. Afterwards, the injured and the control limb were treated with ESWT. The results showed an improved healing of the treated limbs related to stimulation of fibroblasts, a greater number of mitochondria, with an

increase in TGF b1, as well as improvement in their ultrasonographic appearance, reduced size and increased neovascularization [40,41]. The positive responses to ESWT mentioned in the previous reports, together with our results, suggest the possibility to translate these findings into treatments for soft tissue injuries in horses, specially tendon injuries. However, the cells fate and biodistribution after intralesional administration remain to be described [42]. It has been shown that low percentages of MSCs are found after intra-lesional administration, and the MSCs can be found up to 90 days after injection but it has been difficult to prove that they integrate into the tissue [42,43].

Our study was a proof of concept in an *in vitro* model and the biggest limitation is that we did not investigate the effect of the combination of ESWT and equine CB-MSCs *in vivo*. There are more limitations worth mentioning. First the exact energy of ESWT that needs to be applied to equine CB-MSCs is unknown. Despite the fact that the study showed a significant increase in metabolic activity and increased adipogenic and osteogenic potential, the results are limited to the specific experimental settings selected here and should not be applied to clinical cases. Prospective clinical trials are needed to evaluate the effects of ESWT on different sources of MSCs *in vitro* and *in vivo*.

### **3.7 Conclusion**

In conclusion, our findings indicate that ESWT can enhance the metabolic activity and does not adversely affect the proliferation and immunomodulatory properties of equine CB-MSCs. For trilineage differentiation, we showed that ESWT allowed equine CB-MSCs to maintain their differentiation capacity, even more, treated cells showed an increased potency

towards adipogenic and osteogenic lineages. The combined effect of these therapies remains to be seen, but the anecdotal clinical impression of improved healing after the combination of these two therapies can be partially explained through the increased metabolic activity and no effect on their immunomodulatory properties observed in this study.

Further *in vitro* and *in vivo* studies will be required to fully investigate the effects of ESWT on CB-MSCs and the mechanisms by which the combination of this treatments appear to improve healing of soft tissue injuries.

### **3.8 References**

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### 3.9 Figures



Figure 3 - Photograph of the adapted water bath for in vitro extracorporeal shock wave treatment.

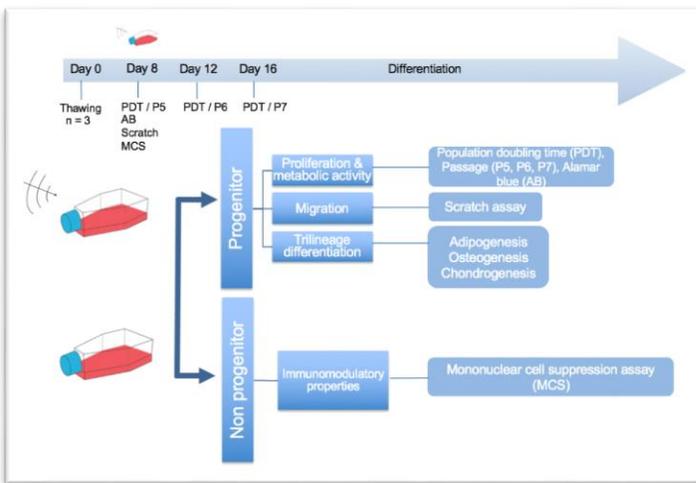


Figure 6 - Experimental design. Time points for ESWT, respective passage numbers (P5, P6, P7) and population doubling time (PDT), Alamar blue assay (AB), scratch (migration) assay and mononuclear cell suppression assay (MCS). Differentiation was initiated after treatment on day 8.

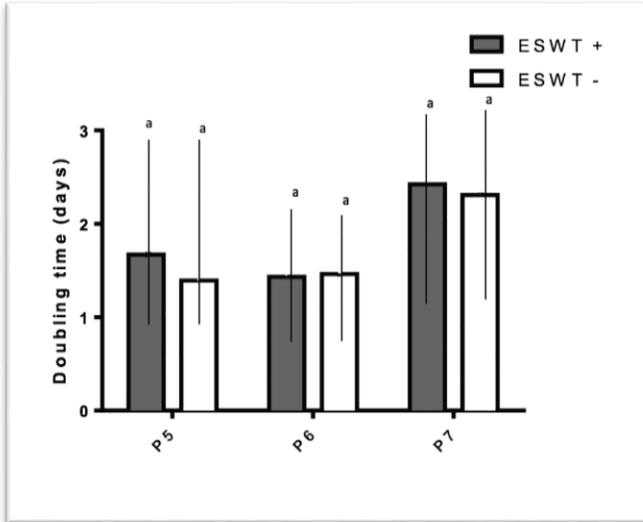


Figure 7 - Proliferation. Population doubling times (days) for treated (ESWT +) and untreated (ESWT -) equine CB-MSCs for passage 5 (P5), 6 (P6) and 7 (P7). Results are shown as mean and CI 95% ( $p > 0.05$ ). No differences were observed.

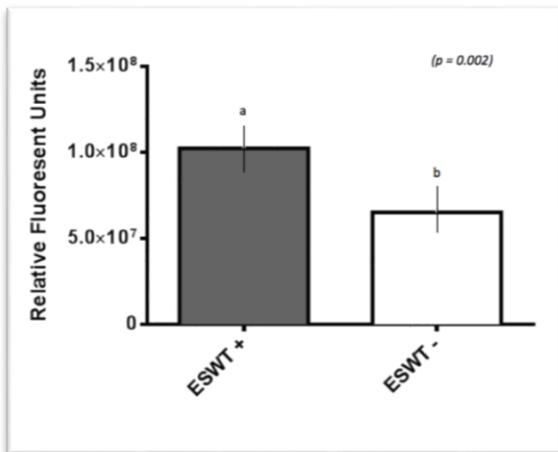


Figure 8 - Metabolic activity. Relative fluorescent units for treated (ESWT +) and untreated (ESWT -) equine CB-MSCs. Results are shown as mean and CI 95% ( $p = 0.0002$ ). Values with different letters are statistically different.

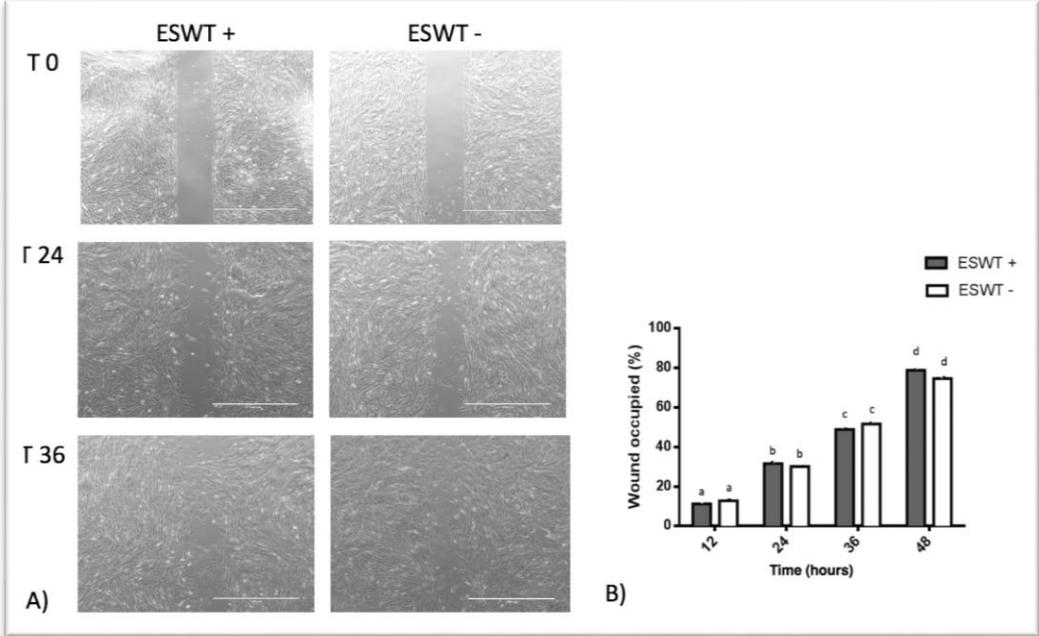


Figure 9 - Migration. A) Representative scratch assay for treated (ESWT +) and untreated (ESWT -) equine CB-MSC. Images were obtained using a motorized inverted microscope until closure of the scratch in the cell layer. B) Percentage of wound occupied, calculated by dividing the non recovered area at 12, 24, 36 and 48 hours respectively by the initial wound area at 0 hour and subtracting this value as a percentage from 100%. There were no differences ( $p > 0.05$ ) between the treated (ESWT +) and untreated (ESWT -) equine CB-MSC at each time point.

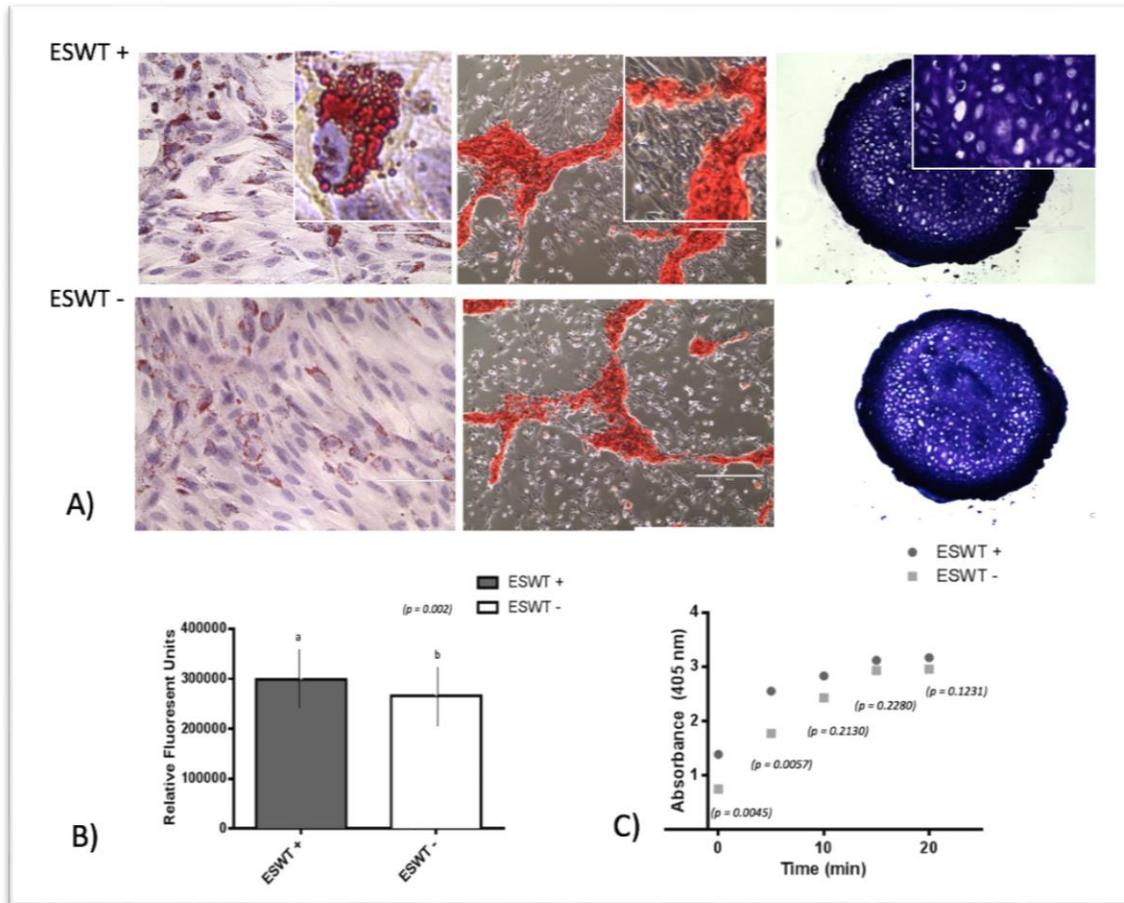


Figure 10 - Trilineage differentiation. A) Positive trilineage potency for adipogenesis (Oil Red O), osteogenesis (Alizarin Red S) and chondrogenesis (Toluidine blue) for treated (ESWT +) and untreated (ESWT -) equine CB-MSC. B) Quantification of intracellular lipid droplets by relative fluorescent units for treated (ESWT +) and untreated (ESWT -) equine CB-MSC. Results are shown as mean and CI 95% ( $p = 0.0002$ ). Values with different letters are statistically different ( $p = 0.002$ ). C) Alkaline phosphatase activity measured in relative fluorescent units in treated (ESWT +) and untreated (ESWT -) equine CB-MSC. Differences were found at time 0 ( $p = 0.0045$ ) and 5 minutes ( $p = 0.0057$ ).

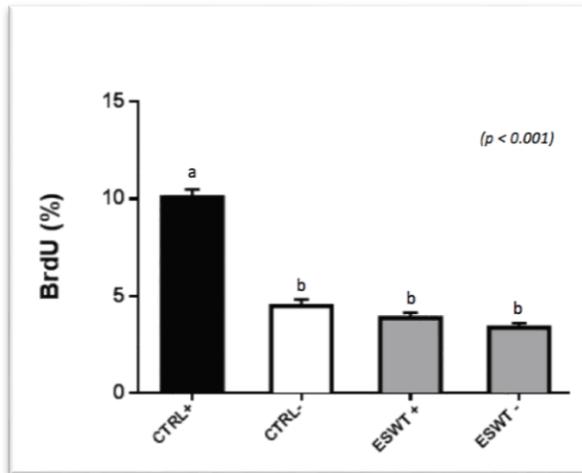


Figure 11 - Mononuclear cell suppression assay. MNC suppression assay to determine MNC proliferation suppression capacity of equine CB-MSC. Both treated (ESWT +) and untreated (ESWT -) equine CB-MSC were able to suppress of the proliferation of stimulated lymphocytes to the level of unstimulated lymphocytes (CTRL -). Values with different letters are statistically different.

## CHAPTER IV

### Summary and Conclusions

Tendon and ligament injuries are among the most common musculoskeletal injuries in the sport horse. Healing is challenging and reinjury rates are high [1]. A large amount of treatments have been reported in the literature and the results have been variable [1,2]. Therefore, there is a continuous search for better treatments of these conditions in the horse.

Extracorporeal shock wave therapy (ESWT) has been shown to induce different biological effects on a variety of cells, including regulation and stimulation of their function and metabolism. ESWT can promote different biological responses such as proliferation, migration, and regenerations of cells [3]. Recent studies have shown that mesenchymal stromal cells (MSCs) secrete factors that enhance regeneration of tissues, stimulate proliferation and differentiation of cells and decrease inflammatory and immune-reactions [4,5]. The combinations of these two therapies *in vitro* have shown that ESWT have an effect on different types of MSCs, which goes from an increased proliferation and differentiation potency to a reduce rate of apoptosis [6,7,8]. Many authors have investigated different settings and protocols to apply ESWT to MSCs. For example, Suhr *et al.* (2013) used freshly prepared pork skin as an absorbing support surface between the applicator and the flask, with an energy flux density of 0.2 mJ/mm<sup>2</sup>, 4 Hz and 1000 pulses. Schuch *et al.* (2014) and Weihs *et al.* (2014) used the setup described by Holfeld with an EFD of 0.09 mJ/mm<sup>2</sup> 3 Hz 200, pulses and 0.03 – 1.9 mJ/mm<sup>2</sup> 3 Hz, 100 – 300 impulses respectively. There are no standardized protocols which makes comparison of results challenging.

Nowadays, the model described by Holfeld *et al.* (2014) appears to be the most consistent in the literature, which can make future comparison easier.

The main objective of this study was to evaluate the effects of ESWT to equine umbilical blood cord MSCs (UC-MSC) *in vitro*. To the authors knowledge this is the first time this specific type of MSCs has been evaluated after ESWT. Additionally, not only their progenitor function was evaluated but their non progenitor functions as well.

The results of the study showed that ESWT produces a biological response in equine CB-MSCs *in vitro* that is likely to be favorable when treating tendon and ligament injuries in the equine patient *in vivo*, compared to non treated equine CB-MSC. Overall, the treated cells showed an increased metabolic activity, increased potency towards the adipogenic and osteogenic lineages and their proliferation and immune - modulatory functions were not adversely affected.

Some limitations are to be recognized. First, our study was a proof of concept in an *in vitro* model and the biggest limitation is that we did not investigate the effect of the combination of ESWT and CB-MSC *in vivo*. Future investigations are needed in order to draw any conclusions about the combination of those treatments in a clinical setting. Additionally, the present study was purely descriptive and does not define the specific cellular response of equine CB-MSC to ESWT. The exact energy of ESWT that needs to be applied to equine CB-MSCs is unknown. Despite the fact that the study showed a significant increase in metabolic activity and increased adipogenic and osteogenic potential, the results are limited to the specific experimental settings selected here and should not be applied to clinical cases. Further *in vitro* and *in vivo* studies are required to fully investigate the effects that ESWT have on MSCs of different species and sources. It would also be

beneficial to explore further the mechanisms by which this combination of therapies mediates its effects in order to formulate evidence-based recommendations.

Our study showed an increased in adipogenic potency of the treated cells with ESWT. Previous studies have shown an increase in lipid droplets formation in cells that have been exposed to cellular stress; adaptive responses for cellular survival are thought to be triggered by cellular stress [9]. It would be important to continue investigate what is the influence of ESWT in the formation of lipid droplets and what is the association of ESWT to cellular stress.

The survival and persistence of the MSCs after intra-lesionally administration remains a concern. Furthermore, finding a technique that is reliable in tracking the cells after administration is challenging. Therefore, different techniques have been explored to label and track MSCs [10,11]. A follow up study will evaluate the effect that ESWT has on equine CB-MSc that have been labeled.

In conclusion, this study demonstrated that equine CB-MSCs are sensitive to ESWT treatment by showing an increased metabolic, adipogenic and osteogenic activity, and unaltered immunosuppressive properties. The positive results of the effects of these combined treatments *in vivo* remains to be proven; however, the anecdotal clinical impression of improved healing after their usage can be partially explained through the increased metabolic activity and no effect on their immunomodulatory properties observed in this study.

## 4.1 References

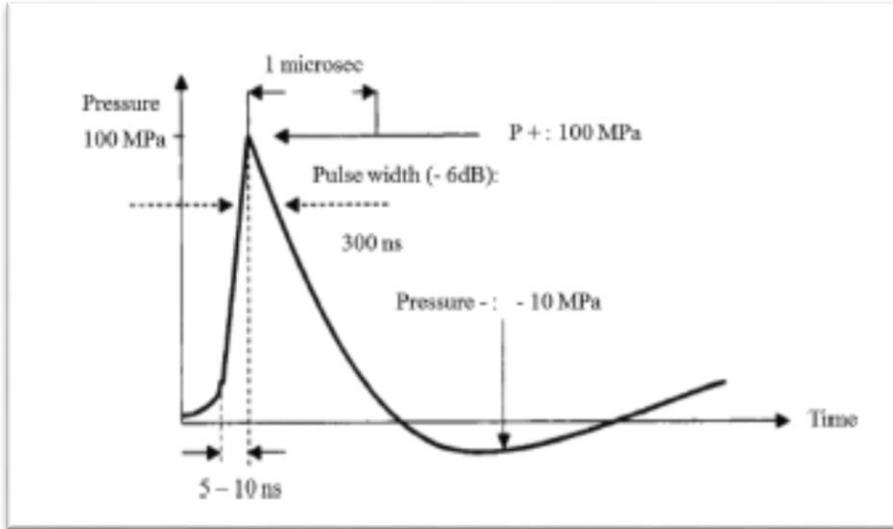
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## CHAPTER V

### Appendices

#### 5.1 Figures



- 1) Figure 1 - Physical characteristics of extracorporeal shock waves. (from: Ogden JA, Toth-Kischkat A, Schultheiss R. Principles of shock wave therapy. *Clin Orthop Relat Res.* 2001; 387:8-17.)

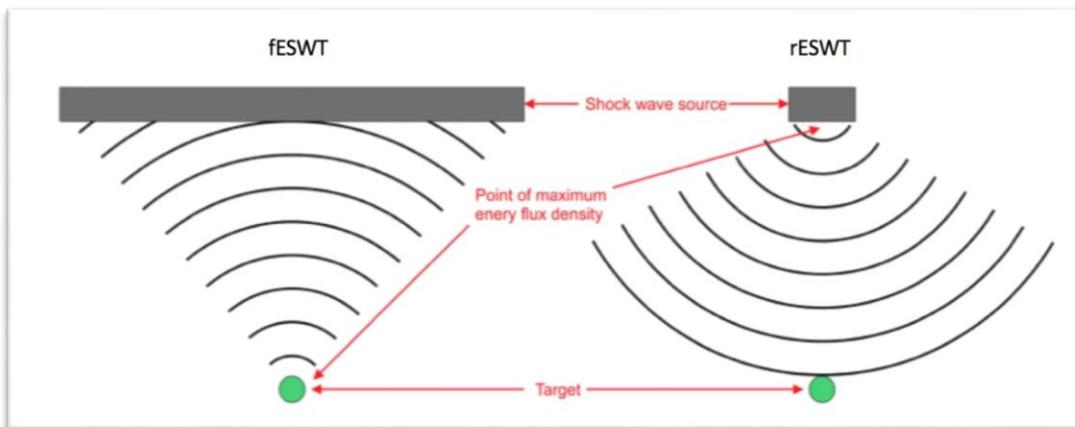


Figure 2 – Differences in wave propagation. (Adapted from: Kiessling MC, Milz S, Frank HG, et al. Radial extracorporeal shock wave treatment harms developing chicken embryos. *Sci Rep.* 2015;6;5:8281)

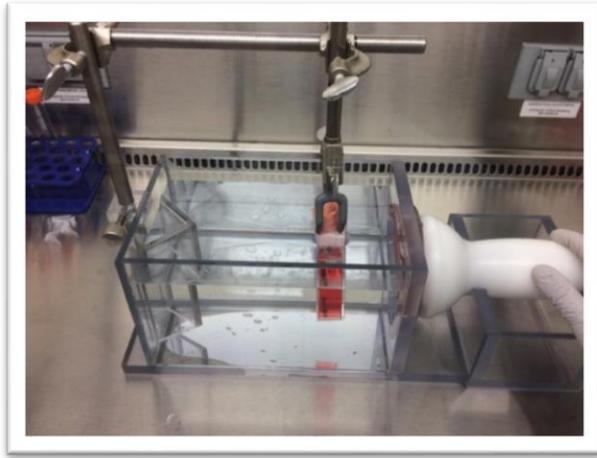


Figure 3 – Photograph of the adapted water bath for *in vitro* extracorporeal shock wave treatment.

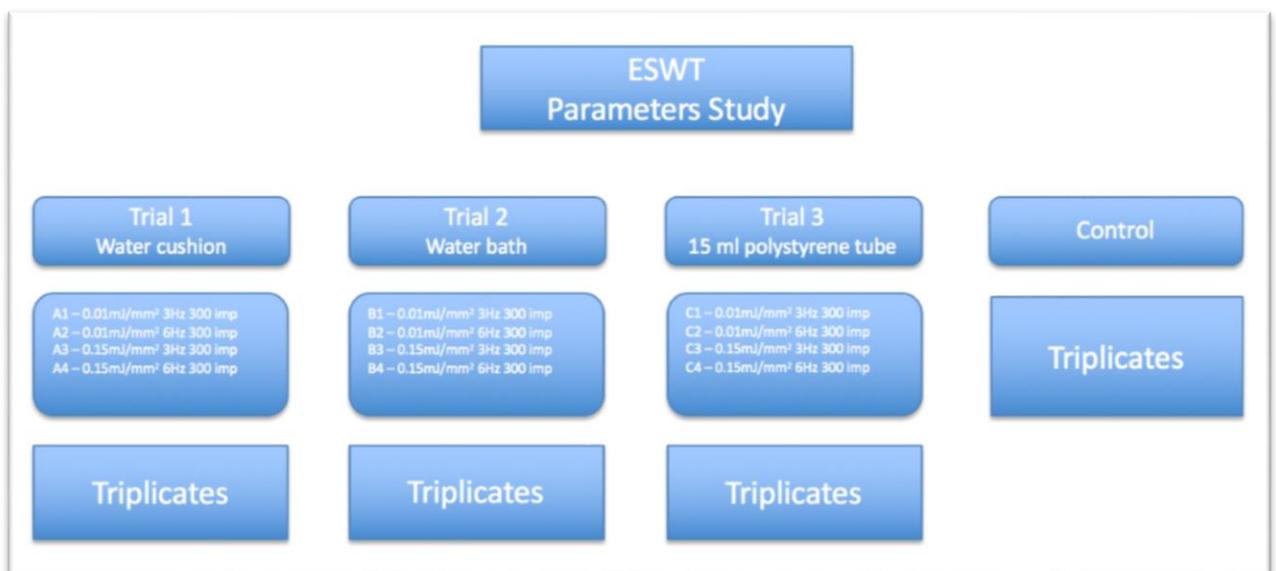


Figure 4. Pilot study experimental design. Three different set ups with four different energy protocols were evaluated. All experiments were performed in triplicates.

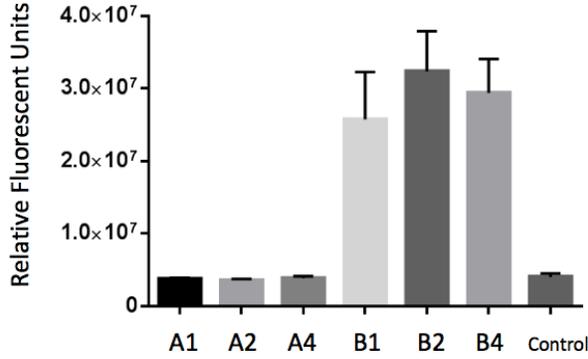


Figure 5 - Metabolic activity. Pilot study. Water cushion (A) and water bath (B) setups were tested with different energy settings. Relative fluorescent units for treated (A1, A2, A4, B1, B2, B4) and control equine CB-MSCs.

## 5.2 Tables

Table 1 - Extracorporeal shock wave generators used in equine veterinary medicine. ALDDFT:

Accessory ligament deep digital flexor tendon; OA: Osteoarthritis.

Indication	Type of study	Device	Technology	Reference
Suspensory Desmitis	Clinical	Swiss DolorClast Vet (EMS)	Radial	Boening <i>et al.</i>
	Clinical	Swiss DolorClast Vet (EMS)	Radial	Crowe <i>et al.</i>
	Experimental	Equitron (HMT)	Electrohydraulic	McClure <i>et al.</i>
	Experimental	Equitron (HMT)	Electrohydraulic	McClure <i>et al.</i>
	Clinical	Swiss DolorClast Vet (EMS)	Radial	Crowe <i>et al.</i>
	Experimental	Equitron (HMT)	Electrohydraulic	Caminoto <i>et al.</i>
	Experimental	Equitron (HMT)	Electrohydraulic	Ringer <i>et al.</i>

	Clinical	Equitron (HMT)	Electrohydraulic	Imboden et al.
	Clinical	Swiss DolorClast Vet (EMS)	Radial	Palmer SE.
	Experimental	Equitron (HMT)	Electrohydraulic	Bischofberger <i>et al.</i>
	Clinical	Equitron (HMT)	Electrohydraulic	Lischer <i>et al.</i>
Desmitis ALDDFT	Experimental	Versatron (HMT)	Electrohydraulic	Waguespack <i>et al.</i>
OA tarsus	Clinical		Electrohydraulic	McCarroll <i>et al.</i>
OA Navicular syndrome	Clinical	Versatron (HMT)	Electrohydraulic	Frisbie <i>et al.</i>
	Clinical	Orthowave (MTS)	Electrohydraulic	McClure <i>et al.</i>
OA	Clinical	Equitron, Versatron (HMT)	Electrohydraulic	Revenaugh MS.
OA	Experimental	Versatron (HMT)	Electrohydraulic	Frisbie <i>et al.</i>
	Experimental	Versatron (HMT)	Electrohydraulic	Kawcak <i>et al.</i>
Tendinitis	Experimental	Equitron (HMT)		Kersh <i>et al.</i>
	Experimental	Equitron (HMT)		Bosch <i>et al.</i>
	Experimental	Equitron (HMT)	Electrohydraulic	Bosch et al.
Bone	Experimental	Equitron (HMT)	Electrohydraulic	Da Costa <i>et al.</i>
	Experimental	Equitron (HMT)		Pawels <i>et al.</i>
		Equitron (HMT)	Electrohydraulic	Bischofberger <i>et al.</i>
Analgesia	Experimental	Equitron (HMT)		McClure <i>et al.</i>
		Swiss DolorClast Vet (EMS)	Radial	